HEPATITIS B VIRUS (HBV) iRNA COMPOSITIONS AND METHODS OF USE THEREOF

Related Applications

This application claims the benefit of priority to U.S. Provisional Application, 62/077,799, filed on November 10, 2014, and U.S. Provisional Application, 62/137,464, filed on March 24, 2015. The entire contents of each of the foregoing patent applications are incorporated herein by reference.

This application also claims priority to U.S. Provisional Application, 62/077,672, filed on November 10, 2014, the entire contents of which are hereby incorporated herein by reference.

This application is related to International Patent Application, PCT/US2015/XXXXX, entitled "Hepatitis D Viris (HDV) iRNA Compositions and Methods of Use Thereof," filed on November 10, 2015, the entire contents of which are incorporated herein by reference.

Sequence Listing

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 5, 2015, is named 121301-02320_SL.txt and is 385,791 bytes in size.

Background of the Invention

Worldwide more than 400 million people are chronically infected with HBV and are, thus, at increased risk of developing serious liver disease, such as chronic hepatitis, cirrhosis, liver failure and hepatocellular carcinoma (HCC) resulting in an estimated 600,000 deaths each year.

The natural evolution of chronic HBV infection includes four consecutive phases: (1) early 'immunotolerant' phase, high levels of virus replication and minimal liver inflammation; (2) immune reactive phase, significant hepatic inflammation and elevated serum aminotransferases; with some patients progressing to (3) 'non-replicative ' phase, seroconversion to anti-HBe; undetectable or low level of viremia (below 2000 IU/ml by PCR-based assays); resolution of hepatic inflammation; and (4) HBeAg-negative chronic hepatitis B, due to the emergence of specific viral mutations, which prevent the production of HBeAg but do not hamper virus replication. This form of chronic hepatitis B (CHB) is characterized by fluctuating serum HBV DNA and serum aminostransferases (ALT and AST) levels, and progressive liver disease. It is important to note that CHB may present either as HBeAg-positive or HBeAg-negative CHB. Longitudinal studies of patients with CHB indicate that the 5-year cumulative incidence of

developing cirrhosis ranges from 8 to 20%. The 5-year cumulative incidence of hepatic decompensation is approximately 20%. The worldwide incidence of HCC has increased and presently constitutes the fifth most common cancer. The annual incidence of HBV-related HCC is high, ranging from 2-5% when cirrhosis is established.

The primary goal of treatment for HBV is to permanently suppress HBV replication and improve liver disease. Clinically important short-term goals are to achieve HBeAg-seroconversion, normalization of serum ALT and AST, resolution of liver inflammation and to prevent hepatic decompensation. The ultimate goal of treatment is to achieve durable response to prevent development of cirrhosis, liver cancer and prolong survival. HBV infection cannot be eradicated completely due to persistence of a particular form of viral covalently closed circular DNA (ccc HBV DNA) in the nuclei of infected hepatocytes. However, treatment-induced clearance of serum HBsAg is a marker of termination of chronic HBV infection and has been associated with the best long-term outcome.

The current standard methods of treatment for HBV include interferon or thymosin a1 -based immunotherapies and the suppression of viral production by inhibition of the HBV polymerase. HBV polymerase inhibitors are effective in reducing viral production but have little to no effect in rapidly reducing HBsAg or can slowly reduce HBsAg with long term treatment in a limited number of patients (as is the case with tenofovir disoproxil fumarate). Interferon based immunotherapy can achieve a reduction of both viral production and early removal of HBsAg from the blood but only in a small percentage of treated subjects. The generally accepted role of HBsAg in the blood is to sequester anti-HBsAg antibodies and allow infectious viral particles to escape immune detection which is likely one of the reasons why HBV infection remains a chronic condition. In addition HBsAg, HBeAg and HBcAg all have immuno-inhibitory properties and the persistence of these viral proteins in the blood of patients following the administration of any of the currently available treatments for HBV is likely having a significant impact in preventing patients from achieving immunological control of their HBV infection.

Although the three primary HBV proteins (HBsAg, HBeAg and HBcAg) all have immunoinhibitory properties, HBsAg comprises the overwhelming majority of HBV protein in the circulation of HBV infected subjects. Additionally, while the removal (*via* seroconversion) of HBeAg or reductions in serum viremia are not correlated with the development of sustained control of HBV infection off treatment, the removal of serum HBsAg from the blood (and seroconversion) in HBV infection is a well-recognized prognostic indicator of antiviral response on treatment which will lead to control of HBV infection off treatment (although this only occurs in a small fraction of patients receiving immunotherapy). Thus, while reduction of all three major HBV proteins (HBsAg, HBeAg and HBcAg) may result in the optimal removal of inhibitory effect, the removal of HBsAg alone is likely sufficient in and of itself to remove the bulk of the viral inhibition of immune function in subjects with HBV infection.

Therefore, in the absence of any current treatment regimen which can restore immunological control of HBV in a large proportion of patients, there is a need for an effective treatment against HBV infection which can inhibit viral replication as well as restore immunological control in the majority of patients. Accordingly, there is a need in the art for alternative therapies and combination therapies for subjects infected with HBV and/or having an HBV-associated disease.

Summary

The present invention refers to subject matter as defined in the attached set of claims. Disclosed herein are iRNA compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a Hepatitis B virus (HBV) gene. The HBV gene may be within a cell, *e.g.,* a cell within a subject, such as a human.

The present disclosure also provides methods and therapies for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of an HBV gene, *e.g.,* an HBV infection and/or an HBV-associated disease, such as chronic Hepatitis B infection (CHB), cirrhosis, liver failure, and hepatocellular carcinoma (HCC), using iRNA compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of an HBV gene for inhibiting the expression of an HBV gene.

The RNAi agents of the disclosure have been designed to target regions in the HBV genome that are conserved across all 8 serotypes of HBV. In addition, the RNAi agents of the disclosure have been designed to inhibit all steps of the HBV life cycle, *e.g.,* replication, assembly, secretion of virus, and secretion of sub-viral antigens, by inhibiting expression of more than one HBV gene. In particular, since transcription of the HBV genome results in polycistronic, overlapping RNAs, an RNAi agent of the disclosure targeting a single HBV gene results in significant inhibition of expression of most or all HBV transcripts. For example, because the HBV genome is transcribed into a single mRNA, an RNAi agent of the disclosure targeting the S gene will result in inhibition of not only S gene expression but also the expression of the "downstream" reverse transcriptase gene. Furthermore, the RNAi agents of the disclosure have been designed to inhibit HBV viral replication by targeting HBV structural genes, and the HBV X gene thereby permitting a subject's immune system to detect and respond to the presence of HBsAg to produce anti-HBV antibodies to clear an HBV infection. Without intending to be limited by theory, it is believed that a combination or sub-combination of the foregoing properties and the specific target sites and/or the specific modifications in these RNAi agents confer to the RNAi agents of the disclosure improved efficacy, stability, safety, potency, and durability.

Accordingly, also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region,

wherein said sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1, and said antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one aspect, the present invention provides, a double stranded RNAi agent for inhibiting expression of hepatitis B virus (HBV) in a cell, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region of complementarity which comprises at least 15 contiguous nucleotides, wherein said sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:39 (GUGUGCACUUCGCUUCACA), and said antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:40 (UGUGAAGCGAAGUGCACACUU), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one example, the one or more of the 3 nucleotide differences in the nucleotide sequence of the antisense strand is a nucleotide mismatch in the antisense strand.

In another example, the one or more of the 3 nucleotide differences in the nucleotide sequence of the antisense strand is a nucleotide mismatch in the sense strand.

In one example, all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand are modified nucleotides.

In one example, the sense strand and the antisense strand comprise a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the sequences listed in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26.

In one example, the at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified

nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

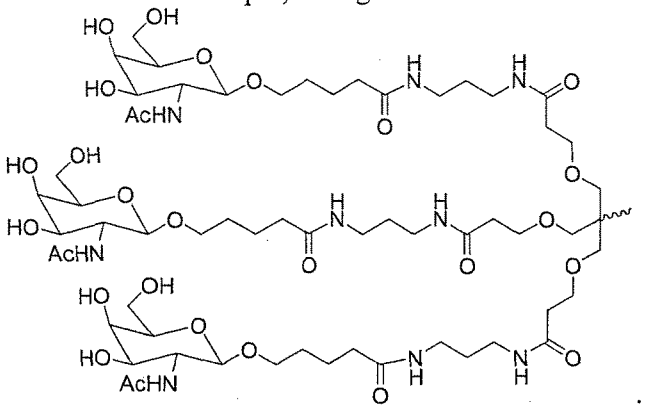
In one example, the at least one strand comprises a 3' overhang of at least 1 nucleotide. In another example, the at least one strand comprises a 3' overhang of at least 2 nucleotides.

In one example, the double-stranded region is 15-30 nucleotide pairs in length. In another example, the double-stranded region is 17-23 nucleotide pairs in length. In yet another example, the double-stranded region is 17-25 nucleotide pairs in length. In one example, the e double-stranded region is 23-27 nucleotide pairs in length. In another

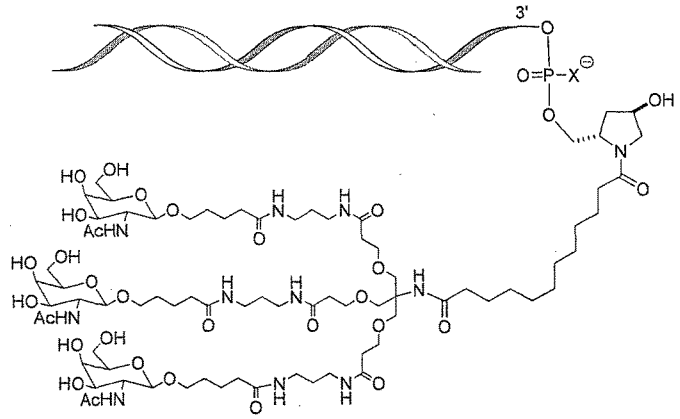
example, the double-stranded region is 19-21 nucleotide pairs in length. In yet another example, the double-stranded region is 21-23 nucleotide pairs in length.

In one example, each strand has 15-30 nucleotides. In another example, each strand has 19-30 nucleotides.

In one example, the ligand is



In one example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

In one example, the RNAi agent is selected from the group of RNAi agents listed in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26.

Also disclosed are RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'-UCGUGGUGGACUUCUCUCA -3' (SEQ ID NO:5), and said antisense strand comprises 5'-UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

The present disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

Also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'-GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7), and said antisense strand comprises 5'-UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. The present disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

Also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'-CGUGGUGGACUUCUCUCAAUU -3' (SEQ ID NO:9), and said antisense strand comprises 5'-AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. The present disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

Also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein the sense strand comprises 5'-CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37), and the antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38), wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. The present

disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

Also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'-GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11), and said antisense strand comprises 5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. The present disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

Also disclosed are double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell. The double stranded RNAi agents include a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'-GUGUGCACUUCGCUUCACA -3' (SEQ ID NO:39), and said antisense strand comprises 5'-UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. The present disclosure also provides RNAi agents comprising sense and antisense nucleotide sequences which are at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over their entire length to the foregoing sense and antisense nucleotide sequences.

In another aspect, the present invention provides A double stranded RNAi agent for inhibiting expression of hepatitis B virus (HBV) in a cell, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39), and said antisense strand comprises 5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one example, all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand comprise a modification.

In one example, at least one of said modified nucleotides is selected from the group consisting of a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified

nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In one example, the 5'-phosphate mimic is a 5'-vinyl phosphate (5'-VP).

In one example, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13) and the antisense strand comprises 5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15) and the antisense strand comprises 5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17) and the antisense strand comprises 5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19) and the antisense strand comprises 5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21) and the antisense strand comprises 5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23) and the antisense strand comprises 5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

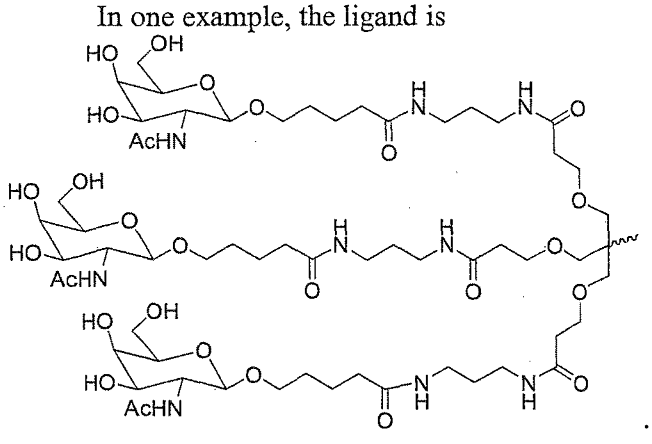
In another example, the sense strand comprises 5'-csgsuggudGgucdTucucuaaauu – 3' (SEQ ID NO:35) and the antisense strand comprises 5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; dA, dC, dG, and dT are deoxyribose A, C, G, and T; and s is a phosphorothioate linkage.

In one example, the sense strand comprises 5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25) and the antisense strand comprises 5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27) and the antisense strand comprises 5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

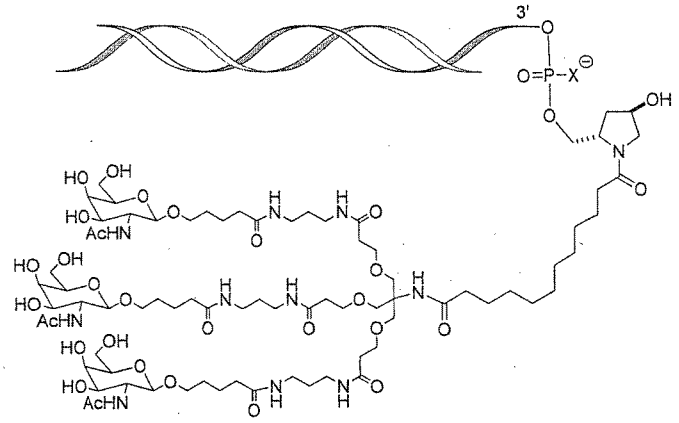
In another example, the sense strand comprises 5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41) and the antisense strand comprises 5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In one example, the ligand is



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In one example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

In one example, the P is a 5'-phosphate mimic. In one example, the 5'-phosphate mimic is a 5'-vinyl phosphate (5'-VP).

Also disclosed are compositions comprising two or more double stranded RNAi agents for inhibiting expression of hepatitis B virus (HBV) in a cell, wherein each double stranded RNAi agent independently comprises a sense strand and an antisense strand forming a double-stranded region, wherein each of said sense strands independently comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1, and each of said antisense strands independently comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of each of said sense strands and substantially all of the nucleotides of each of said antisense strands are independently modified nucleotides, wherein each of said sense strands are independently conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one example, the one or more of the 3 nucleotide differences in the nucleotide sequence of the antisense strand is a nucleotide mismatch in the antisense strand. In another example, the one or more of the 3 nucleotide differences in the nucleotide sequence of the antisense strand is a nucleotide mismatch in the sense strand.

In one example, all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand are modified nucleotides.

In one example, the sense strand and said antisense strand comprise a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the sequences listed in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26.

In one example, the at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-

methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

Also disclosed are compositions for inhibiting expression of hepatitis B virus (HBV) in a cell, the composition comprising (a) a first double-stranded RNAi agent comprising a first sense strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said first sense strand and substantially all of the nucleotides of the first antisense strand are modified nucleotides, wherein said first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and (b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said second sense strand and substantially all of the nucleotides of said second antisense strand are modified nucleotides, wherein said second sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first and second sense strands each independently comprise a sequence selected from the group consisting of
5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),
5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ IDNO:7),
5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9),
5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ IDNO:37),
5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ IDNO:11), and
5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequences), and wherein the first and second antisense strands each independently comprise a sequence selected from the group consisting of
5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6),
5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8),

5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10),
5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),
5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and
5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequences).

In one example, the first and second sense strand and/or all of the nucleotides of the first and second antisense strand comprise a modification.

In one example, the at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In one example, the first and second RNAi agents are selected from the group consisting of:
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13)
5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14);
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)
5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17)
5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19)
5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20);
5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)
5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22);
5'- csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23)
5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24);
5'-csgsuggudGgucdTucucuaaaauu – 3' (SEQ ID NO:35)
5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36);
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)
5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26);

5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27)
5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28); and
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; dA, dC, dG, and dT are deoxyribose A, C, G, and T; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the first and second RNAi agents are
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)
5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16);
5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)
5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In another example, the first and second RNAi agents are
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)
5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26); and
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

Also disclosed is a double stranded RNAi agent comprising the RNAi agents listed in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26.

The present disclosure also provides vectors and cells comprising the double stranded RNAi agent of the disclosure.

Also disclosed are pharmaceutical compositions comprising the double stranded RNAi agents of the disclosure, or the compositions of the disclosure, or the vectors of the disclosure.

In yet another aspect, the present invention provides a pharmaceutical composition comprising the double stranded RNAi agent disclosed herein.

In one example, the double stranded RNAi agent is administered in an unbuffered solution. In one example, the unbuffered solution is saline or water.

In another example, the double stranded RNAi agent is administered with a buffer solution. In one example, the buffer solution comprises acetate, citrate,

prolamine, carbonate, or phosphate or any combination thereof. In another example, the buffer solution is phosphate buffered saline (PBS).

Also disclosed are methods of inhibiting Hepatitis B virus (HBV) gene expression in a cell. The methods include contacting the cell with the double

stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure; and maintaining the cell produced for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby inhibiting expression of the HBV gene in the cell.

In one example, the HBV gene is selected from the group consisting of C, X, P, S, and a combination thereof.

In yet another aspect, the present invention provides the use of a double stranded RNAi agent disclosed herein, or a pharmaceutical composition disclosed herein in the manufacture of a medicament for inhibiting Hepatitis B virus (HBV) gene expression or replication of HBV in a cell, wherein: (a) the agent or the composition is to be contacted with the the cell; and (b) the cell produced in step (a) is to be maintained for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby expression of the HBV gene in the cell is inhibited, wherein the HBV gene is optionally selected from the group consisting of C, X, P, S, and a combination thereof.

Also disclosed are methods of inhibiting replication of a Hepatitis B virus (HBV) in a cell. The methods include contacting the cell with the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure; and maintaining the cell produced for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby inhibiting replication of the HBV in the cell.

In one example, the cell is within a subject. In one example, the subject is a human.

In one example, the subject suffers from an HBV-associated disease.

In one example, HBV gene expression is inhibited by at least about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

In one example, replication of HBV in the cell is inhibited by at least about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

Also disclosed are methods of reducing the level of Hepatitis B virus (HBV) DNA in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby reducing the level of HBV ccc DNA in the subject.

Also disclosed are methods of reducing the level of a Hepatitis B virus (HBV) antigen in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby reducing the level of the HBV antigen in the subject.

In one example, the HBV antigen is HBsAg. In abother example, the HBV antigen is HBeAg.

Also disclosed are methods of reducing the viral load of Hepatitis B virus (HBV) in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the

disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby reducing the viral load of HBV in the subject.

In yet another aspect, the present invention provides the use of a therapeutically effective amount of the double stranded RNAi agent disclosed herein, or the composition disclosed herein in the manufacture of a medicament for reducing the viral load of Hepatitis B virus (HBV) in a subject infected with HBV, wherein the agent or the composition is to be administered to the subject, thereby the viral load of HBV in the subject is reduced.

Also disclosed are methods of reducing the level of alanine aminotransferase (ALT) in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby reducing the level of ALT in the subject.

Also disclosed are methods of reducing the level of aspartate aminotransferase (AST) in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby reducing the level of AST in the subject.

Also disclosed are methods of increasing the level of anti-Hepatitis B virus (HBV) antibodies in a subject infected with HBV. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby increasing the level of anti- HBV antibodies in the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby treating said subject.

In yet another aspect, the present invention provides the use of a therapeutically effective amount of the double stranded RNAi agent disclosed herein, or the pharmaceutical composition disclosed herein for treating a subject having a Hepatitis B virus (HBV) infection, wherein the agent or the composition is to be administered to the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of the double stranded RNAi agent of the disclosure, or the composition of the disclosure, or the vector of the disclosure, or the pharmaceutical composition of the disclosure, thereby treating said subject.

In one example, the HBV-associated disorder is selected from the group consisting of hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma.

In one example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg positive. In another example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg negative.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- UCGUGGUGGACUUCUCUCA -3' (SEQ ID NO:5) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- UCGUGGUGGACUUCUCUCA -3' (SEQ ID NO:5) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and

said antisense strand comprises 5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are provides methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region,

wherein said sense strand comprises 5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region,

wherein said sense strand comprises 5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ ID NO:9) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more

GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ ID NO:9) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein the sense strand comprises 5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37), (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and the antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a

double-stranded region, wherein the sense strand comprises 5'-CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and the antisense strand comprises 5'-AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence),

wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GUGUGCACUUCGCUUCACA -3' (SEQ ID NO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region, wherein said sense strand comprises 5'- GUGUGCACUUCGCUUCACA -3' (SEQ ID NO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), and said antisense strand comprises 5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequence), wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides, wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

In one example, all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand comprise a modification.

In one example, the at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In one example, the 5'-phosphate mimic is a 5'-vinyl phosphate (5'-VP).

In one example, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13) and the antisense strand comprises 5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15) and the antisense strand comprises 5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17) and the antisense strand comprises 5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19) and the antisense strand comprises 5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21) and the antisense strand comprises 5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In another example, the sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23) and the antisense strand comprises 5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

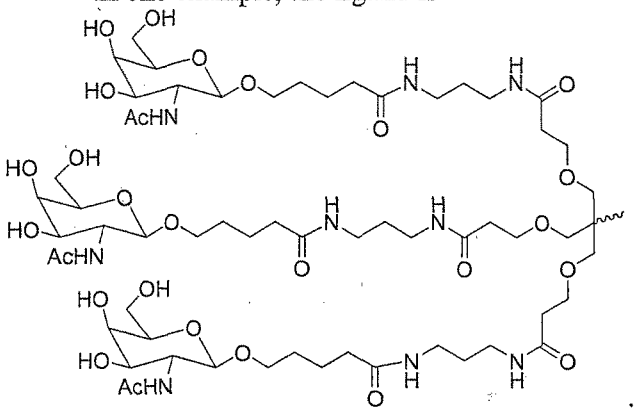
In another example, the sense strand comprises 5'-csgsuggudGgucdTucucuaaauu – 3' (SEQ ID NO:35) and the antisense strand comprises 5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; dA, dC, dG, and dT are deoxyribose A, C, G, and T; and s is a phosphorothioate linkage.

In one example, the sense strand comprises 5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25) and the antisense strand comprises 5'-usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

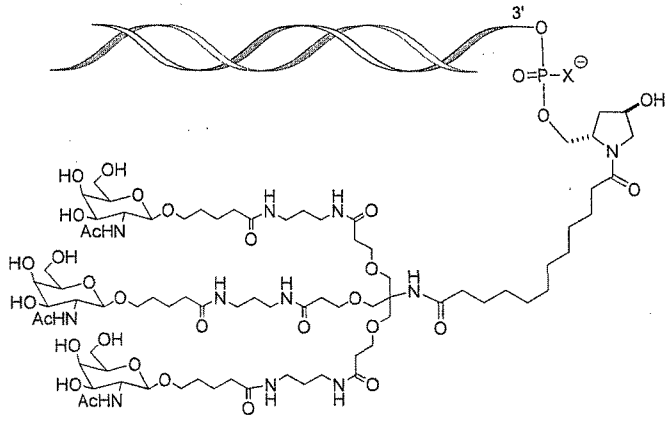
In another example, the sense strand comprises 5'-gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27) and the antisense strand comprises 5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In another example, the sense strand comprises
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41) and the antisense strand comprises 5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In one example, the ligand is



In one example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

In one example, the HBV-associated disorder is selected from the group consisting of hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma.

In one example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg positive. In another example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg negative.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV) infection. The methods include administering to the subject a therapeutically effective amount of a composition for inhibiting expression of hepatitis B virus (HBV) in a cell. The composition includes: (a) a first double-stranded RNAi agent comprising a first sense strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said first sense strand and substantially all of the nucleotides of said first antisense strand are modified nucleotides, wherein said first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and

(b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said second sense strand and substantially all of the nucleotides of said second antisense strand are modified nucleotides, wherein said second sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first and second sense strands each independently comprise a sequence selected from the group consisting of
5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),
5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ IDNO:7),
5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9),
5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ IDNO:37),
5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ IDNO:11), and
5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), and wherein the first and second antisense strands each independently comprise a sequence selected from the group consisting of
5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);
5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);
5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);
5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),
5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and
5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), thereby treating the subject.

Also disclosed are methods of treating a subject having a Hepatitis B virus (HBV)-associated disorder. The methods include administering to the subject a therapeutically effective amount of a composition for inhibiting expression of hepatitis B virus (HBV) in a cell. The composition includes: (a) a first double-stranded RNAi agent comprising a first sense strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said first sense strand and substantially all of the nucleotides of said first antisense strand are modified nucleotides, wherein said first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and (b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said second sense strand and substantially all of the nucleotides of said second antisense strand are modified nucleotides, wherein said second sense strand is conjugated to a

ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first and second sense strands each independently comprise a sequence selected from the group consisting of
5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),
5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ IDNO:7),
5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9),
5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ IDNO:37),
5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ IDNO:11), and
5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), and wherein the first and second antisense strands each independently comprise a sequence selected from the group consisting of
5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);
5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);
5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);
5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),
5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and
5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), thereby treating the subject.

In one example, all of the nucleotides of the first and second sense strand and all of the nucleotides of the first and second antisense strand comprise a modification.

In one example, the at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

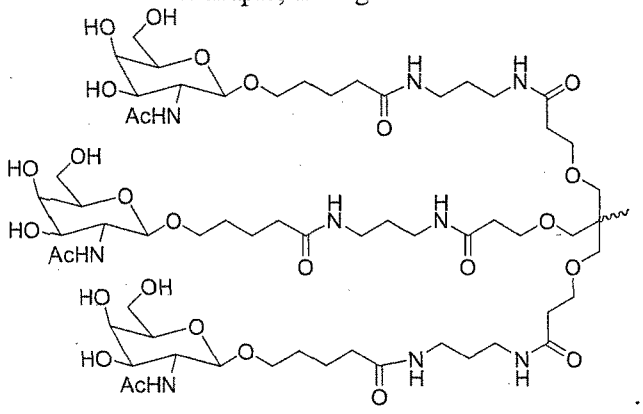
In one example, the first and second RNAi agent are selected from the group consisting of:
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13)

5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14);
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)
5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17)
5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19)
5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20);
5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)
5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22);
5'- csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23)
5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24);
5'-csgsuggudGgucdTucucuaaauu – 3' (SEQ ID NO:35)
5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36);
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)
5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26);
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27)
5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28); and
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; dA, dC, dG, and dT are deoxyribose A, C, G, and T; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

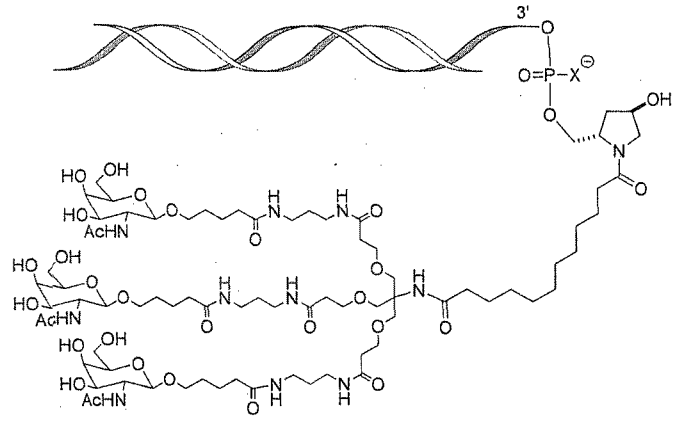
In one example, the first and second RNAi agents are
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)
5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16); and
5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)
5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In another example, the first and second RNAi agents are
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)
5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26); and
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In one example, the ligand is



In one example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

In one example, the subject is a human.

In one example, the HBV-associated disorder is selected from the group consisting of hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma.

In one example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg positive. In another example, the HBV-associated disorder is chronic hepatitis and the subject is HBeAg negative.

In one example, the double stranded RNAi agent is administered at a dose of about 0.01 mg/kg to about 10 mg/kg or about 0.5 mg/kg to about 50 mg/kg.

In one example, the double stranded RNAi agent is administered at a dose of about 10 mg/kg to about 30 mg/kg. In another example, the double stranded RNAi agent is administered at a dose of about 3 mg/kg. In one example, the double stranded RNAi agent is administered at a dose of about 10 mg/kg.

In one example, the double stranded RNAi agent is administered at a dose of about 0.5 mg/kg twice per week.

In one example, the double stranded RNAi agent is administered at a fixed dose of about 50 mg to 200 mg.

In one example, the double stranded RNAi agent is administered subcutaneously. In another example, the double stranded RNAi agent is administered intravenously.

In one example, the RNAi agent is administered in two or more doses.

In one example, the RNAi agent is administered at intervals selected from the group consisting of once every about 12 hours, once every about 24 hours, once every about 48 hours, once every about 72 hours, and once every about 96 hours.

In one example, the RNAi agent is administered twice per week. In another example, the RNAi agent is administered every other week.

In one example, the methods of the disclosure further include administering to the subject an additional therapeutic agent.

In one example, the additional therapeutic agent is selected from the group consisting of an antiviral agent, a reverse transcriptase inhibitor, an immune stimulator, a therapeutic vaccine, a viral entry inhibitor, an oligonucleotide that inhibits the secretion or release of HbsAg, a capsid inhibitor, a cccDNA inhibitor, and a combination of any of the foregoing.

In another example, the methods of the disclosure further include administering administering to the subject a reverse transcriptase inhibitor. In yet another example, the methods of the disclosure further include administering to the subject a reverse transcriptase inhibitor and an immune stimulator.

In one example, the reverse transcriptase inhibitor is slected from the group consisting of Tenofovir disoproxil fumarate (TDF), Tenofovir alafenamide, Lamivudine, Adefovir dipivoxil, Entecavir (ETV), Telbivudine, and AGX-1009.

In some examples, the methods of the disclosure further comprise treatment of hepatitis D virus (HDV) in the subject. Methods of treatment can include any methods of treatment known in the art. In certain examples, HDV is treated in the subject using one of more of the iRNA agents targeting HBV as described herein.

In some examples, the methods of the disclosure further include methods to modulate, *e.g.*, decrease, the expression of PD-L1. Compositions and methods to reduce the expression of PD-L1 are provided, for example, in PCT publication no. WO2011/127180, the entire contents of which are hereby incorporated herein by reference.

In one example, the immune stimulator is selected from the group consisting of pegylated interferon alfa 2a (PEG-IFN-α2a), Interferon alfa-2b, a recombinant human interleukin-7, and aToll-like receptor 7 (TLR7) agonist.

Also disclosed is a method of treating a subject having a Hepatitis B virus (HBV)-associated disorder, comprising administering to the subject a therapeutically effective amount of a double stranded RNAi agent,

wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region,

wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:29, and said antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:30,

wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides,

wherein the sense strand is conjugated to a ligand attached at the 3'-terminus, and

wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

Also disclosed is a method of treating a subject having a Hepatitis B virus (HBV) infection, comprising administering to the subject a therapeutically effective amount of a composition for inhibiting expression of hepatitis B virus (HBV) in a cell, said composition comprising

(a) a first double-stranded RNAi agent comprising a first strand and a first antisense strand forming a double-stranded region,

wherein substantially all of the nucleotides of the first sense strand and substantially all of the nucleotides of the first antisense strand are modified nucleotides,

wherein said first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and

(b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region,

wherein substantially all of the nucleotides of the second sense strand and substantially all of the nucleotides of the second antisense strand are modified nucleotides,

wherein the second sense strand is conjugated to a ligand attached at the 3'-terminus, and

wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker;

wherein the first sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1, and said first antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2,

wherein the sense second strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:29, and the second antisense strand comprises at least 15 contiguous nucleotides differing by no more

than 3 nucleotides from the nucleotide sequence of SEQ ID NO:30, thereby treating the subject.

In some examples, the first sense strand comprises a sequence selected from the group consisting of

5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),

5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ IDNO:7),

5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9),

5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ IDNO:37)

5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ IDNO:11), and

5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39), (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequences), and the second antisense strand comprises a sequence selected from the group consisting of

5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);

5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);

5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);

5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38);

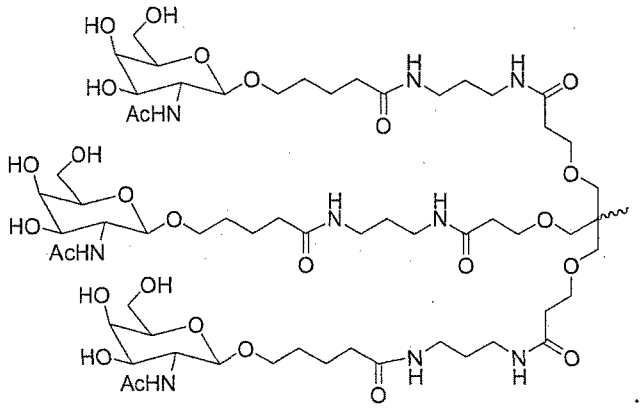
5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12); and

5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to the foregoing nucleotide sequences).

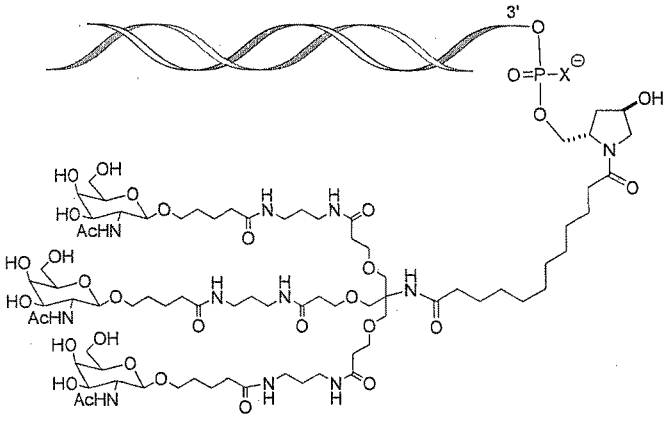
Also disclosed, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

In certain examples, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In some examples, the ligand is



In a specific example, the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

In certain examples, the double stranded RNAi agents and compositions provided herein are used for treatment of an HDV infection and/or an HDV-associated disorder.

Accordingly, the present disclosure provides methods of inhibiting replication of a Hepatitis D virus (HDV) in a cell. The methods include (a) contacting the cell with a double stranded RNAi agent, composition, vector, or the pharmaceutical composition provided herein; and (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby inhibiting replication of the HDV in the cell. In certain examples, the cell is within a subject. In certain examples, the subject is a human.

The disclosure further provides methods of reducing the level of a Hepatitis D virus (HDV) antigen in a subject infected with HDV. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, composition, vector, or the pharmaceutical composition provided herein, thereby reducing the level of the HDV antigen, *e.g.*, S-HDAg or L-HDAg, in the subject.

In yet another aspect, the present invention provides the use of a therapeutically effective amount of the double stranded RNAi agent disclosed herein, or the pharmaceutical composition disclosed herein in the manufacture of a medicament for reducing the level of a Hepatitis D virus (HDV) antigen or reducing the viral load of HDV in a subject infected with HDV, wherein the agent or the composition is to be administered to the subject, thereby the level of the HDV antigen in the subject is reduced, wherein the HDV antigen is optionally S-HDAg or L-HDAg.

The disclosure also provides methods of reducing the viral load of Hepatitis D virus (HDV) in a subject infected with HDV. The methods include administering to the subject a therapeutically effective amount of a double stranded RNAi agent, composition, vector, or pharmaceutical composition provided herein, thereby reducing the viral load of HDV in the subject.

The disclosure also provides methods of treating a subject having a Hepatitis D virus (HDV) infection, comprising administering to the subject a therapeutically effective amount of a double stranded RNAi agent, composition, vector, or pharmaceutical composition provided herein, thereby treating the subject.

In certain examples, the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region. Sense strand and antisense strands can be selected from the following RNAi agents wherein, the sense strand comprises 5'-UCGUGGUGGACUUCUCUCA -3' (SEQ ID NO:5), and the antisense strand comprises 5'-UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6); the sense strand comprises 5'-GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7), and the antisense strand comprises 5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8); the sense strand comprises 5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9), and the antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10); the sense strand comprises 5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37), and the antisense strand comprises 5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38); the sense strand comprises 5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11), and the antisense strand comprises 5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12); or the sense strand comprises 5'- GUGUGCACUUCGCUUCACA -3' (SEQ ID NO:39), and the antisense strand comprises 5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40), wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, wherein the sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, thereby treating the subject.

In certain examples, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification. In certain

examples, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate,

non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic. In certain examples, the 5'-phosphate mimic is a 5'-vinyl phosphate (5'-VP).

In certain examples, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13) and the antisense strand comprises 5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In certain examples, the sense strand comprises 5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15) and the antisense strand comprises 5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17) and the antisense strand comprises 5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In certain examples, the sense strand comprises 5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19) and the antisense strand comprises 5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2'-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21) and the antisense strand comprises 5'-AfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are
2'-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In certain examples, the sense strand comprises 5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23) and the antisense strand comprises 5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2'-O-methyl (2'-OMe) A, U, C, or G; Af, Cf, Gf or Uf are

2′-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, the sense strand comprises

5'-csgsuggudGgucdTucucuaaauu – 3' (SEQ ID NO:35) and the antisense strand comprises

5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; dA, dC, dG, and dT are deoxyribose A, C, G, and T; and s is a phosphorothioate linkage.

In certain examples, the sense strand comprises

5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25) and the antisense strand comprises

5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are

2′-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In certain examples, the sense strand comprises

5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27) and the antisense strand comprises

5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28) , wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are

2′-fluoro A, G, C or U; and s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

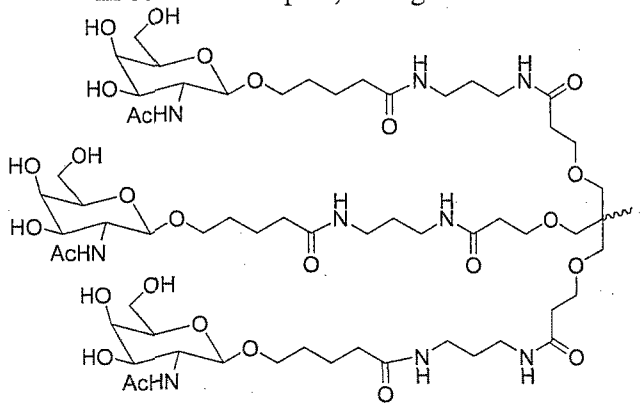
In certain examples, sense strand comprises

5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41) and the antisense strand comprises

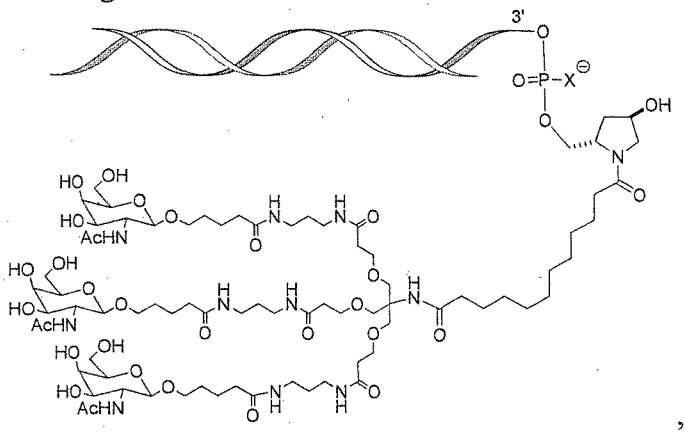
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are

2′-fluoro A, G, C or U; and s is a phosphorothioate linkage.

In certain examples, the ligand is



In certain examples, the RNAi agent is conjugated to the ligand as shown in the following schematic



, wherein X is O or S.

The disclosure provides methods of treating a subject having a Hepatitis D virus (HDV) infection. The methods include administering to the subject a therapeutically effective amount of a composition for inhibiting expression of hepatitis B virus (HBV) in a cell, the composition comprising (a) a first double-stranded RNAi agent comprising a first sense strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of the first sense strand and substantially all of the nucleotides of the first antisense strand are modified nucleotides, wherein the first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and (b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of the second sense strand and substantially all of the nucleotides of the second antisense strand are modified nucleotides, wherein the second sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first and second sense strands each independently comprise a sequence selected from the group consisting of

5'- UCGUGGUGGACUUCUCUCA -3' (SEQ ID NO:5),

5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7),

5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ ID NO:9),

5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37),

5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11), and

5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39), and wherein the first and second antisense strands each independently comprise a sequence selected from the group consisting of

5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);

5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);

5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);

5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),
5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and
5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40), thereby treating the subject.

In certain examples, all of the nucleotides of the first and second sense strand and all of the nucleotides of the first and second antisense strand comprise a modification. In certain examples, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In certain examples, the first and second RNAi agent are selected from the group:
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:13)
5'-usGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:14);
5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)
5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:17)
5'-usAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:18);
5'-gsusgcacUfuCfGfCfuucaccucua – 3' (SEQ ID NO:19)
5'-PusAfsgagGfugaagcgAfaGfugcacsusu – 3' (SEQ ID NO:20);
5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)
5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22);
5'- csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:23)
5'-PasAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:24);
5'-csgsuggudGgucdTucucuaaaauu – 3' (SEQ ID NO:35)
5'- asdAsuugagagdAagudCcaccagcsusu – 3' (SEQ ID NO:36);
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)
5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26);
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:27)
5'- PusAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:28); and
5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)

5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; dA, dC, dG, and dT are deoxyribose A, C, G, and T; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, the first and second RNAi agents are

5'-uscsguGfgUfGfGfacuucucuca – 3' (SEQ ID NO:15)

5'-PusGfsagaGfaAfGfuccaCfcAfcgasusu – 3' (SEQ ID NO:16); and

5'-csgsugguGfgAfCfUfucucUfCfaauu – 3' (SEQ ID NO:21)

5'-asAfsuugAfgAfgAfaguCfcAfccagcsasg – 3' (SEQ ID NO:22), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, the first and second RNAi agents are

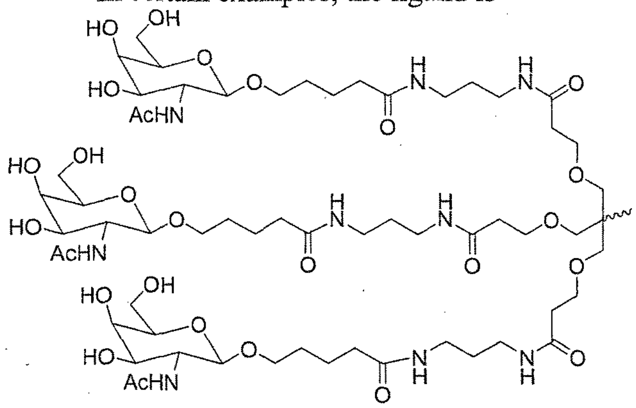
5'- gsgsuggaCfuUfCfUfcucaAfUfuuua – 3' (SEQ ID NO:25)

5'- usAfsaaaUfuGfAfgagaAfgUfccaccsasc – 3' (SEQ ID NO:26); and

5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41)

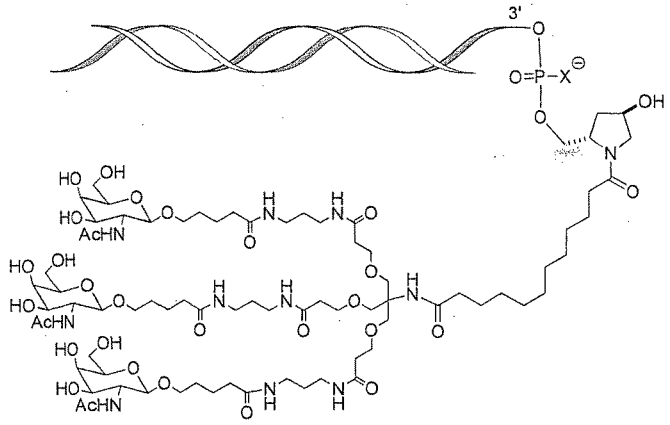
5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42), wherein A, C, G, and U are ribose A, C, G or U; a, g, c and u are 2′-O-methyl (2′-OMe) A, U, C, or G; Af, Cf, Gf or Uf are 2′-fluoro A, G, C or U; s is a phosphorothioate linkage; and P is a 5'-phosphate or 5'phosphate mimic.

In certain examples, the ligand is



.

In certain examples, the RNAi agent is conjugated to the ligand as shown in the following schematic



, wherein X is O or S.

In certain examples, the subject is a human.

In certain examples, the double stranded RNAi agent is administered at a dose of about 0.01 mg/kg to about 10 mg/kg or about 0.5 mg/kg to about 50 mg/kg. In certain examples, the double stranded RNAi agent is administered at a dose of about 10 mg/kg to about 30 mg/kg. In certain examples, the double stranded RNAi agent is administered at a dose of about 3 mg/kg. In certain examples, the double stranded RNAi agent is administered at a dose of about 10 mg/kg. In certain examples, the double stranded RNAi agent is administered at a dose of about 0.5 mg/kg twice per week. In certain examples, the double stranded RNAi agent is administered at a fixed dose of about 50 mg to 200 mg.

In certain examples, the double stranded RNAi agent is administered subcutaneously. In certain examples, the double stranded RNAi agent is administered intravenously.

In certain examples, the RNAi agent is administered in two or more doses. In certain examples, the RNAi agent is administered at intervals selected from the group consisting of once every about 12 hours, once every about 24 hours, once every about 48 hours, once every about 72 hours, and once every about 96 hours. In certain examples, the RNAi agent is administered twice per week. In certain examples, the RNAi agent is administered every other week. In certain examples, the RNAi agent is administered once per month. In certain examples, the RNAi agent is administered once every other month. In certain examples, the RNAi agent is administered once every three months.

In certain examples, the RNAi agent is administered to the subject with an additional therapeutic agent. Additional therapeutic agents include, for example, an antiviral agent, a reverse transcriptase inhibitor, an immune stimulator, a therapeutic vaccine, a viral entry inhibitor, an oligonucleotide that inhibits the secretion or release of HbsAg, a capsid inhibitor, a covalently closed circular (ccc) HBV DNA inhibitor, and a combination of any of the foregoing.

In certain examples, the additional agent is a reverse transcriptase inhibitor. In certain examples, the additional agent is a reverse transcriptase inhibitor and an immune stimulator. Exemplary reverse transcriptase inhibitors include Tenofovir disoproxil fumarate (TDF), Tenofovir alafenamide, Lamivudine, Adefovir dipivoxil, Entecavir (ETV), Telbivudine, and AGX-1009. Exemplary immune stimulators include pegylated interferon alfa 2a (PEG-IFN-α2a), Interferon alfa-2b, a recombinant human interleukin-7, and a Toll-like receptor 7 (TLR7) agonist.

The disclosure further provides methods of treating a subject having a Hepatitis D virus (HDV) infection, comprising administering to the subject a therapeutically effective amount of a composition for inhibiting expression of hepatitis B virus (HBV) in a cell, the composition comprising (a) a first double-stranded RNAi agent comprising a first strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of the first sense strand and substantially all of the nucleotides of the first antisense strand are modified nucleotides, wherein the first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and (b) a second double-stranded RNAi agent comprising a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of the second sense strand and substantially all of the nucleotides of the second antisense strand are modified nucleotides, wherein the second sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1, and the first antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein the sense second strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:29, and the second antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:30, thereby treating the subject.

In certain examples, the first sense strand comprises a sequence selected from the group consisting of

5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),

5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ ID NO:7),

5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ ID NO:9),

5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ ID NO:37),

5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ ID NO:11), and

5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39), and

the second antisense strand comprises a sequence selected from the group consisting of

5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);

5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);

5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);

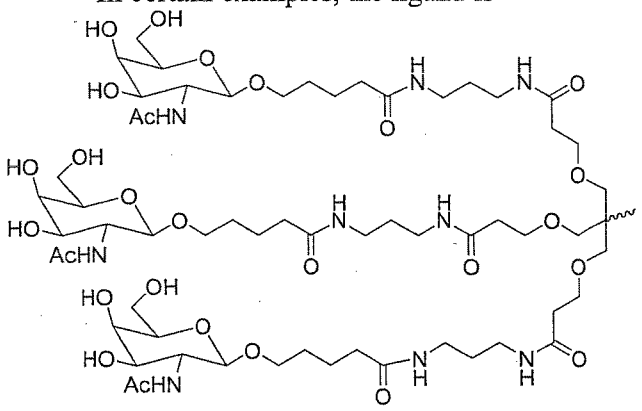
5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),

5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and

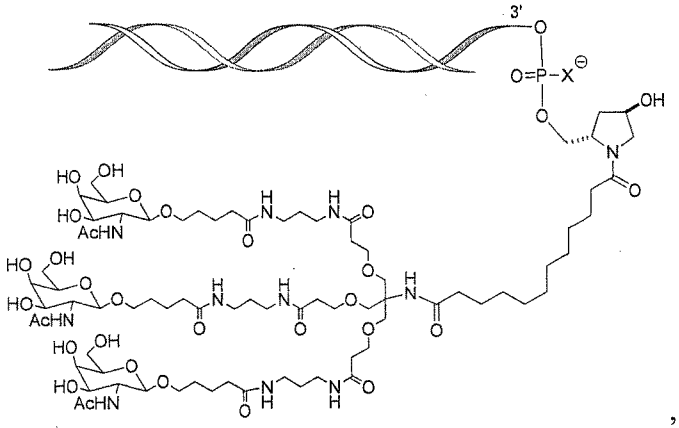
5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40).

In certain examples, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification. In certain examples, the additional agent is at least one of the modified nucleotides is selected from the group consisting of a dexoy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In certain examples, the ligand is



In certain examples, the RNAi agent is conjugated to the ligand as shown in the following schematic



, wherein X is O or S.

The present invention is further illustrated by the following detailed description and drawings.

Brief Description of the Drawings

Figure 1 schematically depicts the structure of the approximately 3.2 kb double-stranded HBV genome. Replication of the HBV genome occurs through an RNA intermediate and produces 4 overlapping viral transcripts (an about 3.5 kb transcript, an about 2.4 kb transcript, an about 2.1 kb transcript, and an about 0.7 kb transcript) encoding seven viral proteins (pre-S1, pre-S2, S, P, X, pre-C and C) translated across three reading frames.

Figure 2 is a graph depicting the log decrease of HBsAg serum levels normalized to pre-dose HBsAg serum levels following administration of a single 3 mg/kg dose of the indicated iRNA agents.

Figure 3 is a graph depicting the log decrease of HBsAg serum levels normalized to pre-dose HBsAg serum levels following administration of a single 3 mg/kg dose of the indicated iRNA agents.

Figure 4 is a graph depicting the percent of pre-dose HBsAg remaining at days 5 and 10 following administration of a single 3 mg/kg dose of the indicated iRNA agents. Figure 4 also depicts the percent of HBsAG remaining at day 10 post-dose relative to the percent of HBsAG remaining at day 10 post-dose in an animal administered 3 mg/kg of a control dsRNA targeting mouse/rat transtherytin (mrTTR).

Figure 5 is a graph depicting the log decrease of HBsAg serum levels normalized to pre-dose HBsAg serum levels following administration of a single 3 mg/kg dose of AD-65403.

Figure 6A is a graph depicting the decrease of HBsAg serum levels normalized to pre-dose HBsAg serum levels on a standard linear scale following administration of a single subcutaneous 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 9 mg/kg dose of AD-66810.

Figure 6B is a graph depicting the decrease of HBsAg serum levels normalized to pre-dose HBsAg serum levels on a $\log_{10}$ scale following administration of a single subcutaneous 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 9 mg/kg dose of AD-66810.

Figure 7 is a graph depicting the decrease of HBsAg plasma levels normalized to pre-dose HBsAg plasma levels on a $\log_{10}$ scale following administration of three weekly subcutaneous 3 mg/kg doses of AD-66810.

Detailed Description of the Invention

The present invention provides iRNA compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a Hepatitis B virus (HBV) gene. The gene may be within a cell, *e.g.*, a cell within a subject, such as a human. The use of these iRNAs enables the targeted degradation of mRNAs of the correponding gene (HBV gene) in mammals.

The RNAi agents of the invention have been designed to target regions in the HBV genome that are conserved across all 8 serotypes of HBV. In addition, the RNAi agents of the invention have been designed to inhibit all steps of the HBV life cycle, *e.g.*, replication, assembly, secretion of virus, and secretion of sub-viral antigens, by inhibiting expression of more than one HBV gene. In particular, since transcription of the HBV genome results in polycistronic, overlapping RNAs, an RNAi agent of the invention targeting a single HBV gene results in significant inhibition of expression of most or all HBV transcripts. For example, because the HBV genome is transcribed into a single mRNA, an RNAi agent of the invention targeting the S gene will result in inhibition of not only S gene expression but also the expression of the "downstream" polymerase gene. Furthermore, the RNAi agents of the invention have been designed to inhibit HBV viral replication by targeting HBV structural genes, and the HBV X gene thereby permiting a subject's immune sytem to detect and respond to the presence of HBsAg to produce anti-HBV antibodies to clear an HBV infection. Without intending to be limited by theory, it is believed that a combination or sub-combination of the foregoing properties and the specific target sites and/or the specific modifications in these RNAi agents confer to the RNAi agents of the invention improved efficacy, stability, safety, potency, and durability.

Using *in vitro* and *in vivo* assays, the present inventors have demonstrated that iRNAs targeting an HBV gene can potently mediate RNAi, resulting in significant inhibition of expression of more than one HBVgene. The present inventors have also demonstrated that the RNAi agents of the invention are exceptionally stable in the cytoplasm and lysosme. Thus, methods and compositions including these iRNAs are useful for treating a subject having an HBV infection and/or an HBV-associated disease, such as chronic hepatitis B (CHB).

Accordingly, the present invention also provides methods for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of an HBV gene, *e.g.*, an HBV-associated disease, such as chronic Hepatitis B virus infection (CHB), using iRNA compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of an HBV gene.

Very low dosages of the iRNAs of the invention, in particular, can specifically and efficiently mediate RNA interference (RNAi), resulting in significant inhibition of expression of the correponding gene (HBV gene).

The iRNAs of the invention include an RNA strand (the antisense strand) having a region which is about 30 nucleotides or less in length, *e.g.*, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of an HBV gene.

The following detailed description discloses how to make and use compositions containing iRNAs to inhibit the expression of an HBV gene as well as compositions, uses, and methods for treating subjects having diseases and disorders that would benefit from inhibition and/or reduction of the expression of an HBV gene.

I. Definitions

In order that the present invention may be more readily understood, certain terms are first defined. In addition, it should be noted that whenever a value or range of values of a parameter are recited, it is intended that values and ranges intermediate to the recited values are also intended to be part of this invention.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element, *e.g.*, a plurality of elements.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

As used herein, "Hepatitis B virus," used interchangeably with the term "HBV" refers to the well-known noncytopathic, liver-tropic DNA virus belonging to the Hepadnaviridae family.

The HBV genome is partially double-stranded, circular DNA with overlapping reading frames (see, *e.g.*, Figure 1).

There are four known genes encoded by the HBC genome, called C, X, P, and S. The core protein is coded for by gene C (HBcAg). Hepatitis B antigen (HBeAg) is produced by proteolytic processing of the pre-core (pre-C) protein. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the surface antigen (HBsAg). The HBsAg gene is one long open reading frame but contains three in frame "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small (pre-S1 + pre-S2 + S, pre-S2 + S, or S) are produced. The function of the non-structural protein coded for by gene X is not fully understood but it is associated with the development of liver cancer and encodes a decoy protein which permits HBsAg in the blood to sequester anti-HBsAg antibodies and allow infectious viral particles to escape immune detection.

The proteins encoded by the HBV genome include: envelope proteins - i) small, Hepatitis B surface antigen (HBsAg); ii) middle - preS2 plus HBsAg; iii) large - preSl plus preS2 plus HBsAg; nucleocapsid protein, hepatitis B core antigen (HBcAg). Hepatitis B e antigen (HBeAg) is a non-structural protein produced during the HBV replication which shares 90% amino acids with the nucleocapsid HBcAg; and the X protein is a nonstructural protein (HBx) which functions in the cytoplasm to activate various signaling pathways, many of which are controlled by modulation of cytosolic calcium and in the nucleus to regulate transcription through a direct interaction with different transcription factors and, in some cases, enhance their binding to specific transcription elements.

HBV is one of the few DNA viruses that utilize reverse transcriptase in the replication process which involves multiple stages including entry, uncoating and transport of the virus genome to the nucleus. Initially, replication of the HBV genome involves the generation of an RNA intermediate that is then reverse transcribed to produce the DNA viral genome.

Upon infection of a cell with HBV, the viral genomic relaxed circular DNA (rcDNA) is transported into the cell nucleus and converted into episomal covalently closed circular DNA (cccDNA), which serves as the transcription template for the viral mRNAs. After transcription and nuclear export, cytoplasmic viral pregenomic RNA (pgRNA) is assembled with HBV polymerase and capsid proteins to form the nucleocapsid, inside which polymerase-catalyzed reverse transcription yields minus-strand DNA, which is subsequently copied into plus-strand DNA to form the progeny rcDNA genome. The mature nucleocapsids are then either packaged with viral envelope proteins to egress as virion particles or shuttled to the nucleus to amplify the cccDNA reservoir through the intracellular cccDNA amplification pathway. cccDNA is an

essential component of the HBV replication cycle and is responsible for the establishment of infection and viral persistence.

HBV infection results in the production of two different particles: 1) the HBV virus itself (or Dane particle) which includes a viral capsid assembled from the HBcAg and is covered by the HBsAg and is capable of reinfecting cells and 2) subviral particles (or SVPs) which are high density lipoprotein-like particles comprised of lipids, cholesterol, cholesterol esters and the small and medium forms of the hepatitis B surface antigen HBsAg which are non-infectious. For each viral particle produced, 1,000-10,000 SVPs are released into the blood. As such SVPs (and the HBsAg protein they carry) represent the overwhelming majority of viral protein in the blood. HBV infected cells also secrete a soluble proteolytic product of the pre-core protein called the HBV e-antigen (HBeAg).

Eight genotypes of HBV, designated A to H, have been determined, each having a distinct geographical distribution. The virus is non-cytopathic, with virus-specific cellular immunity being the main determinant for the outcome of exposure to HBV - acute infection with resolution of liver diseases with 6 months, or chronic HBV infection that is frequently associated with progressive liver injury.

The term "HBV" includes any of the eight genotypes of HBV (A to H). The amino acid and complete coding sequence of the reference sequence of the HBV genome may be found in for example, GenBank Accession Nos. GI:21326584 (SEQ ID NO:1) and GI:3582357 (SEQ ID NO:3).

Additional examples of HBV mRNA sequences are readily available using publicly available databases, *e.g.*, GenBank, UniProt, and OMIM.

The term"HBV," as used herein, also refers to naturally occurring DNA sequence variations of the HBV genome.

As used herein, "Hepatitis D virus," used interchangeably with the term "HDV" refers to the well-known noncytopathic, liver-tropic DNA virus belonging to the Hepadnaviridae family. See, e.g., Ciancio and Rizzetto, Nat. Rev. 11:68-71, 2014; Le Gal et al., Emerg. Infect. Dis. 12:1447-1450, 2006; and Abbas and afzal, World J. Hep., 5:666-675, 2013, all of which are incorporated by reference. Unless otherwise indicated, HDV refers to all clades and variants of HDV.

HDV produces one protein, namely HDAg. It comes in two forms; a 27kDa large-HDAg (also referred to herein as lHD, L-HDAg, and large HDV antigen), and a small-HDAg of 24kDa (also referred to herein as sHD, S-HDAg, and small HDV antigen). The N-terminals of the two forms are identical, they differ by 19 amino acids in the C-terminal of the large HDAg. Both isoforms are produced from the same reading frame which contains an UAG stop codon at codon 196, which normally produces only the small-HDAg. However, editing by cellular enzyme adenosine deaminase-1 changes the stop codon to UCG, allowing the large-HDAg to be produced. Despite having 90% identical sequences, these two proteins

play diverging roles during the course of an infection. HDAg-S is produced in the early stages of an infection and enters the nucleus and supports viral replication. HDAg-L, in contrast, is produced during the later stages of an infection, acts as an inhibitor of viral replication, and is required for assembly of viral particles.

Additional examples of HDV mRNA sequences are readily available using publicly available databases, *e.g.*, GenBank, UniProt, and OMIM.

The term"HDV," as used herein, also refers to naturally occurring DNA sequence variations of the HDV genome.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of an HBV gene, including mRNA that is a product of RNA processing of a primary transcription product. In one embodiment, the target portion of the sequence will be at least long enough to serve as a substrate for iRNA-directed cleavage at or near that portion of the nucleotide sequence of an mRNA molecule formed during the transcription of an HBV gene.

The target sequence may be from about 9-36 nucleotides in length, *e.g.*, about 15-30 nucleotides in length. For example, the target sequence can be from about 15-30 nucleotides, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

As used herein, the term "strand comprising a sequence" refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

"G," "C," "A," "T" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine and uracil as a base, respectively. However, it will be understood that the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety (see, *e.g.,* Table 2). The skilled person is well aware that guanine, cytosine, adenine, and uracil can be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured in the invention by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form

G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the invention.

The terms "iRNA", "RNAi agent," "iRNA agent,", "RNA interference agent" as used interchangeably herein, refer to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript *via* an RNA-induced silencing complex (RISC) pathway. iRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The iRNA modulates, *e.g.,* inhibits, the expression of an HBV gene (*e.g.,* one or more HBV genes) in a cell, *e.g.,* a cell within a subject, such as a mammalian subject.

In one embodiment, an RNAi agent of the invention includes a single stranded RNA that interacts with a target RNA sequence, *e.g.,* an HBV target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory it is believed that long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev.* 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) *Nature* 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) *Cell* 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, *et al.*, (2001) *Genes Dev.* 15:188). Thus, in one aspect the invention relates to a single stranded siRNA (ssRNA) generated within a cell and which promotes the formation of a RISC complex to effect silencing of the target gene, *i.e.,* an HBV gene. Accordingly, the term "siRNA" is also used herein to refer to an RNAi as described above.

In another embodiment, the RNAi agent may be a single-stranded siRNA that is introduced into a cell or organism to inhibit a target mRNA. Single-stranded RNAi agents bind to the RISC endonuclease, Argonaute 2, which then cleaves the target mRNA. The single-stranded siRNAs are generally 15-30 nucleotides and are chemically modified. The design and testing of single-stranded siRNAs are described in U.S. Patent No. 8,101,348 and in Lima *et al.*, (2012) *Cell* 150:883-894, the entire contents of each of which are hereby incorporated herein by reference. Any of the antisense nucleotide sequences described herein may be used as a single-stranded siRNA as described herein or as chemically modified by the methods described in Lima *et al.*, (2012) *Cell* 150:883-894.

In another embodiment, an "iRNA" for use in the compositions, uses, and methods of the invention is a double-stranded RNA and is referred to herein as a "double stranded RNAi agent," "double-stranded RNA (dsRNA) molecule," "dsRNA agent," or "dsRNA". The term "dsRNA", refers to a complex of ribonucleic acid molecules, having a duplex structure comprising two anti-parallel and substantially complementary nucleic acid strands, referred

to as having "sense" and "antisense" orientations with respect to a target RNA, *i.e.,* an HBV gene. In some embodiments of the invention, a double-stranded RNA (dsRNA) triggers the degradation of a target RNA, *e.g.,* an mRNA, through a post-transcriptional gene-silencing mechanism referred to herein as RNA interference or RNAi.

In general, the majority of nucleotides of each strand of a dsRNA molecule are ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, an "RNAi agent" may include ribonucleotides with chemical modifications; an RNAi agent may include substantial modifications at multiple nucleotides. As used herein, the term "modified nucleotide" refers to a nucleotide having, independently, a modified sugar moiety, a modified internucleotide linkage, and/or modified nucleobase. Thus, the term modified nucleotide encompasses substitutions, additions or removal of, *e.g.,* a functional group or atom, to internucleoside linkages, sugar moieties, or nucleobases. The modifications suitable for use in the agents of the invention include all types of modifications disclosed herein or known in the art. Any such modifications, as used in a siRNA type molecule, are encompassed by "RNAi agent" for the purposes of this specification and claims.

The duplex region may be of any length that permits specific degradation of a desired target RNA through a RISC pathway, and may range from about 9 to 36 base pairs in length, *e.g.*, about 15-30 base pairs in length, for example, about 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, or 36 base pairs in length, such as about 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting RNA chain is referred to as a "hairpin loop." A hairpin loop can comprise at least one unpaired nucleotide. In some embodiments, the hairpin loop can comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides.

Where the two substantially complementary strands of a dsRNA are comprised by separate RNA molecules, those molecules need not, but can be covalently connected. Where the two strands are connected covalently by means other than an uninterrupted chain of

nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, an RNAi may comprise one or more nucleotide overhangs.

In one embodiment, an RNAi agent of the invention is a dsRNA, each strand of which comprises 24-30 nucleotides, that interacts with a target RNA sequence, *e.g.*, an HBV target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev.* 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) *Nature* 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) *Cell* 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, *et al.*, (2001) *Genes Dev.* 15:188).

As used herein, the term "nucleotide overhang" refers to at least one unpaired nucleotide that protrudes from the duplex structure of an iRNA, *e.g.*, a dsRNA. For example, when a 3'-end of one strand of a dsRNA extends beyond the 5'-end of the other strand, or *vice versa*, there is a nucleotide overhang. A dsRNA can comprise an overhang of at least one nucleotide; alternatively the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end or both ends of either an antisense or sense strand of a dsRNA.

In one embodiment, the antisense strand of a dsRNA has a 1-10 nucleotide, *e.g.,* a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide, overhang at the 3'-end and/or the 5'-end. In one embodiment, the sense strand of a dsRNA has a 1-10 nucleotide, *e.g.,* a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide, overhang at the 3'-end and/or the 5'-end. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

"Blunt" or "blunt end" means that there are no unpaired nucleotides at that end of the double stranded RNAi agent, *i.e.*, no nucleotide overhang. A "blunt ended" RNAi agent is a dsRNA that is double-stranded over its entire length, *i.e.*, no nucleotide overhang at either end of the molecule. The RNAi agents of the invention include RNAi agents with nucleotide

overhangs at one end (*i.e.*, agents with one overhang and one blunt end) or with nucleotide overhangs at both ends.

The term "antisense strand" or "guide strand" refers to the strand of an iRNA, *e.g.*, a dsRNA, which includes a region that is substantially complementary to a target sequence, *e.g.*, a HBV mRNA. As used herein, the term "region of complementarity" refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, *e.g.*, an HBV nucleotide sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches can be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, *e.g.*, within 5, 4, 3, 2, or 1 nucleotides of the 5'- and/or 3'-terminus of the iRNA. In one embodiment, a double-stranded RNAi agent of the invention includea a nucleotide mismatch in the antisense strand. In another embodiment, a double-stranded RNAi agent of the invention includea a nucleotide mismatch in the sense strand. In one embodiment, the nucleotide mismatch is, for example, within 5, 4, 3, 2, or 1 nucleotides from the 3'-terminus of the iRNA. In another embodiment, the nucleotide mismatch is, for example, in the 3'-terminal nucleotide of the iRNA.

The term "sense strand," or "passenger strand" as used herein, refers to the strand of an iRNA that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

As used herein, the term "cleavage region" refers to a region that is located immediately adjacent to the cleavage site. The cleavage site is the site on the target at which cleavage occurs. In some embodiments, the cleavage region comprises three bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage region comprises two bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage site specifically occurs at the site bound by nucleotides 10 and 11 of the antisense strand, and the cleavage region comprises nucleotides 11, 12 and 13.

As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing (see, *e.g.*, "Molecular Cloning: A Laboratory Manual, Sambrook, *et al.* (1989) Cold Spring Harbor Laboratory Press). Other conditions, such as physiologically relevant conditions as can be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of

complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

Complementary sequences within an iRNA, *e.g.*, within a dsRNA as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as "fully complementary" with respect to each other herein. However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3 or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs, while retaining the ability to hybridize under the conditions most relevant to their ultimate application, *e.g.*, inhibition of gene expression via a RISC pathway. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, can yet be referred to as "fully complementary" for the purposes described herein.

"Complementary" sequences, as used herein, can also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in so far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs include, but are not limited to, G:U Wobble or Hoogstein base pairing.

The terms "complementary," "fully complementary" and "substantially complementary" herein can be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of an iRNA agent and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide that is "substantially complementary to at least part of" a messenger RNA (mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding an HBV gene). For example, a polynucleotide is complementary to at least a part of an HBV mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding an HBV gene.

Accordingly, in some embodiments, the antisense strand polynucleotides disclosed herein are fully complementary to the target HBV sequence. In other embodiments, the antisense strand polynucleotides disclosed herein are substantially complementary to the target HBV sequence and comprise a contiguous nucleotide sequence which is at least about

80% complementary over its entire length to the equivalent region of the nucleotide sequence of SEQ ID NO:1, or a fragment of SEQ ID NO:1, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about % 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In one embodiment, an RNAi agent of the invention includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is complementary to a target HBV sequence, and wherein the sense strand polynucleotide comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:6, 8, 10, 12, 38, and 40, or a fragment of any one of SEQ ID NOs:6, 8, 10, 12, 38, and 40, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about % 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary. In another embodiment, an RNAi agent of the invention includes an antisense strand that is substantially complementary to the target HBV sequence and comprise a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:5, 7, 9, 11, 37, and 39, or a fragment of any one of SEQ ID NOs:5, 7, 9, 11, 37, and 39, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about % 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide and/or a modified nucleotide. In addition, an "iRNA" may include ribonucleotides with chemical modifications. Such modifications may include all types of modifications disclosed herein or known in the art. Any such modifications, as used in an iRNA molecule, are encompassed by "iRNA" for the purposes of this specification and claims.

In one aspect of the invention, an agent for use in the methods and compositions of the invention is a single-stranded antisense nucleic acid molecule that inhibits a target mRNA *via* an antisense inhibition mechanism. The single-stranded antisense RNA molecule is complementary to a sequence within the target mRNA. The single-stranded antisense oligonucleotides can inhibit translation in a stoichiometric manner by base pairing to the mRNA and physically obstructing the translation machinery, see Dias, N. *et al.*, (2002) *Mol Cancer Ther* 1:347-355. The single-stranded antisense RNA molecule may be about 15 to about 30 nucleotides in length and have a sequence that is complementary to a target sequence. For example, the single-stranded antisense RNA molecule may comprise a sequence that is at least about 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from any one of the antisense sequences described herein.

As used herein, a "subject" is an animal, such as a mammal, including a primate (such as a human, a non-human primate, *e.g.*, a monkey, and a chimpanzee), a non-primate (such as a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, a horse, and a whale), or a bird (*e.g.*, a duck or a goose). In an embodiment, the subject is a human, such as a human being treated or assessed for a disease, disorder or condition that would benefit from reduction in HBV gene expression and/or replication; a human at risk for a disease, disorder or condition that would benefit from reduction in HBV gene expression and/or replication; a human having a disease, disorder or condition that would benefit from reduction in HBV gene expression and/or replication; and/or human being treated for a disease, disorder or condition that would benefit from reduction in HBV gene expression and/or replication, as described herein. In another embodiment, the subject has a hepatitis B virus (HBV) infection. In another embodiment, the subject has both a hepatitis B virus (HBV) infection and a hepatitis D virus (HDV) infection.

As used herein, the terms "treating" or "treatment" refer to a beneficial or desired result including, but not limited to, alleviation or amelioration of one or more symptoms associated with unwanted HBV gene expression and/or HBV replication, *e.g.,* the presence of serum and/or liver HBV ccc DNA, the presence of serum and/or liver HBV antigen, *e.g.*, HBsAg and/or HBeAg, elevated ALT, elevated AST, the absence or low level of anti-HBV antibodies, liver injury; cirrhosis; delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma; serum sickness–like syndrome; anorexia; nausea; vomiting, low-grade fever; myalgia; fatigability; disordered gustatory acuity and smell sensations (aversion to food and cigarettes); and/or right upper quadrant and epigastric pain (intermittent, mild to moderate); hepatic encephalopathy; somnolence; disturbances in sleep pattern; mental confusion; coma; ascites; gastrointestinal bleeding; coagulopathy; jaundice; hepatomegaly (mildly enlarged, soft liver); splenomegaly; palmar erythema; spider nevi; muscle wasting; spider angiomas; vasculitis; variceal bleeding; peripheral edema; gynecomastia; testicular atrophy; abdominal collateral veins (caput medusa); high levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), within a range of 1000-2000 IU/mL, although values 100 times above the upper limit of normal (ULN) can be also be identified; ALT levels higher than AST levels; elevated gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) levels (*e.g.,* not more than 3 times the ULN); slightly low albumin levels; elevated serum iron levels; leukopenia (*i.e.,* granulocytopenia); lymphocytosis; increased erythrocyte sedimentation rate (ESR); shortened red blood cell survival; hemolysis; thrombocytopenia; a prolongation of the international normalized ratio (INR); the presence of serum and/ or liverHBsAg, HBeAg, Hepatitis B core antibody (anti-HBc) immunoglobulin M (IgM); hepatitis B surface antibody (anti-HBs), hepatitis B e antibody (anti-HBe), and/or HBV DNA; elevation of the aminotransferases (≤5 times the ULN); ALT levels higher than the

AST levels; increased bilirubin levels, prolonged prothrombin time(PT); hyperglobulinemia; the presence of tissue-nonspecific antibodies, such as anti–smooth muscle antibodies (ASMAs) or antinuclear antibodies (ANAs) (10-20%); the presence of tissue-specific antibodies, such as antibodies against the thyroid gland (10-20%); elevated levels of rheumatoid factor (RF); hyperbilirubinemia, prolonged PT, low platelet and white blood cell counts, AST levels higher than ALT levels; elevated alkaline phosphatase (ALP) and GGT levels; lobular, with degenerative and regenerative hepatocellular changes, and accompanying inflammation; predominantly centrilobular necrosis whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival in the absence of treatment.

The term "lower" in the context of the level of HBV gene expression and/or HBV replication in a subject or a disease marker or symptom refers to a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or more and is preferably down to a level accepted as within the range of normal for an individual without such disorder. In certain embodiments, the expression of the target is normalized, *i.e.*, decreased to a level accepted as within the range of normal for an individual without such disorder, *e.g.*, the level of a disease marker, such as, ALT or AST, is decreased to a level accepted as within the range of normal for an individual without such disorder.

As used herein, "prevention" or "preventing," when used in reference to a disease, disorder or condition thereof, that would benefit from a reduction in expression of an HBV gene and/or replication, refers to a reduction in the likelihood that a subject will develop a symptom associated with such a disease, disorder, or condition, *e.g.,* a symptom of unwanted HBV infection, such as the presence of serum and/or liver HBV ccc DNA, the presence of serum HBV DNA, the presence of serum and/or liver HBV antigen, *e.g.*, HBsAg and/or HBeAg, elevated ALT, elevated AST, the absence or low level of anti-HBV antibodies, a liver injury; cirrhosis; delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma; serum sickness–like syndrome; anorexia; nausea; vomiting, low-grade fever; myalgia; fatigability; disordered gustatory acuity and smell sensations (aversion to food and cigarettes); and/or right upper quadrant and epigastric pain (intermittent, mild to moderate); hepatic encephalopathy; somnolence; disturbances in sleep pattern; mental confusion; coma; ascites; gastrointestinal bleeding; coagulopathy; jaundice; hepatomegaly (mildly enlarged, soft liver); splenomegaly; palmar erythema; spider nevi; muscle wasting; spider angiomas; vasculitis; variceal bleeding; peripheral edema; gynecomastia; testicular atrophy; abdominal collateral veins (caput medusa); high levels of alanine aminotransferase (ALT) and aspartate aminotransferase

(AST), within a range of 1000-2000 IU/mL, although values 100 times above the upper limit of normal (ULN) can be also be identified; ALT levels higher than AST levels; elevated gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) levels (*e.g.,* not more than 3 times the ULN); slightly low albumin levels; elevated serum iron levels; leukopenia (*i.e.,* granulocytopenia); lymphocytosis; increased erythrocyte sedimentation rate (ESR); shortened red blood cell survival; hemolysis; thrombocytopenia; a prolongation of the international normalized ratio (INR); the presence of serum and/ or liverHBsAg, HBeAg, Hepatitis B core antibody (anti-HBc) immunoglobulin M (IgM); hepatitis B surface antibody (anti-HBs), hepatitis B e antibody (anti-HBe), and/or HBV DNA; elevation of the aminotransferases (≤5 times the ULN); ALT levels higher than the AST levels; increased bilirubin levels, prolonged prothrombin time(PT); hyperglobulinemia; the presence of tissue-nonspecific antibodies, such as anti–smooth muscle antibodies (ASMAs) or antinuclear antibodies (ANAs) (10-20%); the presence of tissue-specific antibodies, such as antibodies against the thyroid gland (10-20%); elevated levels of rheumatoid factor (RF); hyperbilirubinemia, prolonged PT, low platelet and white blood cell counts, AST levels higher than ALT levels; elevated alkaline phosphatase (ALP) and GGT levels; lobular, with degenerative and regenerative hepatocellular changes, and accompanying inflammation; predominantly centrilobular necrosis, whether detectable or undetectable. The likelihood of developing, *e.g.,* liver fibrosis, is reduced, for example, when an individual having one or more risk factors for liver fibrosis, *e.g.,* chronic hepatitis B infection, either fails to develop liver fibrosis or develops liver fibrosis with less severity relative to a population having the same risk factors and not receiving treatment as described herein. The failure to develop a disease, disorder or condition, or the reduction in the development of a symptom associated with such a disease, disorder or condition (*e.g.*, by at least about 10% on a clinically accepted scale for that disease or disorder), or the exhibition of delayed symptoms delayed (*e.g.*, by days, weeks, months or years) is considered effective prevention.

As used herein, the term "Hepatitis B virus-associated disease" or "HBV-associated disease," is a disease or disorder that is caused by, or associated with HBV infection and/or replication. The term "HBV-associated disease" includes a disease, disorder or condition that would benefit from reduction in HBV gene expression and/or replication. Non-limiting examples of HBV-associated diseases include, for example, hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma.

In one embodiment, an HBV-associated disease is hepatitis D virus infection. Hepatitis D virus or hepatitis delta virus (HDV) is a human pathogen. However, the virus is defective and depends on obligatory helper functions provided by hepatitis B virus (HBV) for transmission; indeed, HDV requires an associated or pre-existing HBV infection to become infectious and thrive, in particular, the viral envelope containing the surface antigen of

hepatitis B. HDV can lead to severe acute and chronic forms of liver disease in association with HBV. Hepatitis D infection and/or delta hepatitis is highly endemic to several African countries, the Amazonian region, and the Middle East, while its prevalence is low in industrialized countries, except in the Mediterranean.

Transmission of HDV can occur either via simultaneous infection with HBV (coinfection) or superimposed on chronic hepatitis B or hepatitis B carrier state (superinfection). Both superinfection and coinfection with HDV results in more severe complications compared to infection with HBV alone. These complications include a greater likelihood of experiencing liver failure in acute infections and a rapid progression to liver cirrhosis, with an increased chance of developing liver cancer in chronic infections. In combination with hepatitis B virus, hepatitis D has the highest fatality rate of all the hepatitis infections, at 20%.

In one embodiment, an HBV-associated disease is acute hepatitis B. Acute hepatitis B includes inflammation of the liver that lasts less than six months. Typical symptoms of acute hepatitis B are fatigue, anorexia, nausea, and vomiting. Very high aminotransferase values (>1000 U/L) and hyperbilirubinemia are often observed. Severe cases of acute hepatitis B may progress rapidly to acute liver failure, marked by poor hepatic synthetic function. This is often defined as a prothrombin time (PT) of 16 seconds or an international normalized ratio (INR) of 1.5 in the absence of previous liver disease. Acute hepatitis B may evolve into chronic hepatitis B.

In one embodiment, an HBV-associated disease is chronic hepatitis. Chronic hepatitis B (CHB) includes inflammation of the liver that lasts more than six months. Subjects having chronic hepatitis B disease can be immune tolerant or have an inactive chronic infection without any evidence of active disease, and they are also asymptomatic. Patients with chronic active hepatitis, especially during the replicative state, may have symptoms similar to those of acute hepatitis. The persistence of HBV infection in CHB subjects is the result of ccc HBV DNA. In one embodiment, a subject having CHB is HBeAg positive. In another embodiment, a subject having CHB is HBeAg negative. Subjects having CHB have a level of serum HBV DNA of less than about 10^5 and a persistent elevation in transaminases, for examples ALT, AST and gamma-glutamyl transferase. A subject having CHB may have a liver biopsy score of less than about 4 (*e.g.,* a necroinflammatory score). In addition, a subject having CHB may have

In one embodiment, an HBV-associated disease is acute fulminant hepatitis B. A subject having acute fulminant hepatitis B has symptoms of acute hepatitis and the additional symptoms of confusion or coma (due to the liver's failure to detoxify chemicals) and bruising or bleeding (due to a lack of blood clotting factors).

Subjects having an HBV infection, *e.g.,* CHB, may develop liver fibrosis. Accordingly, in one embodiment, an HBV-associated disease is iver fibrosis. Liver fibrosis, or cirrhosis, is defined histologically as a diffuse hepatic process characterized by fibrosis (excess fibrous connective tissue) and the conversion of normal liver architecture into structurally abnormal nodules.

Subjects having an HBV infection, *e.g.,* CHB, may develop end-stage liver disease. Accordingly, in one embodiment, an HBV-associated disease is end-stage liver disease. For example, liver fibrosis may progress to a point where the body may no longer be able to compensate for, *e.g.,* reduced liver function, as a result of liver fibrosis, and result in, *e.g.,* mental and neurological symptoms and liver failure.

Subjects having an HBV infection, *e.g.,* CHB, may develop hepatocellular carcinoma (HCC), also referred to as malignant hepatoma. Accordingly, in one embodiment, an HBV-associated disease isHCC. HCC commonly develops in subjects having CHB and may be fibrolamellar, pseudoglandular (adenoid), pleomorphic (giant cell) or clear cell.

An "HDV-associated disorder" or a Hepatitis D-virus-associated disorder" is a disease or disorder associated with expression of an HDV. Exemplary HDV-associated disorders include, hepatitis B virus infection, acute hepatits B, acute hepatitis D; acute fulminant hepatitis D; chronic hepatitis D; liver fibrosis; end-stage liver disease; and hepatocellular carcinoma.

"Therapeutically effective amount," as used herein, is intended to include the amount of an RNAi agent that, when administered to a patient for treating a subject having an HBV infection and/or HBV-associated disease, is sufficient to effect treatment of the disease (*e.g.,* by diminishing, ameliorating or maintaining the existing disease or one or more symptoms of disease). The "therapeutically effective amount" may vary depending on the RNAi agent, how the agent is administered, the disease and its severity and the history, age, weight, family history, genetic makeup, stage of pathological processes mediated by HBV gene expression, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

"Prophylactically effective amount," as used herein, is intended to include the amount of an RNAi agent that, when administered to a subject who does not yet experience or display symptoms of an HBV infection and/or HBV-associated disease, but who may be predisposed, is sufficient to prevent or ameliorate the disease or one or more symptoms of the disease. Ameliorating the disease includes slowing the course of the disease or reducing the severity of later-developing disease. The "prophylactically effective amount" may vary depending on the RNAi agent, how the agent is administered, the degree of risk of disease, and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

A "therapeutically-effective amount" or "prophylacticaly effective amount" also includes an amount of an RNAi agent that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. RNAi agents employed in the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The term "sample," as used herein, includes a collection of similar fluids, cells, or tissues isolated from a subject, as well as fluids, cells, or tissues present within a subject. Examples of biological fluids include blood, serum and serosal fluids, plasma, cerebrospinal fluid, ocular fluids, lymph, urine, saliva, and the like. Tissue samples may include samples from tissues, organs or localized regions. For example, samples may be derived from particular organs, parts of organs, or fluids or cells within those organs. In certain embodiments, samples may be derived from the liver (*e.g.*, whole liver or certain segments of liver or certain types of cells in the liver, such as, *e.g.*, hepatocytes), the retina or parts of the retina (*e.g.*, retinal pigment epithelium), the central nervous system or parts of the central nervous system (*e.g.*, ventricles or choroid plexus), or the pancreas or certain cells or parts of the pancreas. In some embodiments, a "sample derived from a subject" refers tocerebrospinal fluid obtained from the subject. In preferred embodiments, a "sample derived from a subject" refers to blood or plasma drawn from the subject. In further embodiments, a "sample derived from a subject" refers to liver tissue (or subcomponents thereof) or retinal tissue (or subcomponents thereof) derived from the subject.

II. iRNAs of the Invention

The present invention provides iRNAs which inhibit the expression of one or more HBV genes. In one embodiment, the iRNA agent includes double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of an HBV gene in a cell, such as a cell within a subject, *e.g.,* a mammal, such as a human having an HBV-associated disease, *e.g.,* chronic hepatitis B. The dsRNA includes an antisense strand having a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of an HBV gene. The region of complementarity is about 30 nucleotides or less in length (*e.g.,* about 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 nucleotides or less in length). Upon contact with a cell expressing the HBV gene, the iRNA inhibits the expression of the HBV gene by at least about 10% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by immunofluorescence analysis, using, for example, western blotting or flowcytometric techniques.

A dsRNA includes two RNA strands that are complementary and hybridize to form a duplex structure under conditions in which the dsRNA will be used. One strand of a dsRNA (the antisense strand) includes a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence. The target

sequence can be derived from the sequence of an mRNA formed during the expression of an HBV gene. The other strand (the sense strand) includes a region that is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. As described elsewhere herein and as known in the art, the complementary sequences of a dsRNA can also be contained as self-complementary regions of a single nucleic acid molecule, as opposed to being on separate oligonucleotides.

Generally, the duplex structure is between 15 and 30 base pairs in length, *e.g.*, between, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

Similarly, the region of complementarity to the target sequence is between 15 and 30 nucleotides in length, *e.g.*, between 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

In some embodiments, the dsRNA is between about 15 and about 20 nucleotides in length, or between about 25 and about 30 nucleotides in length. In general, the dsRNA is long enough to serve as a substrate for the Dicer enzyme. For example, it is well-known in the art that dsRNAs longer than about 21-23 nucleotides in length may serve as substrates for Dicer. As the ordinarily skilled person will also recognize, the region of an RNA targeted for cleavage will most often be part of a larger RNA molecule, often an mRNA molecule. Where relevant, a "part" of an mRNA target is a contiguous sequence of an mRNA target of sufficient length to allow it to be a substrate for RNAi-directed cleavage (*i.e.*, cleavage through a RISC pathway).

One of skill in the art will also recognize that the duplex region is a primary functional portion of a dsRNA, *e.g.*, a duplex region of about 9 to 36 base pairs, *e.g.*, about 10-36, 11-36, 12-36, 13-36, 14-36, 15-36, 9-35, 10-35, 11-35, 12-35, 13-35, 14-35, 15-35, 9-34, 10-34, 11-34, 12-34, 13-34, 14-34, 15-34, 9-33, 10-33, 11-33, 12-33, 13-33, 14-33, 15-33, 9-32, 10-32, 11-32, 12-32, 13-32, 14-32, 15-32, 9-31, 10-31, 11-31, 12-31, 13-32, 14-31, 15-31, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27,

20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs. Thus, in one embodiment, to the extent that it becomes processed to a functional duplex, of *e.g.*, 15-30 base pairs, that targets a desired RNA for cleavage, an RNA molecule or complex of RNA molecules having a duplex region greater than 30 base pairs is a dsRNA. Thus, an ordinarily skilled artisan will recognize that in one embodiment, a miRNA is a dsRNA. In another embodiment, a dsRNA is not a naturally occurring miRNA. In another embodiment, an iRNA agent useful to target HBV gene expression is not generated in the target cell by cleavage of a larger dsRNA.

A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs *e.g.*, 1, 2, 3, or 4 nucleotides. dsRNAs having at least one nucleotide overhang can have unexpectedly superior inhibitory properties relative to their blunt-ended counterparts. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end or both ends of either an antisense or sense strand of a dsRNA.

A dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc.

iRNA compounds of the invention may be prepared using a two-step procedure. First, the individual strands of the double-stranded RNA molecule are prepared separately. Then, the component strands are annealed. The individual strands of the siRNA compound can be prepared using solution-phase or solid-phase organic synthesis or both. Organic synthesis offers the advantage that the oligonucleotide strands comprising unnatural or modified nucleotides can be easily prepared. Single-stranded oligonucleotides of the invention can be prepared using solution-phase or solid-phase organic synthesis or both.

In one aspect, a dsRNA of the invention includes at least two nucleotide sequences, a sense sequence and an anti-sense sequence. The sense strand is selected from the group of sequences provided in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26, and the corresponding antisense strand of the sense strand is selected from the group of sequences of any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26. In this aspect, one of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of an HBV gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26, and the second oligonucleotide is described as the corresponding antisense strand of the sense strand in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26. In one embodiment, the substantially complementary sequences of the dsRNA are contained on

separate oligonucleotides. In another embodiment, the substantially complementary sequences of the dsRNA are contained on a single oligonucleotide.

It will be understood that, although some of the sequences in Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 are described as modified and/or conjugated sequences, the RNA of the iRNA of the invention *e.g.*, a dsRNA of the invention, may comprise any one of the sequences set forth in Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 that is un-modified, un-conjugated, and/or modified and/or conjugated differently than described therein.

The skilled person is well aware that dsRNAs having a duplex structure of between about 20 and 23 base pairs, *e.g.*, 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al.*, *EMBO* 2001, 20:6877-6888). However, others have found that shorter or longer RNA duplex structures can also be effective (Chu and Rana (2007) *RNA* 14:1714-1719; Kim *et al.* (2005) *Nat Biotech* 23:222-226). In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26, dsRNAs described herein can include at least one strand of a length of minimally 21 nucleotides. It can be reasonably expected that shorter duplexes having one of the sequences of any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 minus only a few nucleotides on one or both ends can be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides derived from one of the sequences of any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26, and differing in their ability to inhibit the expression of a HBV gene by not more than about 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated to be within the scope of the present invention.

In addition, the RNAs provided in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 identify a site(s) in a HBV transcript that is susceptible to RISC-mediated cleavage. As such, the present invention further features iRNAs that target within one of these sites. As used herein, an iRNA is said to target within a particular site of an RNA transcript if the iRNA promotes cleavage of the transcript anywhere within that particular site. Such an iRNA will generally include at least about 15 contiguous nucleotides from one of the sequences provided in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a HBV gene.

While a target sequence is generally about 15-30 nucleotides in length, there is wide variation in the suitability of particular sequences in this range for directing cleavage of any given target RNA. Various software packages and the guidelines set out herein provide guidance for the identification of optimal target sequences for any given gene target, but an empirical approach can also be taken in which a "window" or "mask" of a given size (as a non-limiting example, 21 nucleotides) is literally or figuratively (including, *e.g.*, in silico)

placed on the target RNA sequence to identify sequences in the size range that can serve as target sequences. By moving the sequence "window" progressively one nucleotide upstream or downstream of an initial target sequence location, the next potential target sequence can be identified, until the complete set of possible sequences is identified for any given target size selected. This process, coupled with systematic synthesis and testing of the identified sequences (using assays as described herein or as known in the art) to identify those sequences that perform optimally can identify those RNA sequences that, when targeted with an iRNA agent, mediate the best inhibition of target gene expression. Thus, while the sequences identified, for example, in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26 represent effective target sequences, it is contemplated that further optimization of inhibition efficiency can be achieved by progressively "walking the window" one nucleotide upstream or downstream of the given sequences to identify sequences with equal or better inhibition characteristics.

Further, it is contemplated that for any sequence identified, *e.g.*, in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26, further optimization could be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those sequences generated by walking a window of the longer or shorter size up or down the target RNA from that point. Again, coupling this approach to generating new candidate targets with testing for effectiveness of iRNAs based on those target sequences in an inhibition assay as known in the art and/or as described herein can lead to further improvements in the efficiency of inhibition. Further still, such optimized sequences can be adjusted by, *e.g.*, the introduction of modified nucleotides as described herein or as known in the art, addition or changes in overhang, or other modifications as known in the art and/or discussed herein to further optimize the molecule (*e.g.*, increasing serum stability or circulating half-life, increasing thermal stability, enhancing transmembrane delivery, targeting to a particular location or cell type, increasing interaction with silencing pathway enzymes, increasing release from endosomes) as an expression inhibitor.

An iRNA as described herein can contain one or more mismatches to the target sequence. In one embodiment, an iRNA as described herein contains no more than 3 mismatches. If the antisense strand of the iRNA contains mismatches to a target sequence, it is preferable that the area of mismatch is not located in the center of the region of complementarity. If the antisense strand of the iRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, for a 23 nucleotide iRNA agent the strand which is complementary to a region of an HBV gene, generally does not contain any mismatch within the central 13 nucleotides. The methods described herein or methods known in the art can be used to determine whether an iRNA containing a mismatch to a target sequence is effective in inhibiting the expression of an

HBV gene. Consideration of the efficacy of iRNAs with mismatches in inhibiting expression of an HBV gene is important, especially if the particular region of complementarity in an HBV gene is known to have polymorphic sequence variation within the population.

III. Modified iRNAs of the Invention

In one embodiment, the RNA of the iRNA of the invention *e.g.*, a dsRNA, is un-modified, and does not comprise, *e.g.*, chemical modifications and/or conjugations known in the art and described herein. In another embodiment, the RNA of an iRNA of the invention, *e.g.*, a dsRNA, is chemically modified to enhance stability or other beneficial characteristics. In certain embodiments of the invention, substantially all of the nucleotides of an iRNA of the invention are modified. In other embodiments of the invention, all of the nucleotides of an iRNA of the invention are modified. iRNAs of the invention in which "substantially all of the nucleotides are modified" are largely but not wholly modified and can include not more than 5, 4, 3, 2, or 1 unmodified nucleotides.

The nucleic acids featured in the invention can be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, end modifications, *e.g.*, 5'-end modifications (phosphorylation, conjugation, inverted linkages) or 3'-end modifications (conjugation, DNA nucleotides, inverted linkages, *etc.*); base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases; sugar modifications (*e.g.*, at the 2'-position or 4'-position) or replacement of the sugar; and/or backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of iRNA compounds useful in the embodiments described herein include, but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In some embodiments, a modified iRNA will have a phosphorus atom in its internucleoside backbone.

Modified RNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and

aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5'-linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6, 239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and US Pat RE39464, the entire contents of each of which are hereby incorporated herein by reference.

Modified RNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, the entire contents of each of which are hereby incorporated herein by reference.

In other embodiments, suitable RNA mimetics are contemplated for use in iRNAs, in which both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of

the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Nos. 5,539,082; 5,714,331; and 5,719,262, the entire contents of each of which are hereby incorporated herein by reference. Additional PNA compounds suitable for use in the iRNAs of the invention are described in, for example, in Nielsen *et al.*, *Science*, 1991, 254, 1497-1500.

Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2-, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O--P--O--CH_2--] of the above-referenced U.S. Patent No. 5,489,677, and the amide backbones of the above-referenced U.S. Patent No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified RNAs can also contain one or more substituted sugar moieties. The iRNAs, *e.g.*, dsRNAs, featured herein can include one of the following at the 2'-position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include O[$(CH_2)_nO$] $_mCH_3$, O$(CH_2)._nOCH_3$, O$(CH_2)_nNH_2$, O(CH_2) $_nCH_3$, O$(CH_2)_nONH_2$, and O$(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an iRNA, or a group for improving the pharmacodynamic properties of an iRNA, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.*, a O$(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--N$(CH_2)_2$.

Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of an iRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. iRNAs can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures

include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application,. The entire contents of each of the foregoing are hereby incorporated herein by reference.

An iRNA can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as deoxy-thymine (dT), 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl anal other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, these disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, dsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., dsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent Nos. 3,687,808, 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610;

7,427,672; and 7,495,088, the entire contents of each of which are hereby incorporated herein by reference.

The RNA of an iRNA can also be modified to include one or more bicyclic sugar moities. A "bicyclic sugar" is a furanosyl ring modified by the bridging of two atoms. A"bicyclic nucleoside" ("BNA") is a nucleoside having a sugar moiety comprising a bridge connecting two carbon atoms of the sugar ring, thereby forming a bicyclic ring system. In certain embodiments, the bridge connects the 4′-carbon and the 2′-carbon of the sugar ring. Thus, in some embodiments an agent of the invention may include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. In other words, an LNA is a nucleotide comprising a bicyclic sugar moiety comprising a 4'-CH2-O-2' bridge. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12):3185-3193). Examples of bicyclic nucleosides for use in the polynucleotides of the invention include without limitation nucleosides comprising a bridge between the 4′ and the 2′ ribosyl ring atoms. In certain embodiments, the antisense polynucleotide agents of the invention include one or more bicyclic nucleosides comprising a 4′ to 2′ bridge. Examples of such 4′ to 2′ bridged bicyclic nucleosides, include but are not limited to 4′-(CH2)—O-2′ (LNA); 4′-(CH2)—S-2′; 4′-(CH2)2—O-2′ (ENA); 4′-CH(CH3)—O-2′ (also referred to as "constrained ethyl" or "cEt") and 4′-CH(CH2OCH3)—O-2′ (and analogs thereof; see, *e.g.*, U.S. Pat. No. 7,399,845); 4′-C(CH3)(CH3)—O-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,283); 4′-CH2—N(OCH3)-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,425); 4′-CH2—O—N(CH3)-2′ (see, e.g.,U.S. Patent Publication No. 2004/0171570); 4′-CH2—N(R)—O-2′, wherein R is H, C1-C12 alkyl, or a protecting group (see, *e.g.*, U.S. Pat. No. 7,427,672); 4′-CH2—C(H)(CH3)-2′ (see, *e.g.*, Chattopadhyaya *et al., J. Org. Chem.*, 2009, 74, 118-134); and 4′-CH2—C(=CH2)-2′ (and analogs thereof; see, *e.g.*, US Patent No. 8,278,426). The entire contents of each of the foregoing are hereby incorporated herein by reference.

Additional representative U.S. Patents and US Patent Publications that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Patent Nos. 6,268,490; 6,525,191; 6,670,461; 6,770,748; 6,794,499; 6,998,484; 7,053,207; 7,034,133;7,084,125; 7,399,845; 7,427,672; 7,569,686; 7,741,457; 8,022,193; 8,030,467; 8,278,425; 8,278,426; 8,278,283; US 2008/0039618; and US 2009/0012281, the entire contents of each of which are hereby incorporated herein by reference.

Any of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α-L-ribofuranose and β-D-ribofuranose (see WO 99/14226).

The RNA of an iRNA can also be modified to include one or more constrained ethyl nucleotides. As used herein, a "constrained ethyl nucleotide" or "cEt" is a locked nucleic acid comprising a bicyclic sugar moiety comprising a 4'-CH(CH3)-0-2' bridge. In one embodiment, a constrained ethyl nucleotide is in the S conformation referred to herein as "S-cEt."

An iRNA of the invention may also include one or more "conformationally restricted nucleotides" ("CRN"). CRN are nucleotide analogs with a linker connecting the C2'and C4' carbons of ribose or the C3 and -C5′ carbons of ribose . CRN lock the ribose ring into a stable conformation and increase the hybridization affinity to mRNA. The linker is of sufficient length to place the oxygen in an optimal position for stability and affinity resulting in less ribose ring puckering.

Representative publications that teach the preparation of certain of the above noted CRN include, but are not limited to, US Patent Publication No. 2013/0190383; and PCT publication WO 2013/036868, the entire contents of each of which are hereby incorporated herein by reference.

One or more of the nucleotides of an iRNA of the invention may also include a hydroxymethyl substituted nucleotide. A "hydroxymethyl substituted nucleotide" is an acyclic 2'-3'-seco-nucleotide, also referred to as an "unlocked nucleic acid" ("UNA") modification.

Representative U.S. publications that teach the preparation of UNA include, but are not limited to, US Patent No. 8,314,227; and US Patent Publication Nos. 2013/0096289; 2013/0011922; and 2011/0313020, the entire contents of each of which are hereby incorporated herein by reference.

Potentially stabilizing modifications to the ends of RNA molecules can include N-(acetylaminocaproyl)-4-hydroxyprolinol (Hyp-C6-NHAc), N-(caproyl-4-hydroxyprolinol (Hyp-C6), N-(acetyl-4-hydroxyprolinol (Hyp-NHAc), thymidine-2'-0-deoxythymidine (ether), N-(aminocaproyl)-4-hydroxyprolinol (Hyp-C6-amino), 2-docosanoyl-uridine-3"-phosphate, inverted base dT(idT) and others. Disclosure of this modification can be found in PCT Publication No. WO 2011/005861.

Other modifications of the nucleotides of an iRNA of the invention include a 5' phosphate or 5' phosphate mimic, *e.g.,* a 5'-terminal phosphate or phosphate mimic on the antisense strand of an RNAi agent. Suitable phosphate mimics are disclosed in, for example US Patent Publication No. 2012/0157511, the entire contents of which are incorporated herein by reference.

A. Modified iRNAs Comprising Motifs of the Invention

In certain aspects of the invention, the double-stranded RNAi agents of the invention include agents with chemical modifications as disclosed, for example, in WO 2013/075035, filed on November 16, 2012, the entire contents of which are incorporated herein by reference. As shown herein and in PCT Publication No. WO 2013/075035, a superior result may be obtained by introducing one or more motifs of three identical modifications on three consecutive nucleotides into a sense strand and/or antisense strand of an RNAi agent, particularly at or near the cleavage site. In some embodiments, the sense strand and antisense strand of the RNAi agent may otherwise be completely modified. The introduction of these motifs interrupts the modification pattern, if present, of the sense and/or antisense strand. The RNAi agent may be optionally conjugated with a GalNAc derivative ligand, for instance on the sense strand. The resulting RNAi agents present superior gene silencing activity.

More specifically, it has been surprisingly discovered that when the sense strand and antisense strand of the double-stranded RNAi agent are completely modified to have one or more motifs of three identical modifications on three consecutive nucleotides at or near the cleavage site of at least one strand of an RNAi agent, the gene silencing acitivity of the RNAi agent was superiorly enhanced.

Accordingly, the invention provides double-stranded RNAi agents capable of inhibiting the expression of a target gene (*i.e.*, HBV gene) *in vivo*. The RNAi agent comprises a sense strand and an antisense strand. Each strand of the RNAi agent may range from 12-30 nucleotides in length. For example, each strand may be between 14-30 nucleotides in length, 17-30 nucleotides in length, 25-30 nucleotides in length, 27-30 nucleotides in length, 17-23 nucleotides in length, 17-21 nucleotides in length, 17-19 nucleotides in length, 19-25 nucleotides in length, 19-23 nucleotides in length, 19-21 nucleotides in length, 21-25 nucleotides in length, or 21-23 nucleotides in length.

The sense strand and antisense strand typically form a duplex double stranded RNA ("dsRNA"), also referred to herein as an "RNAi agent." The duplex region of an RNAi agent may be 12-30 nucleotide pairs in length. For example, the duplex region can be between 14-30 nucleotide pairs in length, 17-30 nucleotide pairs in length, 27-30 nucleotide pairs in length, 17 - 23 nucleotide pairs in length, 17-21 nucleotide pairs in length, 17-19 nucleotide pairs in length, 19-25 nucleotide pairs in length, 19-23 nucleotide pairs in length, 19- 21 nucleotide pairs in length, 21-25 nucleotide pairs in length, or 21-23 nucleotide pairs in length. In another example, the duplex region is selected from 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 nucleotides in length.

In one embodiment, the RNAi agent may contain one or more overhang regions and/or capping groups at the 3'-end, 5'-end, or both ends of one or both strands. The overhang can be 1-6 nucleotides in length, for instance 2-6 nucleotides in length, 1-5 nucleotides in length, 2-5 nucleotides in length, 1-4 nucleotides in length, 2-4 nucleotides in

length, 1-3 nucleotides in length, 2-3 nucleotides in length, or 1-2 nucleotides in length. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be another sequence. The first and second strands can also be joined, *e.g.*, by additional bases to form a hairpin, or by other non-base linkers.

In one embodiment, the nucleotides in the overhang region of the RNAi agent can each independently be a modified or unmodified nucleotide including, but no limited to 2'-sugar modified, such as, 2-F, 2'-Omethyl, thymidine (T), 2`-O-methoxyethyl-5-methyluridine (Teo), 2`-O-methoxyethyladenosine (Aeo), 2`-O-methoxyethyl-5-methylcytidine (m5Ceo), and any combinations thereof. For example, TT can be an overhang sequence for either end on either strand. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be another sequence.

The 5'- or 3'- overhangs at the sense strand, antisense strand or both strands of the RNAi agent may be phosphorylated. In some embodiments, the overhang region(s) contains two nucleotides having a phosphorothioate between the two nucleotides, where the two nucleotides can be the same or different. In one embodiment, the overhang is present at the 3'-end of the sense strand, antisense strand, or both strands. In one embodiment, this 3'-overhang is present in the antisense strand. In one embodiment, this 3'-overhang is present in the sense strand.

The RNAi agent may contain only a single overhang, which can strengthen the interference activity of the RNAi, without affecting its overall stability. For example, the single-stranded overhang may be located at the 3'-terminal end of the sense strand or, alternatively, at the 3'-terminal end of the antisense strand. The RNAi may also have a blunt end, located at the 5'-end of the antisense strand (or the 3'-end of the sense strand) or *vice versa*. Generally, the antisense strand of the RNAi has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. While not wishing to be bound by theory, the asymmetric blunt end at the 5'-end of the antisense strand and 3'-end overhang of the antisense strand favor the guide strand loading into RISC process.

In one embodiment, the RNAi agent is a double ended bluntmer of 19 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 7, 8, 9 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In another embodiment, the RNAi agent is a double ended bluntmer of 20 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 8, 9, 10 from the 5'end. The antisense strand

contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In yet another embodiment, the RNAi agent is a double ended bluntmer of 21 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In one embodiment, the RNAi agent comprises a 21 nucleotide sense strand and a 23 nucleotide antisense strand, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end; the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end, wherein one end of the RNAi agent is blunt, while the other end comprises a 2 nucleotide overhang. Preferably, the 2 nucleotide overhang is at the 3'-end of the antisense strand.

When the 2 nucleotide overhang is at the 3'-end of the antisense strand, there may be two phosphorothioate internucleotide linkages between the terminal three nucleotides, wherein two of the three nucleotides are the overhang nucleotides, and the third nucleotide is a paired nucleotide next to the overhang nucleotide. In one embodiment, the RNAi agent additionally has two phosphorothioate internucleotide linkages between the terminal three nucleotides at both the 5'-end of the sense strand and at the 5'-end of the antisense strand. In one embodiment, every nucleotide in the sense strand and the antisense strand of the RNAi agent, including the nucleotides that are part of the motifs are modified nucleotides. In one embodiment each residue is independently modified with a 2'-O-methyl or 3'-fluoro, *e.g.,* in an alternating motif. Optionally, the RNAi agent further comprises a ligand (preferably $GalNAc_3$).

In one embodiment, the RNAi agent comprises a sense and an antisense strand, wherein the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1) positions 1 to 23 of the first strand comprise at least 8 ribonucleotides; the antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, comprises at least 8 ribonucleotides in the positions paired with positions 1- 23 of sense strand to form a duplex; wherein at least the 3 ' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand, thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a substantially duplexed region

between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene expression when the double stranded nucleic acid is introduced into a mammalian cell; and wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

In one embodiment, the RNAi agent comprises sense and antisense strands, wherein the RNAi agent comprises a first strand having a length which is at least 25 and at most 29 nucleotides and a second strand having a length which is at most 30 nucleotides with at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at position 11, 12, 13 from the 5' end; wherein the 3' end of the first strand and the 5' end of the second strand form a blunt end and the second strand is 1-4 nucleotides longer at its 3' end than the first strand, wherein the duplex region region which is at least 25 nucleotides in length, and the second strand is sufficiently complemenatary to a target mRNA along at least 19 nucleotide of the second strand length to reduce target gene expression when the RNAi agent is introduced into a mammalian cell, and wherein dicer cleavage of the RNAi agent preferentially results in an siRNA comprising the 3' end of the second strand, thereby reducing expression of the target gene in the mammal. Optionally, the RNAi agent further comprises a ligand.

In one embodiment, the sense strand of the RNAi agent contains at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at the cleavage site in the sense strand.

In one embodiment, the antisense strand of the RNAi agent can also contain at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at or near the cleavage site in the antisense strand

For an RNAi agent having a duplex region of 17-23 nucleotide in length, the cleavage site of the antisense strand is typically around the 10, 11 and 12 positions from the 5'-end. Thus the motifs of three identical modifications may occur at the 9, 10, 11 positions; 10, 11, 12 positions; 11, 12, 13 positions; 12, 13, 14 positions; or 13, 14, 15 positions of the antisense strand, the count starting from the 1st nucleotide from the 5'-end of the antisense strand, or, the count starting from the 1st paired nucleotide within the duplex region from the 5'- end of the antisense strand. The cleavage site in the antisense strand may also change according to the length of the duplex region of the RNAi from the 5'-end.

The sense strand of the RNAi agent may contain at least one motif of three identical modifications on three consecutive nucleotides at the cleavage site of the strand; and the antisense strand may have at least one motif of three identical modifications on three consecutive nucleotides at or near the cleavage site of the strand. When the sense strand and

the antisense strand form a dsRNA duplex, the sense strand and the antisense strand can be so aligned that one motif of the three nucleotides on the sense strand and one motif of the three nucleotides on the antisense strand have at least one nucleotide overlap, *i.e.*, at least one of the three nucleotides of the motif in the sense strand forms a base pair with at least one of the three nucleotides of the motif in the antisense strand. Alternatively, at least two nucleotides may overlap, or all three nucleotides may overlap.

In one embodiment, the sense strand of the RNAi agent may contain more than one motif of three identical modifications on three consecutive nucleotides. The first motif may occur at or near the cleavage site of the strand and the other motifs may be a wing modification. The term "wing modification" herein refers to a motif occurring at another portion of the strand that is separated from the motif at or near the cleavage site of the same strand. The wing modification is either adajacent to the first motif or is separated by at least one or more nucleotides. When the motifs are immediately adjacent to each other then the chemistry of the motifs are distinct from each other and when the motifs are separated by one or more nucleotide than the chemistries can be the same or different. Two or more wing modifications may be present. For instance, when two wing modifications are present, each wing modification may occur at one end relative to the first motif which is at or near cleavage site or on either side of the lead motif.

Like the sense strand, the antisense strand of the RNAi agent may contain more than one motifs of three identical modifications on three consecutive nucleotides, with at least one of the motifs occurring at or near the cleavage site of the strand. This antisense strand may also contain one or more wing modifications in an alignment similar to the wing modifications that may be present on the sense strand.

In one embodiment, the wing modification on the sense strand or antisense strand of the RNAi agent typically does not include the first one or two terminal nucleotides at the 3'-end, 5'-end or both ends of the strand.

In another embodiment, the wing modification on the sense strand or antisense strand of the RNAi agent typically does not include the first one or two paired nucleotides within the duplex region at the 3'-end, 5'-end or both ends of the strand.

When the sense strand and the antisense strand of the RNAi agent each contain at least one wing modification, the wing modifications may fall on the same end of the duplex region, and have an overlap of one, two or three nucleotides.

When the sense strand and the antisense strand of the RNAi agent each contain at least two wing modifications, the sense strand and the antisense strand can be so aligned that two modifications each from one strand fall on one end of the duplex region, having an overlap of one, two or three nucleotides; two modifications each from one strand fall on the other end of the duplex region, having an overlap of one, two or three nucleotides; two

modifications one strand fall on each side of the lead motif, having an overlap of one, two or three nucleotides in the duplex region.

In one embodiment, every nucleotide in the sense strand and antisense strand of the RNAi agent, including the nucleotides that are part of the motifs, may be modified. Each nucleotide may be modified with the same or different modification which can include one or more alteration of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens; alteration of a constituent of the ribose sugar, *e.g.*, of the 2′ hydroxyl on the ribose sugar; wholesale replacement of the phosphate moiety with "dephospho" linkers; modification or replacement of a naturally occurring base; and replacement or modification of the ribose-phosphate backbone.

As nucleic acids are polymers of subunits, many of the modifications occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, *e.g.,* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of a RNA or may only occur in a single strand region of a RNA. For example, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal region, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

It may be possible, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. For example, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang may be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' position of the ribose sugar with modifications that are known in the art, *e.g.*, the use of deoxyribonucleotides, , 2'-deoxy-2'-fluoro (2'-F) or 2'-O-methyl modified instead of the ribosugar of the nucleobase , and modifications in the phosphate group, *e.g.*, phosphorothioate modifications. Overhangs need not be homologous with the target sequence.

In one embodiment, each residue of the sense strand and antisense strand is independently modified with LNA, CRN, cET, UNA, HNA, CeNA, 2'-methoxyethyl, 2'- O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-deoxy, 2'-hydroxyl, or 2'-fluoro. The strands can contain more than one modification. In one embodiment, each residue of the sense strand and antisense strand is independently modified with 2'- O-methyl or 2'-fluoro.

At least two different modifications are typically present on the sense strand and antisense strand. Those two modifications may be the 2'- O-methyl or 2'-fluoro modifications, or others.

In one embodiment, the N_a and/or N_b comprise modifications of an alternating pattern. The term "alternating motif" as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B and C each represent one type of modification to the nucleotide, the alternating motif can be "ABABABABABAB...," "AABBAABBAABB...," "AABAABAABAAB...," "AAABAAABAAAB...," "AAABBBAAABBB...," or "ABCABCABCABC...," *etc.*

The type of modifications contained in the alternating motif may be the same or different. For example, if A, B, C, D each represent one type of modification on the nucleotide, the alternating pattern, *i.e.*, modifications on every other nucleotide, may be the same, but each of the sense strand or antisense strand can be selected from several possibilities of modifications within the alternating motif such as "ABABAB...", "ACACAC..." "BDBDBD..." or "CDCDCD...," *etc.*

In one embodiment, the RNAi agent of the invention comprises the modification pattern for the alternating motif on the sense strand relative to the modification pattern for the alternating motif on the antisense strand is shifted. The shift may be such that the modified group of nucleotides of the sense strand corresponds to a differently modified group of nucleotides of the antisense strand and *vice versa*. For example, the sense strand when paired with the antisense strand in the dsRNA duplex, the alternating motif in the sense strand may start with "ABABAB" from 5'-3' of the strand and the alternating motif in the antisense strand may start with "BABABA" from 5'-3'of the strand within the duplex region. As another example, the alternating motif in the sense strand may start with "AABBAABB" from 5'-3' of the strand and the alternating motif in the antisenese strand may start with "BBAABBAA" from 5'-3' of the strand within the duplex region, so that there is a complete or partial shift of the modification patterns between the sense strand and the antisense strand.

In one embodiment, the RNAi agent comprises the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the sense strand initially has a shift relative to the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the antisense strand initially, *i.e.*, the 2'-O-methyl modified nucleotide on the sense strand base pairs with a 2'-F modified nucleotide on the antisense strand and vice versa. The 1 position of the sense strand may start with the 2'-F modification, and the 1 position of the antisense strand may start with the 2'- O-methyl modification.

The introduction of one or more motifs of three identical modifications on three consecutive nucleotides to the sense strand and/or antisense strand interrupts the initial

modification pattern present in the sense strand and/or antisense strand. This interruption of the modification pattern of the sense and/or antisense strand by introducing one or more motifs of three identical modifications on three consecutive nucleotides to the sense and/or antisense strand surprisingly enhances the gene silencing acitivty to the target gene.

In one embodiment, when the motif of three identical modifications on three consecutive nucleotides is introduced to any of the strands, the modification of the nucleotide next to the motif is a different modification than the modification of the motif. For example, the portion of the sequence containing the motif is "...N_aYYYN_b...," where "Y" represents the modification of the motif of three identical modifications on three consecutive nucleotide, and "N_a" and "N_b" represent a modification to the nucleotide next to the motif "YYY" that is different than the modification of Y, and where N_a and N_b can be the same or different modifications. Altnernatively, N_a and/or N_b may be present or absent when there is a wing modification present.

The RNAi agent may further comprise at least one phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand or antisense strand or both strands in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand and/or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand and/or antisense strand; or the sense strand or antisense strand may contain both internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand. In one embodiment, a double-standed RNAi agent comprises 6-8 phosphorothioate internucleotide linkages. In one embodiment, the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus, and the sense strand comprises at least two phosphorothioate internucleotide linkages at either the 5'-terminus or the 3'-terminus.

In one embodiment, the RNAi comprises a phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region may contain two nucleotides having a phosphorothioate or methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the overhang nucleotides with the terminal paired nucleotides within the duplex region. For example, at least 2, 3, 4, or all the overhang nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide

linkages linking the overhang nucleotide with a paired nucleotide that is next to the overhang nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are overhang nucleotides, and the third is a paired nucleotide next to the overhang nucleotide. These terminal three nucleotides may be at the 3'-end of the antisense strand, the 3'-end of the sense strand, the 5'-end of the antisense strand, and/or the 5'end of the antisense strand.

In one embodiment, the 2 nucleotide overhang is at the 3'-end of the antisense strand, and there are two phosphorothioate internucleotide linkages between the terminal three nucleotides, wherein two of the three nucleotides are the overhang nucleotides, and the third nucleotide is a paired nucleotide next to the overhang nucleotide. Optionally, the RNAi agent may additionally have two phosphorothioate internucleotide linkages between the terminal three nucleotides at both the 5'-end of the sense strand and at the 5'-end of the antisense strand.

In one embodiment, the RNAi agent comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mistmatch may occur in the overhang region or the duplex region. The base pair may be ranked on the basis of their propensity to promote dissociation or melting (*e.g.*, on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C is preferred over G:C (I=inosine). Mismatches, *e.g.*, non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a universal base are preferred over canonical pairings.

In one embodiment, the RNAi agent comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'- end of the antisense strand independently selected from the group of: A:U, G:U, I:C, and mismatched pairs, *e.g.*, non-canonical or other than canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

In one embodiment, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2 or 3 base pair within the duplex region from the 5'-end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair.

In another embodiment, the nucleotide at the 3'-end of the sense strand is deoxy-thymine (dT). In another embodiment, the nucleotide at the 3'-end of the antisense strand is deoxy-thymine (dT). In one embodiment, there is a short sequence of deoxy-thymine nucleotides, for example, two dT nucleotides on the 3'-end of the sense and/or antisense strand.

In one embodiment, the sense strand sequence may be represented by formula (I):

5' n_p-N_a-(X X X)$_i$-N_b-Y Y Y -N_b-(Z Z Z)$_j$-N_a-n_q 3' (I)

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein Nb and Y do not have the same modification; and

XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. Preferably YYY is all 2'-F modified nucleotides.

In one embodiment, the N_a and/or N_b comprise modifications of alternating pattern.

In one embodiment, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the RNAi agent has a duplex region of 17-23 nucleotides in length, the YYY motif can occur at or the vicinity of the cleavage site (*e.g.*: can occur at positions 6, 7, 8, 7, 8, 9, 8, 9, 10, 9, 10, 11, 10, 11,12 or 11, 12, 13) of - the sense strand, the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end.

In one embodiment, i is 1 and j is 0, or i is 0 and j is 1, or both i and j are 1. The sense strand can therefore be represented by the following formulas:

5' n_p-N_a-YYY-N_b-ZZZ-N_a-n_q 3' (Ib);

5' n_p-N_a-XXX-N_b-YYY-N_a-n_q 3' (Ic); or

5' n_p-N_a-XXX-N_b-YYY-N_b-ZZZ-N_a-n_q 3' (Id).

When the sense strand is represented by formula (Ib), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Ic), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Id), each N_b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6 Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X, Y and Z may be the same or different from each other.

In other embodiments, i is 0 and j is 0, and the sense strand may be represented by the formula:

5' n_p-N_a-YYY- N_a-n_q 3' (Ia).

When the sense strand is represented by formula (Ia), each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

In one embodiment, the antisense strand sequence of the RNAi may be represented by formula (II):

5' $n_{q'}$-N_a'-(Z'Z'Z')$_k$-N_b'-Y'Y'Y'-N_b'-(X'X'X')$_l$-N'_a-$n_{p'}$ 3' (II)

wherein:

k and l are each independently 0 or 1;

p' and q' are each independently 0-6;

each N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each $n_{p'}$ and $n_{q'}$ independently represent an overhang nucleotide;

wherein N_b' and Y' do not have the same modification; and

X'X'X', Y'Y'Y' and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, the N_a' and/or N_b' comprise modifications of alternating pattern.

The Y'Y'Y' motif occurs at or near the cleavage site of the antisense strand. For example, when the RNAi agent has a duplex region of 17-23nucleotidein length, the Y'Y'Y' motif can occur at positions 9, 10, 11;10, 11, 12; 11, 12, 13; 12, 13, 14 ; or 13, 14, 15 of the antisense strand, with the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end. Preferably, the Y'Y'Y' motif occurs at positions 11, 12, 13.

In one embodiment, Y'Y'Y' motif is all 2'-OMe modified nucleotides.

In one embodiment, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

The antisense strand can therefore be represented by the following formulas:

5' $n_{q'}$-N_a'-Z'Z'Z'-N_b'-Y'Y'Y'-N_a'-$n_{p'}$ 3' (IIb);

5' $n_{q'}$-N_a'-Y'Y'Y'-N_b'-X'X'X'-$n_{p'}$ 3' (IIc); or

5' $n_{q'}$-N_a'- Z'Z'Z'-N_b'-Y'Y'Y'-N_b'- X'X'X'-N_a'-$n_{p'}$ 3' (IId).

When the antisense strand is represented by formula (IIb), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIc), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IId), each N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6.

In other embodiments, k is 0 and l is 0 and the antisense strand may be represented by the formula:

5' $n_{p'}$-$N_{a'}$-Y'Y'Y'- $N_{a'}$-$n_{q'}$ 3' (Ia).

When the antisense strand is represented as formula (IIa), each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X′, Y′ and Z′ may be the same or different from each other.

Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, CRN, UNA, cEt, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-hydroxyl, or 2'-fluoro. For example, each nucleotide of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. Each X, Y, Z, X′, Y′ and Z′, in particular, may represent a 2'-O-methyl modification or a 2'-fluoro modification.

In one embodiment, the sense strand of the RNAi agent may contain YYY motif occurring at 9, 10 and 11 positions of the strand when the duplex region is 21 nt, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y represents 2'-F modification. The sense strand may additionally contain XXX motif or ZZZ motifs as wing modifications at the opposite end of the duplex region; and XXX and ZZZ each independently represents a 2'-OMe modification or 2'-F modification.

In one embodiment the antisense strand may contain Y′Y′Y′ motif occurring at positions 11, 12, 13 of the strand, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y′ represents 2'-O-methyl modification. The antisense strand may additionally contain X′X′X′ motif or Z′Z′Z′ motifs as wing modifications at the opposite end of the duplex region; and X′X′X′ and Z′Z′Z′ each independently represents a 2'-OMe modification or 2'-F modification.

The sense strand represented by any one of the above formulas (Ia), (Ib), (Ic), and (Id) forms a duplex with a antisense strand being represented by any one of formulas (IIa), (IIb), (IIc), and (IId), respectively.

Accordingly, the RNAi agents for use in the methods of the invention may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the RNAi duplex represented by formula (III):

sense: 5' n_p -N_a-(X X X)$_i$ -N_b- Y Y Y -N_b -(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'-n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p', q, and q' are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein each n_p', n_p, n_q', and n_q, each of which may or may not be present, independently represents an overhang nucleotide; and

XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, i is 0 and j is 0; or i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 0; or both i and j are 1. In another embodiment, k is 0 and l is 0; or k is 1 and l is 0; k is 0 and l is 1; or both k and l are 0; or both k and l are 1.

Exemplary combinations of the sense strand and antisense strand forming a RNAi duplex include the formulas below:

5' n_p - N_a -Y Y Y -N_a-n_q 3'

3' n_p'-N_a'-Y'Y'Y' -N_a'n_q' 5'

(IIIa)

5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-Y'Y'Y'-N_b'-Z'Z'Z'-N_a'n_q' 5'

(IIIb)

5' n_p-N_a- X X X -N_b -Y Y Y - N_a-n_q 3'

3' n_p'-N_a'-X'X'X'-N_b'-Y'Y'Y'-N_a'-n_q' 5'

(IIIc)

5' n_p -N_a -X X X -N_b-Y Y Y -N_b- Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-X'X'X'-N_b'-Y'Y'Y'-N_b'-Z'Z'Z'-N_a-n_q' 5'

(IIId)

When the RNAi agent is represented by formula (IIIa), each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented by formula (IIIb), each N_b independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5 or 1-4 modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIIc), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIId), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a, N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N_a, N_a', N_b and N_b' independently comprises modifications of alternating pattern.

Each of X, Y and Z in formulas (III), (IIIa), (IIIb), (IIIc), and (IIId) may be the same or different from each other.

When the RNAi agent is represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), at least one of the Y nucleotides may form a base pair with one of the Y′ nucleotides. Alternatively, at least two of the Y nucleotides form base pairs with the corresponding Y′ nucleotides; or all three of the Y nucleotides all form base pairs with the corresponding Y′ nucleotides.

When the RNAi agent is represented by formula (IIIb) or (IIId), at least one of the Z nucleotides may form a base pair with one of the Z′ nucleotides. Alternatively, at least two of the Z nucleotides form base pairs with the corresponding Z′ nucleotides; or all three of the Z nucleotides all form base pairs with the corresponding Z′ nucleotides.

When the RNAi agent is represented as formula (IIIc) or (IIId), at least one of the X nucleotides may form a base pair with one of the X′ nucleotides. Alternatively, at least two of the X nucleotides form base pairs with the corresponding X′ nucleotides; or all three of the X nucleotides all form base pairs with the corresponding X′ nucleotides.

In one embodiment, the modification on the Y nucleotide is different than the modification on the Y' nucleotide, the modification on the Z nucleotide is different than the modification on the Z' nucleotide, and/or the modification on the X nucleotide is different than the modification on the X' nucleotide.

In one embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications. In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications and $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide a via

phosphorothioate linkage. In yet another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker (described below). In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, when the RNAi agent is represented by formula (IIIa), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, the RNAi agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, the RNAi agent is a multimer containing three, four, five, six or more duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, two RNAi agents represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId) are linked to each other at the 5' end, and one or both of the 3' ends and are optionally conjugated to to a ligand. Each of the agents can target the same gene or two different genes; or each of the agents can target same gene at two different target sites.

Various publications describe multimeric RNAi agents that can be used in the methods of the invention. Such publications include WO2007/091269, US Patent No. 7858769, WO2010/141511, WO2007/117686, WO2009/014887 and WO2011/031520 the entire contents of each of which are hereby incorporated herein by reference.

As described in more detail below, the RNAi agent that contains conjugations of one or more carbohydrate moieties to a RNAi agent can optimize one or more properties of the RNAi agent. In many cases, the carbohydrate moiety will be attached to a modified subunit of the RNAi agent. For example, the ribose sugar of one or more ribonucleotide subunits of a

dsRNA agent can be replaced with another moiety, *e.g.*, a non-carbohydrate (preferably cyclic) carrier to which is attached a carbohydrate ligand. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier may be a carbocyclic ring system, *i.e.*, all ring atoms are carbon atoms, or a heterocyclic ring system, *i.e.*, one or more ring atoms may be a heteroatom, *e.g.*, nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system, or may contain two or more rings, *e.g.* fused rings. The cyclic carrier may be a fully saturated ring system, or it may contain one or more double bonds.

The ligand may be attached to the polynucleotide *via* a carrier. The carriers include (i) at least one "backbone attachment point," preferably two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a functional group, *e.g.* a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, *e.g.*, the phosphate, or modified phosphate, *e.g.*, sulfur containing, backbone, of a ribonucleic acid. A "tethering attachment point" (TAP) in some embodiments refers to a constituent ring atom of the cyclic carrier, *e.g.*, a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, *e.g.*, a carbohydrate, *e.g.* monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide and polysaccharide. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, the cyclic carrier will often include a functional group, *e.g.*, an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, *e.g.*, a ligand to the constituent ring.

The RNAi agents may be conjugated to a ligand *via* a carrier, wherein the carrier can be cyclic group or acyclic group; preferably, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl and and decalin; preferably, the acyclic group is selected from serinol backbone or diethanolamine backbone.

In certain specific embodiments, the RNAi agent for use in the methods of the invention is an agent selected from the group of agents listed in any one of Tables 3, 4, 6, 7, 12, 13, 22, 23, 25, and 26. These agents may further comprise a ligand.

IV. iRNAs Conjugated to Ligands

Another modification of the RNA of an iRNA of the invention involves chemically linking to the RNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the iRNA. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acid. Sci. USA*, 1989, 86: 6553-6556), cholic acid (Manoharan *et al.*, *Biorg. Med. Chem. Let.*, 1994, 4:1053-

1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660:306-309; Manoharan *et al.*, *Biorg. Med. Chem. Let.*, 1993, 3:2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20:533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J*, 1991, 10:1111-1118; Kabanov *et al.*, *FEBS Lett.*, 1990, 259:327-330; Svinarchuk *et al.*, *Biochimie*, 1993, 75:49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651-3654; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14:969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264:229-237), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al.*, J. *Pharmacol. Exp. Ther.*, 1996, 277:923-937).

In one embodiment, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Preferred ligands will not take part in duplex pairing in a duplexed nucleic acid.

Ligands can include a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin, N-acetylgalactosamine, or hyaluronic acid); or a lipid. The ligand can also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucoseamine multivalent mannose, multivalent fucose,

glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, vitamin A, biotin, or an RGD peptide or RGD peptide mimetic.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a hepatic cell. Ligands can also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In some embodiments, a ligand attached to an iRNA as described herein acts as a pharmacokinetic modulator (PK modulator). PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins *etc*. Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin *etc*. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, *e.g.*, oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbone are also amenable to the present invention as ligands (*e.g.* as PK modulating ligands). In addition, aptamers that bind

serum components (*e.g.* serum proteins) are also suitable for use as PK modulating ligands in the embodiments described herein.

Ligand-conjugated oligonucleotides of the invention may be synthesized by the use of an oligonucleotide that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the oligonucleotide (described below). This reactive oligonucleotide may be reacted directly with commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto.

The oligonucleotides used in the conjugates of the present invention may be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates and alkylated derivatives.

In the ligand-conjugated oligonucleotides and ligand-molecule bearing sequence-specific linked nucleosides of the present invention, the oligonucleotides and oligonucleosides may be assembled on a suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

When using nucleotide-conjugate precursors that already bear a linking moiety, the synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. In some embodiments, the oligonucleotides or linked nucleosides of the present invention are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to the standard phosphoramidites and non-standard phosphoramidites that are commercially available and routinely used in oligonucleotide synthesis.

A. Lipid Conjugates

In one embodiment, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, naproxen or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

A lipid based ligand can be used to inhibit, *e.g.*, control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a target cell, *e.g.*, a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, *e.g.*, of the malignant or non-malignant type, *e.g.*, cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by target cells such as liver cells. Also included are HSA and low density lipoprotein (LDL).

B. Cell Permeation Agents

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF

having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO: 43). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO: 44) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO: 45) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 46) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, Nature, 354:82-84, 1991). Examples of a peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit for cell targeting purposes is an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide for use in the compositions and methods of the invention may be linear or cyclic, and may be modified, *e.g.,* glycosylated or methylated, to facilitate targeting to a specific tissue(s). RGD-containing peptides and peptidiomimemtics may include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Preferred conjugates of this ligand target PECAM-1 or VEGF.

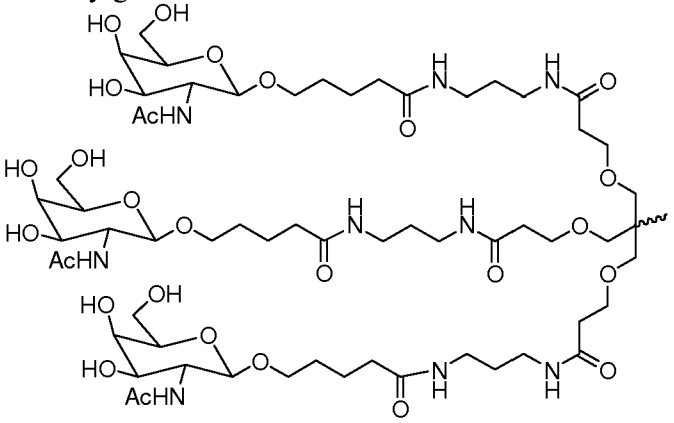
A "cell permeation peptide" is capable of permeating a cell, *e.g.,* a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

C. Carbohydrate Conjugates

In some embodiments of the compositions and methods of the invention, an iRNA oligonucleotide further comprises a carbohydrate. The carbohydrate conjugated iRNA are advantageous for the *in vivo* delivery of nucleic acids, as well as compositions suitable for *in vivo* therapeutic use, as described herein. As used herein, "carbohydrate" refers to a compound which is either a carbohydrate *per se* made up of one or more monosaccharide units having at least 6 carbon atoms (which can be linear, branched or cyclic) with an oxygen,

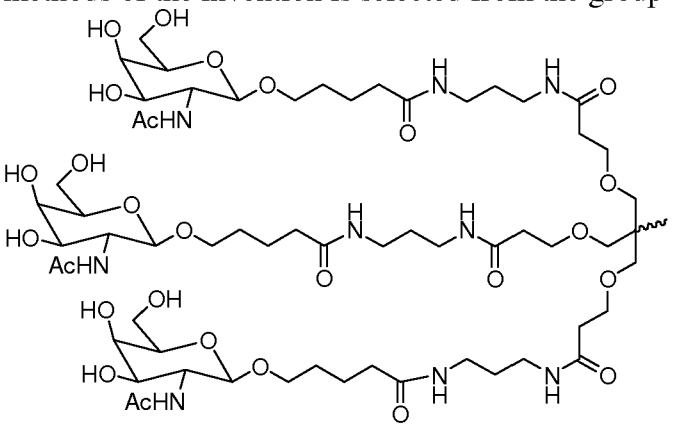
nitrogen or sulfur atom bonded to each carbon atom; or a compound having as a part thereof a carbohydrate moiety made up of one or more monosaccharide units each having at least six carbon atoms (which can be linear, branched or cyclic), with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Representative carbohydrates include the sugars (mono-, di-, tri- and oligosaccharides containing from about 4, 5, 6, 7, 8, or 9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include HBV and above (*e.g.,* HBV, C6, C7, or C8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (*e.g.,* HBV, C6, C7, or C8).

In one embodiment, a carbohydrate conjugate for use in the compositions and methods of the invention is a monosaccharide. In one embodiment, the monosaccharide is an N-acetylgalactosamine, such as

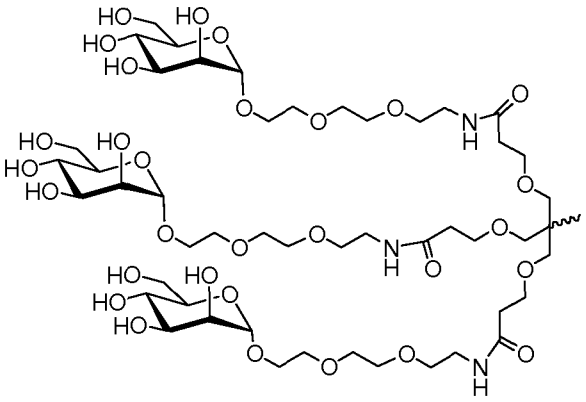


Formula II.

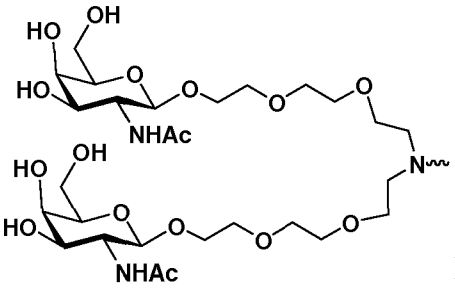
In another embodiment, a carbohydrate conjugate for use in the compositions and methods of the invention is selected from the group consisting of:



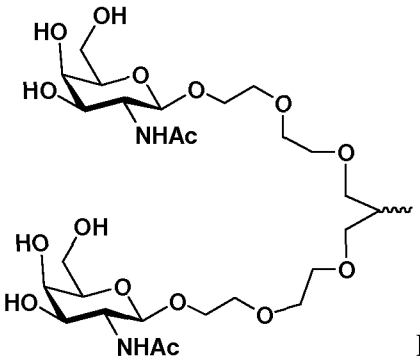
Formula II,



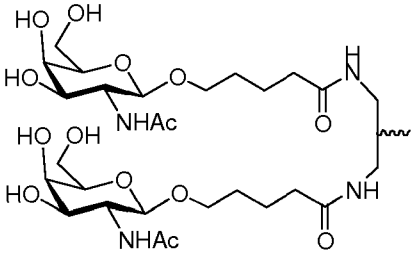
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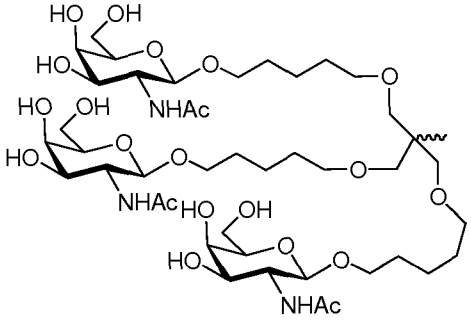
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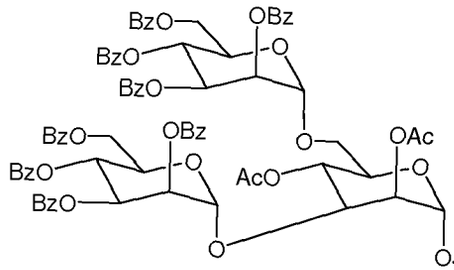
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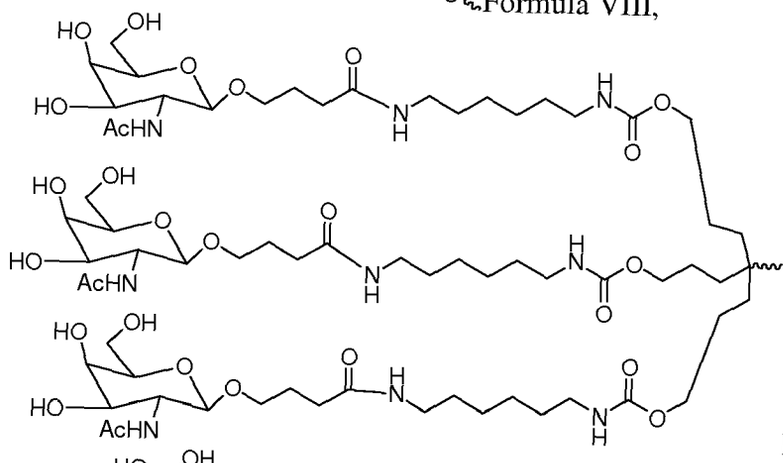
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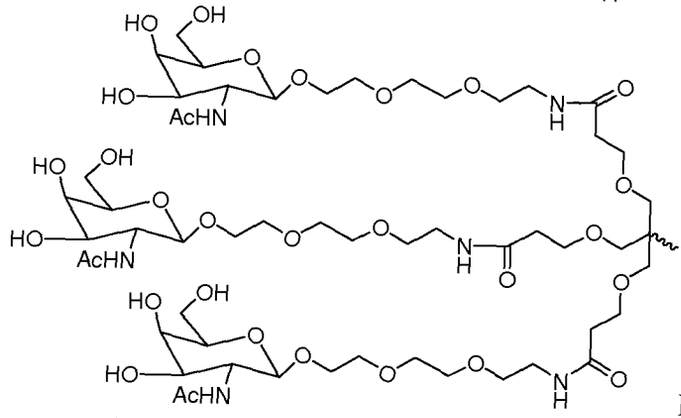
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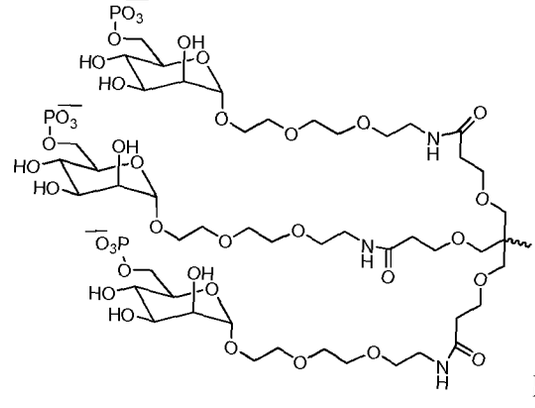
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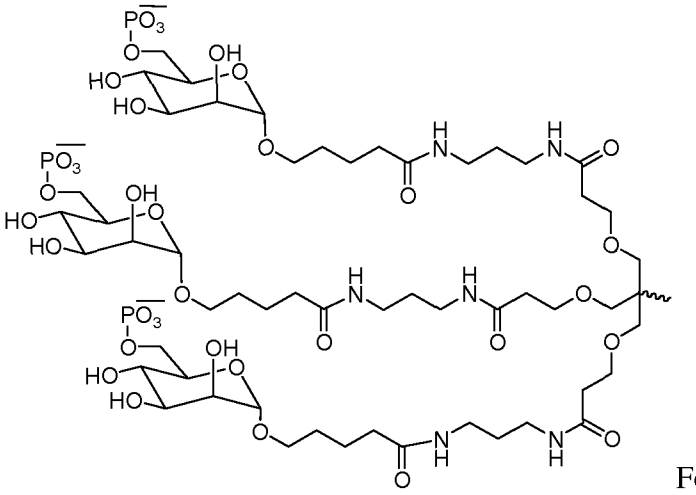
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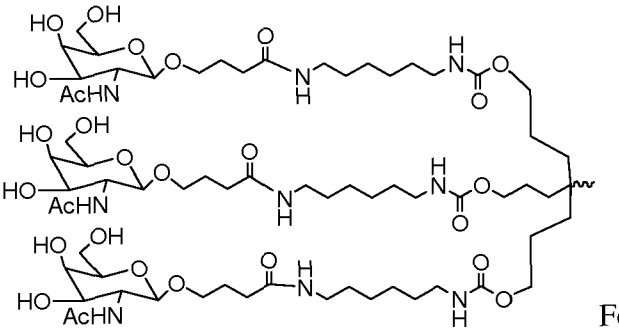
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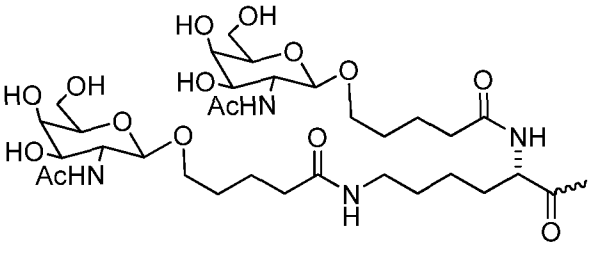
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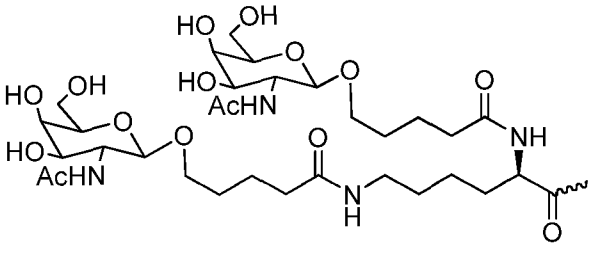
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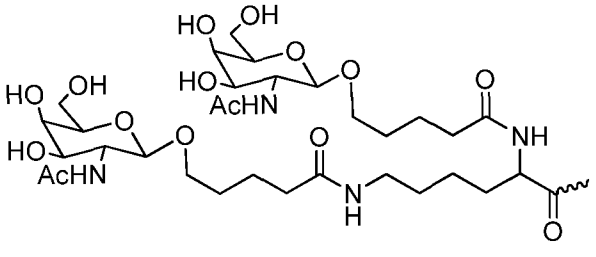
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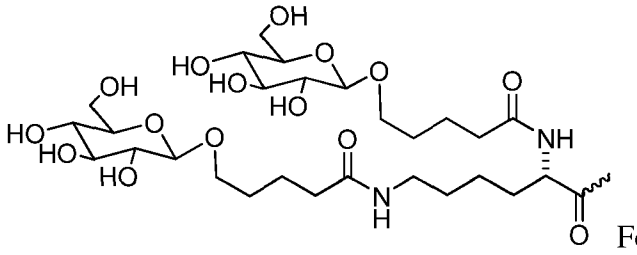
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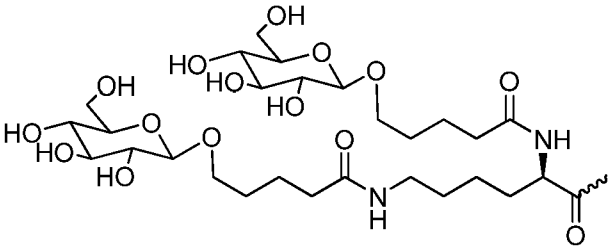
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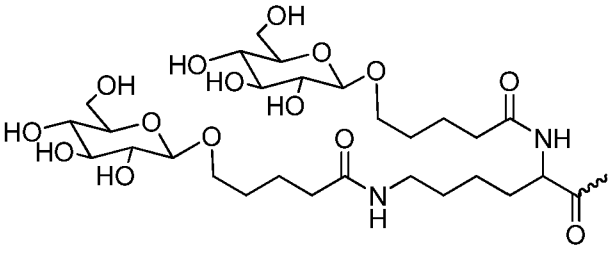
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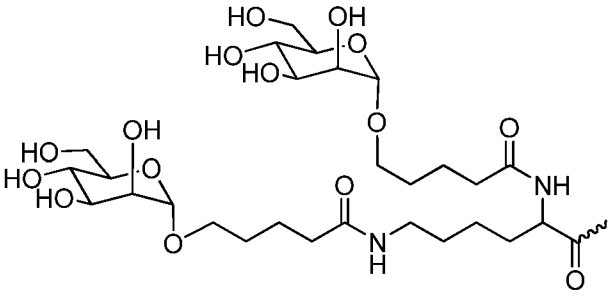
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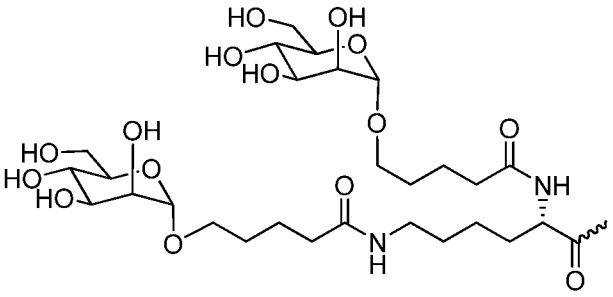
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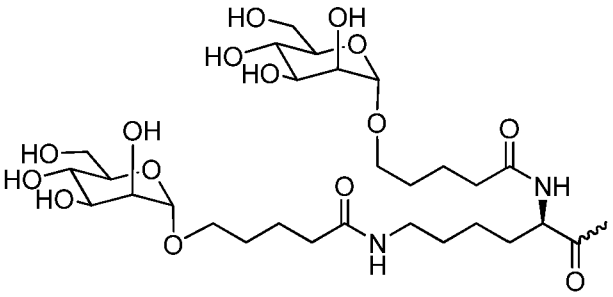
Formula XIX,



Formula XX,

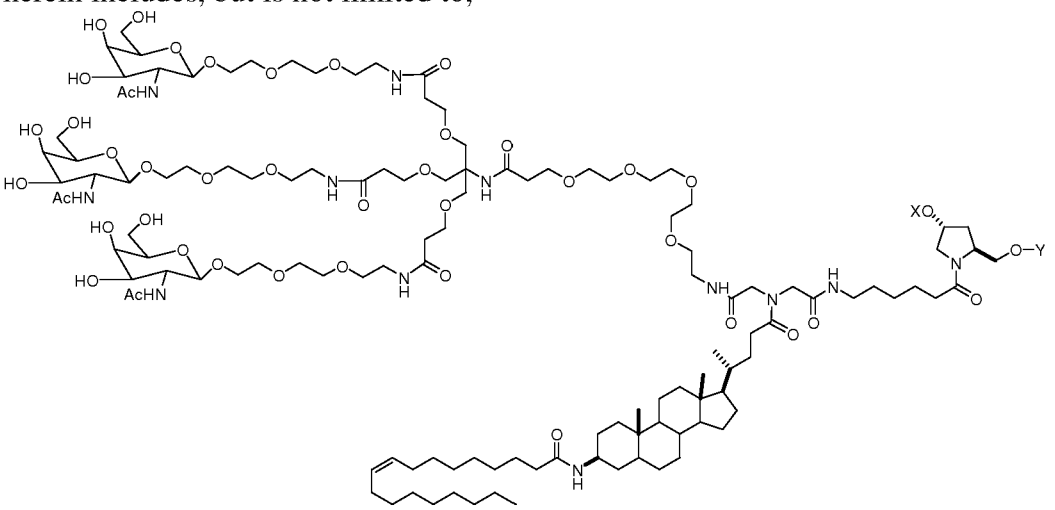


Formula XXI,



Formula XXII.

Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to,



(Formula XXIII), when one of X or Y is an oligonucleotide, the other is a hydrogen.

In some embodiments, the carbohydrate conjugate further comprises one or more additional ligands as described above, such as, but not limited to, a PK modulator and/or a cell permeation peptide.

Additional carbohydrate conjugates (and linkers) suitable for use in the present invention include those described in PCT Publication Nos. WO 2014/179620 and WO 2014/179627, the entire contents of each of which are incorporated herein by reference.

D. Linkers

In some embodiments, the conjugate or ligand described herein can be attached to an iRNA oligonucleotide with various linkers that can be cleavable or non-cleavable.

The term "linker" or “linking group” means an organic moiety that connects two parts of a compound, *e.g.,* covalently attaches two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR8, C(O), C(O)NH, SO, SO_2, SO_2NH or a chain of atoms, such as, but not limited to, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, which one or

more methylenes can be interrupted or terminated by O, S, S(O), SO_2, N(R8), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R8 is hydrogen, acyl, aliphatic or substituted aliphatic. In one embodiment, the linker is between about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18 atoms, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least about 10 times, 20, times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times or more, or at least about 100 times faster in a target cell or under a first reference condition (which can, *e.g.*, be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, *e.g.*, be selected to mimic or represent conditions found in the blood or serum).

Cleavable linking groups are susceptible to cleavage agents, *e.g.*, pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, *e.g.*, oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, *e.g.*, those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing a cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, a liver-targeting ligand can be linked to a cationic lipid through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, *e.g.*, blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It can be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

i. Redox cleavable linking groups

In one embodiment, a cleavable linking group is a redox cleavable linking group that is cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, *e.g.*, a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In one, candidate compounds are cleaved by at most about 10% in the blood. In other embodiments, useful candidate compounds are degraded at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

ii. Phosphate-based cleavable linking groups

In another embodiment, a cleavable linker comprises a phosphate-based cleavable linking group. A phosphate-based cleavable linking group is cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-

P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

iii. Acid cleavable linking groups

In another embodiment, a cleavable linker comprises an acid cleavable linking group. An *a*cid cleavable linking group is a linking group that is cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (*e.g.*, about 6.0, 5.75, 5.5, 5.25, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

iv. Ester-based linking groups

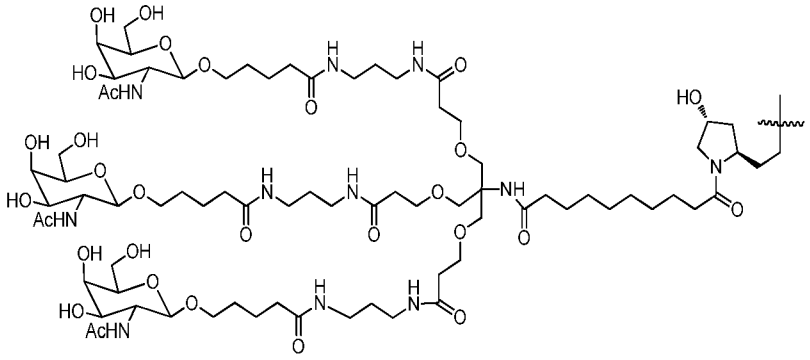
In another embodiment, a cleavable linker comprises an ester-based cleavable linking group. An ester-based cleavable linking group is cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

v. Peptide-based cleaving groups

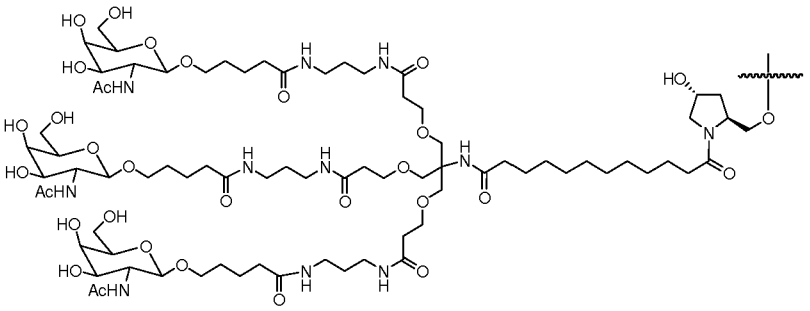
In yet another embodiment, a cleavable linker comprises a peptide-based cleavable linking group. A peptide-based cleavable linking group is cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (*e.g.*, dipeptides, tripeptides *etc.*) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (*i.e.*, the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general

formula – NHCHRAC(O)NHCHRBC(O)-, where RA and RB are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

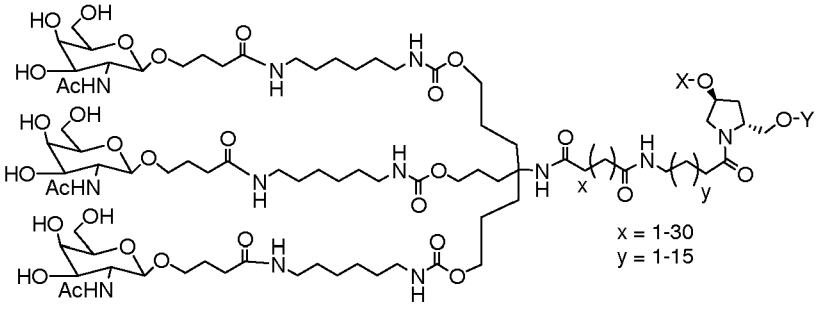
In one embodiment, an iRNA of the invention is conjugated to a carbohydrate through a linker. Non-limiting examples of iRNA carbohydrate conjugates with linkers of the compositions and methods of the invention include, but are not limited to,



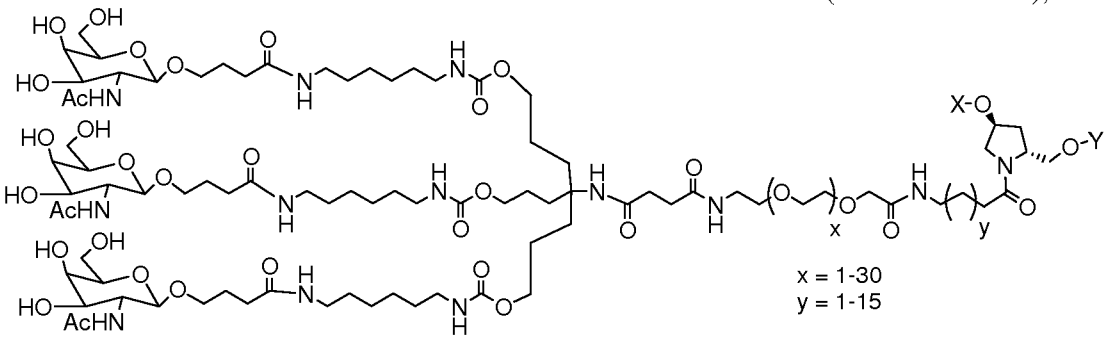
(Formula XXIV),



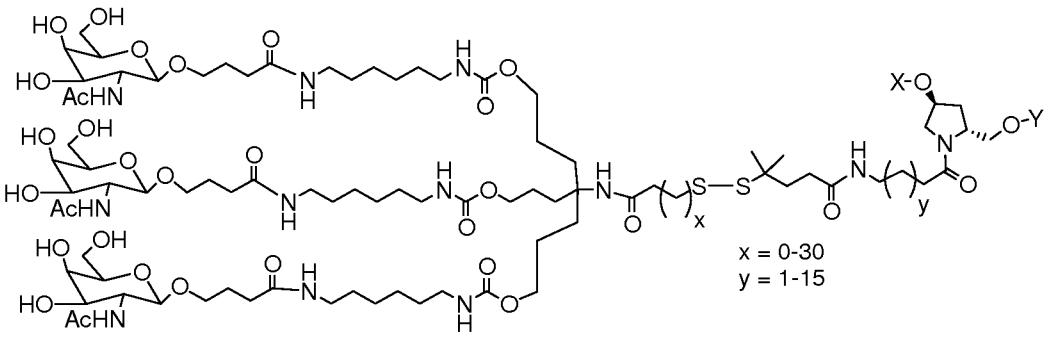
(Formula XXV),



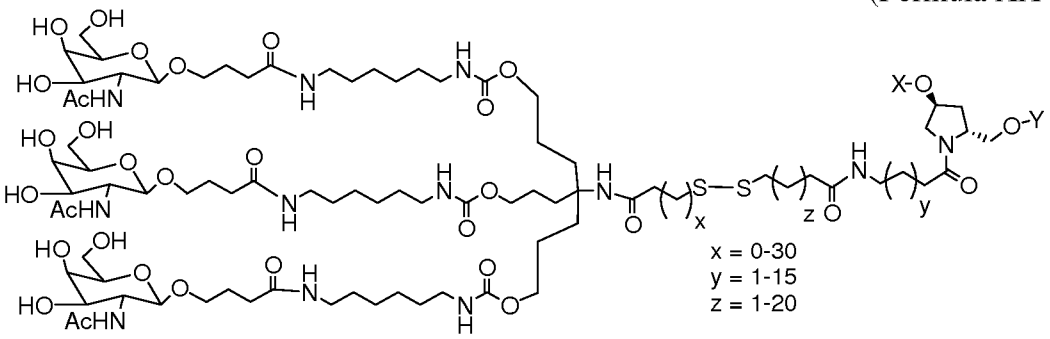
(Formula XXVI),



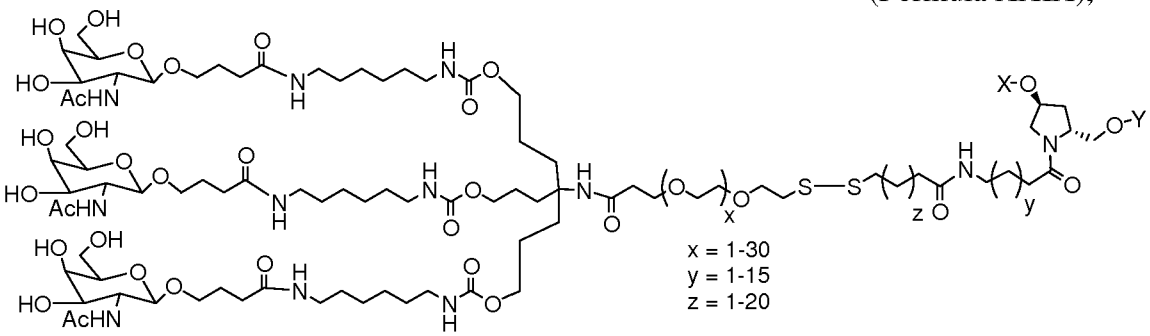
(Formula XXVII),



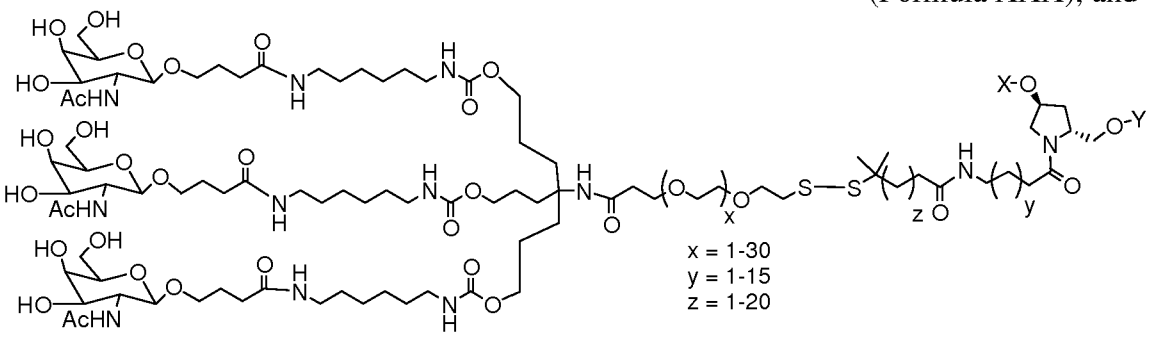
(Formula XXVIII),



(Formula XXIX),



(Formula XXX), and



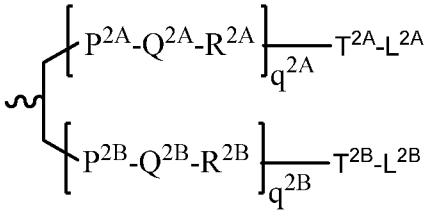
(Formula XXXI),

when one of X or Y is an oligonucleotide, the other is a hydrogen.

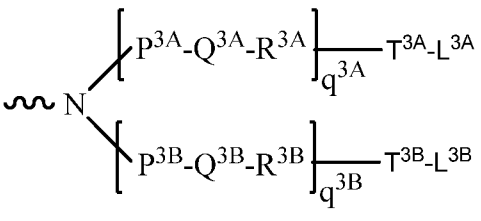
In certain embodiments of the compositions and methods of the invention, a ligand is one or more "GalNAc" (N-acetylgalactosamine) derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, a dsRNA of the invention is conjugated to a bivalent or trivalent branched linker selected from the group of structures shown in any of formula (XXXII) – (XXXV):

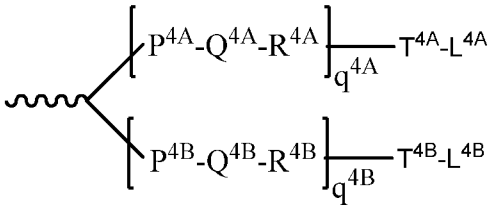
Formula XXXII



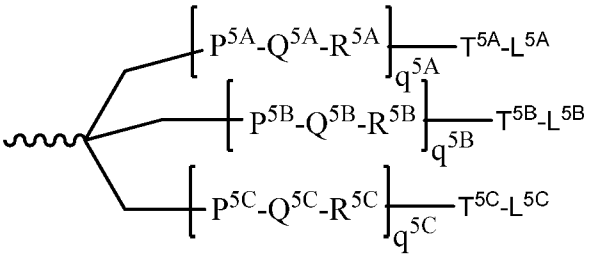
Formula XXXIII



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Formula XXXIV

Formula XXXV

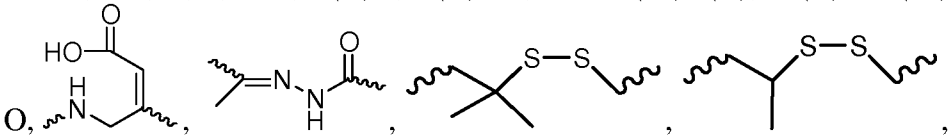
wherein:

q2A, q2B, q3A, q3B, q4A, q4B, q5A, q5B and q5C represent independently for each occurrence 0-20 and wherein the repeating unit can be the same or different;

P^{2A}, P^{2B}, P^{3A}, P^{3B}, P^{4A}, P^{4B}, P^{5A}, P^{5B}, P^{5C}, T^{2A}, T^{2B}, T^{3A}, T^{3B}, T^{4A}, T^{4B}, T^{4A}, T^{5B}, T^{5C} are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH or CH_2O;

Q^{2A}, Q^{2B}, Q^{3A}, Q^{3B}, Q^{4A}, Q^{4B}, Q^{5A}, Q^{5B}, Q^{5C} are independently for each occurrence absent, alkylene, substituted alkylene wherin one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO_2, $N(R^N)$, C(R')=C(R''), C≡C or C(O);

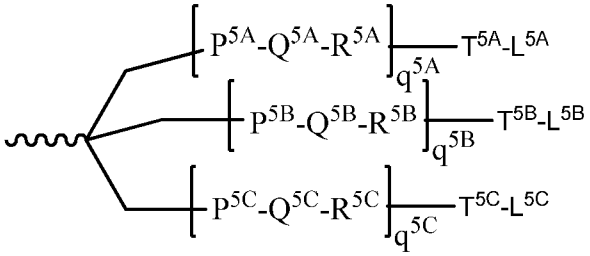
R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C} are each independently for each occurrence absent, NH, O, S, CH_2, C(O)O, C(O)NH, $NHCH(R^a)C(O)$, -C(O)-$CH(R^a)$-NH-, CO, CH=N-



or heterocyclyl;

L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B} and L^{5C} represent the ligand; *i.e.* each independently for each occurrence a monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; andR^a is H or amino acid side chain.Trivalent conjugating GalNAc derivatives are particularly useful for use with RNAi agents for inhibiting the expression of a target gene, such as those of formula (XXXVI):

Formula XXXVI



wherein L^{5A}, L^{5B} and L^{5C} represent a monosaccharide, such as GalNAc derivative.

Examples of suitable bivalent and trivalent branched linker groups conjugating GalNAc derivatives include, but are not limited to, the structures recited above as formulas II, VII, XI, X, and XIII.

Representative U.S. patents that teach the preparation of RNA conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; 8,106,022, the entire contents of each of which are hereby incorporated herein by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single compound or even at a single nucleoside within an iRNA. The present invention also includes iRNA compounds that are chimeric compounds.

"Chimeric" iRNA compounds or "chimeras," in the context of this invention, are iRNA compounds, preferably dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of a dsRNA compound. These iRNAs typically contain at least one region wherein the RNA is modified so as to confer upon the iRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the iRNA can serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of iRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter iRNAs when chimeric

dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the RNA of an iRNA can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to iRNAs in order to enhance the activity, cellular distribution or cellular uptake of the iRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Kubo, T. *et al., Biochem. Biophys. Res. Comm.*, 2007, 365(1):54-61; Letsinger *et al., Proc. Natl. Acad. Sci. USA*, 1989, 86:6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan *et al., Bioorg. Med. Chem. Let.*, 1993, 3:2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al., EMBO J.*, 1991, 10:111; Kabanov *et al., FEBS Lett.*, 1990, 259:327; Svinarchuk *et al., Biochimie*, 1993, 75:49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.*, 1995, 36:3651; Shea *et al., Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al., J. Pharmacol. Exp. Ther.*, 1996, 277:923). Representative United States patents that teach the preparation of such RNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of an RNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction can be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

V. Delivery of an iRNA of the Invention

The delivery of an iRNA of the invention to a cell *e.g.,* a cell within a subject, such as a human subject (*e.g.,* a subject in need thereof, such as a subject having a disease, disorder or condition associated with HBV infection) can be achieved in a number of different ways. For example, delivery may be performed by contacting a cell with an iRNA of the invention either *in vitro* or *in vivo*. *In vivo* delivery may also be performed directly by administering a composition comprising an iRNA, *e.g.,* a dsRNA, to a subject. Alternatively, *in vivo* delivery may be performed indirectly by administering one or more vectors that encode and direct the expression of the iRNA. These alternatives are discussed further below.

In general, any method of delivering a nucleic acid molecule (*in vitro* or *in vivo*) can be adapted for use with an iRNA of the invention (see *e.g.*, Akhtar S. and Julian RL. (1992) *Trends Cell. Biol.* 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties). For *in vivo* delivery, factors to consider in order to deliver an iRNA molecule include, for example, biological stability of the delivered molecule, prevention of non-specific effects, and accumulation of the delivered molecule in the target tissue. The non-specific effects of an iRNA can be minimized by local administration, for example, by direct injection or implantation into a tissue or topically administering the preparation. Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that can otherwise be harmed by the agent or that can degrade the agent, and permits a lower total dose of the iRNA molecule to be administered. Several studies have shown successful knockdown of gene products when an iRNA is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ., *et al* (2004) *Retina* 24:132-138) and subretinal injections in mice (Reich, SJ., *et al* (2003) *Mol. Vis.* 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a dsRNA in mice reduces tumor volume (Pille, J., *et al* (2005) *Mol. Ther.*11:267-274) and can prolong survival of tumor-bearing mice (Kim, WJ., *et al* (2006) *Mol. Ther.* 14:343-350; Li, S., *et al* (2007) *Mol. Ther.* 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G., *et al.* (2004) *Nucleic Acids* 32:e49; Tan, PH., *et al* (2005) *Gene Ther.* 12:59-66; Makimura, H., *et al* (2002) *BMC Neurosci.* 3:18; Shishkina, GT., *et al* (2004) *Neuroscience* 129:521-528; Thakker, ER., *et al* (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101:17270-17275; Akaneya,Y., *et al* (2005) *J. Neurophysiol.* 93:594-602) and to the lungs by intranasal administration (Howard, KA., *et al* (2006) *Mol. Ther.* 14:476-484; Zhang, X., *et al* (2004) *J. Biol. Chem.* 279:10677-10684; Bitko, V., *et al* (2005) *Nat. Med.* 11:50-55). For administering an iRNA systemically for the treatment of a disease, the RNA can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the dsRNA by endo- and exo-nucleases *in vivo*. Modification of the RNA or the pharmaceutical carrier can also permit targeting of the iRNA composition to the target tissue and avoid undesirable off-target effects. iRNA molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, an iRNA directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and jejunum (Soutschek, J., *et al* (2004) *Nature* 432:173-178). Conjugation of an iRNA to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO., *et al* (2006) *Nat. Biotechnol.* 24:1005-1015). In an alternative embodiment, the iRNA can be delivered using

drug delivery systems such as a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of an iRNA molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an iRNA by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an iRNA, or induced to form a vesicle or micelle (see *e.g.*, Kim SH., *et al* (2008) *Journal of Controlled Release* 129(2):107-116) that encases an iRNA. The formation of vesicles or micelles further prevents degradation of the iRNA when administered systemically. Methods for making and administering cationic- iRNA complexes are well within the abilities of one skilled in the art (see *e.g.*, Sorensen, DR., *et al* (2003) *J. Mol. Biol* 327:761-766; Verma, UN., *et al* (2003) Clin. *Cancer Res.* 9:1291-1300; Arnold, AS *et al* (2007) *J. Hypertens.* 25:197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of iRNAs include DOTAP (Sorensen, DR., *et al* (2003), supra; Verma, UN., *et al* (2003), *supra*), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS., *et al* (2006) *Nature* 441:111-114), cardiolipin (Chien, PY., *et al* (2005) *Cancer Gene Ther.* 12:321-328; Pal, A., *et al* (2005) *Int J. Oncol.* 26:1087-1091), polyethyleneimine (Bonnet ME., *et al* (2008) *Pharm. Res.* Aug 16 Epub ahead of print; Aigner, A. (2006) *J. Biomed. Biotechnol.* 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) *Mol. Pharm.* 3:472-487), and polyamidoamines (Tomalia, DA., *et al* (2007) *Biochem. Soc. Trans.* 35:61-67; Yoo, H., *et al* (1999) *Pharm. Res.* 16:1799-1804). In some embodiments, an iRNA forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of iRNAs and cyclodextrins can be found in U.S. Patent No. 7,427,605, which is herein incorporated by reference in its entirety.

A. Vector encoded iRNAs of the Invention

iRNA targeting the HBV gene can be expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al.*, *TIG.* (1996), 12:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). Expression can be transient (on the order of hours to weeks) or sustained (weeks to months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:1292).

The individual strand or strands of an iRNA can be transcribed from a promoter on an expression vector. Where two separate strands are to be expressed to generate, for example, a dsRNA, two separate expression vectors can be co-introduced (*e.g.*, by transfection or infection) into a target cell. Alternatively each individual strand of a dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one

embodiment, a dsRNA is expressed as inverted repeat polynucleotides joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

iRNA expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of an iRNA as described herein. Eukaryotic cell expression vectors are well known in the art and are available from a number of commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired nucleic acid segment. Delivery of iRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

iRNA expression plasmids can be transfected into target cells as a complex with cationic lipid carriers (*e.g.,* Oligofectamine) or non-cationic lipid-based carriers (*e.g.,* Transit-TKO™). Multiple lipid transfections for iRNA-mediated knockdowns targeting different regions of a target RNA over a period of a week or more are also contemplated by the invention. Successful introduction of vectors into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of cells *ex vivo* can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (*e.g.*, antibiotics and drugs), such as hygromycin B resistance.

Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, *etc.*; (c) adeno- associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, *e.g.*, vaccinia virus vectors or avipox, *e.g.* canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. Replication-defective viruses can also be advantageous. Different vectors will or will not become incorporated into the cells' genome. The constructs can include viral sequences for transfection, if desired. Alternatively, the construct can be incorporated into vectors capable of episomal replication, *e.g.* EPV and EBV vectors. Constructs for the recombinant expression of an iRNA will generally require regulatory elements, *e.g.*, promoters, enhancers, *etc.*, to ensure the expression of the iRNA in target cells. Other aspects to consider for vectors and constructs are further described below.

Vectors useful for the delivery of an iRNA will include regulatory elements (promoter, enhancer, *etc.*) sufficient for expression of the iRNA in the desired target cell or tissue. The regulatory elements can be chosen to provide either constitutive or regulated/inducible expression.

Expression of the iRNA can be precisely regulated, for example, by using an inducible regulatory sequence that is sensitive to certain physiological regulators, *e.g.*, circulating glucose levels, or hormones (Docherty *et al.*, 1994, *FASEB J.* 8:20-24). Such inducible expression systems, suitable for the control of dsRNA expression in cells or in mammals include, for example, regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-beta-D1 - thiogalactopyranoside (IPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the iRNA transgene.

Viral vectors that contain nucleic acid sequences encoding an iRNA can be used. For example, a retroviral vector can be used (see Miller *et al.*, *Meth. Enzymol.* 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding an iRNA are cloned into one or more vectors, which facilitate delivery of the nucleic acid into a patient. More detail about retroviral vectors can be found, for example, in Boesen *et al.*, *Biotherapy* 6:291-302 (1994), which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, *J. Clin. Invest.* 93:644-651 (1994); Kiem *et al.*, *Blood* 83:1467-1473 (1994); Salmons and Gunzberg, *Human Gene Therapy* 4:129-141 (1993); and Grossman and Wilson, *Curr. Opin. in Genetics and Devel.* 3:110-114 (1993). Lentiviral vectors contemplated for use include, for example, the HIV based vectors described in U.S. Patent Nos. 6,143,520; 5,665,557; and 5,981,276, which are herein incorporated by reference.

Adenoviruses are also contemplated for use in delivery of iRNAs of the invention. Adenoviruses are especially attractive vehicles, *e.g.*, for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, *Current Opinion in Genetics and Development* 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout *et al.*, *Human Gene Therapy* 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, *Science* 252:431-434 (1991); Rosenfeld *et al.*, *Cell* 68:143-155 (1992); Mastrangeli *et al.*, *J. Clin. Invest.* 91:225-234 (1993); PCT Publication WO94/12649; and Wang, *et al.*, *Gene Therapy* 2:775-783 (1995). A suitable AV vector for expressing an iRNA featured in the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H *et al.* (2002), *Nat. Biotech.* 20: 1006-1010.

Adeno-associated virus (AAV) vectors may also be used to delivery an iRNA of the invention (Walsh *et al.*, Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146). In one embodiment, the iRNA can be expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector having, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter. Suitable AAV vectors for expressing the dsRNA featured in the invention, methods for constructing the recombinant AV vector, and methods for delivering the vectors into target cells are described in Samulski R *et al.* (1987), *J. Virol.* 61: 3096-3101; Fisher K J *et al.* (1996), *J. Virol*, 70: 520-532; Samulski R *et al.* (1989), *J. Virol.* 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

Another viral vector suitable for delivery of an iRNA of the inevtion is a pox virus such as a vaccinia virus, for example an attenuated vaccinia such as Modified Virus Ankara (MVA) or NYVAC, an avipox such as fowl pox or canary pox.

The tropism of viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate. For example, lentiviral vectors can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors can be made to target different cells by engineering the vectors to express different capsid protein serotypes; see, *e.g.*, Rabinowitz J E *et al.* (2002), *J Virol* 76:791-801, the entire disclosure of which is herein incorporated by reference.

The pharmaceutical preparation of a vector can include the vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

VI. Pharmaceutical Compositions of the Invention

The present invention also includes pharmaceutical compositions and formulations which include the iRNAs of the invention. In one embodiment, provided herein are pharmaceutical compositions containing an iRNA, as described herein, and a pharmaceutically acceptable carrier. The pharmaceutical compositions containing the iRNA are useful for treating a disease or disorder associated with the expression or activity of an HBV gene. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration *via* parenteral delivery, *e.g.*, by subcutaneous (SC) or intravenous (IV) delivery. Another example is

compositions that are formulated for direct delivery into the brain parenchyma, *e.g.*, by infusion into the brain, such as by continuous pump infusion. The pharmaceutical compositions of the invention may be administered in dosages sufficient to inhibit expression of an HBV gene.

In one embodiment, an iRNA agent of the invention is administered to a subject as a weight-based dose. A "weight-based dose" (*e.g.,* a dose in mg/kg) is a dose of the iRNA agent that will change depending on the subject's weight. In another embodiement, an iRNA agent is administered to a subject as a fixed dose. A "fixed dose" (*e.g.,* a dose in mg) means that one dose of an iRNA agent is used for all subjects regardless of any specific subject-related factors, such as weight. In one particular embodiment, a fixed dose of an iRNA agent of the invention is based on a predetermined weight or age.

In general, a suitable dose of an iRNA of the invention will be in the range of about 0.001 to about 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of about 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at about 0.01 mg/kg, about 0.05 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 3 mg/kg, about 10 mg/kg, about 20 mg/kg, about 30 mg/kg, about 40 mg/kg, or about 50 mg/kg per single dose.

For example, the dsRNA may be administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, or about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In another embodiment, the dsRNA is administered at a dose of about 0.1 to about 50 mg/kg, about 0.25 to about 50 mg/kg, about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/kg, about 1.5 to about 50 mg/kg, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.1 to about 45 mg/kg, about 0.25 to about 45 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/kg, about 1.5 to about 45 mg/kg, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg,

about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.1 to about 40 mg/kg, about 0.25 to about 40 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/kg, about 1.5 to about 40 mg/kg, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.1 to about 30 mg/kg, about 0.25 to about 30 mg/kg, about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/kg, about 1.5 to about 30 mg/kg, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.1 to about 20 mg/kg, about 0.25 to about 20 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/kg, about 1.5 to about 20 mg/kg, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, the dsRNA may be administered at a dose of about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, or about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In another embodiment, the dsRNA is administered at a dose of about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/kg, about 1.5 to about 50 mg/kg, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/kg, about 1.5 to about 45

mg/kg, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg, about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/kg, about 1.5 to about 40 mg/kg, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/kg, about 1.5 to about 30 mg/kg, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/kg, about 1.5 to about 20 mg/kg, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. In one embodiment, the dsRNA is administered at a dose of about 10mg/kg to about 30 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, subjects can be administered, *e.g.,* subcutaneously or intravenously, a single therapeutic amount of iRNA, such as about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In some embodiments, subjects are administered, *e.g.,* subcutaneously or intravenously, multiple doses of a therapeutic amount of iRNA, such as a dose about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. A multi-dose regimine may include administration of a therapeutic amount of iRNA daily, such as for two days, three days, four days, five days, six days, seven days, or longer.

In other embodiments, subjects are administered, *e.g.,* subcutaneously or intravenously, a repeat dose of a therapeutic amount of iRNA, such as a dose about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. A repeat-dose regimine may include administration of a therapeutic amount of iRNA on a regular basis, such as every other day, every third day, every fourth day, twice a week, once a week, every other week, or once a month.

In certain embodiments, for example, when a composition of the invention comprises a dsRNA as described herein and a lipid, subjects can be administered a therapeutic amount of iRNA, such as about 0.01 mg/kg to about 5 mg/kg, about 0.01 mg/kg to about 10 mg/kg, about 0.05 mg/kg to about 5 mg/kg, about 0.05 mg/kg to about 10 mg/kg, about 0.1 mg/kg to about 5 mg/kg, about 0.1 mg/kg to about 10 mg/kg, about 0.2 mg/kg to about 5 mg/kg, about 0.2 mg/kg to about 10 mg/kg, about 0.3 mg/kg to about 5 mg/kg, about 0.3 mg/kg to about 10 mg/kg, about 0.4 mg/kg to about 5 mg/kg, about 0.4 mg/kg to about 10 mg/kg, about 0.5 mg/kg to about 5 mg/kg, about 0.5 mg/kg to about 10 mg/kg, about 1 mg/kg to about 5 mg/kg, about 1 mg/kg to about 10 mg/kg, about 1.5 mg/kg to about 5 mg/kg, about 1.5 mg/kg

to about 10 mg/kg, about 2 mg/kg to about about 2.5 mg/kg, about 2 mg/kg to about 10 mg/kg, about 3 mg/kg to about 5 mg/kg, about 3 mg/kg to about 10 mg/kg, about 3.5 mg/kg to about 5 mg/kg, about 4 mg/kg to about 5 mg/kg, about 4.5 mg/kg to about 5 mg/kg, about 4 mg/kg to about 10 mg/kg, about 4.5 mg/kg to about 10 mg/kg, about 5 mg/kg to about 10 mg/kg, about 5.5 mg/kg to about 10 mg/kg, about 6 mg/kg to about 10 mg/kg, about 6.5 mg/kg to about 10 mg/kg, about 7 mg/kg to about 10 mg/kg, about 7.5 mg/kg to about 10 mg/kg, about 8 mg/kg to about 10 mg/kg, about 8.5 mg/kg to about 10 mg/kg, about 9 mg/kg to about 10 mg/kg, or about 9.5 mg/kg to about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, the dsRNA may be administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, or about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In certain embodiments of the invention, for example, when a double-stranded RNAi agent includes a modification (*e.g.,* one or more motifs of three identical modifications on three consecutive nucleotides), including one such motif at or near the cleavage site of the agent, six phosphorothioate linkages, and a ligand, such an agent is administered at a dose of about 0.01 to about 0.5 mg/kg, about 0.01 to about 0.4 mg/kg, about 0.01 to about 0.3 mg/kg, about 0.01 to about 0.2 mg/kg, about 0.01 to about 0.1 mg/kg, about 0.01 mg/kg to about 0.09 mg/kg, about 0.01 mg/kg to about 0.08 mg/kg, about 0.01 mg/kg to about 0.07 mg/kg, about 0.01 mg/kg to about 0.06 mg/kg, about 0.01 mg/kg to about 0.05 mg/kg, about 0.02 to about 0.5 mg/kg, about 0.02 to about 0.4 mg/kg, about 0.02 to about 0.3 mg/kg, about 0.02 to about 0.2 mg/kg, about 0.02 to about 0.1 mg/kg, about 0.02 mg/kg to about 0.09 mg/kg, about 0.02 mg/kg to about 0.08 mg/kg, about 0.02 mg/kg to about 0.07 mg/kg, about 0.02 mg/kg to about 0.06 mg/kg, about 0.02 mg/kg to about 0.05 mg/kg, about 0.03 to about 0.5 mg/kg, about 0.03 to about 0.4 mg/kg, about 0.03 to about 0.3 mg/kg, about 0.03 to about 0.2 mg/kg, about 0.03 to about 0.1 mg/kg, about 0.03 mg/kg to about 0.09 mg/kg, about 0.03 mg/kg to about 0.08 mg/kg, about 0.03 mg/kg to about 0.07 mg/kg, about 0.03 mg/kg to about 0.06 mg/kg, about 0.03 mg/kg to about 0.05 mg/kg, about 0.04 to about 0.5 mg/kg, about 0.04 to about 0.4 mg/kg, about 0.04 to about 0.3 mg/kg, about 0.04 to about 0.2 mg/kg, about 0.04 to about 0.1 mg/kg, about 0.04 mg/kg to about 0.09 mg/kg, about 0.04 mg/kg to about 0.08 mg/kg, about 0.04 mg/kg to about 0.07 mg/kg, about 0.04 mg/kg to about 0.06 mg/kg, about 0.05 to about 0.5 mg/kg, about 0.05 to about 0.4 mg/kg, about 0.05 to about 0.3 mg/kg, about 0.05 to about 0.2 mg/kg, about 0.05 to about 0.1 mg/kg, about 0.05 mg/kg to about 0.09 mg/kg, about 0.05 mg/kg to about 0.08 mg/kg, or about 0.05 mg/kg to about 0.07 mg/kg.

Values and ranges intermediate to the foregoing recited values are also intended to be part of this invention, *e.g.*, the RNAi agent may be administered to the subject at a dose of about 0.015 mg/kg to about 0.45 mg/kg.

For example, the RNAi agent, *e.g.*, RNAi agent in a pharmaceutical composition, may be administered at a dose of about 0.01 mg/kg, 0.0125 mg/kg, 0.015 mg/kg, 0.0175 mg/kg, 0.02 mg/kg, 0.0225 mg/kg, 0.025 mg/kg, 0.0275 mg/kg, 0.03 mg/kg, 0.0325 mg/kg, 0.035 mg/kg, 0.0375 mg/kg, 0.04 mg/kg, 0.0425 mg/kg, 0.045 mg/kg, 0.0475 mg/kg, 0.05 mg/kg, 0.0525 mg/kg, 0.055 mg/kg, 0.0575 mg/kg, 0.06 mg/kg, 0.0625 mg/kg, 0.065 mg/kg, 0.0675 mg/kg, 0.07 mg/kg, 0.0725 mg/kg, 0.075 mg/kg, 0.0775 mg/kg, 0.08 mg/kg, 0.0825 mg/kg, 0.085 mg/kg, 0.0875 mg/kg, 0.09 mg/kg, 0.0925 mg/kg, 0.095 mg/kg, 0.0975 mg/kg, 0.1 mg/kg, 0.125 mg/kg, 0.15 mg/kg, 0.175 mg/kg, 0.2 mg/kg, 0.225 mg/kg, 0.25 mg/kg, 0.275 mg/kg, 0.3 mg/kg, 0.325 mg/kg, 0.35 mg/kg, 0.375 mg/kg, 0.4 mg/kg, 0.425 mg/kg, 0.45 mg/kg, 0.475 mg/kg, or about 0.5 mg/kg. Values intermediate to the foregoing recited values are also intended to be part of this invention.

In some embodiments, the RNAi agent is administered as a fixed dose of between about 100 mg to about 900 mg, e.g., between about 100 mg to about 850 mg, between about 100 mg to about 800 mg, between about 100 mg to about 750 mg, between about 100 mg to about 700 mg, between about 100 mg to about 650 mg, between about 100 mg to about 600 mg, between about 100 mg to about 550 mg, between about 100 mg to about 500 mg, between about 200 mg to about 850 mg, between about 200 mg to about 800 mg, between about 200 mg to about 750 mg, between about 200 mg to about 700 mg, between about 200 mg to about 650 mg, between about 200 mg to about 600 mg, between about 200 mg to about 550 mg, between about 200 mg to about 500 mg, between about 300 mg to about 850 mg, between about 300 mg to about 800 mg, between about 300 mg to about 750 mg, between about 300 mg to about 700 mg, between about 300 mg to about 650 mg, between about 300 mg to about 600 mg, between about 300 mg to about 550 mg, between about 300 mg to about 500 mg, between about 400 mg to about 850 mg, between about 400 mg to about 800 mg, between about 400 mg to about 750 mg, between about 400 mg to about 700 mg, between about 400 mg to about 650 mg, between about 400 mg to about 600 mg, between about 400 mg to about 550 mg, or between about 400 mg to about 500 mg.

In some embodiments, the RNAi agent is administered as a fixed dose of about 100 mg, about 125 mg, about 150 mg, about 175 mg, 200 mg, about 225 mg, about 250 mg, about 275 mg, about 300 mg, about 325 mg, about 350 mg, about 375 mg, about 400 mg, about 425 mg, about 450 mg, about 475 mg, about 500 mg, about 525 mg, about 550 mg, about 575 mg, about 600 mg, about 625 mg, about 650 mg, about 675 mg, about 700 mg, about 725 mg, about 750 mg, about 775 mg, about 800 mg, about 825 mg, about 850 mg, about 875 mg, or about 900 mg.

The pharmaceutical composition can be administered by intravenous infusion over a period of time, such as over a 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21, 22, 23, 24, or about a 25 minute period. The administration may be repeated, for example, on a regular basis, such as weekly, biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration weekly or biweekly for three months, administration can be repeated once per month, for six months or a year or longer.

The pharmaceutical composition can be administered once daily, or the iRNA can be administered as two, three, or more sub-doses at appropriate intervals throughout the day or even using continuous infusion or delivery through a controlled release formulation. In that case, the iRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the iRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

In other embodiments, a single dose of the pharmaceutical compositions can be long lasting, such that subsequent doses are administered at not more than 3, 4, or 5 day intervals, or at not more than 1, 2, 3, or 4 week intervals. In some embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered once per week. In other embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered bi-monthly. In some embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered once per month, once every other month, or once quarterly (*i.e.*, every three months).

The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and *in vivo* half-lives for the individual iRNAs encompassed by the invention can be made using conventional methodologies or on the basis of *in vivo* testing using an appropriate animal model, as described elsewhere herein.

The pharmaceutical compositions of the present invention can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration can be topical (*e.g.*, by a transdermal patch), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer;

intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; subdermal, *e.g.*, via an implanted device; or intracranial, *e.g.*, by intraparenchymal, intrathecal or intraventricular, administration.

The iRNA can be delivered in a manner to target a particular tissue, such as the liver (*e.g.*, the hepatocytes of the liver).

Pharmaceutical compositions and formulations for topical administration can include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like can be necessary or desirable. Coated condoms, gloves and the like can also be useful. Suitable topical formulations include those in which the iRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (*e.g.,* dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (*e.g.,* dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.,* dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). iRNAs featured in the invention can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, iRNAs can be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-20} alkyl ester (*e.g.,* isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof). Topical formulations are described in detail in U.S. Patent No. 6,747,014, which is incorporated herein by reference.

A. iRNA Formulations Comprising Membranous Molecular Assemblies

An iRNA for use in the compositions and methods of the invention can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term "liposome" refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the iRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the iRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of

the liposome and cell progresses, the internal aqueous contents that include the iRNA are delivered into the cell where the iRNA can specifically bind to a target RNA and can mediate iRNA. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the iRNA to particular cell types.

A liposome containing an iRNA agent can be prepared by a variety of methods. In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The iRNA agent preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the iRNA agent and condense around the iRNA agent to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of iRNA agent.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also adjusted to favor condensation.

Methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are further described in, e.g., WO 96/37194, the entire contents of which are incorporated herein by reference. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al., Proc. Natl. Acad. Sci., USA* 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, e*t al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging iRNA agent preparations into liposomes.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged nucleic acid molecules to form a stable complex. The positively charged nucleic acid/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the

endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap nucleic acids rather than complex with it. Since both the nucleic acid and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver nucleic acids encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu *et al. S.T.P.Pharma. Sci.*, 1994, 4(6) 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing

gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, 1987, 223, 42; Wu *et al.*, *Cancer Research*, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al*).

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver iRNA agents to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated iRNA agents in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of iRNA agent (see, e.g., Felgner, P. L. et al., Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes

prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, e.g., U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., *Biochim. Biophys. Res. Commun.* 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. et al., *Biochim. Biophys. Acta* 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer iRNA agent into the skin. In some implementations, liposomes are used for delivering iRNA agent to epidermal cells and also to enhance the penetration of iRNA agent into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al., Journal of Drug Targeting,* 1992, vol. 2,405-410 and du Plessis *et al., Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., *Biotechniques* 6:682-690, 1988; Itani, T. *et al. Gene* 56:267-276. 1987; Nicolau, C. *et al. Meth. Enz.* 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. *Meth. Enz.* 101:512-527, 1983; Wang, C. Y. and Huang, L., P*roc. Natl. Acad. Sci. USA* 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and

cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with iRNA agent are useful for treating a dermatological disorder.

Liposomes that include iRNA can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include iRNA agent can be delivered, for example, subcutaneously by infection in order to deliver iRNA agent to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present invention are described in, for example, PCT Publication No. WO 2008/042973, the entire contents of which are incorporated herein by reference.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes can be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.,* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in “Pharmaceutical Dosage Forms”, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms", Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

The iRNA for use in the methods of the invention can also be provided as micellar formulations. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof,

glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the siRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the siRNA composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.,* there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.,* through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

Propellants may include hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. In certain embodiments, HFA 134a (1,1,1,2 tetrafluoroethane) may be used.

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.,* at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

B. *Lipid particles*

iRNAs, *e.g.,* dsRNAs of in the invention may be fully encapsulated in a lipid formulation, *e.g.,* a LNP, or other nucleic acid-lipid particle.

As used herein, the term "LNP" refers to a stable nucleic acid-lipid particle. LNPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). LNPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.)

injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). LNPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid- lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; U.S. Publication No. 2010/0324120 and PCT Publication No. WO 96/40964.

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. Ranges intermediate to the above recited ranges are also contemplated to be part of the invention.

The cationic lipid can be, for example, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(I -(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(I -(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-DiLinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-Dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1), or a mixture thereof. The cationic lipid can

comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle.

In another embodiment, the compound 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane can be used to prepare lipid-siRNA nanoparticles. Synthesis of 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

In one embodiment, the lipid-siRNA particle includes 40% 2, 2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 siRNA/Lipid Ratio.

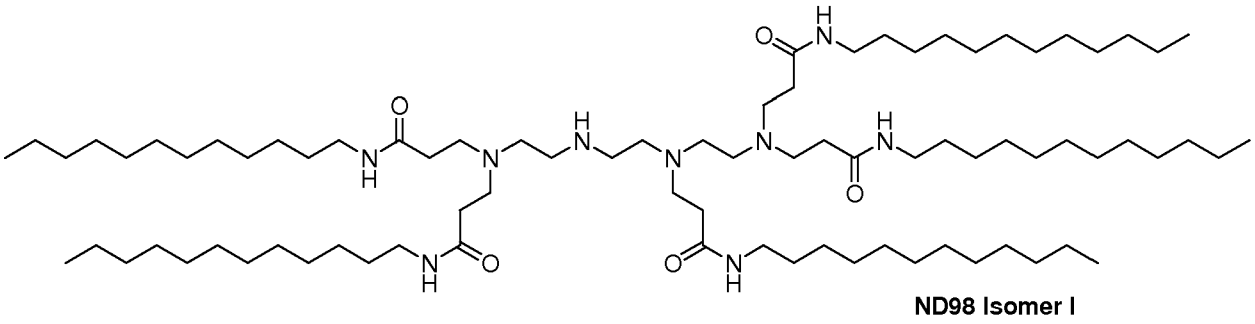
The ionizable/non-cationic lipid can be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl- phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1 -trans PE, 1 -stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid can be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

The conjugated lipid that inhibits aggregation of particles can be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate can be, for example, a PEG-dilauryloxypropyl (Ci_2), a PEG-dimyristyloxypropyl (Ci_4), a PEG-dipalmityloxypropyl (Ci_6), or a PEG-distearyloxypropyl ($C]_8$). The conjugated lipid that prevents aggregation of particles can be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle.

In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, *e.g.*, about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in the particle.

In one embodiment, the lipidoid ND98·4HCl (MW 1487) (see U.S. Patent Application No. 12/056,230, filed 3/26/2008, which is incorporated herein by reference), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-dsRNA nanoparticles (*i.e.,* LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be

combined in a, *e.g.*, 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous dsRNA (*e.g.*, in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-dsRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (*e.g.*, 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, *e.g.*, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



Formula 1

LNP01 formulations are described, *e.g.*, in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-dsRNA formulations are described in Table 1.

Table 1

	Ionizable/Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio**
SNALP-1	l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
2-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1

LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1)	Tech G1/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 33:1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid:siRNA: 11:1

LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 12:1
LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:siRNA: 8:1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1

DSPC: distearoylphosphatidylcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyristoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000)

PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of 2000)

PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000)

SNALP (l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed April 15, 2009, which is hereby incorporated by reference.

XTC comprising formulations are described, *e.g.*, in PCT Publication No. WO 2010/088537, the entire contents of which are incorporated herein by reference.

MC3 comprising formulations are described, *e.g.*, in U.S. Publication No. 2010/0324120, filed June 10, 2010, the entire contents of which are incorporated herein by reference.

ALNY-100 comprising formulations are described, *e.g.*, PCT Publication No. WO 2010/054406, the entire contents of which are incorporated herein by reference.

C12-200 comprising formulations are described in PCT Publication No. WO 2010/129709, the entire contents of which are incorporated herein by reference.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders can be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancer surfactants and chelators. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.*, sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs featured in the invention can be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (*e.g.*, p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are

described in detail in U.S. Patent 6,887,906, US Publn. No. 20030027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration can include sterile aqueous solutions which can also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions can be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the liver when treating hepatic disorders such as hepatic carcinoma.

The pharmaceutical formulations of the present invention, which can conveniently be presented in unit dosage form, can be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention can be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention can also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions can further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension can also contain stabilizers.

C. Additional Formulations

i. Emulsions

The compositions of the present invention can be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1μm in diameter (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in Remington's Pharmaceutical

Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions can be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions can contain additional components in addition to the dispersed phases, and the active drug which can be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Pharmaceutical emulsions can also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion can be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that can be incorporated into either phase of the emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant

has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants can be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that can readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used can be free radical scavengers such as tocopherols, alkyl gallates, butylated

hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

ii. Microemulsions

In one embodiment of the present invention, the compositions of iRNAs and nucleic acids are formulated as microemulsions. A microemulsion can be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions can, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase can typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase can include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see e.g., U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides et al., Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see e.g., U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ho et al., J. Pharm. Sci.,

1996, 85, 138-143). Often microemulsions can form spontaneously when their components are brought together at ambient temperature. This can be particularly advantageous when formulating thermolabile drugs, peptides or iRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of iRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of iRNAs and nucleic acids.

Microemulsions of the present invention can also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the iRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention can be classified as belonging to one of five broad categories--surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

iii. Microparticles

An iRNA agent of the invention may be incorporated into a particle, e.g., a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques.

iv. Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly iRNAs, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs can cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers can be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of iRNAs

through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., J. Pharm. Pharmacol., 1988, 40, 252).

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C1-20 alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (see e.g., Touitou, E., et al. Enhancement in Drug Delivery, CRC Press, Danvers, MA, 2006; Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri et al., J. Pharm. Pharmacol., 1992, 44, 651-654).

The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (see e.g., Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579-583).

Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of iRNAs through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(see e.g., Katdare, A. et al., Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers, MA, 2006; Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51).

As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of iRNAs through the alimentary mucosa (see e.g., Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of iRNAs at the cellular level can also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of dsRNAs. Examples of commercially available transfection reagents include, for example Lipofectamine™ (Invitrogen; Carlsbad, CA), Lipofectamine 2000™ (Invitrogen; Carlsbad, CA), 293fectin™ (Invitrogen; Carlsbad, CA), Cellfectin™ (Invitrogen; Carlsbad, CA), DMRIE-C™ (Invitrogen; Carlsbad, CA), FreeStyle™ MAX (Invitrogen; Carlsbad, CA), Lipofectamine™ 2000 CD (Invitrogen; Carlsbad, CA), Lipofectamine™ (Invitrogen; Carlsbad, CA), iRNAMAX (Invitrogen; Carlsbad, CA), Oligofectamine™ (Invitrogen; Carlsbad, CA), Optifect™ (Invitrogen; Carlsbad, CA), X-tremeGENE Q2 Transfection Reagent (Roche; Grenzacherstrasse, Switzerland), DOTAP Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), DOSPER Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), or Fugene (Grenzacherstrasse, Switzerland), Transfectam® Reagent (Promega; Madison, WI),

TransFast™ Transfection Reagent (Promega; Madison, WI), Tfx™-20 Reagent (Promega; Madison, WI), Tfx™-50 Reagent (Promega; Madison, WI), DreamFect™ (OZ Biosciences; Marseille, France), EcoTransfect (OZ Biosciences; Marseille, France), TransPass[a] D1 Transfection Reagent (New England Biolabs; Ipswich, MA, USA), LyoVec™/LipoGen™ (Invitrogen; San Diego, CA, USA), PerFectin Transfection Reagent (Genlantis; San Diego, CA, USA), NeuroPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), GenePORTER Transfection reagent (Genlantis; San Diego, CA, USA), GenePORTER 2 Transfection reagent (Genlantis; San Diego, CA, USA), Cytofectin Transfection Reagent (Genlantis; San Diego, CA, USA), BaculoPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), TroganPORTER™ transfection Reagent (Genlantis; San Diego, CA, USA), RiboFect (Bioline; Taunton, MA, USA), PlasFect (Bioline; Taunton, MA, USA), UniFECTOR (B-Bridge International; Mountain View, CA, USA), SureFECTOR (B-Bridge International; Mountain View, CA, USA), or HiFect™ (B-Bridge International, Mountain View, CA, USA), among others.

Other agents can be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

v. Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., DsRNA Res. Dev., 1995, 5, 115-121; Takakura et al., DsRNA & Nucl. Acid Drug Dev., 1996, 6, 177-183.

vi. Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient can be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other

components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc).

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids can include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions can also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

vii. Other Components

The compositions of the present invention can additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions can contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or can contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions can contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension can also contain stabilizers.

In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more iRNA compounds and (b) one or more agents which function by a non-iRNA mechanism and which are useful in treating an HBV infection. Examples of such agents include, but are not lmited to antiviral agents aimed at suppressing or destroying HBV by interfering with viral replication; and immune modulators aimed at helping the human immune system mount a defence against the virus. In contrast, immune modulators, such as corticosteroids, which induce an enhanced expression of virus and viral antigens, and a suppression of T-lymphocyte function, or adenine arabinoside, acyclovir, or dideoxyinosine, are not beneficial for the treatment of chronic hepatitis B. Suitable agents are discussed in more detail below.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured herein in the invention lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (*e.g.*, achieving a decreased concentration of the polypeptide) that includes the IC50 (*i.*e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

In addition to their administration, as discussed above, the iRNAs featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by HBV expression. In any event, the administering physician can adjust the amount and timing of iRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

VII. Methods of the Invention

The present invention provides therapeutic and prophylactic methods which include administering to a subject having an HBV infection and/or HBV-associated disease, disorder, and/or condition, or prone to developing, an HBV-associated disease, disorder, and/or condition (*e.g.*, CHB), compositions comprising an iRNA agent, or pharmaceutical compositions comprising an iRNA agent, or vectors comprising an iRNA of the invention.

The methods of the invention are useful for treating a subject having an HBV infection, *e.g.,* a subject that would benefit from reduction in HBV gene expression and/or HBV replication. In one aspect, the present invention provides methods of reducing the level of Hepatis B virus ccc DNA in a subject infected with HBV. In another aspect, the present invention provides methods of reducing the level of HBV antigen, *e.g.,* HBsAg and/or HBeAg, in a subject infected with HBV. In another aspect, the present invention provides methods of reducing the viral load of HBV in a subject infected with HBV. The present invention also provides methods of reducing the level of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) in a subject infected with HBV. In one aspect, the present invention provides methods for increasing the level of anti-HBV antibodies in a subject infected with HBV. In another aspect, the present invention provides methods of treating a subject having an HBV infection. In one aspect, the present invention provides methods of treating a subject having an HBV-associated disease, *e.g.,* hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma. Furthermore, as HDV infection depends on obligatory helper functions provided by HBV for transmission, and subjects having an HBV infection may also have an HDV infection, the methods for treatment described herein are also useful for treating a subject having an HDV infection and/or an HDV-associated disorder, such as hepatitis B virus infection, chronic hepatitis B infection (CHB), chronic Hepatitis B infection (CHB), cirrhosis, liver failure, and hepatocellular carcinoma (HCC). The treatment methods (and uses) of the invention include administering to the subject, *e.g.,* a human, a therapeutically effective amount of an iRNA agent of the invention targeting an HBV gene or a pharmaceutical composition comprising an iRNA agent of the invention targeting an HBV gene or a vector of the invention comprising an iRNA agent targeting an HBV gene.

In one aspect, the invention provides methods of preventing at least one symptom in a subject having an HBV infection, *e.g.,* the presence of serum and/or liver HBV ccc DNA, the presence of serum HBV DNA the presence of serum and/or liver HBV antigen, *e.g.*, HBsAg and/or HBeAg, elevated ALT, elevated AST, the absence or low level of anti-HBV antibodies, a liver injury; cirrhosis; hepatitis D virus infection, delta hepatitis, acute hepatitis B; acute fulminant hepatitis B; chronic hepatitis B; liver fibrosis; end-stage liver disease; hepatocellular carcinoma; serum sickness–like syndrome; anorexia; nausea; vomiting, low-

grade fever; myalgia; fatigability; disordered gustatory acuity and smell sensations (aversion to food and cigarettes); and/or right upper quadrant and epigastric pain (intermittent, mild to moderate); hepatic encephalopathy; somnolence; disturbances in sleep pattern; mental confusion; coma; ascites; gastrointestinal bleeding; coagulopathy; jaundice; hepatomegaly (mildly enlarged, soft liver); splenomegaly; palmar erythema; spider nevi; muscle wasting; spider angiomas; vasculitis; variceal bleeding; peripheral edema; gynecomastia; testicular atrophy; abdominal collateral veins (caput medusa); high levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), within a range of 1000-2000 IU/mL, although values 100 times above the upper limit of normal (ULN) can be also be identified; ALT levels higher than AST levels; elevated gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) levels (*e.g.,* not more than 3 times the ULN); slightly low albumin levels; elevated serum iron levels; leukopenia (*i.e.,* granulocytopenia); lymphocytosis; increased erythrocyte sedimentation rate (ESR); shortened red blood cell survival; hemolysis; thrombocytopenia; a prolongation of the international normalized ratio (INR); the presence of serum and/ or liverHBsAg, HBeAg, Hepatitis B core antibody (anti-HBc) immunoglobulin M (IgM); hepatitis B surface antibody (anti-HBs), hepatitis B e antibody (anti-HBe), and/or HBV DNA; elevation of the aminotransferases (≤5 times the ULN); ALT levels higher than the AST levels; increased bilirubin levels, prolonged prothrombin time(PT); hyperglobulinemia; the presence of tissue-nonspecific antibodies, such as anti–smooth muscle antibodies (ASMAs) or antinuclear antibodies (ANAs) (10-20%); the presence of tissue-specific antibodies, such as antibodies against the thyroid gland (10-20%); elevated levels of rheumatoid factor (RF); hyperbilirubinemia, prolonged PT, low platelet and white blood cell counts, AST levels higher than ALT levels; elevated alkaline phosphatase (ALP) and GGT levels; lobular, with degenerative and regenerative hepatocellular changes, and accompanying inflammation; predominantly centrilobular necrosis. The methods include administering to the subject a therapeutically effective amount of the iRNA agent, *e.g.,* dsRNA, pharmaceutical compositions, or vectors of the invention, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in HBV gene expression, such as a subject having an HBV infection or a subject having both an HBV and an HDV infection.

In another aspect, the present invention provides uses of a therapeutically effective amount of an iRNA agent of the invention for treating a subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of HBV gene expression, such as a subject having an HBV infection or a subject having both an HBV and an HDV infection.

In a further aspect, the present invention provides uses of an iRNA agent, *e.g.*, a dsRNA, of the invention targeting an HBV gene or pharmaceutical composition comprising an iRNA agent targeting an HBV gene in the manufacture of a medicament for treating a subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of HBV gene

expression and/or HBV replication,such as a subject having an HBV infection or a subject having both an HBV and an HDV infection, and a subject having a disorder that would benefit from reduction in HBV gene expression, *e.g.*, a HBV-associated disease.

In another aspect, the invention provides uses of an iRNA, *e.g.*, a dsRNA, of the invention for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of HBV gene expression and/or HBV replication.

In a further aspect, the present invention provides uses of an iRNA agent of the invention in the manufacture of a medicament for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of HBV gene expression and/or HBV replication, such as a HBV-associated disease.

In one embodiment, an iRNA agent targeting HBV is administered to a subject having an HBV infection or both and HBV and an HDV infection, and/or an HBV-associated disease such that the expression of one or more HBV genes, HBV ccc DNA levels, HBV antigen levels, HBV viral load levels, ALT, and/or AST, *e.g.*, in a cell, tissue, blood or other tissue or fluid of the subject are reduced by at least about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 62%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more when the dsRNA agent is administered to the subject.

In one embodiment, an iRNA agent targeting HBV is administered to a subject having an HBV infection or both and HBV and an HDV infection, and/or an HBV-associated disease such that the level of anti-HBV antibodies, *e.g.*, in a cell, tissue, blood or other tissue or fluid of the subject are increased by at least about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 62%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more when the dsRNA agent is administered to the subject.

The methods and uses of the invention include administering a composition described herein such that expression of the target HBV gene is decreased, such as for about 1, 2, 3, 4 5, 6, 7, 8, 12, 16, 18, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, or about 80 hours. In one embodiment, expression of the target HBV gene is decreased for an extended duration,

e.g., at least about two, three, four, five, six, seven days or more, *e.g.*, about one week, two weeks, three weeks, or about four weeks or longer.

Administration of the dsRNA according to the methods and uses of the invention may result in a reduction of the severity, signs, symptoms, and/or markers of such diseases or disorders in a patient with an HBV infection or both and HBV and an HDV infection, and/or HBV-associated disease. By "reduction" in this context is meant a statistically significant decrease in such level. The reduction can be, for example, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100%.

Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. For example, efficacy of treatment of CHB may be assessed, for example, by periodic monitoring of viral load and transaminase levels. Comparison of the later readings with the initial readings provide a physician an indication of whether the treatment is effective. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of an iRNA targeting HBV or pharmaceutical composition thereof, "effective against" an HBV-associated disease indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as improvement of symptoms, a cure, a reduction in disease, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating HBV infection and/or an HBV-associated disease and the related causes.

A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given iRNA drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

Subjects can be administered a therapeutic amount of iRNA, such as about 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.15 mg/kg, 0.2 mg/kg, 0.25 mg/kg, 0.3 mg/kg, 0.35 mg/kg, 0.4 mg/kg, 0.45 mg/kg, 0.5 mg/kg, 0.55 mg/kg, 0.6 mg/kg, 0.65 mg/kg, 0.7 mg/kg, 0.75 mg/kg, 0.8 mg/kg, 0.85 mg/kg, 0.9 mg/kg, 0.95 mg/kg, 1.0 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2.0 mg/kg, 2.1 mg/kg, 2.2 mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg dsRNA, 2.6 mg/kg dsRNA, 2.7 mg/kg dsRNA, 2.8 mg/kg dsRNA, 2.9 mg/kg dsRNA, 3.0 mg/kg dsRNA, 3.1 mg/kg dsRNA, 3.2 mg/kg dsRNA, 3.3 mg/kg dsRNA, 3.4 mg/kg dsRNA, 3.5 mg/kg dsRNA, 3.6 mg/kg dsRNA, 3.7 mg/kg dsRNA, 3.8 mg/kg dsRNA, 3.9 mg/kg dsRNA, 4.0 mg/kg dsRNA, 4.1 mg/kg dsRNA, 4.2 mg/kg dsRNA, 4.3 mg/kg dsRNA, 4.4 mg/kg dsRNA, 4.5 mg/kg dsRNA, 4.6 mg/kg dsRNA, 4.7 mg/kg dsRNA, 4.8 mg/kg dsRNA, 4.9 mg/kg dsRNA, 5.0 mg/kg dsRNA, 5.1 mg/kg dsRNA, 5.2 mg/kg dsRNA, 5.3 mg/kg dsRNA, 5.4 mg/kg dsRNA, 5.5 mg/kg dsRNA, 5.6 mg/kg dsRNA, 5.7 mg/kg dsRNA, 5.8 mg/kg dsRNA, 5.9 mg/kg dsRNA, 6.0 mg/kg dsRNA, 6.1 mg/kg dsRNA, 6.2 mg/kg dsRNA, 6.3 mg/kg dsRNA, 6.4 mg/kg dsRNA, 6.5 mg/kg dsRNA, 6.6 mg/kg dsRNA, 6.7 mg/kg dsRNA, 6.8 mg/kg dsRNA, 6.9 mg/kg dsRNA, 7.0 mg/kg dsRNA, 7.1 mg/kg dsRNA, 7.2 mg/kg dsRNA, 7.3 mg/kg dsRNA, 7.4 mg/kg dsRNA, 7.5 mg/kg dsRNA, 7.6 mg/kg dsRNA, 7.7 mg/kg dsRNA, 7.8 mg/kg dsRNA, 7.9 mg/kg dsRNA, 8.0 mg/kg dsRNA, 8.1 mg/kg dsRNA, 8.2 mg/kg dsRNA, 8.3 mg/kg dsRNA, 8.4 mg/kg dsRNA, 8.5 mg/kg dsRNA, 8.6 mg/kg dsRNA, 8.7 mg/kg dsRNA, 8.8 mg/kg dsRNA, 8.9 mg/kg dsRNA, 9.0 mg/kg dsRNA, 9.1 mg/kg dsRNA, 9.2 mg/kg dsRNA, 9.3 mg/kg dsRNA, 9.4 mg/kg dsRNA, 9.5 mg/kg dsRNA, 9.6 mg/kg dsRNA, 9.7 mg/kg dsRNA, 9.8 mg/kg dsRNA, 9.9 mg/kg dsRNA, 9.0 mg/kg dsRNA, 10 mg/kg dsRNA, 15 mg/kg dsRNA, 20 mg/kg dsRNA, 25 mg/kg dsRNA, 30 mg/kg dsRNA, 35 mg/kg dsRNA, 40 mg/kg dsRNA, 45 mg/kg dsRNA, or about 50 mg/kg dsRNA. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In certain embodiments, for example, when a composition of the invention comprises a dsRNA as described herein and a lipid, subjects can be administered a therapeutic amount of iRNA, such as about 0.01 mg/kg to about 5 mg/kg, about 0.01 mg/kg to about 10 mg/kg, about 0.05 mg/kg to about 5 mg/kg, about 0.05 mg/kg to about 10 mg/kg, about 0.1 mg/kg to about 5 mg/kg, about 0.1 mg/kg to about 10 mg/kg, about 0.2 mg/kg to about 5 mg/kg, about 0.2 mg/kg to about 10 mg/kg, about 0.3 mg/kg to about 5 mg/kg, about 0.3 mg/kg to about 10 mg/kg, about 0.4 mg/kg to about 5 mg/kg, about 0.4 mg/kg to about 10 mg/kg, about 0.5 mg/kg to about 5 mg/kg, about 0.5 mg/kg to about 10 mg/kg, about 1 mg/kg to about 5 mg/kg, about 1 mg/kg to about 10 mg/kg, about 1.5 mg/kg to about 5 mg/kg, about 1.5 mg/kg to about 10 mg/kg, about 2 mg/kg to about 2.5 mg/kg, about 2 mg/kg to about 10 mg/kg, about 3 mg/kg to about 5 mg/kg, about 3 mg/kg to about 10 mg/kg, about 3.5 mg/kg to about 5 mg/kg, about 4 mg/kg to about 5 mg/kg, about 4.5 mg/kg to about 5 mg/kg, about 4 mg/kg

to about 10 mg/kg, about 4.5 mg/kg to about 10 mg/kg, about 5 mg/kg to about 10 mg/kg, about 5.5 mg/kg to about 10 mg/kg, about 6 mg/kg to about 10 mg/kg, about 6.5 mg/kg to about 10 mg/kg, about 7 mg/kg to about 10 mg/kg, about 7.5 mg/kg to about 10 mg/kg, about 8 mg/kg to about 10 mg/kg, about 8.5 mg/kg to about 10 mg/kg, about 9 mg/kg to about 10 mg/kg, or about 9.5 mg/kg to about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, the dsRNA may be administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, or about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In other embodiments, for example, when a composition of the invention comprises a dsRNA as described herein and an N-acetylgalactosamine, subjects can be administered a therapeutic amount of iRNA, such as a dose of about 0.1 to about 50 mg/kg, about 0.25 to about 50 mg/kg, about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/kg, about 1.5 to about 50 mg/kg, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.1 to about 45 mg/kg, about 0.25 to about 45 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/kg, about 1.5 to about 45 mg/kg, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg, about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.1 to about 40 mg/kg, about 0.25 to about 40 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/kg, about 1.5 to about 40 mg/kg, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.1 to about 30 mg/kg, about 0.25 to about 30 mg/kg,

about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/kg, about 1.5 to about 30 mg/kg, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.1 to about 20 mg/kg, about 0.25 to about 20 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/kg, about 1.5 to about 20 mg/kg, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. In one embodiment, when a composition of the invention comprises a dsRNA as described herein and an N-acetylgalactosamine, subjects can be administered a therapeutic amount of about 10 to about 30 mg/kg of dsRNA. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, subjects can be administered a therapeutic amount of iRNA, such as about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In certain embodiments of the invention, for example, when a double-stranded RNAi agent includes a modification (*e.g.,* one or more motifs of three identical modifications on three consecutive nucleotides), including one such motif at or near the cleavage site of the agent, six phosphorothioate linkages, and a ligand, such an agent is administered at a dose of about 0.01 to about 0.5 mg/kg, about 0.01 to about 0.4 mg/kg, about 0.01 to about 0.3 mg/kg, about 0.01 to about 0.2 mg/kg, about 0.01 to about 0.1 mg/kg, about 0.01 mg/kg to about 0.09 mg/kg, about 0.01 mg/kg to about 0.08 mg/kg, about 0.01 mg/kg to about 0.07 mg/kg, about 0.01 mg/kg to about 0.06 mg/kg, about 0.01 mg/kg to about 0.05 mg/kg, about 0.02 to about 0.5 mg/kg, about 0.02 to about 0.4 mg/kg, about 0.02 to about 0.3 mg/kg, about 0.02 to about 0.2 mg/kg, about 0.02 to about 0.1 mg/kg, about 0.02 mg/kg to about 0.09 mg/kg, about 0.02 mg/kg to about 0.08 mg/kg, about 0.02 mg/kg to about 0.07 mg/kg, about 0.02 mg/kg to about 0.06 mg/kg, about 0.02 mg/kg to about 0.05 mg/kg, about 0.03 to about 0.5 mg/kg, about 0.03 to about 0.4 mg/kg, about 0.03 to about 0.3 mg/kg, about 0.03 to about 0.2 mg/kg, about 0.03 to about 0.1 mg/kg, about 0.03 mg/kg to about 0.09 mg/kg, about 0.03 mg/kg to

about 0.08 mg/kg, about 0.03 mg/kg to about 0.07 mg/kg, about 0.03 mg/kg to about 0.06 mg/kg, about 0.03 mg/kg to about 0.05 mg/kg, about 0.04 to about 0.5 mg/kg, about 0.04 to about 0.4 mg/kg, about 0.04 to about 0.3 mg/kg, about 0.04 to about 0.2 mg/kg, about 0.04 to about 0.1 mg/kg, about 0.04 mg/kg to about 0.09 mg/kg, about 0.04 mg/kg to about 0.08 mg/kg, about 0.04 mg/kg to about 0.07 mg/kg, about 0.04 mg/kg to about 0.06 mg/kg, about 0.05 to about 0.5 mg/kg, about 0.05 to about 0.4 mg/kg, about 0.05 to about 0.3 mg/kg, about 0.05 to about 0.2 mg/kg, about 0.05 to about 0.1 mg/kg, about 0.05 mg/kg to about 0.09 mg/kg, about 0.05 mg/kg to about 0.08 mg/kg, or about 0.05 mg/kg to about 0.07 mg/kg. Values and ranges intermediate to the foregoing recited values are also intended to be part of this invention, *e.g.*, the RNAi agent may be administered to the subject at a dose of about 0.015 mg/kg to about 0.45 mg/kg.

For example, the RNAi agent, *e.g.*, RNAi agent in a pharmaceutical composition, may be administered at a dose of about 0.01 mg/kg, 0.0125 mg/kg, 0.015 mg/kg, 0.0175 mg/kg, 0.02 mg/kg, 0.0225 mg/kg, 0.025 mg/kg, 0.0275 mg/kg, 0.03 mg/kg, 0.0325 mg/kg, 0.035 mg/kg, 0.0375 mg/kg, 0.04 mg/kg, 0.0425 mg/kg, 0.045 mg/kg, 0.0475 mg/kg, 0.05 mg/kg, 0.0525 mg/kg, 0.055 mg/kg, 0.0575 mg/kg, 0.06 mg/kg, 0.0625 mg/kg, 0.065 mg/kg, 0.0675 mg/kg, 0.07 mg/kg, 0.0725 mg/kg, 0.075 mg/kg, 0.0775 mg/kg, 0.08 mg/kg, 0.0825 mg/kg, 0.085 mg/kg, 0.0875 mg/kg, 0.09 mg/kg, 0.0925 mg/kg, 0.095 mg/kg, 0.0975 mg/kg, 0.1 mg/kg, 0.125 mg/kg, 0.15 mg/kg, 0.175 mg/kg, 0.2 mg/kg, 0.225 mg/kg, 0.25 mg/kg, 0.275 mg/kg, 0.3 mg/kg, 0.325 mg/kg, 0.35 mg/kg, 0.375 mg/kg, 0.4 mg/kg, 0.425 mg/kg, 0.45 mg/kg, 0.475 mg/kg, or about 0.5 mg/kg. Values intermediate to the foregoing recited values are also intended to be part of this invention.

In some embodiments, the RNAi agent is administered as a fixed dose of between about 100 mg to about 900 mg, e.g., between about 100 mg to about 850 mg, between about 100 mg to about 800 mg, between about 100 mg to about 750 mg, between about 100 mg to about 700 mg, between about 100 mg to about 650 mg, between about 100 mg to about 600 mg, between about 100 mg to about 550 mg, between about 100 mg to about 500 mg, between about 200 mg to about 850 mg, between about 200 mg to about 800 mg, between about 200 mg to about 750 mg, between about 200 mg to about 700 mg, between about 200 mg to about 650 mg, between about 200 mg to about 600 mg, between about 200 mg to about 550 mg, between about 200 mg to about 500 mg, between about 300 mg to about 850 mg, between about 300 mg to about 800 mg, between about 300 mg to about 750 mg, between about 300 mg to about 700 mg, between about 300 mg to about 650 mg, between about 300 mg to about 600 mg, between about 300 mg to about 550 mg, between about 300 mg to about 500 mg, between about 400 mg to about 850 mg, between about 400 mg to about 800 mg, between about 400 mg to about 750 mg, between about 400 mg to about 700 mg, between about 400 mg to about 650 mg, between about 400 mg to about 600 mg, between about 400 mg to about 550 mg, or between about 400 mg to about 500 mg.

In some embodiments, the RNAi agent is administered as a fixed dose of about 100 mg, about 125 mg, about 150 mg, about 175 mg, 200 mg, about 225 mg, about 250 mg, about 275 mg, about 300 mg, about 325 mg, about 350 mg, about 375 mg, about 400 mg, about 425 mg, about 450 mg, about 475 mg, about 500 mg, about 525 mg, about 550 mg, about 575 mg, about 600 mg, about 625 mg, about 650 mg, about 675 mg, about 700 mg, about 725 mg, about 750 mg, about 775 mg, about 800 mg, about 825 mg, about 850 mg, about 875 mg, or about 900 mg.

The iRNA can be administered by intravenous infusion over a period of time, such as over a 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or about a 25 minute period. The administration may be repeated, for example, on a regular basis, such as weekly, biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration weekly or biweekly for three months, administration can be repeated once per month, for six months or a year or longer.

Administration of the iRNA can reduce the presence of serum and/or liver HBV ccc DNA, the presence of serum and/or liver HBV antigen, *e.g.,* HBsAg and/or HBeAg, ALT levels, and/or AST levels, *e.g.*, in a cell, tissue, blood, urine or other compartment of the patient by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more, *e.g.*, to below the level of detection of the assay.

Administration of the iRNA can increase the presence of serum and/or liver anti-HBV antibodies, *e.g.*, in a cell, tissue, blood, urine or other compartment of the patient by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more.

Before administration of a full dose of the iRNA, patients can be administered a smaller dose, such as a 5% infusion, and monitored for adverse effects, such as an allergic reaction. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (*e.g.*, TNF-alpha or INF-alpha) levels.

Owing to the inhibitory effects on HBV expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

An iRNA of the invention may be administered in "naked" form, where the modified or unmodified iRNA agent is directly suspended in aqueous or suitable buffer solvent, as a "free iRNA." A free iRNA is administered in the absence of a pharmaceutical composition. The free iRNA may be in a suitable buffer solution. The buffer solution may comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. In one embodiment, the buffer solution is phosphate buffered saline (PBS). The pH and osmolarity of the buffer solution containing the iRNA can be adjusted such that it is suitable for administering to a subject.

Alternatively, an iRNA of the invention may be administered as a pharmaceutical composition, such as a dsRNA liposomal formulation.

Subjects that would benefit from a reduction and/or inhibition of HBV gene expression are those having an HBV infection and/or an HBV-associated disease or disorder as described herein.

Treatment of a subject that would benefit from a reduction and/or inhibition of HBV gene expression includes therapeutic and prophylactic treatment.

The invention further provides methods and uses of an iRNA agent or a pharmaceutical composition thereof for treating a subject that would benefit from reduction and/or inhibition of HBV gene expression, *e.g.,* a subject having a HBV-associated disease, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.*, with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders.

For example, in certain embodiments, an iRNA targeting one or more HBV genes is administered in combination with, *e.g.*, an agent useful in treating an HBV-associated disease as described elsewhere herein. For example, additional therapeutics and therapeutic methods suitable for treating a subject that would benefit from reduction in HBV expression, *e.g.,* a subject having a HBV-associated disease, include an iRNA agent targeting a different portion of the HBV genome, an antiviral agent, a reverse transcriptase inhibitor (*e.g.,* Tenofovir disoproxil fumarate (TDF), Tenofovir alafenamide, Lamivudine, Adefovir dipivoxil, Entecavir (ETV), Telbivudine, and AGX-1009), an immune stimulator (*e.g.,* pegylated interferon alfa 2a (PEG-IFN-α2a), Interferon alfa-2b, a recombinant human interleukin-7, and aToll-like receptor 7 (TLR7) agonist), a therapeutic vaccine (*e.g.,* GS-4774, DV-601, and TG1050), a viral entry inhibitor (*e.g.,* Myrcludex), an oligonucleotide that inhibits the secretion or release of HbsAg (*e.g.,* REP 9AC), a capsid inhibitor (*e.g.,* Bay41-4109 and NVR-1221), a cccDNA inhibitor (*e.g.,* IHVR-25), or other therapeutic agents and/or

procedures, *e.g.,* liver transplant, chemotherapy, for treating a HBV-associated disease, a combination of any of the foregoing.

In certain embodiments, a first iRNA agent targeting one or more HBV genes is administered in combination with a second iRNA agent targeting a different portion of the HBV genome. For example, a first iRNA agent targeting one or more structural genes may be administered in combination with a second RNAi agent targeting the X gene. For example, the first RNAi agent comprises a first sense strand and a first antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of said first sense strand and substantially all of the nucleotides of the first antisense strand are modified nucleotides, wherein said first sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and the second RNAi agent comprises a second sense strand and a second antisense strand forming a double-stranded region, wherein substantially all of the nucleotides of the second sense strand and substantially all of the nucleotides of the second antisense strand are modified nucleotides, wherein the second sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; wherein the first sense strand comprises a sequences selected from the group consisting of

5'- UCGUGGUGGACUUCUCUCA -3' (SEQ IDNO:5),

5'- GUGCACUUCGCUUCACCUCUA -3' (SEQ IDNO:7),

5'- CGUGGUGGACUUCUCUCAAUU -3' (SEQ IDNO:9),

5'- CGUGGUGGUCUUCUCUAAAUU -3' (SEQ IDNO:37),

5'- GGUGGACUUCUCUCAAUUUUA -3' (SEQ IDNO:11), and

5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), and wherein the first and second antisense strands each independently comprise a sequence selected from the group consisting of

5'- UGAGAGAAGUCCACCACGAUU -3' (SEQ ID NO:6);

5'- UAGAGGUGAAGCGAAGUGCACUU -3' (SEQ ID NO:8);

5'- AAUUGAGAGAAGUCCACCAGCAG -3' (SEQ ID NO:10);

5'- AAUUGAGAGAAGUCCACCAGCUU -3' (SEQ ID NO:38),

5'- UAAAAUUGAGAGAAGUCCACCAC -3' (SEQ ID NO:12), and

5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40) (or a nucleotide sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical over its entire length to any of the foregoing nucleotide sequences), thereby treating the subject.

In one embodiment, all of the nucleotides of the first and second sense strand and/or all of the nucleotides of the first and second antisense strand comprise a modification.

In one embodiment, the at least one of the modified nucleotides is selected from the group consisting of a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

In certain embodiments, a first iRNA agent targeting one or more HBV genes is administered in combination with a second iRNA agent targeting a gene that is different from one or more HBV genes. For example, the iRNA agent targeting one or more HBV genes may be administered in combination with an iRNA agent targeting a CD274/PD-L1 gene. Examples of iRNA agents targeting a CD274/PD-L1 gene are described in WO 2011/127180, the entire contents of which are incorporated herein by reference. The first iRNA agent targeting one or more HBV genes and the second iRNA agent targeting a gene different from one or more HBV genes, *e.g.*, a CD274/PD-L1 gene and/or an HDV gene, may be administred as parts of the same pharmaceutical composition. Alternatively, the first iRNA agent targeting one or more HBV genes and the second iRNA agent targeting a gene different from one or more HBV genes, *e.g.*, a CD274/PD-L1 gene and/or an HDV gene, may be administered as parts of different pharmaceutical compositions.

CD274 or PD-L1 is a 290 amino acid type I transmembrane protein encoded by the CD274 gene on mouse chromosome 19 and human chromosome 9. CD274/PD-L1 expression is implicated in evasion of immune responses involved in chronic infection, *e.g.*, by viruses (including, for example, HIV, HBV, HCV and HTLV, among others), by bacteria (including, for example, *Helicobacter pylori*, among others) and by parasites (including, for example, *Schistosoma mansoni*).

PD-L1 can influence immune responses by engaging PD-1 or B7-1 (CD80) and modifying TCR or BCR signaling, but can also deliver signals into PD-L1 expressing cells, *i.e.*, reverse signaling through PD-L1. Surface plasmon resonance studies demonstrate specific and unique interaction between both PD-L1 and B7-1, with an affinity of 1.7 μM, and an affinity of 0.5 μM for the interaction between PD-L1 and PD-1. Chemical cross-linking studies indicate that PD-L1 and B7-1, like PD-L1 and PD-1, can also interact through their IgV-like domains. The PD-L1:B7-1 interface overlaps at least partially with the putative PD-L1:PD-1 interface. B7-1:PD-L1 interactions can induce an inhibitory signal into

T cells. Ligation of PD-L1 on CD4 T cells by B7-1, or ligation of B7-1 on CD4 T cells by PD-L1, delivers a functionally significant, inhibitory signal. Because both PD-L1 and B7-1 are expressed on T cells, B cells, DCs, and macrophages, there is the potential for bidirectional interactions between B7-1 and PD-L1 on these cell types. In addition, PD-L1 on nonhematopoietic cells may interact with B7-1 as well as PD-1 on T cells to regulate cells (Keir ME *et al.*, 2008. *Annu Rev Immunol.* 26:677-704).

In chronic viral infections in humans, several groups have shown that PD-1 expression is high on HIV-specific (Petrovas C *et al.*, 2006, *J. Exp. Med.* 203:2281-92; Day CL *et al.*, 2006, *Nature* 443:350-54; Trautmann L *et al.*, 2006, *Nat. Med.* 12: 1198-202), HBV-specific (Boettler T *et al.*, 2006, *J. Virol.* 80:3532-40; Boni C *et al.* 2007, *J. Virol.* 81:4215-25), and HCV-specific T cells (Urbani S *et al.*, 2006, *J. Virol.* 80: 11398-403). PD-L1 is also upregulated on peripheral blood CD14+ monocytes and myeloid DCs in patients with chronic HBV infection (Chen L *et al.*, 2007, *J. Immunol.* 178:6634-41; Ceng L *et al.*, 2006, *J. Viral Hepat.* 13:725-33), and on CD14+ cells and T cells in HIV patients (Trabattoni D *et al.*, 2003. *Blood*101 :2514-20). Blocking PD-LPD-L interactions in vitro reverses the exhaustion of HIV-specific, HBV-specific (Boni C *et al.* 2007, *J. Virol.* 81 :4215-25), HCV-specific, and SIV-specific (Velu V *et al.*, 2007, *J. Virol.*81 :5819-28) CD8 and CD4 T cells and restores proliferation and cytokine production (Petrovas C *et al.*, 2006, *J. Exp. Med.* 203:2281-92; Day CL *et al.*, 2006, *Nature* 443:350-54; Trautmann L *et al.*, 2006, *Nat. Med.* 12: 1198-202; Urbani S *et al.*, 2006, *J. Virol.* 80: 11398-403). Recent work shows that the HCV core, a nucleocapsid protein, can upregulate PD-1 and PD-L1 expression on healthy donor T cells and that upregulation of PD-1 is mediated by interaction of the HCV core with the complement receptor C1QBP (Yao ZQ *et al.*, 2007, *Viral Immunol.* 20:276-87).

A subject administered a first RNAi agent or a first and second RNAi agent of the invention may further be administered with one or more other therapeutics which function by a non- iRNA mechanism and which are useful in treating an HBV infection. Exemplary therapeutics that may be used in a combination therapy of the invention include immune modulators which stimulate the immune system by, for example, enhancing T-cell helper activity, maturation of B lymphocytes, inhibiting T-cell suppressors, and enhancing HLA type I expression. Suitable immune modulators include interferons which have a variety of properties that include antiviral, immunomodulatory, and antiproliferative effects.

For example, the current treatment for chronic hepatitis B is interferon therapy, which is administered to subjects who have a documented HBV infection for at least six months, elevated liver enzymes (AST and ALT) and an actively dividing virus in their blood (HBeAg, and/or HBV DNA positive tests). Interferon-α therapy produces a long-term, sustained remission of the disease in about 35% of those with chronic hepatitis B, with normalization of liver enzymes and loss of the three markers for an active infection (HBeAg, HBV DNA, and

HBsAg). Subjects with an acute HBV infection, end stage cirrhosis or other major medical problems are typically not treated with interferon.

In addition, interferon therapy for patients with HBV-related cirrhosis decreases significantly the hepatocellular carcinoma (HCC) rate, particularly in patients with a larger amount of serum HBV DNA. In patients with HBeAg-positive compensated cirrhosis, virological and biochemical remission following interferon therapy is associated with improved survival. In patients with chronic HBV infection, the clearance of HBeAg after treatment with interferon-α is associated with improved clinical outcomes.

The standard duration of therapy is considered 16 weeks. Patients who exhibit a low level of viral replication at the end of the standard regimen benefit most from prolonged treatment.

Other exemplary therapeutic agents which can be used in a combination therapy of the invention include, for example, an antiviral agent, a nucleotide analog, a nucleoside analog, a reverse transcriptase inhibitor (*e.g.,* Tenofovir disoproxil fumarate (TDF), Tenofovir alafenamide, Lamivudine, Adefovir dipivoxil, Entecavir (ETV), Telbivudine, AGX-1009, emtricitabine, clevudine, ritonavir, dipivoxil, lobucavir, famvir, FTC, N-Acetyl-Cysteine (NAC), PC1323, theradigm-HBV, thymosin-alpha, and ganciclovir), an immune stimulator (*e.g.,* pegylated interferon alfa 2a (PEG-IFN-α2a), Interferon alfa-2b, a recombinant human interleukin-7, and aToll-like receptor 7 (TLR7) agonist), a therapeutic vaccine (*e.g.,* GS-4774, DV-601, and TG1050), a viral entry inhibitor (*e.g.,* Myrcludex), an oligonucleotide that inhibits the secretion or release of HbsAg (*e.g.,* REP 9AC), a capsid inhibitor (*e.g.,* Bay41-4109 and NVR-1221), a cccDNA inhibitor (*e.g.,* IHVR-25), or other therapeutic agents and/or procedures, *e.g.,* liver transplant, chemotherapy, for treating a HBV-associated disease, a combination of any of the foregoing.

In one embodiment, the methods of the invention include administering to a subject having an HBV infection and/or HBV-associate disease a reverse transcriptase inhibitor. In another embodiment, the methods of the invention include administering to a subject having an HBV infection and/or HBV-associate disease a reverse transcriptase inhibitor and an immune stimulator.

The iRNA agent(s) and an additional therapeutic agent and/or treatment may be administered at the same time and/or in the same combination, *e.g.*, parenterally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times and/or by another method known in the art or described herein.

The present invention also provides methods of using an iRNA agent of the invention and/or a composition containing an iRNA agent of the invention to reduce and/or inhibit HBV expression in a cell. In other aspects, the present invention provides an iRNA of the invention and/or a composition comprising an iRNA of the invention for use in reducing and/or inhibiting HBV gene expression in a cell. In yet other aspects, use of an iRNA of the

invention and/or a composition comprising an iRNA of the invention for the manufacture of a medicament for reducing and/or inhibiting HBV gene expression in a cell are provided. In still other aspect, the the present invention provides an iRNA of the invention and/or a composition comprising an iRNA of the invention for use in reducing and/or inhibiting HBV replication in a cell. In yet other aspects, use of an iRNA of the invention and/or a composition comprising an iRNA of the invention for the manufacture of a medicament for reducing and/or inhibiting HBV replication in a cell are provided. The methods and uses include contacting the cell with an iRNA, *e.g.,* a dsRNA, of the invention and maintaining the cell for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby inhibiting expression of the HBV gene or inhibiting HBV replication in the cell.

Reduction in gene expression can be assessed by any methods known in the art. For example, a reduction in the expression of HBV may be determined by determining the mRNA expression level of HBV using methods routine to one of ordinary skill in the art, *e.g.,* northern blotting, qRT-PCR, by determining the protein level of HBV using methods routine to one of ordinary skill in the art, such as western blotting, immunological techniques, flow cytometry methods, ELISA, and/or by determining a biological activity of HBV.

In the methods and uses of the invention the cell may be contacted *in vitro* or *in vivo*, *i.e.*, the cell may be within a subject.

A cell suitable for treatment using the methods of the invention may be any cell that expresses an HBV gene, *e.g.,* a cell infected with HBV or a cell comprising an expression vector comprising an HBV genome or portion of an HBV gene. A cell suitable for use in the methods and uses of the invention may be a mammalian cell, *e.g.,* a primate cell (such as a human cell or a non-human primate cell, *e.g.*, a monkey cell or a chimpanzee cell), a non-primate cell (such as a cow cell, a pig cell, a camel cell, a llama cell, a horse cell, a goat cell, a rabbit cell, a sheep cell, a hamster, a guinea pig cell, a cat cell, a dog cell, a rat cell, a mouse cell, a lion cell, a tiger cell, a bear cell, or a buffalo cell), a bird cell (*e.g.*, a duck cell or a goose cell), or a whale cell. In one embodiment, the cell is a human cell, *e.g.,* a human liver cell.

HBV gene expression may be inhibited in the cell by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100%, *i.e.*, to below the level of detection of the assay.

HBV replication may be inhibited in the cell by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100%, *i.e.*, to below the level of detection of the assay.

The *in vivo* methods and uses of the invention may include administering to a subject a composition containing an iRNA, where the iRNA includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the HBV gene of the mammal to be treated. When the organism to be treated is a human, the composition can be administered by any means known in the art including, but not limited to subcutaneous, intravenous, oral, intraperitoneal, or parenteral routes, including intracranial (*e.g.*, intraventricular, intraparenchymal and intrathecal), intramuscular, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by subcutaneous injection. In some embodiments, the compositions are administered by intravenous infusion or injection. In other embodiments, the compositions are administered by intramuscular injection.

In some embodiments, the administration is *via* a depot injection. A depot injection may release the iRNA in a consistent way over a prolonged time period. Thus, a depot injection may reduce the frequency of dosing needed to obtain a desired effect, *e.g.*, a desired inhibition of HBV, or a therapeutic or prophylactic effect. A depot injection may also provide more consistent serum concentrations. Depot injections may include subcutaneous injections or intramuscular injections. In preferred embodiments, the depot injection is a subcutaneous injection.

In some embodiments, the administration is *via* a pump. The pump may be an external pump or a surgically implanted pump. In certain embodiments, the pump is a subcutaneously implanted osmotic pump. In other embodiments, the pump is an infusion pump. An infusion pump may be used for intravenous, subcutaneous, arterial, or epidural infusions. In preferred embodiments, the infusion pump is a subcutaneous infusion pump. In other embodiments, the pump is a surgically implanted pump that delivers the iRNA to the liver.

The mode of administration may be chosen based upon whether local or systemic treatment is desired and based upon the area to be treated. The route and site of administration may be chosen to enhance targeting.

In one aspect, the present invention also provides methods for inhibiting the expression of an HBV gene in a mammal, *e.g.,* a human. The present invention also provides a composition comprising an iRNA, *e.g.,* a dsRNA, that targets an HBV gene in a cell of a mammal for use in inhibiting expression of the HBV gene in the mammal. In another aspect, the present invention provides use of an iRNA, *e.g.,* a dsRNA, that targets an HBV gene in a cell of a mammal in the manufacture of a medicament for inhibiting expression of the HBV gene in the mammal.

The methods and uses include administering to the mammal, *e.g.,* a human, a composition comprising an iRNA, *e.g.,* a dsRNA, that targets an HBV gene in a cell of the mammal and maintaining the mammal for a time sufficient to obtain degradation of the mRNA transcript of the HBV gene, thereby inhibiting expression of the HBV gene in the mammal.

Reduction in gene expression can be assessed in peripheral blood sample of the iRNA-administered subject by any methods known it the art, *e.g.* qRT-PCR, described herein. Reduction in protein production can be assessed by any methods known it the art and by methods, *e.g.*, ELISA or western blotting, described herein. In one embodiment, a puncture liver biopsy sample serves as the tissue material for monitoring the reduction in HBV gene and/or protein expression. In another embodiment, a blood sample serves as the tissue material for monitoring the reduction in HBV gene and/or protein expression.

In one embodiment, verification of RISC medicated cleavage of target *in vivo* following administration of iRNA agent is done by performing 5'-RACE or modifications of the protocol as known in the art (Lasham A *et al.*, (2010) *Nucleic Acid Res.*, 38 (3) p-e19) (Zimmermann *et al.* (2006) *Nature* 441: 111-4).

This invention is further illustrated by the following examples which should not be construed as limiting. The entire contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are hereby incorporated herein by reference.

EXAMPLES

Example 1. iRNA Synthesis

Source of reagents

Where the source of a reagent is not specifically given herein, such reagent can be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Transcripts

siRNA Design

The selection of siRNA designs targeting HBV was driven by two primary factors: a) potency and b), the desire to employ siRNA with near-perfect matches with greater than 90% fractional coverage of the large number of public HBV sequences of all known serotypes (A through H). The coordinates for the siRNA selection were determined relative to the NCBI HBV reference genome sequence NC_003977.1 (GenBank Accession No. GI:21326584 (SEQ ID NO:1). A first set of siRNAs containing structure-activity modifications, including various 2'-O-methyl and 2'-fluoro substitution patterns, centered on two adjacent regions of the HBV genome coding for surface antigen (HbSAg) and the HBV polymerase, were designed, synthesized and screened *in-vitro*. A second set of siRNAs were designed, synthesized and screened targeting additional target regions with particular attention to positions 1581-1599 of SEQ ID NO:1 that code, in addition to the HbSAg and polymerase, the X gene.

A detailed list of the unmodified HBV sense and antisense strand sequences is shown in Table 3.

A detailed list of the modified HBV sense and antisense strand sequences is shown in Table 4.

siRNA Synthesis

HBV siRNA sequences were synthesized at 1 μmol scale on Mermade 192 synthesizer (BioAutomation) using the solid support mediated phosphoramidite chemistry. The solid support was controlled pore glass (500 A) loaded with custom GalNAc ligand or universal solid support (AM biochemical). Ancillary synthesis reagents, 2'-F and 2'-O-Methyl RNA and deoxy phosphoramidites were obtained from Thermo-Fisher (Milwaukee, WI) and Hongene (China). 2'F 2'-O-Methyl, GNA (glycol nucleic acids), 5'phosphate and abasic modifications were introduced employing the corresponding phosphoramidites. Synthesis of 3' GalNAc conjugated single strands was performed on a GalNAc modified CPG support. Custom CPG universal solid support was used for the synthesis of antisense single strands. Coupling time for all phosphoramidites (100 mM in acetonitrile) was 5 min employing 5-Ethylthio-1H-tetrazole (ETT) as activator (0.6 M in acetonitrile). Phosphorothioate linkages were generated using a 50 mM solution of 3-((Dimethylamino-methylidene) amino)-3H-1,2,4-dithiazole-3-thione (DDTT, obtained from Chemgenes (Wilmington, MA, USA)) in anhydrous acetonitrile/pyridine (1:1 v/v). Oxidation time was 3 minutes. All sequences were synthesized with final removal of the DMT group ("DMT off").

Upon completion of the solid phase synthesis, oligoribonucleotides were cleaved from the solid support and deprotected in sealed 96 deep well plates using 200 μL Aqueous Methylamine reagents at 60°C for 20 minutes. At the end of cleavage and deprotection step,

the synthesis plate was allowed to come to room temperature and was precipitated by addition of 1mL of acetontile: ethanol mixture (9:1). The plates were cooled at -80 C for 2 hrs, superanatant decanted carefully with the aid of a multi channel pipette. The oligonucleotide pellet was re-suspended in 20mM NaOAc buffer and were desalted using a 5 mL HiTrap size exclusion column (GE Healthcare) on an AKTA Purifier System equipped with an A905 autosampler and a Frac 950 fraction collector. Desalted samples were collected in 96-well plates. Samples from each sequence were analyzed by LC-MS to confirm the identity, UV (260 nm) for quantification and a selected set of samples by IEX chromatography to determine purity.

Annealing of HBV single strands was performed on a Tecan liquid handling robot. Equimolar mixture of sense and antisense single strands were combined and annealed in 96 well plates. After combining the complementary single strands, the 96-well plate was sealed tightly and heated in an oven at 100°C for 10 minutes and allowed to come slowly to room temperature over a period 2-3 hours. The concentration of each duplex was normalized to 10μM in 1X PBS.

Example 2. *In vitro* screening of siRNA duplexes

Cell culture and transfections

Cos7 cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO2 in DMEM (ATCC) supplemented with 10% FBS, before being released from the plate by trypsinization. Dual-Glo® Luciferase constructs generated in the psiCHECK2 plasmid containing approximately 1.1 kb of HBV genomic sequences were transfected into approximately $15x10^4$ cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA. cat # 11668-019). For each well of a 96 well plate, 0.2 μl of Lipofectamine was added to 10 ng of plasmid vector in 14.8 μl of Opti-MEM and allowed to complex at room temperature for 15 minutes. The mixture was then added to the cells which were resuspended in 80 μl of fresh complete media. After approximately 24 hours, the media were removed and the cells re-transfected with siRNA. Each siRNA was transfected into cells that had previously been transfected with the psiCHECK2-HBV vector that had a perfect match for the siRNA. siRNA transfection was carried out by adding 14.8 μl of Opti-MEM plus 0.2 μ l of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5μl of siRNA duplexes per well into a 96-well plate and incubated at room temperature for 15 minutes. The mixture was then added to the cells previously transfected with the psiCHECK2-HBV plasmid that had a perfect match to the siRNA sequence. Cells were incubated for 24 hours before luciferase was measured.

Single dose experiments were performed at 10nM and 0.01nM final duplex concentration.

Dual-Glo® Luciferase assay

Twenty-four hours after the siRNAs were transfected, Firefly (transfection control) and Rinella (fused to HBV target sequence) luciferase were measured. First, media was removed from cells. Then Firefly luciferase activity was measured by adding75 µl of Dual-Glo® Luciferase Reagent equal to the culture medium volume to each well and mix. The mixture was incubated at room temperature for 30 minutes before lunimescense (500 nm) was measured on a Spectramax (Molecular Devices) to detect the Firefly luciferase signal. Renilla luciferase activity was measured by adding 75 µl of room temperature of Dual-Glo® Stop & Glo® Reagent was added to each well and the plates were incubated for 10-15 minutes before luminescence was again measured to determine the Renilla luciferase signal. The Dual-Glo® Stop & Glo® Reagent , quench the firefl y luciferase signal and sustain luminescence for the Renilla luciferase reaction. siRNA activity was determined by normalizing the Renilla (HBV) signal to the Firefly (control) signal within each well. The magnitude of siRNA activity was then assessed relative to cells that were transfected with the same vector but were not treated with siRNA or were treated with a non-targeting siRNA. All transfections were done at n=2 or greater.

Table 5 shows the results of a single dose screen in Cos7 cells transfected with the indicated HBV iRNAs. Data are expressed as percent of mRNA remaining relative to negative control.

Table 2. Abbreviations of nucleotide monomers used in nucleic acid sequence representation. It will be understood that, unless otherwise indicated, these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds.

Abbreviation	**Nucleotide(s)**
A	Adenosine-3'-phosphate
Af	2'-fluoroadenosine-3'-phosphate
Afs	2'-fluoroadenosine-3'-phosphorothioate
As	adenosine-3'-phosphorothioate
C	cytidine-3'-phosphate
Cf	2'-fluorocytidine-3'-phosphate
Cfs	2'-fluorocytidine-3'-phosphorothioate
Cs	cytidine-3'-phosphorothioate
G	guanosine-3'-phosphate
Gf	2'-fluoroguanosine-3'-phosphate
Gfs	2'-fluoroguanosine-3'-phosphorothioate

Gs	guanosine-3'-phosphorothioate
T	5'-methyluridine-3'-phosphate
Tf	2'-fluoro-5-methyluridine-3'-phosphate
Tfs	2'-fluoro-5-methyluridine-3'-phosphorothioate
Ts	5-methyluridine-3'-phosphorothioate
U	Uridine-3'-phosphate
Uf	2'-fluorouridine-3'-phosphate
Ufs	2'-fluorouridine -3'-phosphorothioate
Us	uridine -3'-phosphorothioate
N	any nucleotide (G, A, C, T or U)
a	2'-O-methyladenosine-3'-phosphate
as	2'-O-methyladenosine-3'- phosphorothioate
c	2'-O-methylcytidine-3'-phosphate
cs	2'-O-methylcytidine-3'- phosphorothioate
g	2'-O-methylguanosine-3'-phosphate
gs	2'-O-methylguanosine-3'- phosphorothioate
t	2'-O-methyl-5-methyluridine-3'-phosphate
ts	2'-O-methyl-5-methyluridine-3'-phosphorothioate
u	2'-O-methyluridine-3'-phosphate
us	2'-O-methyluridine-3'-phosphorothioate
s	phosphorothioate linkage
L96	N-[tris(GalNAc-alkyl)-amidodecanoyl)]-4-hydroxyprolinol Hyp-(GalNAc-alkyl)3
(dT)	2`-deoxythymidine-3`-phosphate
Y34	2-hydroxymethyl-tetrahydrofurane-4-methoxy-3-phosphate (abasic 2'-OMe furanose)
Y44	2-hydroxymethyl-tetrahydrofurane-5-phosphate
(Agn)	Adenosine-glycol nucleic acid (GNA)
(Tgn)	Thymidine-glycol nucleic acid (GNA) S-Isomer
(Cgn)	Cytidine-glycol nucleic acid (GNA)
P	Phosphate
VP	Vinyl-phosphate

Table 3. Unmodified Sense and Antisense Strand Sequences of HBV dsRNAs

Duple Name	Sense Oligo Name	Sense Sequence (5' to 3')	SEQ ID NO	Antisense Oligo Name	Antisense Sequence (5' to 3')	SEQ ID NO:	Position in NC_003977.1
AD-61522.2	A-123463.2	AGUUAUAUGGAUGAUGUGGUA	47	A-123464.2	UACCACAUCAUCCAUAUAACUGA	263	731_753
AD-61547.2	A-123487.2	GGAUGUGUCUGCGGCGUUUUA	48	A-123488.2	UAAAACGCCGCAGACACAUCCAG	264	373_395
AD-63938.2	A-127896.1	ACUCGUGGUGGACUUCUCUCA	49	A-127897.1	UGAGAGAAGUCCACCACGAGUCU	265	250_272
AD-63939.2	A-127909.1	ACUCGUGGUGGACUUCUCUCA	50	A-127906.3	UGAGAGAAGUCCACCACGAGUCU	266	250_272
AD-63940.2	A-127917.1	ACUCGUGGUGGACUUCTCUCA	51	A-127906.11	UGAGAGAAGUCCACCACGAGUCU	267	250_272
AD-63941.2	A-127905.8	ACUCGUGGUGGACUUCUCUCA	52	A-127925.1	UGAGAGAAGUCCACCACGAGUCU	268	250_272
AD-63942.2	A-127933.1	UCGUGGUGGACUUCUCUCA	53	A-127934.1	UGAGAGAAGUCCACCACGAGU	269	252_274
AD-63943.2	A-127944.2	ACUCGUGGUGGACUUCUCUCA	54	A-127942.2	UGAGAGAAGUCCACCACGAGUCU	270	250_272
AD-63945.2	A-127910.1	ACUCGUGGUGGACUUCUCUCA	55	A-127906.4	UGAGAGAAGUCCACCACGAGUCU	271	250_272
AD-63946.2	A-127918.1	ACUCGUGGUGGACUUCUCUCA	56	A-127906.12	UGAGAGAAGUCCACCACGAGUCU	272	250_272
AD-63947.2	A-127905.9	ACUCGUGGUGGACUUCUCUCA	57	A-127926.1	UGAGAGAAGUCCACCACGAGUCU	273	250_272
AD-63948.2	A-127935.1	GUGGUGGACUUCUCUCA	58	A-127936.1	UGAGAGAAGUCCACCACGA	274	254_276
AD-63949.2	A-127944.3	ACUCGUGGUGGACUUCUCUCA	59	A-127906.14	UGAGAGAAGUCCACCACGAGUCU	275	250_272
AD-63950.2	A-127900.1	UCGUGGUGGACUUCUCUCAUU	60	A-127901.1	UGAGAGAAGUCCACCACGAUU	276	252_274
AD-63951.2	A-127911.1	ACUCGUGGUGGACUUCUCUCA	61	A-127906.5	UGAGAGAAGUCCACCACGAGUCU	277	250_272
AD-63952.2	A-127905.2	ACUCGUGGUGGACUUCUCUCA	62	A-127919.1	UGAGAGAAGUCCACCACGAGUCU	278	250_272
AD-63953.2	A-127905.10	ACUCGUGGUGGACUUCUCUCA	63	A-127927.1	UGAGAGAAGUCCACCACGAGUCU	279	250_272
AD-63955.2	A-127945.1	ACUCGUGGUGGACUUCUCUCA	64	A-127940.3	UGAGAGAAGUCCACCACGAGUCU	280	250_272
AD-63956.2	A-127902.1	UCGUGGUGGACUUCUCUCA	65	A-127903.1	UGAGAGAAGUCCACCACGAUU	281	252_274
AD-63957.2	A-127912.1	ACUCGUGGUGGACUUCUCUCA	66	A-127906.6	UGAGAGAAGUCCACCACGAGUCU	282	250_272
AD-63958.2	A-127905.3	ACUCGUGGUGGACUUCUCUCA	67	A-127920.1	UGAGAGAAGUCCACCACGAGUCU	283	250_272

AD-63959.2	A-127905.11	ACUCGUGGUGGACUUCUCUCA	68	A-127928.1	UGAGAGAAGUCCACCACGAGUCU	284	250_272
AD-63960.2	A-126619.2	UAUUUCCUAGGGUACAA	69	A-127938.1	UGAGAGAAGUCCACCACGA	285	254_276
AD-63961.2	A-127945.2	ACUCGUGGUGGACUUCUCUCA	70	A-127942.3	UGAGAGAAGUCCACCACGAGUCU	286	250_272
AD-63962.2	A-127902.2	UCGUGGUGGACUUCUCUCA	71	A-127904.1	UGAGAGAAGUCCACCACGAUU	287	252_274
AD-63963.2	A-127913.1	ACUCGUGGUGGACUUCUCUCA	72	A-127906.7	UGAGAGAAGUCCACCACGAGUCU	288	250_272
AD-63964.2	A-127905.4	ACUCGUGGUGGACUUCUCUCA	73	A-127921.1	UGAGAGAAGUCCACCACGAGUCU	289	250_272
AD-63965.2	A-127905.12	ACUCGUGGUGGACUUCUCUCA	74	A-127929.1	UGAGAGAAGUCCACCACGAGUCU	290	250_272
AD-63966.2	A-127939.1	ACUCGUGGUGGACUUCUCUCA	75	A-127940.1	UGAGAGAAGUCCACCACGAGUCU	291	250_272
AD-63967.2	A-127945.3	ACUCGUGGUGGACUUCUCUCA	76	A-127906.15	UGAGAGAAGUCCACCACGAGUCU	292	250_272
AD-63968.2	A-127905.1	ACUCGUGGUGGACUUCUCUCA	77	A-127906.1	UGAGAGAAGUCCACCACGAGUCU	293	250_272
AD-63968.4	A-127905.15	ACUCGUGGUGGACUUCUCUCA	78	A-127906.17	UGAGAGAAGUCCACCACGAGUCU	294	250_272
AD-63968.5	A-127905.17	ACUCGUGGUGGACUUCUCUCA	79	A-127906.18	UGAGAGAAGUCCACCACGAGUCU	295	250_272
AD-63969.2	A-127914.1	ACUCGUGGUGGACUUCUCUCA	80	A-127906.8	UGAGAGAAGUCCACCACGAGUCU	296	250_272
AD-63970.2	A-127905.5	ACUCGUGGUGGACUUCUCUCA	81	A-127922.1	UGAGAGAAGUCCACCACGAGUCU	297	250_272
AD-63971.2	A-127905.13	ACUCGUGGUGGACUUCUCUCA	82	A-127930.1	UGAGAGAAGUCCACCACGAGUCU	298	250_272
AD-63972.2	A-127941.1	ACUCGUGGUGGACUUCUCUCA	83	A-127942.1	UGAGAGAAGUCCACCACGAGUCU	299	250_272
AD-63973.2	A-127946.1	ACUCGUGGUGGACUUCUCUCA	84	A-127947.1	UGAGAGAAGTCCACCACGAGUCU	300	250_272
AD-63975.2	A-127915.1	ACUCGUGGUGGACUUCTCUCA	85	A-127906.9	UGAGAGAAGUCCACCACGAGUCU	301	250_272
AD-63976.2	A-127905.6	ACUCGUGGUGGACUUCUCUCA	86	A-127923.1	UGAGAGAAGUCCACCACGAGUCU	302	250_272
AD-63977.2	A-127917.2	ACUCGUGGUGGACUUCTCUCA	87	A-127931.1	UGAGAGAAGUCCACCACGAGUCU	303	250_272
AD-63978.2	A-127943.1	ACUCGUGGUGGACUUCUCUCA	88	A-127906.13	UGAGAGAAGUCCACCACGAGUCU	304	250_272
AD-63979.2	A-127908.1	ACUCGUGGUGGACUUCUCUCA	89	A-127906.2	UGAGAGAAGUCCACCACGAGUCU	305	250_272
AD-63980.2	A-127916.1	ACUCGUGGUGGACUUCTCUCA	90	A-127906.10	UGAGAGAAGUCCACCACGAGUCU	306	250_272
AD-63981.2	A-127905.7	ACUCGUGGUGGACUUCUCUCA	91	A-127924.1	UGAGAGAAGUCCACCACGAGUCU	307	250_272
AD-63982.2	A-127917.3	ACUCGUGGUGGACUUCTCUCA	92	A-127932.1	UGAGAGAAGUCCACCACGAGUCU	308	250_272
AD-63983.2	A-127944.1	ACUCGUGGUGGACUUCUCUCA	93	A-127940.2	UGAGAGAAGUCCACCACGAGUCU	309	250_272

AD-63985.2	A-127961.1	GUGGUGGACUUCUCUCUCAAUUU	94	A-127956.4	AAAUUGAGAGAAGUCCACCACGA	310	254_276
AD-63986.2	A-127969.1	GUGGUGGACUUCUCUCUCAAUUU	95	A-127956.12	AAAUUGAGAGAAGUCCACCACGA	311	254_276
AD-63987.2	A-127955.9	GUGGUGGACUUCUCUCUCAAUUU	96	A-127977.1	AAAUUGAGAGAAGUCCACCACGA	312	254_276
AD-63988.2	A-127986.1	UGGACUUCUCUCAAUUU	97	A-127987.1	AAAUUGAGAGAAGUCCACC	313	258_280
AD-63989.2	A-127996.1	GUGGUGGACUUCUCUCUCAAUUU	98	A-127992.2	AAAUUGAGAGAAGUCCACCACGA	314	254_276
AD-63990.2	A-127950.1	GGUGGACUUCUCUCAAUUUUU	99	A-127951.1	AAAUUGAGAGAAGUCCACCUU	315	256_278
AD-63991.2	A-127962.1	GUGGUGGACUUCUCUCUCAAUUU	100	A-127956.5	AAAUUGAGAGAAGUCCACCACGA	316	254_276
AD-63992.2	A-127955.2	GUGGUGGACUUCUCUCUCAAUUU	101	A-127970.1	AAAUUGAGAGAAGUCCACCACGA	317	254_276
AD-63993.2	A-127955.10	GUGGUGGACUUCUCUCUCAAUUU	102	A-127978.1	AAAUUGAGAGAAGUCCACCACGA	318	254_276
AD-63994.2	A-127984.2	GGUGGACUUCUCUCUCAAUUU	103	A-127988.1	AAAUUGAGAGAAGUCCACCAC	319	256_278
AD-63995.2	A-127996.2	GUGGUGGACUUCUCUCUCAAUUU	104	A-127993.2	AAAUUGAGAGAAGUCCACCACGA	320	254_276
AD-63996.2	A-127952.1	GGUGGACUUCUCUCUCAAUUU	105	A-127953.1	AAAUUGAGAGAAGUCCACCUU	321	256_278
AD-63997.2	A-127963.1	GUGGUGGACUUCUCUCUCAAUUU	106	A-127956.6	AAAUUGAGAGAAGUCCACCACGA	322	254_276
AD-63999.2	A-127955.11	GUGGUGGACUUCUCUCUCAAUUU	107	A-127979.1	AAAUUGAGAGAAGUCCACCACGA	323	254_276
AD-64000.2	A-127986.2	UGGACUUCUCUCUCAAUUUU	108	A-127989.1	AAAUUGAGAGAAGUCCACC	324	258_280
AD-64001.2	A-127996.3	GUGGUGGACUUCUCUCUCAAUUU	109	A-127994.2	AAAUUGAGAGAAGUCCACCACGA	325	254_276
AD-64002.2	A-127952.2	GGUGGACUUCUCUCUCAAUUU	110	A-127954.1	AAAUUGAGAGAAGUCCACCUU	326	256_278
AD-64003.2	A-127964.1	GUGGUGGACUUCUCUCUCAAUUU	111	A-127956.7	AAAUUGAGAGAAGUCCACCACGA	327	254_276
AD-64004.2	A-127955.4	GUGGUGGACUUCUCUCUCAAUUU	112	A-127972.1	AAAUUGAGAGAAGUCCACCACGA	328	254_276
AD-64005.2	A-127955.12	GUGGUGGACUUCUCUCUCAAUUU	113	A-127980.1	AAAUUGAGAGAAGUCCACCACGA	329	254_276
AD-64006.2	A-127990.1	GUGGUGGACUUCUCUCUCAAUUU	114	A-127991.1	AAAUUGAGAGAAGUCCACCACGA	330	254_276
AD-64007.2	A-127996.4	GUGGUGGACUUCUCUCUCAAUUU	115	A-127995.2	AAAUUGAGAGAAGUCCACCACGA	331	254_276
AD-64008.2	A-127955.1	GUGGUGGACUUCUCUCUCAAUUU	116	A-127956.1	AAAUUGAGAGAAGUCCACCACGA	332	254_276
AD-64008.4	A-127955.15	GUGGUGGACUUCUCUCUCAAUUU	117	A-127956.14	AAAUUGAGAGAAGUCCACCACGA	333	254_276
AD-64009.2	A-127965.1	GUGGUGGACUUCUCUCUCAAUUU	118	A-127956.8	AAAUUGAGAGAAGUCCACCACGA	334	254_276
AD-64010.2	A-127955.5	GUGGUGGACUUCUCUCUCAAUUU	119	A-127973.1	AAAUUGAGAGAAGUCCACCACGA	335	254_276

AD-64011.2	A-127955.13	GUGGUGGACUUCUCUCAAUUU	120	A-127981.1	AAAUUGAGAGAAGUCCACCACGA	336	254_276
AD-64012.2	A-127990.2	GUGGUGGACUUCUCUCAAUUU	121	A-127992.1	AAAUUGAGAGAAGUCCACCACGA	337	254_276
AD-64013.2	A-127997.1	GUGGUGGACTTCUCUCAAUUU	122	A-127998.1	AAAUUGAGAGAAGTCCACCACGA	338	254_276
AD-64014.2	A-127957.1	GUGGUGGACUUCUCUCAAUUU	123	A-127958.1	AAAUUGAGAGAAGUCCACCACGA	339	254_276
AD-64015.2	A-127966.1	GUGGUGGACUUCUCUCAAUUU	124	A-127956.9	AAAUUGAGAGAAGUCCACCACGA	340	254_276
AD-64016.2	A-127955.6	GUGGUGGACUUCUCUCAAUUU	125	A-127974.1	AAAUUGAGAGAAGUCCACCACGA	341	254_276
AD-64017.2	A-127968.2	GUGGUGGACUTCUCUCAAUUU	126	A-127982.1	AAAUUGAGAGAAGTCCACCACGA	342	254_276
AD-64018.2	A-127990.3	GUGGUGGACUUCUCUCAAUUU	127	A-127993.1	AAAUUGAGAGAAGUCCACCACGA	343	254_276
AD-64019.2	A-127959.1	GUGGUGGACUUCUCUCAAUUU	128	A-127956.2	AAAUUGAGAGAAGUCCACCACGA	344	254_276
AD-64020.2	A-127967.1	GUGGUGGACUUCUCUCAAUUU	129	A-127956.10	AAAUUGAGAGAAGUCCACCACGA	345	254_276
AD-64021.2	A-127955.7	GUGGUGGACUUCUCUCAAUUU	130	A-127975.1	AAAUUGAGAGAAGUCCACCACGA	346	254_276
AD-64022.2	A-127968.3	GUGGUGGACUTCUCUCAAUUU	131	A-127983.1	AAAUUGAGAGAAGTCCACCACGA	347	254_276
AD-64023.2	A-127990.4	GUGGUGGACUUCUCUCAAUUU	132	A-127994.1	AAAUUGAGAGAAGUCCACCACGA	348	254_276
AD-64024.2	A-127960.1	GUGGUGGACUUCUCUCAAUUU	133	A-127956.3	AAAUUGAGAGAAGUCCACCACGA	349	254_276
AD-64025.2	A-127968.1	GUGGUGGACUTCUCUCAAUUU	134	A-127956.11	AAAUUGAGAGAAGUCCACCACGA	350	254_276
AD-64026.2	A-127955.8	GUGGUGGACUUCUCUCAAUUU	135	A-127976.1	AAAUUGAGAGAAGUCCACCACGA	351	254_276
AD-64027.2	A-127984.1	GGUGGACUUCUCUCAAUUU	136	A-127985.1	AAAUUGAGAGAAGUCCACCAC	352	256_278
AD-64028.2	A-127990.5	GUGGUGGACUUCUCUCAAUUU	137	A-127995.1	AAAUUGAGAGAAGUCCACCACGA	353	254_276
AD-64272.2	A-128001.2	GUGCACUUCGCUUCACCUCUG	138	A-128002.2	CAGAGGUGAAGCGAAGUGCACAC	354	1577_1599
AD-64274.1	A-128363.1	GUUGACAAAAAUCCUCACAAU	139	A-128364.1	AUUGUGAGGAUUUUUGUCAACAA	355	215_237
AD-64275.1	A-128377.1	UGUUGACAAAAAUCCUCACAA	140	A-128378.1	UUGUGAGGAUUUUUGUCAACAAG	356	214_236
AD-64276.1	A-128393.1	GGUGGACUUCUCUCAAUUUUA	141	A-128394.1	UAAAAUUGAGAGAAGUCCACCAC	357	256_278
AD-64277.1	A-128407.1	UCUUUUGGAGUGUGGAUUCGA	142	A-128408.1	UCGAAUCCACACUCCAAAAGACA	358	2259_2281
AD-64277.1	A-128407.1	UCUUUUGGAGUGUGGAUUCGA	143	A-128408.1	UCGAAUCCACACUCCAAAAGACA	359	2259_2281
AD-64278.1	A-128423.1	ACUGUUCAAGCCUCCAAGCUA	144	A-128424.1	UAGCUUGGAGGCUUGAACAAGAC	360	1857_1879
AD-64279.1	A-128435.1	UCUGCCGAUCCAUACUGCGGA	145	A-128436.1	UCCGCAGUAUGGAUCGGCAGAGG	361	1255_1277

AD-64280.1	A-128379.1	AUGUGUCUGCGGCGUUUUAUA	146	A-128380.1	UAUAAAACGCCGCAGACACAUCC	362	375_397
AD-64281.1	A-128395.1	CCCCGUCUGUGCCUUCUCAUA	147	A-128396.1	UAUGAGAAGGCACAGACGGGGAG	363	1545_1567
AD-64282.1	A-128409.1	GCCUAAUCAUCUCUUGUUCAU	148	A-128410.1	AUGAACAAGAGAUGAUUAGCGAG	364	1831_1853
AD-64283.1	A-128425.1	UCUAGACUCGUGGUGGACUUC	149	A-128426.1	GAAGUCCACCACGAGUCUAGACU	365	245_267
AD-64284.1	A-128437.1	CUGCCGAUCCAUACUGCGGAA	150	A-128438.1	UUCCGCAGUAUGGAUCGGCAGAG	366	1256_1278
AD-64285.1	A-128365.1	UUUUUCUUGUUGACAAAAAUA	151	A-128366.1	UAUUUUUGUCAACAAGAAAAACC	367	207_229
AD-64286.1	A-128381.1	AUCUUCUUGUUGGUUCUUCUA	152	A-128382.1	UAGAAGAACCAACAAGAAGAUGA	368	426_448
AD-64289.1	A-128367.1	GUUUUUCUUGUUGACAAAAAU	153	A-128368.1	AUUUUUGUCAACAAGAAAAACCC	369	206_228
AD-64290.1	A-128383.1	CUGCCUAAUCAUCUCUUGUUA	154	A-128384.1	UAACAAGAGAUGAUUAGGCAGAG	370	1829_1851
AD-64291.1	A-128399.1	UCCUCACAAUACCACAGAGUA	155	A-128400.1	UACUCUGUGGUAUUGUGAGGAUU	371	226_248
AD-64292.1	A-128413.1	CUUGUUGACAAAAAAUCCUCAA	156	A-128414.1	UUGAGGAUUUUUGUCAACAAGAA	372	212_234
AD-64293.1	A-128439.1	GCAACUUUUUUCACCUCUGCCU	157	A-128440.1	AGGCAGAGGUGAAAAAGUUGCAU	373	1814_1836
AD-64294.1	A-128369.1	GGGAACAAGAGCUACAGCAUA	158	A-128370.1	UAUGCUGUAGCUCUUGUUCCCAA	374	2828_2850
AD-64295.1	A-128385.1	CGUGGUGGACUUCUCUCAAUU	159	A-128386.1	AAUUGAGAGAAGUCCACCAGCAG	375	253_275
AD-64297.1	A-128415.1	CUGCUGCUAUGCCUCAUCUUA	160	A-128416.1	UAAGAUGAGGCAUAGCAGCAGGA	376	411_433
AD-64298.1	A-128427.1	GUUGGAUGUGUCUGCGGCGUU	161	A-128428.1	AACGCCGCAGACACAUCCAACGA	377	370_392
AD-64299.1	A-128441.1	UUCAUCCUGCUGCUAUGCCUA	162	A-128442.1	UAGGCAUAGCAGCAGGAUGAAGA	378	405_427
AD-64300.1	A-128371.1	UUCUUGUUGACAAAAAUCCUA	163	A-128372.1	UAGGAUUUUUUGUCAACAAGAAAA	379	210_232
AD-64302.1	A-128417.1	UAUAUGGAUGAUGUGGUAUUA	164	A-128418.1	UAAUACCACACAUCAUCCAUAUAAC	380	734_756
AD-64303.1	A-128429.1	UUCAUCCUGCUGCUAUGCCUC	165	A-128430.1	GAGGCAUAGCAGCAGGAUGAAGA	381	405_427
AD-64304.1	A-128443.1	GUGCACUUCGCUUCACCUCUA	166	A-128444.1	UAGAGGUGAAGCGAAGUGCACAC	382	1577_1599
AD-64305.1	A-128373.1	UUGACAAAAAUCCUCACAAUA	167	A-128374.1	UAUUGUGAGGAUUUUUGUCAACA	383	216_238
AD-64307.1	A-128403.1	AAGCCUCCAAGCUGUGCCUUA	168	A-128404.1	UAAGGCACAGCUUGGAGGCUUGA	384	1864_1886
AD-64308.1	A-128419.1	CCUCUUCAUCCUGCUGCUAUA	169	A-128420.1	UAUAGCAGCAGGAUGAAGAGGAA	385	401_423
AD-64309.1	A-128431.1	CCUGCUGCUAUGCCUCAUCUU	170	A-128432.1	AAGAUGAGGCAUAGCAGCAGGAU	386	410_432
AD-64310.1	A-128375.1	CAUCUUCUUGUUGGUUCUUCU	171	A-128376.1	AGAAGAACCAACAAGAAGAUGAG	387	425_447

AD-64311.1	A-128391.1	CCGUCUGUGCCUUCUCAUCUA	172	A-128392.1	UAGAUGAGAAGGCACAGACGGGG	388	1547_1569
AD-64312.1	A-128405.1	CCUCAUCUUCUUGUUGGUUCU	173	A-128406.1	AGAACCAACAAGAAGAUGAGGCA	389	422_444
AD-64313.1	A-128421.1	CCACCAAAUGCCCCUAUCUUA	174	A-128422.1	UAAGAUAGGGGCAUUUGGUGGUC	390	2298_2320
AD-64314.1	A-128433.1	GCUCCUCUGCCGAUCCAUACU	175	A-128434.1	AGUAUGGAUCGGCAGAGGAGCCA	391	1250_1272
AD-64315.1	A-128363.2	GUUGACAAAAAAUCCUCACAAU	176	A-128445.1	AUUGUGAGGAUUUUUGUCAACAA	392	215_237
AD-64316.1	A-128377.2	UGUUGACAAAAAAUCCUCACAA	177	A-128453.1	UUGUGAGGAUUUUUGUCAACAAG	393	214_236
AD-64317.1	A-128393.2	GGUGGACUUCUCUCAAUUUUA	178	A-128461.1	UAAAAUUGAGAGAAGUCCACCAC	394	256_278
AD-64318.1	A-128407.2	UCUUUUGGAGUGUGGAUUCGA	179	A-128469.1	UCGAAUCCACACUCCAAAAGACA	395	2259_2281
AD-64318.1	A-128407.2	UCUUUUGGAGUGUGGAUUCGA	180	A-128469.1	UCGAAUCCACACUCCAAAAGACA	396	2259_2281
AD-64319.1	A-128423.2	ACUGUUCAAGCCUCCAAGCUA	181	A-128477.1	UAGCUUGGAGGCUUGAACAAGAC	397	1857_1879
AD-64320.1	A-128435.2	UCUGCCGAUCCAUACUGCGGA	182	A-128483.1	UCCGCAGUAUGGAUCGGCAGAGG	398	1255_1277
AD-64321.1	A-123463.3	AGUUAUAUGGAUGAUGUGGUA	183	A-128446.1	UACCACAUCAUCCAUAUAACUGA	399	731_753
AD-64322.1	A-128379.2	AUGUGUCUGCGGCGUUUUAUA	184	A-128454.1	UAUAAAAACGCCGCAGACACAUCC	400	375_397
AD-64323.1	A-128395.2	CCCCGUCUGUGCCUUCUCAUA	185	A-128462.1	UAUGAGAAGGCACAGACGGGGAG	401	1545_1567
AD-64324.1	A-128409.2	GCCUAAUCAUCUCUUGUUCAU	186	A-128470.1	AUGAACAAGAGAUGAUUAGCGAG	402	1831_1853
AD-64325.1	A-128425.2	UCUAGACUCGUGGUGGACUUC	187	A-128478.1	GAAGUCCACCACGAGUCUAGACU	403	245_267
AD-64326.1	A-128437.2	CUGCCGAUCCAUACUGCGGAA	188	A-128484.1	UUCCGCAGUAUGGAUCGGCAGAG	404	1256_1278
AD-64328.1	A-128381.2	AUCUUCUUGUUGGUUCUUCUA	189	A-128455.1	UAGAAGAACCAACAAGAAGAUGA	405	426_448
AD-64330.1	A-128411.2	UUCUCUCAAUUUUUCUAGGGGA	190	A-128471.1	UCCCCUAGAAAAAUUGAGAGAAGU	406	263_285
AD-64331.1	A-127905.16	ACUCCGUGGUGGACUUCUCUCA	191	A-127907.2	UGAGAGAAGUCCACCACGAGUCU	407	250_272
AD-64332.1	A-128001.3	GUGCACUUCGCUUCACCUCUG	192	A-128485.1	CAGAGGUGAAGCGAAGUGCACAC	408	1577_1599
AD-64333.1	A-128367.2	GUUUUUCUUGUUGACAAAAAU	193	A-128448.1	AUUUUUGUCAACAAGAAAAACCC	409	206_228
AD-64334.1	A-128383.2	CUGCCUAAUCAUCUCUUGUUA	194	A-128456.1	UAACAAGAGAUGAUUAGGCAGAG	410	1829_1851
AD-64335.1	A-128399.2	UCCUCACAAUACCACAGAGUA	195	A-128464.1	UACUCUGUGGUAUUGUGAGGAUU	411	226_248
AD-64336.1	A-128413.2	CUUGUUGACAAAAAAUCCUCAA	196	A-128472.1	UUGAGGAUUUUUGUCAACAAGAA	412	212_234
AD-64337.1	A-127955.16	GUGGUGGACUUCUCUCAAUUU	197	A-127958.2	AAAUUGAGAGAAGUCCACCACGA	413	254_276

AD-64338.1	A-128439.2	GCAACUUUUUUCACCUCUGCCU	198	A-128486.1	AGGCAGAGGUGAAAAAGUUGCAU	414	1814_1836
AD-64339.1	A-128369.2	GGGAACAAGAGCUACAGCAUA	199	A-128449.1	UAUGCUGUAGCUCUUGUUCCCAA	415	2828_2850
AD-64341.1	A-128401.2	UCAUCUUCUUGUUGGUUCUUA	200	A-128465.1	UAAGAACCAACAAGAAGAUGAGG	416	424_446
AD-64342.1	A-128415.2	CUGCUGCUAUGCCUCAUCUUA	201	A-128473.1	UAAGAUGAGGCAUAGCAGCAGGA	417	411_433
AD-64343.1	A-128427.2	GUUGGAUGUGUCUGCGGCGUU	202	A-128479.1	AACGCCGCAGACACAUCCAACGA	418	370_392
AD-64344.1	A-128441.2	UUCAUCCUGCUGCUAUGCCUA	203	A-128487.1	UAGGCAUAGCAGCAGGAUGAAGA	419	405_427
AD-64345.1	A-128371.2	UUCUUGUUGACAAAAAAUCCUA	204	A-128450.1	UAGGAUUUUUGUCAACAAGAAAA	420	210_232
AD-64347.1	A-123487.3	GGAUGUGUCUGCGGCGUUUUA	205	A-128466.1	UAAAACGCCGCAGACACAUCCAG	421	373_395
AD-64348.1	A-128417.2	UAUAUGGAUGAUGUGGUAUUA	206	A-128474.1	UAAUACCACAUCAUCCAUAUAAC	422	734_756
AD-64349.1	A-128429.2	UUCAUCCUGCUGCUAUGCCUC	207	A-128480.1	GAGGCAUAGCAGCAGGAUGAAGA	423	405_427
AD-64350.1	A-128443.2	GUGCACUUCCGCUUCACCUCUA	208	A-128488.1	UAGAGGUGAAGCGAAGUGCACAC	424	1577_1599
AD-64351.1	A-128373.2	UUGACAAAAAAUCCUCACAAUA	209	A-128451.1	UAUUGUGAGGAUUUUUGUCAACA	425	216_238
AD-64352.1	A-128389.2	CCAAGUGUUUUGCUGACGCAAA	210	A-128459.1	UUUGCGUCAGCAAACACUUGGCA	426	1174_1196
AD-64352.1	A-128389.2	CCAAGUGUUUUGCUGACGCAAA	211	A-128459.1	UUUGCGUCAGCAAACACUUGGCA	427	1174_1196
AD-64353.1	A-128403.2	AAGCCUCCAAGCUGUGCCUUA	212	A-128467.1	UAAGGCACAGCUUGGAGGCUUGA	428	1864_1886
AD-64354.1	A-128419.2	CCUCUUCAUCCUGCUGCUAUA	213	A-128475.1	UAUAGCAGCAGGAUGAAGAGGAA	429	401_423
AD-64355.1	A-128431.2	CCUGCUGCUAUGCCUCAUCUU	214	A-128481.1	AAGAUGAGGCAUAGCAGCAGGAU	430	410_432
AD-64356.1	A-128375.2	CAUCUUCUUGUUGGUUCUUCU	215	A-128452.1	AGAAGAACCAACAAGAAGAUGAG	431	425_447
AD-64357.1	A-128391.2	CCGUCUGUGCCUUCUCAUCUA	216	A-128460.1	UAGAUGAGAAGGCACAGACGGGG	432	1547_1569
AD-64358.1	A-128405.2	CCUCAUCUUCUUGUUGGUUCU	217	A-128468.1	AGAACCAACAAGAAGAUGAGGCA	433	422_444
AD-64359.1	A-128421.2	CCACCAAAUGCCCCUAUCUUA	218	A-128476.1	UAAGAUAGGGGCAUUUGGUGGUC	434	2298_2320
AD-64360.1	A-128433.2	GCUCCUCUGCCGAUCCAUACU	219	A-128482.1	AGUAUGGAUCGGCAGAGGAGCCA	435	1250_1272
AD-64700.1	A-129379.1	ACUCGUGGUGTACUUCUCUCA	220	A-127906.26	UGAGAGAAGUCCACCACGAGUCU	436	250_272
AD-64701.1	A-127905.20	ACUCGUGGUGGACUUCUCUCA	221	A-129387.1	UGAGAGAAGTCCACCACGAGUCU	437	250_272
AD-64702.1	A-127905.28	ACUCGUGGUGGACUUCUCUCA	222	A-129395.1	UGAGAGAAGUCCACCACGAGUCU	438	250_272
AD-64703.1	A-129376.2	ACUCGUGGUGGACUUCACUCA	223	A-129385.5	UGAGAGAAGTCCACCACGAGUCU	439	250_272

AD-64704.1	A-129381.3	ACUCGUGGUGTGTACUUCACUCA	224	A-129389.6	UGAGAGAAGUCCACCACGAGUCU	440	250_272
AD-64705.1	A-129380.1	ACUCGUGGUGUGTACUUCACUCA	225	A-127906.27	UGAGAGAAGUCCACCACGAGUCU	441	250_272
AD-64706.1	A-127905.21	ACUCGUGGUGGACUUCUCUCA	226	A-129388.1	UGAGAGAAGUCCACCACGAGUCU	442	250_272
AD-64707.1	A-127905.29	ACUCGUGGUGGACUUCUCUCA	227	A-129396.1	UGAGAGAAGTCCACCACGAGUCU	443	250_272
AD-64708.1	A-129382.2	ACUCGUGGTGGACUUCTCUCA	228	A-129385.6	UGAGAGAAGTCCACCACGAGUCU	444	250_272
AD-64709.1	A-129373.4	ACUCGUGGUGGACUUCUCUCA	229	A-129391.2	UGAGAGAAGTCCACCACGAGUCU	445	250_272
AD-64710.1	A-129373.1	ACUCGUGGUGGACUUCUCUCA	230	A-127906.20	UGAGAGAAGUCCACCACGAGUCU	446	250_272
AD-64711.1	A-129381.1	ACUCGUGGTGTACUUCACUCA	231	A-127906.28	UGAGAGAAGUCCACCACGAGUCU	447	250_272
AD-64712.1	A-127905.22	ACUCGUGGUGGACUUCUCUCA	232	A-129389.1	UGAGAGAAGUCCACCACGAGUCU	448	250_272
AD-64713.1	A-127905.30	ACUCGUGGUGGACUUCUCUCA	233	A-129397.1	UGAGAGAAGTCCACCACGAGUCU	449	250_272
AD-64714.1	A-129384.2	ACUCGUGGTGGACUUCACUCA	234	A-129385.7	UGAGAGAAGTCCACCACGAGUCU	450	250_272
AD-64715.1	A-129376.4	ACUCGUGGUGGACUUCACUCA	235	A-129391.3	UGAGAGAAGTCCACCACGAGUCU	451	250_272
AD-64716.1	A-129374.1	ACUCGUGGUGGACUUCUCUCA	236	A-127906.21	UGAGAGAAGUCCACCACGAGUCU	452	250_272
AD-64717.1	A-129382.1	ACUCGUGGTGGACUUCTCUCA	237	A-127906.29	UGAGAGAAGUCCACCACGAGUCU	453	250_272
AD-64718.1	A-127905.23	ACUCGUGGUGGACUUCUCUCA	238	A-129390.1	UGAGAGAAGUCCACCACGAGUCU	454	250_272
AD-64719.1	A-127917.5	ACUCGUGGUGGACUUCTCUCA	239	A-129385.2	UGAGAGAAGTCCACCACGAGUCU	455	250_272
AD-64720.1	A-129381.2	ACUCGUGGTGTACUUCACUCA	240	A-129385.8	UGAGAGAAGTCCACCACGAGUCU	456	250_272
AD-64721.1	A-129382.4	ACUCGUGGTGGACUUCTCUCA	241	A-129391.4	UGAGAGAAGTCCACCACGAGUCU	457	250_272
AD-64722.1	A-129375.1	ACUCGUGGUGGACUUCCUCA	242	A-127906.22	UGAGAGAAGUCCACCACGAGUCU	458	250_272
AD-64723.1	A-129383.1	ACUCGUGGUGGACUUCTCUCA	243	A-127906.30	UGAGAGAAGUCCACCACGAGUCU	459	250_272
AD-64725.1	A-127917.6	ACUCGUGGUGGACUUCTCUCA	244	A-129398.1	UGAGAGAAGTCCACCACGAGUCU	460	250_272
AD-64726.1	A-129373.3	ACUCGUGGUGGACUUCUCUCA	245	A-129389.2	UGAGAGAAGUCCACCACGAGUCU	461	250_272
AD-64727.1	A-129384.4	ACUCGUGGTGGACUUCACUCA	246	A-129391.5	UGAGAGAAGTCCACCACGAGUCU	462	250_272
AD-64728.1	A-129376.1	ACUCGUGGUGGACUUCACUCA	247	A-127906.23	UGAGAGAAGUCCACCACGAGUCU	463	250_272
AD-64729.1	A-129384.1	ACUCGUGGTGGACUUCACUCA	248	A-127906.31	UGAGAGAAGUCCACCACGAGUCU	464	250_272
AD-64730.1	A-127905.25	ACUCGUGGUGGACUUCUCUCA	249	A-129392.1	UGAGAGAAGTCCACCACGAGUCU	465	250_272

AD-64731.1	A-129399.1	ACUCGUGGUGGACUUCTCUCA	250	A-129385.3	UGAGAGAAGTCCACCACGAGUCU	466	250_272
AD-64732.1	A-129376.3	ACUCGUGGUGGACUUCACUCA	251	A-129389.3	UGAGAGAAGUCCACCACGAGUCU	467	250_272
AD-64733.1	A-129381.4	ACUCGUGGTGTACUUCACUCA	252	A-129391.6	UGAGAGAAGTCCACCACGAGUCU	468	250_272
AD-64734.1	A-129377.1	ACUCGUGGUGGACUUCCCUCA	253	A-127906.24	UGAGAGAAGUCCACCACGAGUCU	469	250_272
AD-64735.1	A-127905.18	ACUCGUGGUGGACUUCUCUCA	254	A-129385.1	UGAGAGAAGTCCACCACGAGUCU	470	250_272
AD-64736.1	A-127905.26	ACUCGUGGUGGACUUCUCUCA	255	A-129393.1	UGAGAGAAGTCCACCACGAGUCU	471	250_272
AD-64737.1	A-129399.2	ACUCGUGGUGGACUUCTCUCA	256	A-129398.2	UGAGAGAAGTCCACCACGAGUCU	472	250_272
AD-64738.1	A-129382.3	ACUCGUGGTGGACUUCTCUCA	257	A-129389.4	UGAGAGAAGUCCACCACGAGUCU	473	250_272
AD-64739.1	A-129378.1	ACUCGUGGUGGACUUCGCUCA	258	A-127906.25	UGAGAGAAGUCCACCACGAGUCU	474	250_272
AD-64740.1	A-127905.19	ACUCGUGGUGGACUUCUCUCA	259	A-129386.1	UGAGAGAAGTCCACCACGAGUCU	475	250_272
AD-64741.1	A-127905.27	ACUCGUGGUGGACUUCUCUCA	260	A-129394.1	UGAGAGAAGTCCACCACGAGUCU	476	250_272
AD-64742.1	A-129373.2	ACUCGUGGUGGACUUCUCUCA	261	A-129385.4	UGAGAGAAGTCCACCACGAGUCU	477	250_272
AD-64743.1	A-129384.3	ACUCGUGGTGGACUUCACUCA	262	A-129389.5	UGAGAGAAGUCCACCACGAGUCU	478	250_272

Table 4. Modified Sense and Antisense Strand Sequences of HBV dsRNAs

Duplex Name	Sense Oligo Name	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Oligo Name	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-61522.2	A-123463.2	AfsgsUfuAfuAfuGfGfAfuGfaUfgUfgGfuAfL96	479	A-123464.2	usAfscCfaCfaUfcAfuccAfuAfuAfaCfusgsa	694
AD-61547.2	A-123487.2	GfsgsAfuGfuGfuCfUfGfcGfgCfgUfuUfuAfL96	480	A-123488.2	usAfsaAfaCfgCfcGfcagAfcAfcAfuCfcsasg	695
AD-63938.2	A-127896.1	Y44ACUCGUGGUGGACUUCUCUCA	481	A-127897.1	UGAGAGAAGUCCACCACGAGUCU	696
AD-63939.2	A-127909.1	ascsucGfuGfgUfGfGfaCfuucUfcucaL96	482	A-127906.3	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	697
AD-63940.2	A-127917.1	ascsucguggugdGacuuc(Tgn)cucaL96	483	A-127906.11	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	698
AD-63941.2	A-127905.8	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	484	A-127925.1	usGfsaGfagaAfguccaCfcAfcgaGfuscsu	699
AD-63942.2	A-127933.1	uscsGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	485	A-127934.1	usGfsaGfaGfaAfgUfccaCfcAfcGfasgsu	700
AD-63943.2	A-127944.2	ascsucGfuGfguGfGfaCfuucucucaL96	486	A-127942.2	usGfsAfgaGfaAfgUfccaCfcAfcGfaguscsu	701
AD-63945.2	A-127910.1	ascsucguGfgUfGfGfaCfuucUfcucaL96	487	A-127906.4	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	702
AD-63946.2	A-127918.1	ascsucguGfgUfGfGfacuuCfucucaL96	488	A-127906.12	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	703
AD-63947.2	A-127905.9	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	489	A-127926.1	usGfsaGfagaagUfccaCfcAfcgaGfuscsu	704
AD-63948.2	A-127935.1	gsusGfgUfGfGfaCfuUfcUfcUfcAfL96	490	A-127936.1	usGfsaGfaGfaAfgUfccaCfcAfcsgsa	705
AD-63949.2	A-127944.3	ascsucGfuGfguGfGfaCfuucucucaL96	491	A-127906.14	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	706
AD-63950.2	A-127900.1	Y44UfcGfuGfgUfgGfaCfuUfcUfcUfcAfusuY44	492	A-127901.1	usGfsasGfaGfaAfgUfcCfaCfcAfcGfausu	707
AD-63951.2	A-127911.1	ascsucguGfgUfGfGfaCfuucucucaL96	493	A-127906.5	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	708
AD-63952.2	A-127905.2	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	494	A-127919.1	usGfsaGfaGfaagUfccaCfcAfcGfaGfuscsu	709
AD-63953.2	A-127905.10	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	495	A-127927.1	usGfsagagaAfgUfccaCfcAfcgaguscsu	710
AD-63955.2	A-127945.1	ascsucgugguGfGfacuucucucaL96	496	A-127940.3	usGfsAfgAfgAfaGfuccaCfCfaCfgAfguscsu	711
AD-63956.2	A-127902.1	Y44uscsGfuGfgUfgGfaCfuUfcUfcUfcAfY44	497	A-127903.1	usGfsaGfaGfaAfgUfcCfaCfcAfcGfasusu	712
AD-63957.2	A-127912.1	ascsucguGfgUfGfGfacuucucucaL96	498	A-127906.6	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	713
AD-63958.2	A-127905.3	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	499	A-127920.1	usGfsagaGfaAfgUfccaCfcAfcgaGfuscsu	714

AD-63959.2	A-127905.11	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	500	A-127928.1	usGfsaGfagaAfguccaCfcAfcgaguscsu	715
AD-63960.2	A-126619.2	usasUfuUfCfCfuAfgGfgUfaCfaAfL96	501	A-127938.1	PusGfsaGfaGfaAfgUfccaCfcAfcsgsa	716
AD-63961.2	A-127945.2	ascsucgugguGfGfacuucucucaL96	502	A-127942.3	usGfsAfgaGfaAfgUfccaCfcAfcGfaguscsu	717
AD-63962.2	A-127902.2	Y44uscsGfuGfgUfgGfaCfuUfcUfcUfcAfY44	503	A-127904.1	PusGfsaGfaGfaAfgUfcCfaCfcAfcGfasusu	718
AD-63963.2	A-127913.1	ascsucgugguUfgGfacuucucucaL96	504	A-127906.7	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	719
AD-63964.2	A-127905.4	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	505	A-127921.1	usGfsaGfaGfaAfgUfccaCfcAfcgaguscsu	720
AD-63965.2	A-127905.12	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	506	A-127929.1	usGfsagaGfaaGfuccaCfcAfcgaguscsu	721
AD-63966.2	A-127939.1	ascsUfcGfugguGfGfaCfuuCfuCfucaL96	507	A-127940.1	usGfsAfgAfgAfaGfuccaCfCfaCfgAfguscsu	722
AD-63967.2	A-127945.3	ascsucgugguGfGfacuucucucaL96	508	A-127906.15	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	723
AD-63968.2	A-127905.1	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	509	A-127906.1	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	724
AD-63968.4	A-127905.15	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	510	A-127906.17	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	725
AD-63968.5	A-127905.17	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	511	A-127906.18	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	726
AD-63969.2	A-127914.1	ascsucgugguGfacuucucucaL96	512	A-127906.8	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	727
AD-63970.2	A-127905.5	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	513	A-127922.1	usGfsagaGfaagUfccaCfcAfcgaGfuscsu	728
AD-63971.2	A-127905.13	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	514	A-127930.1	usGfsagaGfaaguccaCfcAfcgaguscsu	729
AD-63972.2	A-127941.1	ascsUfcGfuGfguGfGfaCfuuCfuCfucaL96	515	A-127942.1	usGfsAfgaGfaAfgUfccaCfcAfcGfagguscsu	730
AD-63973.2	A-127946.1	ascsucgugguGdGacuucucucaL96	516	A-127947.1	usdGsaGfaGfaAfgdTccadCcAfcGfaguscsu	731
AD-63975.2	A-127915.1	ascsucgugguUfgGfacuuc(Tgn)cucaL96	517	A-127906.9	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	732
AD-63976.2	A-127905.6	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	518	A-127923.1	usGfsagaGfaAfgUfccaCfcAfcgaguscsu	733
AD-63977.2	A-127917.2	ascsucgugguGdGacuuc(Tgn)cucaL96	519	A-127931.1	usdGsagagaaguccadCcacgaguscsu	734
AD-63978.2	A-127943.1	ascsUfcGfuGfguGfGfaCfuUfcUfcUfcaL96	520	A-127906.13	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	735
AD-63979.2	A-127908.1	ascsucGfuGfgUfGfGfaCfuucUfcucAfL96	521	A-127906.2	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	736
AD-63980.2	A-127916.1	ascsucgugguGfacuuc(Tgn)cucaL96	522	A-127906.10	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	737
AD-63981.2	A-127905.7	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfL96	523	A-127924.1	usGfsaGfagaAfgUfccaCfcAfcgaGfuscsu	738
AD-63982.2	A-127917.3	ascsucgugguGdGacuuc(Tgn)cucaL96	524	A-127932.1	PusdGsagagaaguccadCcacgaguscsu	739
AD-63983.2	A-127944.1	ascsucGfuGfguGfGfaCfuucucucaL96	525	A-127940.2	usGfsAfgAfgAfaGfuccaCfCfaCfgAfguscsu	740

AD-63985.2	A-127961.1	gsusggugGfaCfUfUfcUfcucAfauuuL96	526	A-127956.4	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	741
AD-63986.2	A-127969.1	gsusggugGfaCfUfUfcucuCfaauuuL96	527	A-127956.12	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	742
AD-63987.2	A-127955.9	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	528	A-127977.1	asAfsaUfugagaGfaagUfcCfaccAfcsgsa	743
AD-63988.2	A-127986.1	usgsGfaCfUfUfcUfcUfcAfaUfuUfIL96	529	A-127987.1	asAfsaUfuGfaGfaGfaagUfcCfascsc	744
AD-63989.2	A-127996.1	gsusgguggacUfUfcucucaauuuL96	530	A-127992.2	asAfsAfUfuGfaGfaGfaagUfcCfaCfcacsgsa	745
AD-63990.2	A-127950.1	Y44GfgUfgGfaCfuUfcUfcAfaUfuUfusuY44	531	A-127951.1	asAfsasUfuGfaGfaGfaAfgUfcCfaCfcusu	746
AD-63991.2	A-127962.1	gsusggugGfaCfUfUfcUfcUfcucaauuuL96	532	A-127956.5	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	747
AD-63992.2	A-127955.2	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	533	A-127970.1	asAfsaUfuGfagaGfaagUfcCfaCfcAfcsgsa	748
AD-63993.2	A-127955.10	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	534	A-127978.1	asAfsauugaGfaGfaagUfcCfaccacsgsa	749
AD-63994.2	A-127984.2	gsgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	535	A-127988.1	PasAfsaUfuGfaGfaGfaagUfcCfaCfcsasc	750
AD-63995.2	A-127996.2	gsusgguggacUfUfcucucaauuuL96	536	A-127993.2	asAfsAfuuGfaGfaGfaagUfCfcaCfcacsgsa	751
AD-63996.2	A-127952.1	Y44gsgsUfgGfaCfuUfcUfcUfcAfaUfuUfY44	537	A-127953.1	asAfsaUfuGfaGfaGfaAfgUfcCfaCfcsusu	752
AD-63997.2	A-127963.1	gsusggugGfaCfUfUfcucucaauuuL96	538	A-127956.6	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	753
AD-63999.2	A-127955.11	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	539	A-127979.1	asAfsaUfugaGfagaagUfcCfaccacsgsa	754
AD-64000.2	A-127986.2	usgsGfaCfUfUfcUfcUfcAfaUfuUfIL96	540	A-127989.1	PasAfsaUfuGfaGfaGfaagUfcCfascsc	755
AD-64001.2	A-127996.3	gsusgguggacUfUfcucucaauuuL96	541	A-127994.2	asAfsAfUfuGfaGfaGfaagUfCfcaCfcacsgsa	756
AD-64002.2	A-127952.2	Y44gsgsUfgGfaCfuUfcUfcAfaUfuUfY44	542	A-127954.1	PasAfsaUfuGfaGfaGfaAfgUfcCfaCfcsusu	757
AD-64003.2	A-127964.1	gsusggugGfaCfuUfcucucaauuuL96	543	A-127956.7	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	758
AD-64004.2	A-127955.4	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	544	A-127972.1	asAfsaUfuGfaGfaGfaagUfcCfaccacsgsa	759
AD-64005.2	A-127955.12	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	545	A-127980.1	asAfsauuGfagAfgaagUfcCfaccacsgsa	760
AD-64006.2	A-127990.1	gsusGfgugGfaCfUfUfcUfcUfcAfaUfuuL96	546	A-127991.1	asAfsaUfuGfaGfaGfaagUfcCfaCfcacsgsa	761
AD-64007.2	A-127996.4	gsusgguggacUfUfcucucaauuuL96	547	A-127995.2	asAfsAfUfugaGfaGfaagUfCfcaCfcacsgsa	762
AD-64008.2	A-127955.1	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	548	A-127956.1	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	763
AD-64008.4	A-127955.15	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	549	A-127956.14	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	764
AD-64009.2	A-127965.1	gsusgguggacuUfcucucaauuuL96	550	A-127956.8	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	765
AD-64010.2	A-127955.5	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	551	A-127973.1	asAfsauuGfagaGfaagUfcCfaccAfcsgsa	766

AD-64011.2	A-127955.13	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	552	A-127981.1	asAfsauuGfagagaagUfcCfaccacsgsa	767
AD-64012.2	A-127990.2	gsusGfgugGfaCfUfUfcUfcUfcAfaUfuuL96	553	A-127992.1	asAfsAfUfuGfaGfaGfaagUfcCfaCfcacsgsa	768
AD-64013.2	A-127997.1	gsusgguggacdTdTcucucaauuuL96	554	A-127998.1	asdAsAfuugaGfaGfaagdTdCcaCfcacsgsa	769
AD-64014.2	A-127957.1	Y44GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	555	A-127958.1	PasAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	770
AD-64015.2	A-127966.1	gsusgguggaCfuUfcucuc(Agn)auuuL96	556	A-127956.9	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	771
AD-64016.2	A-127955.6	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	557	A-127974.1	asAfsauuGfaGfaGfaagUfcCfaccacsgsa	772
AD-64017.2	A-127968.2	gsusgguggacudTcucuc(Agn)auuuL96	558	A-127982.1	asdAsauugagagaagdTccaccacsgsa	773
AD-64018.2	A-127990.3	gsusGfgugGfaCfUfUfcUfcUfcAfaUfuuL96	559	A-127993.1	asAfsAfuuGfaGfaGfaagUfCfcaCfcacsgsa	774
AD-64019.2	A-127959.1	gsusggUfgGfaCfUfUfcUfcucAfauuUfIL96	560	A-127956.2	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	775
AD-64020.2	A-127967.1	gsusgguggacuUfcucuc(Agn)auuuL96	561	A-127956.10	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	776
AD-64021.2	A-127955.7	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	562	A-127975.1	asAfsaUfugaGfaGfaagUfcCfaccAfcsgsa	777
AD-64022.2	A-127968.3	gsusgguggacudTcucuc(Agn)auuuL96	563	A-127983.1	PasdAsauugagagaagdTccaccacsgsa	778
AD-64023.2	A-127990.4	gsusGfgugGfaCfUfUfcUfcUfcAfaUfuuL96	564	A-127994.1	asAfsAfUfuGfaGfaGfaagUfCfcaCfcacsgsa	779
AD-64024.2	A-127960.1	gsusggUfgGfaCfUfUfcUfcucAfauuuL96	565	A-127956.3	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	780
AD-64025.2	A-127968.1	gsusgguggacudTcucuc(Agn)auuuL96	566	A-127956.11	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	781
AD-64026.2	A-127955.8	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	567	A-127976.1	asAfsaUfugaGfagaagUfcCfaccAfcsgsa	782
AD-64027.2	A-127984.1	gsgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	568	A-127985.1	asAfsaUfuGfaGfaGfaagUfcCfaCfcsasc	783
AD-64028.2	A-127990.5	gsusGfgugGfaCfUfUfcUfcUfcAfaUfuuL96	569	A-127995.1	asAfsAfUfugaGfaGfaagUfCfcaCfcacsgsa	784
AD-64272.2	A-128001.2	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuGfIL96	570	A-128002.2	csAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	785
AD-64274.1	A-128363.1	GfsusUfgAfcAfaAfAfAfAfuCfcUfcAfcAfaUfIL96	571	A-128364.1	asUfsuGfuGfaGfgAfuuuUfuGfuCfaAfcsasa	786
AD-64275.1	A-128377.1	UfsgsUfuGfaCfaAfAfAfAfaUfcCfuCfaCfaAfL96	572	A-128378.1	usUfsgUfgAfgGfaUfuuuUfgUfcAfaCfasasg	787
AD-64276.1	A-128393.1	GfsgsUfgGfaCfuUfCfUfcUfcAfaUfuUfuAfIL96	573	A-128394.1	usAfsaAfaUfuGfaGfagaAfgUfcCfaCfcsasc	788
AD-64277.1	A-128407.1	UfscsUfuUfuGfgAfGfUfgUfgGfaUfuCfgAfL96	574	A-128408.1	usCfsgAfaUfcCfaCfacuCfcAfaAfaGfascsa	789
AD-64277.1	A-128407.1	UfscsUfuUfuGfgAfGfUfgUfgGfaUfuCfgAfL96	575	A-128408.1	usCfsgAfaUfcCfaCfacuCfcAfaAfaGfascsa	790
AD-64278.1	A-128423.1	AfscsUfgUfuCfaAfGfCfcUfcCfaAfgCfuAfL96	576	A-128424.1	usAfsgCfuUfgGfaGfgcuUfgAfaCfaAfgsasc	791
AD-64279.1	A-128435.1	UfscsUfgCfcGfaUfCfCfaUfaCfuGfcGfgAfL96	577	A-128436.1	usCfscGfcAfgUfaUfgguUfcGfgCfaGfasgsg	792

AD-64280.1	A-128379.1	AfsusGfuGfuCfuGfCfGfgCfgUfuUfuAfuAfL96	578	A-128380.1	usAfsuAfaAfaCfgCfcgcAfgAfcAfcAfuscsc	793
AD-64281.1	A-128395.1	CfscsCfcGfuCfuGfUfGfcCfuUfcUfcAfuAfL96	579	A-128396.1	usAfsuGfaGfaAfgGfcacAfgAfcGfgGfgsasg	794
AD-64282.1	A-128409.1	GfscsCfuAfaUfcAfUfCfuCfuUfgUfuCfaUfL96	580	A-128410.1	asUfsgAfaCfaAfgAfgauGfaUfuAfgCfgsasg	795
AD-64283.1	A-128425.1	UfscsUfaGfaCfuCfGfUfgGfuGfgAfcUfuCfL96	581	A-128426.1	gsAfsaGfuCfcAfcCfacgAfgUfcUfaGfascsu	796
AD-64284.1	A-128437.1	CfsusGfcCfgAfuCfCfAfuAfcUfgCfgGfaAfL96	582	A-128438.1	usUfscCfgCfaGfuAfuggAfuCfgGfcAfgsasg	797
AD-64285.1	A-128365.1	UfsusUfuUfcUfuGfUfUfgAfcAfaAfaAfuAfL96	583	A-128366.1	usAfsuUfuUfuGfuCfaacAfaGfaAfaAfascsc	798
AD-64286.1	A-128381.1	AfsusCfuUfcUfuGfUfUfgGfuUfcUfuCfuAfL96	584	A-128382.1	usAfsgAfaGfaAfcCfaacAfaGfaAfgAfusgsa	799
AD-64289.1	A-128367.1	GfsusUfuUfuCfuUfGfUfuGfaCfaAfaAfaUfL96	585	A-128368.1	asUfsuUfuUfgUfcAfacaAfgAfaAfaAfcscsc	800
AD-64290.1	A-128383.1	CfsusGfcCfuAfaUfCfAfuCfuCfuUfgUfuAfL96	586	A-128384.1	usAfsaCfaAfgAfgAfugaUfuAfgGfcAfgsasg	801
AD-64291.1	A-128399.1	UfscsCfuCfaCfaAfUfAfcCfaCfaGfaGfuAfL96	587	A-128400.1	usAfscUfcUfgUfgGfuauUfgUfgAfgGfasusu	802
AD-64292.1	A-128413.1	CfsusUfgUfuGfaCfAfAfaAfaUfcCfuCfaAfL96	588	A-128414.1	usUfsgAfgGfaUfuUfuugUfcAfaCfaAfgsasa	803
AD-64293.1	A-128439.1	GfscsAfaCfuUfuUfUfCfaCfcUfcUfgCfcUfL96	589	A-128440.1	asGfsgCfaGfaGfgUfgaaAfaAfgUfuGfcsasu	804
AD-64294.1	A-128369.1	GfsgsGfaAfcAfaGfAfGfcUfaCfaGfcAfuAfL96	590	A-128370.1	usAfsuGfcUfgUfaGfcucUfuGfuUfcCfcsasa	805
AD-64295.1	A-128385.1	CfsgsUfgGfuGfgAfCfUfuCfuCfuCfaAfuUfL96	591	A-128386.1	asAfsuUfgAfgAfgAfaguCfcAfcCfaGfcsasg	806
AD-64297.1	A-128415.1	CfsusGfcUfgCfuAfUfGfcCfuCfaUfcUfuAfL96	592	A-128416.1	usAfsaGfaUfgAfgGfcauAfgCfaGfcAfgsgsa	807
AD-64298.1	A-128427.1	GfsusUfgGfaUfgUfGfUfcUfgCfgGfcGfuUfL96	593	A-128428.1	asAfscGfcCfgCfaGfacaCfaUfcCfaAfcsgsa	808
AD-64299.1	A-128441.1	UfsusCfaUfcCfuGfCfUfgCfuAfuGfcCfuAfL96	594	A-128442.1	usAfsgGfcAfuAfgCfagcAfgGfaUfgAfasgsa	809
AD-64300.1	A-128371.1	UfsusCfuUfgUfuGfAfCfaAfaAfaUfcCfuAfL96	595	A-128372.1	usAfsgGfaUfuUfuUfgucAfaCfaAfgAfasasa	810
AD-64302.1	A-128417.1	UfsasUfaUfgGfaUfGfAfuGfuGfgUfaUfuAfL96	596	A-128418.1	usAfsaUfaCfcAfcAfucaUfcCfaUfaUfasasc	811
AD-64303.1	A-128429.1	UfsusCfaUfcCfuGfCfUfgCfuAfuGfcCfuCfL96	597	A-128430.1	gsAfsgGfcAfuAfgCfagcAfgGfaUfgAfasgsa	812
AD-64304.1	A-128443.1	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuAfL96	598	A-128444.1	usAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	813
AD-64305.1	A-128373.1	UfsusGfaCfaAfaAfaAfUfUfcCfuCfaCfaAfuAfL96	599	A-128374.1	usAfsuUfgUfgAfgGfauuUfuUfgUfcAfascsa	814
AD-64307.1	A-128403.1	AfsasGfcCfuCfcAfAfGfcUfgUfgCfcUfuAfL96	600	A-128404.1	usAfsaGfgCfaCfaGfcuuGfgAfgGfcUfusgsa	815
AD-64308.1	A-128419.1	CfscsUfcUfuCfaUfCfCfuGfcUfgCfuAfuAfL96	601	A-128420.1	usAfsuAfgCfaGfcAfggaUfgAfaGfaGfgsasa	816
AD-64309.1	A-128431.1	CfscsUfgCfuGfcUfAfUfgCfcUfcAfuCfuUfL96	602	A-128432.1	asAfsgAfuGfaGfgCfauaGfcAfgCfaGfgsasu	817
AD-64310.1	A-128375.1	CfsasUfcUfuCfuUfGfUfuGfgUfuCfuUfcUfL96	603	A-128376.1	asGfsaAfgAfaCfcAfacaAfgAfaGfaUfgsasg	818

AD-64311.1	A-128391.1	CfscsGfuCfuGfuGfCfCfuUfcUfcAfuCfuAfL96	604	A-128392.1	usAfsgAfuGfaGfaAfggcAfcAfgAfcGfgsgsg	819
AD-64312.1	A-128405.1	CfscsUfcAfuCfuUfCfUfuGfuUfgGfuUfcUfIL96	605	A-128406.1	asGfsaAfcCfaAfcAfagaAfgAfuGfaGfgscsa	820
AD-64313.1	A-128421.1	CfscsAfcCfaAfaUfGfCfcCfcUfaUfcUfuAfL96	606	A-128422.1	usAfsaGfaUfaGfgGfgcaUfuUfgGfuGfgsusc	821
AD-64314.1	A-128433.1	GfscsUfcCfuCfuGfCfCfCfgAfuCfcAfuAfcUfL96	607	A-128434.1	asGfsuAfuGfgAfuCfggcAfgAfgGfaGfcscsa	822
AD-64315.1	A-128363.2	GfsusUfgAfcAfaAfAfAfuCfcUfcAfcAfaUfL96	608	A-128445.1	PasUfsuGfuGfaGfgAfuuuUfuGfuCfaAfcsasa	823
AD-64316.1	A-128377.2	UfsgsUfuGfaCfaAfAfAfaUfcCfuCfaCfaAfL96	609	A-128453.1	PusUfsgUfgAfgGfaUfuuuUfgUfcAfaCfasasg	824
AD-64317.1	A-128393.2	GfsgsUfgGfaCfuUfCfUfcUfcAfaUfuUfuAfL96	610	A-128461.1	PusAfsaAfaUfuGfaGfagaAfgUfcCfaCfcsasc	825
AD-64318.1	A-128407.2	UfscsUfuUfuGfgAfGfUfgUfgGfaUfuCfgAfL96	611	A-128469.1	PusCfsgAfaUfcCfaCfacuCfcAfaAfaGfascsa	826
AD-64319.1	A-128423.2	AfscsUfgUfuCfaAfGfCfcUfcCfaAfgCfuAfL96	612	A-128477.1	PusAfsgCfuUfgGfaGfgcuUfgAfaCfaAfgsasc	827
AD-64320.1	A-128435.2	UfscsUfgCfcGfaUfCfCfCfaUfaCfuGfcGfgAfL96	613	A-128483.1	PusCfscGfcAfgUfaUfgggaUfcGfgCfaGfasgsg	828
AD-64321.1	A-123463.3	AfsgsUfuAfuAfuGfGfAfuGfaUfgUfgGfuAfL96	614	A-128446.1	PusAfscCfaCfaUfcAfuccAfuAfuAfaCfusgsa	829
AD-64322.1	A-128379.2	AfsusGfuGfuCfuGfCfCfGfgCfgUfuUfuAfuAfL96	615	A-128454.1	PusAfsuAfaAfaCfgCfcgcAfgAfcAfcAfuscsc	830
AD-64323.1	A-128395.2	CfscsCfcGfuCfuGfUfGfcCfuUfcUfcAfuAfL96	616	A-128462.1	PusAfsuGfaGfaAfgGfcacAfgAfcGfgGfgsasg	831
AD-64324.1	A-128409.2	GfscsCfuAfaUfcAfUfCfuCfuUfgUfuCfaUfL96	617	A-128470.1	PasUfsgAfaCfaAfgAfgauGfaUfuAfgCfgsasg	832
AD-64325.1	A-128425.2	UfscsUfaGfaCfuCfGfUfgGfuGfgAfcUfuCfL96	618	A-128478.1	PgsAfsaGfuCfcAfcCfacgAfgUfcUfaGfascsu	833
AD-64326.1	A-128437.2	CfsusGfcCfgAfuCfCfCfAfuAfcUfgCfgGfaAfL96	619	A-128484.1	PusUfscCfgCfaGfuAfuggAfuCfgGfcAfgsasg	834
AD-64328.1	A-128381.2	AfsusCfuUfcUfuGfUfUfgGfuUfcUfuCfuAfL96	620	A-128455.1	PusAfsgAfaGfaAfcCfaacAfaGfaAfgAfusgsa	835
AD-64330.1	A-128411.2	UfsusCfuCfuCfaAfUfUfuUfcUfaGfgGfgAfL96	621	A-128471.1	PusCfscCfcUfaGfaAfaauUfgAfgAfgAfasgsu	836
AD-64331.1	A-127905.16	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	622	A-127907.2	PusGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	837
AD-64332.1	A-128001.3	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuGfL96	623	A-128485.1	PcsAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	838
AD-64333.1	A-128367.2	GfsusUfuUfuCfuUfGfUfuGfaCfaAfaAfaUfL96	624	A-128448.1	PasUfsuUfuUfgUfcAfacaAfgAfaAfaAfcscsc	839
AD-64334.1	A-128383.2	CfsusGfcCfuAfaUfCfAfuCfuCfuUfgUfuAfL96	625	A-128456.1	PusAfsaCfaAfgAfgAfugaUfuAfgGfcAfgsasg	840
AD-64335.1	A-128399.2	UfscsCfuCfaCfaAfUfAfcCfaCfaGfaGfuAfL96	626	A-128464.1	PusAfscUfcUfgUfgGfuauUfgUfgAfgGfasusu	841
AD-64336.1	A-128413.2	CfsusUfgUfuGfaCfAfAfaAfaUfcCfuCfaAfL96	627	A-128472.1	PusUfsgAfgGfaUfuUfuUfuugUfcAfaCfaAfgsasa	842
AD-64337.1	A-127955.16	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfIL96	628	A-127958.2	PasAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	843
AD-64338.1	A-128439.2	GfscsAfaCfuUfuUfUfCfaCfcUfcUfgCfcUfL96	629	A-128486.1	PasGfsgCfaGfaGfgUfgaaAfaAfgUfuGfcsasu	844

AD-64339.1	A-128369.2	GfsgsGfaAfcAfaGfAfGfcUfaCfaGfcAfuAfL96	630	A-128449.1	PusAfsuGfcUfgUfaGfcucUfuGfuUfcCfcsasa	845
AD-64341.1	A-128401.2	UfscsAfuCfuUfcUfUfGfuUfgGfuUfcUfuAfL96	631	A-128465.1	PusAfsaGfaAfcCfaAfcaaGfaAfgAfuGfasgsg	846
AD-64342.1	A-128415.2	CfsusGfcUfgCfuAfUfGfcCfuCfaUfcUfuAfL96	632	A-128473.1	PusAfsaGfaUfgAfgGfcauAfgCfaGfcAfgsgsa	847
AD-64343.1	A-128427.2	GfsusUfgGfaUfgUfGfUfcUfgCfgGfcGfuUfL96	633	A-128479.1	PasAfscGfcCfgCfaGfacaCfaUfcCfaAfcsgsa	848
AD-64344.1	A-128441.2	UfsusCfaUfcCfuGfCfUfgCfuAfuGfcCfuAfL96	634	A-128487.1	PusAfsgGfcAfuAfgCfagcAfgGfaUfgAfasgsa	849
AD-64345.1	A-128371.2	UfsusCfuUfgUfuGfAfCfaAfaAfaUfcCfuAfL96	635	A-128450.1	PusAfsgGfaUfuUfuUfgucAfaCfaAfgAfasasa	850
AD-64347.1	A-123487.3	GfsgsAfuGfuGfuCfUfGfcGfgCfgUfuUfuAfL96	636	A-128466.1	PusAfsaAfaCfgCfcGfcagAfcAfcAfuCfcsasg	851
AD-64348.1	A-128417.2	UfsasUfaUfgGfaUfGfAfuGfuGfgUfaUfuAfL96	637	A-128474.1	PusAfsaUfaCfcAfcAfucaUfcCfaUfaUfasasc	852
AD-64349.1	A-128429.2	UfsusCfaUfcCfuGfCfUfgCfuAfuGfcCfuCfL96	638	A-128480.1	PgsAfsgGfcAfuAfgCfagcAfgGfaUfgAfasgsa	853
AD-64350.1	A-128443.2	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuAfL96	639	A-128488.1	PusAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	854
AD-64351.1	A-128373.2	UfsusGfaCfaAfaAfAfUfcCfuCfaCfaAfuAfL96	640	A-128451.1	PusAfsuUfgUfgAfgGfauuUfuUfgUfcAfascsa	855
AD-64352.1	A-128389.2	CfscsAfaGfuGfuUfUfGfcUfgAfcGfcAfaAfL96	641	A-128459.1	PusUfsuGfcGfuCfaGfcaaAfcAfcUfuGfgscsa	856
AD-64352.1	A-128389.2	CfscsAfaGfuGfuUfUfGfcUfgAfcGfcAfaAfL96	642	A-128459.1	PusUfsuGfcGfuCfaGfcaaAfcAfcUfuGfgscsa	857
AD-64353.1	A-128403.2	AfsasGfcCfuCfcAfAfGfcUfgUfgCfcUfuAfL96	643	A-128467.1	PusAfsaGfgCfaCfaGfcuuGfgAfgGfcUfusgsa	858
AD-64354.1	A-128419.2	CfscsUfcUfuCfaUfCfCfuGfcUfgCfuAfuAfL96	644	A-128475.1	PusAfsuAfgCfaGfcAfggaUfgAfaGfaGfgsasa	859
AD-64355.1	A-128431.2	CfscsUfgCfuGfcUfAfAfUfgCfcUfcAfuCfuUfL96	645	A-128481.1	PasAfsgAfuGfaGfgCfauaGfcAfgCfaGfgsasu	860
AD-64356.1	A-128375.2	CfsasUfcUfuCfuUfGfUfuGfgUfuCfuUfcUfL96	646	A-128452.1	PasGfsaAfgAfaCfcAfacaAfgAfaGfaUfgsasg	861
AD-64357.1	A-128391.2	CfscsGfuCfuGfuGfCfCfuUfcUfcAfuCfuAfL96	647	A-128460.1	PusAfsgAfuGfaGfaAfggcAfcAfgAfcGfgsgsg	862
AD-64358.1	A-128405.2	CfscsUfcAfuCfuUfCfUfuGfuUfgGfuUfcUfL96	648	A-128468.1	PasGfsaAfcCfaAfcAfagaAfgAfuGfaGfgscsa	863
AD-64359.1	A-128421.2	CfscsAfcCfaAfaUfuGfCfcCfcUfaUfcUfuAfL96	649	A-128476.1	PusAfsaGfaUfaGfgGfgcaUfuUfgGfuGfgsusc	864
AD-64360.1	A-128433.2	GfscsUfcCfuCfuGfCfCfCfgAfuCfcAfuAfcUfL96	650	A-128482.1	PasGfsuAfuGfgAfuCfggcAfgAfgGfaGfcscsa	865
AD-64700.1	A-129379.1	ascsucguggugdTacuu(Cgn)ucucaL96	651	A-127906.26	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	866
AD-64701.1	A-127905.20	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	652	A-129387.1	PusgsagagaagdTccadCcacgaguscsu	867
AD-64702.1	A-127905.28	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	653	A-129395.1	usGsagadGaaguccaCcacgaguscsu	868
AD-64703.1	A-129376.2	ascsucguggugdGacuucdAcucaL96	654	A-129385.5	usdGsagagaagdTccadCcacgaguscsu	869
AD-64704.1	A-129381.3	ascsucguggdTgdTacuucdAcucaL96	655	A-129389.6	usdGsagadGaaguccadCcacgaguscsu	870

AD-64705.1	A-129380.1	ascsucguggugdTacuucdAcucaL96	656	A-127906.27	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	871
AD-64706.1	A-127905.21	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	657	A-129388.1	usdGsadGagaaguccadCcacgaguscsu	872
AD-64707.1	A-127905.29	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	658	A-129396.1	usgsagadGaagdTccadCcacgaguscsu	873
AD-64708.1	A-129382.2	ascsucguggdTgdGacuuc(Tgn)cucaL96	659	A-129385.6	usdGsagagaagdTccadCcacgaguscsu	874
AD-64709.1	A-129373.4	ascsucguggugdGacuu(Cgn)ucucaL96	660	A-129391.2	usdGsagadGaagdTccadCcacgaguscsu	875
AD-64710.1	A-129373.1	ascsucguggugdGacuu(Cgn)ucucaL96	661	A-127906.20	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	876
AD-64711.1	A-129381.1	ascsucguggdTgdTacuucdAcucaL96	662	A-127906.28	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	877
AD-64712.1	A-127905.22	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	663	A-129389.1	usdGsagadGaaguccadCcacgaguscsu	878
AD-64713.1	A-127905.30	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	664	A-129397.1	PusgsagadGaagdTccadCcacgaguscsu	879
AD-64714.1	A-129384.2	ascsucguggdTgdGacuucdAcucaL96	665	A-129385.7	usdGsagagaagdTccadCcacgaguscsu	880
AD-64715.1	A-129376.4	ascsucguggugdGacuucdAcucaL96	666	A-129391.3	usdGsagadGaagdTccadCcacgaguscsu	881
AD-64716.1	A-129374.1	ascsucguggugdGacuucu(Cgn)ucaL96	667	A-127906.21	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	882
AD-64717.1	A-129382.1	ascsucguggdTgdGacuuc(Tgn)cucaL96	668	A-127906.29	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	883
AD-64718.1	A-127905.23	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	669	A-129390.1	usdGsagagadAguccadCcacgaguscsu	884
AD-64719.1	A-127917.5	ascsucguggugdGacuuc(Tgn)cucaL96	670	A-129385.2	usdGsagagaagdTccadCcacgaguscsu	885
AD-64720.1	A-129381.2	ascsucguggdTgdTacuucdAcucaL96	671	A-129385.8	usdGsagagaagdTccadCcacgaguscsu	886
AD-64721.1	A-129382.4	ascsucguggdTgdGacuuc(Tgn)cucaL96	672	A-129391.4	usdGsagadGaagdTccadCcacgaguscsu	887
AD-64722.1	A-129375.1	ascsucguggugdGacuucY34cucaL96	673	A-127906.22	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	888
AD-64723.1	A-129383.1	ascsucguggugdGdAcuuc(Tgn)cucaL96	674	A-127906.30	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	889
AD-64725.1	A-127917.6	ascsucguggugdGacuuc(Tgn)cucaL96	675	A-129398.1	PusdGsagagaagdTccadCcacgaguscsu	890
AD-64726.1	A-129373.3	ascsucguggugdGacuu(Cgn)ucucaL96	676	A-129389.2	usdGsagadGaaguccadCcacgaguscsu	891
AD-64727.1	A-129384.4	ascsucguggdTgdGacuucdAcucaL96	677	A-129391.5	usdGsagadGaagdTccadCcacgaguscsu	892
AD-64728.1	A-129376.1	ascsucguggugdGacuucdAcucaL96	678	A-127906.23	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	893
AD-64729.1	A-129384.1	ascsucguggdTgdGacuucdAcucaL96	679	A-127906.31	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	894
AD-64730.1	A-127905.25	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	680	A-129392.1	usGsagagaagdTccadCcacgaguscsu	895
AD-64731.1	A-129399.1	Y34ascsucguggugdGacuuc(Tgn)cucaL96	681	A-129385.3	usdGsagagaagdTccadCcacgaguscsu	896

AD-64732.1	A-129376.3	ascsucguggugdGacuucdAcucaL96	682	A-129389.3	usdGsagadGaaguccadCcacgaguscsu	897
AD-64733.1	A-129381.4	ascsucguggdTgdTacuucdAcucaL96	683	A-129391.6	usdGsagadGaagdTccadCcacgaguscsu	898
AD-64734.1	A-129377.1	ascsucguggugdGacuucdCcucaL96	684	A-127906.24	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	899
AD-64735.1	A-127905.18	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfIL96	685	A-129385.1	usdGsagagaagdTccadCcacgaguscsu	900
AD-64736.1	A-127905.26	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfIL96	686	A-129393.1	usdGsagagaagdTccaCcacgaguscsu	901
AD-64737.1	A-129399.2	Y34ascsucguggugdGacuuc(Tgn)cucaL96	687	A-129398.2	PusdGsagagaagdTccadCcacgaguscsu	902
AD-64738.1	A-129382.3	ascsucguggdTgdGacuuc(Tgn)cucaL96	688	A-129389.4	usdGsagadGaaguccadCcacgaguscsu	903
AD-64739.1	A-129378.1	ascsucguggugdGacuucdGcucaL96	689	A-127906.25	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	904
AD-64740.1	A-127905.19	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfIL96	690	A-129386.1	usgsagagaagdTccadCcacgaguscsu	905
AD-64741.1	A-127905.27	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcAfIL96	691	A-129394.1	usGsagagaagdTccaCcacgaguscsu	906
AD-64742.1	A-129373.2	ascsucguggugdGacuu(Cgn)ucucaL96	692	A-129385.4	usdGsagagaagdTccadCcacgaguscsu	907
AD-64743.1	A-129384.3	ascsucguggdTgdGacuucdAcucaL96	693	A-129389.5	usdGsagadGaaguccadCcacgaguscsu	908

Table 5. HBV single dose screen using Dual-Glo Luciferase® Assay

Duplex ID	10nM Avg	0.1nM Avg	10nM SD	0.1nM_SD
AD-63938.2	0.12	ND	0.01	ND
AD-63950.2	0.38	ND	0.04	ND
AD-63956.2	0.31	ND	0.02	ND
AD-63962.2	0.16	ND	0.03	ND
AD-63968.2	0.56	ND	0.10	ND
AD-63968.2	0.79	ND	0.09	ND
AD-63979.2	0.54	ND	0.02	ND
AD-63939.2	0.51	ND	0.01	ND
AD-63945.2	0.54	ND	0.08	ND
AD-63951.2	0.60	ND	0.03	ND
AD-63957.2	0.57	ND	0.02	ND
AD-63963.2	0.91	ND	0.06	ND
AD-63969.2	0.92	ND	0.02	ND
AD-63975.2	0.83	ND	0.01	ND
AD-63980.2	0.77	ND	0.01	ND
AD-63940.2	0.77	ND	0.06	ND
AD-63946.2	0.60	ND	0.10	ND
AD-63952.2	0.48	ND	0.04	ND
AD-63958.2	0.51	ND	0.01	ND
AD-63964.2	0.58	ND	0.04	ND
AD-63970.2	0.69	ND	0.07	ND
AD-63976.2	0.63	ND	0.04	ND
AD-63981.2	0.60	ND	0.04	ND
AD-63941.2	0.56	ND	0.09	ND
AD-63947.2	0.55	ND	0.08	ND
AD-63953.2	0.56	ND	0.06	ND
AD-63959.2	0.51	ND	0.03	ND
AD-63965.2	0.55	ND	0.03	ND
AD-63971.2	0.65	ND	0.02	ND
AD-63977.2	0.88	ND	0.01	ND
AD-63982.2	0.73	ND	0.07	ND
AD-63942.2	0.32	ND	0.09	ND
AD-63948.2	0.57	ND	0.09	ND
AD-63960.2	0.92	ND	0.05	ND
AD-63966.2	0.85	ND	0.06	ND
AD-63972.2	0.82	ND	0.06	ND
AD-63978.2	0.83	ND	0.02	ND
AD-63983.2	0.89	ND	0.02	ND
AD-63943.2	0.86	ND	0.04	ND
AD-63949.2	0.76	ND	0.02	ND

AD-63955.2	0.82	ND	0.02	ND
AD-63961.2	0.83	ND	0.07	ND
AD-63967.2	0.86	ND	0.03	ND
AD-63973.2	0.86	ND	0.03	ND
AD-63990.2	0.27	ND	0.07	ND
AD-63996.2	0.29	ND	0.06	ND
AD-64002.2	0.30	ND	0.11	ND
AD-64008.2	0.28	ND	0.05	ND
AD-64008.2	0.34	ND	0.07	ND
AD-64014.2	0.30	ND	0.03	ND
AD-64019.2	0.36	ND	0.04	ND
AD-64024.2	0.27	ND	0.03	ND
AD-63985.2	0.28	ND	0.06	ND
AD-63991.2	0.33	ND	0.02	ND
AD-63997.2	0.47	ND	0.07	ND
AD-64003.2	0.69	ND	0.06	ND
AD-64009.2	0.91	ND	0.03	ND
AD-64015.2	0.69	ND	0.09	ND
AD-64020.2	0.81	ND	0.06	ND
AD-64025.2	0.77	ND	0.06	ND
AD-63986.2	0.28	ND	0.05	ND
AD-63992.2	0.44	ND	0.04	ND
AD-64004.2	0.45	ND	0.04	ND
AD-64010.2	0.37	ND	0.05	ND
AD-64016.2	0.48	ND	0.05	ND
AD-64021.2	0.39	ND	0.03	ND
AD-64026.2	0.30	ND	0.02	ND
AD-63987.2	0.20	ND	0.02	ND
AD-63993.2	0.33	ND	0.02	ND
AD-63999.2	0.36	ND	0.05	ND
AD-64005.2	0.45	ND	0.11	ND
AD-64011.2	0.39	ND	0.08	ND
AD-64017.2	0.84	ND	0.06	ND
AD-64022.2	0.81	ND	0.03	ND
AD-64027.2	0.38	ND	0.05	ND
AD-63988.2	0.37	ND	0.04	ND
AD-63994.2	0.23	ND	0.01	ND
AD-64000.2	0.29	ND	0.00	ND
AD-64006.2	0.40	ND	0.04	ND
AD-64012.2	0.45	ND	0.17	ND
AD-64018.2	0.65	ND	0.07	ND
AD-64023.2	0.53	ND	0.07	ND
AD-64028.2	0.52	ND	0.07	ND
AD-63989.2	0.47	ND	0.04	ND

AD-63995.2	0.81	ND	0.03	ND
AD-64001.2	0.83	ND	0.04	ND
AD-64007.2	0.87	ND	0.04	ND
AD-64013.2	0.88	ND	0.03	ND
AD-64289.1	0.276	ND	0.009	ND
AD-64333.1	0.208	ND	0.015	ND
AD-64285.1	0.324	ND	0.034	ND
AD-64300.1	0.225	ND	0.005	ND
AD-64345.1	0.102	ND	0.090	ND
AD-64292.1	0.288	ND	0.232	ND
AD-64336.1	0.199	ND	0.056	ND
AD-64275.1	0.287	ND	0.185	ND
AD-64316.1	0.297	ND	0.024	ND
AD-64274.1	0.209	ND	0.033	ND
AD-64315.1	0.199	ND	0.002	ND
AD-64305.1	0.360	ND	0.035	ND
AD-64351.1	0.281	ND	0.014	ND
AD-64291.1	0.725	ND	0.005	ND
AD-64335.1	0.478	ND	0.020	ND
AD-64283.1	0.917	ND	0.018	ND
AD-64304.1	0.937	ND	0.050	ND
AD-64325.1	0.446	ND	0.223	ND
AD-64350.1	0.934	ND	0.055	ND
AD-63968.4	0.748	ND	0.008	ND
AD-64331.1	0.294	ND	0.038	ND
AD-64008.4	0.416	ND	0.028	ND
AD-64337.1	0.318	ND	0.049	ND
AD-64295.1	0.415	ND	0.034	ND
AD-64276.1	0.453	ND	0.073	ND
AD-64317.1	0.203	ND	0.040	ND
AD-64330.1	0.313	ND	0.030	ND
AD-64298.1	0.797	ND	0.007	ND
AD-64343.1	0.667	ND	0.020	ND
AD-61547.2	0.637	ND	0.019	ND
AD-64347.1	0.418	ND	0.066	ND
AD-64280.1	0.754	ND	0.092	ND
AD-64322.1	0.407	ND	0.013	ND
AD-64308.1	0.720	ND	0.055	ND
AD-64354.1	0.315	ND	0.034	ND
AD-64303.1	0.815	ND	0.150	ND
AD-64349.1	0.447	ND	0.030	ND
AD-64299.1	0.831	ND	0.007	ND
AD-64344.1	0.404	ND	0.009	ND
AD-64309.1	0.856	ND	0.005	ND

AD-64355.1	0.498	ND	0.040	ND
AD-64297.1	0.895	ND	0.024	ND
AD-64342.1	0.508	ND	0.006	ND
AD-64312.1	0.590	ND	0.034	ND
AD-64358.1	0.425	ND	0.044	ND
AD-64341.1	0.223	ND	0.119	ND
AD-64310.1	0.301	ND	0.064	ND
AD-64356.1	0.336	ND	0.024	ND
AD-64286.1	0.611	ND	0.012	ND
AD-64328.1	0.317	ND	0.043	ND
AD-61522.2	0.447	ND	0.008	ND
AD-64321.1	0.237	ND	0.009	ND
AD-64302.1	0.523	ND	0.020	ND
AD-64348.1	0.208	ND	0.003	ND
AD-64352.1	0.343	ND	0.224	ND
AD-64352.1	0.567	ND	0.015	ND
AD-64314.1	0.920	ND	0.044	ND
AD-64360.1	0.778	ND	0.029	ND
AD-64279.1	0.882	ND	0.034	ND
AD-64320.1	0.589	ND	0.017	ND
AD-64284.1	0.696	ND	0.119	ND
AD-64326.1	0.552	ND	0.009	ND
AD-64281.1	0.921	ND	0.019	ND
AD-64323.1	0.715	ND	0.097	ND
AD-64311.1	0.815	ND	0.030	ND
AD-64357.1	0.549	ND	0.001	ND
AD-64272.2	0.965	ND	0.024	ND
AD-64332.1	0.548	ND	0.013	ND
AD-64293.1	0.837	ND	0.013	ND
AD-64338.1	0.597	ND	0.031	ND
AD-64290.1	0.489	ND	0.026	ND
AD-64334.1	0.368	ND	0.003	ND
AD-64282.1	0.767	ND	0.009	ND
AD-64324.1	0.726	ND	0.077	ND
AD-64278.1	0.951	ND	0.077	ND
AD-64319.1	0.895	ND	0.029	ND
AD-64307.1	0.890	ND	0.065	ND
AD-64353.1	0.567	ND	0.500	ND
AD-64277.1	0.416	ND	0.019	ND
AD-64277.1	0.839	ND	0.058	ND
AD-64318.1	0.613	ND	0.042	ND
AD-64318.1	0.768	ND	0.042	ND
AD-64313.1	0.698	ND	0.062	ND
AD-64359.1	0.441	ND	0.081	ND

AD-64294.1	0.563	ND	0.066	ND
AD-64339.1	0.486	ND	0.044	ND
AD-63968.5	0.57	0.72	0.07	0.03
AD-63940.3	0.81	0.83	0.11	0.03
AD-64710.1	0.79	0.85	0.12	0.04
AD-64716.1	0.73	0.85	0.08	0.01
AD-64722.1	0.67	0.80	0.06	0.02
AD-64728.1	0.74	0.87	0.06	0.05
AD-64734.1	0.78	0.83	0.08	0.05
AD-64739.1	0.73	0.85	0.07	0.02
AD-64700.1	0.54	0.75	0.13	0.02
AD-64705.1	0.67	0.79	0.15	0.04
AD-64711.1	0.57	0.83	0.13	0.04
AD-64717.1	0.72	0.83	0.13	0.02
AD-64723.1	0.83	0.87	0.12	0.01
AD-64729.1	0.74	0.87	0.08	0.07
AD-64735.1	0.73	0.89	0.05	0.04
AD-64740.1	0.89	0.88	0.05	0.07
AD-64701.1	0.88	0.84	0.07	0.05
AD-64706.1	0.71	0.88	0.12	0.05
AD-64712.1	0.81	0.86	0.13	0.07
AD-64718.1	0.84	0.89	0.16	0.01
AD-64730.1	0.88	0.89	0.02	0.04
AD-64736.1	0.80	0.88	0.10	0.05
AD-64741.1	0.85	0.83	0.06	0.05
AD-64702.1	0.87	0.93	0.02	0.06
AD-64707.1	0.95	0.88	0.05	0.08
AD-64713.1	0.90	0.85	0.08	0.03
AD-64719.1	0.80	0.89	0.09	0.09
AD-64725.1	0.70	0.84	0.09	0.03
AD-64731.1	0.82	0.87	0.04	0.08
AD-64737.1	0.76	0.84	0.09	0.08
AD-64742.1	0.76	0.85	0.09	0.03
AD-64703.1	0.79	0.88	0.05	0.02
AD-64708.1	0.83	0.82	0.08	0.06
AD-64714.1	0.75	0.85	0.12	0.03
AD-64720.1	0.61	0.81	0.17	0.04
AD-64726.1	0.75	0.83	0.07	0.02
AD-64732.1	0.86	0.84	0.14	0.10
AD-64738.1	0.80	0.90	0.04	0.02
AD-64743.1	0.75	0.85	0.12	0.04
AD-64704.1	0.67	0.78	0.16	0.02
AD-64709.1	0.83	0.86	0.16	0.03
AD-64715.1	0.87	0.88	0.09	0.04

AD-64721.1	0.77	0.82	0.12	0.06
AD-64727.1	0.75	0.85	0.14	0.02
AD-64733.1	0.67	0.81	0.14	0.03

Example 3. Synthesis and *In vitro* Screening of Additional siRNA Duplexes

Additional iRNA molecules targeting the HBV genome were synthesized as described above. A detailed list of the additional unmodified HBV sense and antisense strand sequences is shown in Table 6 and a detailed list of the modified HBV sense and antisense strand sequences is shown in Table 7.

Table 6. Unmodified Sense and Antisense Strand Sequences of HBV dsRNAs

Duplex ID	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-65369.1	UCGUGGUGGACUUCUCUCA	909	UGAGAGAAGUCCACCACGAUU	938
AD-65381.1	UCGUGGUGGACUUCUCUCA	910	UGAGAGAAGUCCACCACGAUU	939
AD-63962.1	UCGUGGUGGACUUCUCUCA	911	UGAGAGAAGUCCACCACGAUU	940
AD-63938.1	ACUCGUGGUGGACUUCUCUCA	912	UGAGAGAAGUCCACCACGAGUCU	941
AD-65561.1	UCGUGGUGGACUUCUCUCA	913	UGAGAGAAGUCCACCACGAUU	942
AD-65566.1	UCGUGGUGGACUUCUCUCA	914	UGAGAGAAGUCCACCACGAUU	943
AD-63944.1	UCGUGGUGGACUUCUCUCAUU	915	UGAGAGAAGUCCACCACGAUU	944
AD-63968.1	ACUCGUGGUGGACUUCUCUCA	916	UGAGAGAAGUCCACCACGAGUCU	945
AD-65406.1	UCGUGGUGGACUUCUCUCA	917	UGAGAGAAGUCCACCACGAUU	946
AD-65396.1	ACUCGUGGUGGACUUCUCUCA	918	UGAGAGAAGUCCACCACGAGUUU	947
AD-65427.1	GUGCACUUCGCUUCACCUCUA	919	UAGAGGUGAAGCGAAGUGCACUU	948
AD-65573.1	GUGCACUUCGCUUCACCUCUA	920	UAGAGGUGAAGCGAAGUGCACAC	949
AD-65432.1	GCACUUCGCUUCACCUCUA	921	UAGAGGUGAAGCGAAGUGCAC	950
AD-64332.1	GUGCACUUCGCUUCACCUCUG	922	CAGAGGUGAAGCGAAGUGCACAC	951
AD-64322.1	AUGUGUCUGCGGCGUUUUAUA	923	UAUAAAACCGCCGCAGACACAUCC	952
AD-64272.1	GUGCACUUCGCUUCACCUCUG	924	CAGAGGUGAAGCGAAGUGCACAC	953
AD-65583.1	GCACUUCGCUUCACCUCUA	925	UAGAGGUGAAGCGAAGUGCUU	954
AD-63994.1	GGUGGACUUCUCUCUCAAUUU	926	AAAUUGAGAGAAGUCCACCAC	955
AD-65370.1	CGUGGUGGACUUCUCUCAAUU	927	AAUUGAGAGAAGUCCACCAGCAG	956
AD-65265.1	GUGGUGGACUUCUCUCAAUUU	928	AAAUUGAGAGAAGUCCACCACGA	957
AD-65407.1	CGUGGUGGACUUCUCUCAAUU	929	AAUUGAGAGAAGUCCACCAGCAG	958
AD-64027.1	GGUGGACUUCUCUCAAUUU	930	AAAUUGAGAGAAGUCCACCAC	959
AD-65266.1	GUGGUGGACUUCUCUCAAUUU	931	AAAUUGAGAGAAGUCCACCACGA	960

AD-65389.1	UGGUGGUCTUCUCUAAAUU	932	AAUUGAGAGAAGUCCACCAUU	961
AD-64008.1	GUGGUGGACUUCUCUCAAUUU	933	AAAUUGAGAGAAGUCCACCACGA	962
AD-65377.1	CGUGGUGGUCTUCUCUAAAUU	934	AAUUGAGAGAAGUCCACCAGCUU	963
AD-65409.2	GGUGGACUUCUCUCAAUUUUA	935	UAAAAUUGAGAGAAGUCCACCAC	964
AD-65403.1	GGUGGACUUCUCUCAAUUUUA	936	UAAAAUUGAGAGAAGUCCACCAC	965
AD-65385.1	UGGACUACTCUCAAAUUUA	937	UAAAAUUGAGAGAAGUCCAUU	966

Table 7. Modified Sense and Antisense Strand Sequences of HBV dsRNAs

DuplexID	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-65369	uscsguGfgUfGfGfacuuCfUfcucaL96	967	PusGfsagaGfaAfGfuccaCfcAfcgasusu	996
AD-65381	uscsguGfgUfGfGfacuucucucaL96	968	PusGfsagaGfaAfGfuccaCfcAfcgasusu	997
AD-63962	Y44uscsGfuGfgUfgGfaCfuUfcUfcUfcAfY44	969	PusGfsaGfaGfaAfgUfcCfaCfcAfcGfasusu	998
AD-63938	Y44ACUCGUGGUGGACUUCUCUCA	970	UGAGAGAAGUCCACCACGAGUCU	999
AD-65561	uscsguGfgUfGfGfacuuCfUfcucaL96	971	UfsGfsagaGfaAfGfuccaCfcAfcgasusu	1000
AD-65566	uscsguGfgUfGfGfacuucucucaL96	972	UfsGfsagaGfaAfGfuccaCfcAfcgasusu	1001
AD-63944	Y44ucGuGGuGGAcuucucucAusuY44	973	UfGfagAfgAfAfGUfccaCfCAfcgAusu	1002
AD-63968	AfscsUfcGfuGfgUfGfGfaCfuUfcUfcUfcAfL96	974	usGfsaGfaGfaAfgUfccaCfcAfcGfaGfuscsu	1003
AD-65406	uscsguGfgUfGfGfacuuCfUfcucaL96	975	usGfsagaGfaAfGfuccaCfcAfcgasusu	1004
AD-65396	ascsucguGfgUfGfGfacuucucucaL96	976	usGfsagaGfaaguccaCfcAfcgagususu	1005
AD-65427	gsusgcacUfuCfGfCfuucaccucuaL96	977	PusAfsgagGfugaagcgAfaGfugcacsusu	1006
AD-65573	gsusgcacUfuCfGfCfuucaCfCfucuaL96	978	UfsAfsgagGfuGfAfagcgAfaGfugcacsasc	1007
AD-65432	gscsacUfucGfCfuucacCfucuaL96	979	PusAfsgagGfuGfAfagcgAfaGfugcsasc	1008
AD-64332	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuGfL96	980	PcsAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	1009
AD-64322	AfsusGfuGfuCfuGfCfGfgCfgUfuUfuAfuAfL96	981	PusAfsuAfaAfaCfgCfcgcAfgAfcAfcAfuscsc	1010

AD-64272	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuGfL96	982	csAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	1011
AD-65583	gscsacuucgdCuucac(Cgn)ucuaL96	983	usdAsgagdGugaagcgdAagugcsusu	1012
AD-63994	gsgsUfgGfaCfUfUfcUfcUfcAfaUfuUfL96	984	PasAfsaUfuGfaGfaGfaagUfcCfaCfcsasc	1013
AD-65370	csgsugguGfgAfCfUfucucUfCfaauuL96	985	asAfsuugAfgAfGfaaguCfcAfccagcsasg	1014
AD-65265	gsusggugGfaCfUfUfcUfcucaauuuL96	986	asAfsaUfugagaGfaagUfcCfaccAfcsgsa	1015
AD-65407	csgsugguGfgAfCfUfucucUfCfaauuL96	987	asAfsuugAfgAfgAfaguCfcAfccagcsasg	1016
AD-64027	gsgsUfgGfaCfUfUfcUfcUfcAfaUfuUfL96	988	asAfsaUfuGfaGfaGfaagUfcCfaCfcsasc	1017
AD-65266	gsusggugGfaCfUfUfcucuCfaauuuL96	989	asAfsaUfugagaGfaagUfcCfaccAfcsgsa	1018
AD-65389	usgsgudGgucdTucucuaaaauuL96	990	asdAsuugagagdAagudCcaccasusu	1019
AD-64008	GfsusGfgUfgGfaCfUfUfcUfcUfcAfaUfuUfL96	991	asAfsaUfuGfaGfaGfaagUfcCfaCfcAfcsgsa	1020
AD-65377	csgsuggudGgucdTucucuaaaauuL96	992	asdAsuugagagdAagudCcaccagcsusu	1021
AD-65409	gsgsuggaCfuUfCfUfcucaAfUfuuuaL96	993	PusAfsaaaUfuGfAfgagaAfgUfccaccsasc	1022
AD-65403	gsgsuggaCfuUfCfUfcucaAfUfuuuaL96	994	usAfsaaaUfuGfAfgagaAfgUfccaccsasc	1023
AD-65385	usgsgacuacdTcucaaauuuuaL96	995	usdAsaaauugadGagadAguccasusu	1024

A single dose screen of these duplexes was performed in duplicate by transfecting the duplexes into HepG2.215 and Hep3B cells and measuring HBV viral RNA using primer/probe pairs to detect HBV P open reading frame (ORF) RNA (PORF-1_A and PORF-1_B) and/or primer sets to detect HBV S ORF RNA (SORF-2_A and SORF-2_B). The results of the assays in HepG2.2.15 cells are shown in Table 8 and the results of the assays in Hep3B cells are provided in Table 9.

Table 8. HBV single dose screen In HepG2.2.15 cells

DuplexID	PORF-1 Primer/ Probe Set Experiment A	PORF-1 Primer/ Probe Set Duplicate Experiment B	SORF-2 Primer/ Probe Set Experiment A	SORF-2 Primer/ Probe Set Duplicate Experiment B
AD-65369	0.1875	0.042	0.0446	0.3018
AD-65381	0.086	0.249	0.1008	0.553
AD-63962	0.4838	0.3475	0.2237	0.5258
AD-63938	0.3587	2.1213	0.0501	1.1434
AD-65561	0.1076	0.3801	0.0718	0.6897
AD-65566	0.4127	0.3211	0.185	11.1161
AD-63944	0.9489	0.7098	0.393	0.2771
AD-63968	NoIC50	NoIC50	1.8788	NoIC50
AD-65406	3.3749	18.8396	3.8204	2.2662
AD-65396	NoIC50	6.8758	3.7382	4.2157
AD-65427	0.0089	0.0181	0.0066	0.015
AD-65573	0.0174	0.0332	0.0029	0.0227
AD-65432	0.0211	0.0593	0.0112	0.0366
AD-64332	0.0268	0.0329	0.0624	0.0217
AD-64322	0.0963	0.1077	0.0992	0.0963
AD-64272	0.0773	0.1199	0.0763	0.093
AD-65583	0.1624	0.2228	0.1568	0.1496
AD-63994	0.7019	0.1467	0.0832	0.0385
AD-65370	0.2404	0.7916	0.3952	0.1964
AD-65265	0.2255	0.5008	0.2893	0.318
AD-65407	0.9533	0.261	0.4254	0.1121
AD-64027	0.7692	0.5887	0.5208	0.5697
AD-65266	3.4109	0.5055	0.8532	0.3658
AD-65389	0.9172	0.6514	0.4915	0.2872
AD-64008	1.2738	0.7865	1.9519	0.808
AD-65377	0.6052	1.6	24.9403	0.6065

AD-65409	1.8304	1.6479	0.104	0.0557
AD-65403	12.1516	0.667	1.006	0.233
AD-65385	NoIC50	NoIC50	NoIC50	NoIC50

Table 9. HBV single dose screen In Hep3B cells

DuplexID	PORF-1 Primer/ Probe Set Experiment A	PORF-1 Primer/ Probe Set Experiment B
AD-65369	0.0982	0.0508
AD-65381	0.2392	0.1097
AD-63962	0.0769	0.0706
AD-63938	0.039	0.0111
AD-65561	0.6316	0.6931
AD-65566	0.2747	0.5331
AD-63944	0.1317	0.0566
AD-63968	0.4374	0.8811
AD-65406	1.4961	1.2573
AD-65396	1.9971	0.9952
AD-65427	0.0234	0.006
AD-65573	0.0346	0.0334
AD-65432	0.0352	0.2664
AD-64332	0.0221	0.4541
AD-64322	0.1743	0.1616
AD-64272	0.1885	0.6699
AD-65583	0.1241	8.1611
AD-63994	3.3623	5.2897
AD-65370	0.2281	NoIC50
AD-65265	NoIC50	7.3426
AD-65407	0.1404	1.3833
AD-64027	27.1417	1.1832
AD-65266	NoIC50	NoIC50
AD-65389	NoIC50	NoIC50
AD-64008	NoIC50	NoIC50
AD-65377	NoIC50	NoIC50
AD-65409	1.8065	3.436
AD-65403	0.5113	18.0359
AD-65385	NoIC50	NoIC50

A subset of these duplexes were also assayed for *in vitro* metabolic stability using two assays, a tritosome stability assay and a cytosol stability assay.

For the tritosome stability assays, rat liver tritosomes (Xenotech custom product PR14044) were thawed to room temperature and diluted to 0.5units/mL Acid Phosphatase in 20mM Sodium Citrate pH 5.0 Buffer. Twenty-four hour samples were prepared by mixing 100µL of 0.5units/mL Acid Phosphatase Tritosomes with 25µL of 0.4mg/mL siRNA sample in a microcentrifuge tube and incubating for twenty-four hours in an eppendorf Thermomixer set to 37°C and 300rpm. After twenty-four hours of incubation 300µL of Phenomenex Lysis Loading Buffer (Cat.# ALO-8498) and 12.5µL of a 0.4mg/mL internal standard siRNA were added to each sample. Time 0 hour samples were prepared by by mixing 100µL of 0.5units/mL Acid Phosphatase Tritosomes with 25µL of 0.4mg/mL siRNA sample, 300µL of Phenomenex Lysis Loading Buffer, and 12.5µL of a 0.4mg/mL internal standard siRNA. siRNA was extracted from twenty-four hour samples and 0 hour samples using a Phenomenex Clarity OTX Starter Kit (Cat.# KSO-8494). After the samples were extracted they were transferred to a microcentrifuge tube and dried down using a Labconco CentriVap Concentrator (Cat.# 7810010). The samples were then resuspended with 500µL of nuclease free water. Fifty µL of each sample was run on an Agilent Technologies 1260 Infinity Binary LC with Agilent Technologies 6130 Quadrupole LC/MS. The Quaternary pump method was run for 12.20 minutes at 0.400mL/min with the following timetable:

Time Function	Parameter
0.20	5% Buffer A(16mM TEA 200mM HFIP), 95% Buffer B (100% Methanol)
2.50	5% Buffer A(16mM TEA 200mM HFIP), 95% Buffer B (100% Methanol)
3.00	100% Buffer A(16mM TEA 200mM HFIP)

The Binary Pump method was run for 12.20min at 0.700mL/min with the following timetable:

Time Function	Parameter
0.00	100% Buffer A(16mM TEA 200mM HFIP)
0.40	100% Buffer A(16mM TEA 200mM HFIP)
10.00	60% Buffer A(16mM TEA 200mM HFIP), 40% Buffer B (100% ACN)
10.10	100% Buffer A(16mM TEA 200mM HFIP)
12.20	100% Buffer A(16mM TEA 200mM HFIP)

Both the left and right column was set at 75.00°C. The UV signal was measured at 260nm wavelength. The percent remaining of each strand was calculated using the following equation:

% Strand remaining $=100*(\text{Peak Area}_{\text{Strand 24h}}/\text{Peak Area}_{\text{Strand 0h}}*(\text{Peak Area}_{\text{Standard 24h}}/\text{Peak Area}_{\text{Standard 0h}}))$.

For the cytosol stability assay, female rat liver cytosol (Xenotech Cat. # R1500.C) were thawed to room temperature and diluted to 1mg/mL in 50mM Tris buffer: HCl pH 7.4, 5mM MgCl2. 24 hour samples were prepared by mixing 100uL of 1mg/mL Cytosol with 25uL of 0.4mg/mL siRNA sample in a microcentrifuge tube and incubating for 24 hours in an eppendorf Thermomixer set to 37°C and 300rpm. After 24 hours of incubation 300uL of Phenomenex Lysis Loading Buffer (Cat.# ALO-8498) and 12.5uL of a 0.4mg/mL internal standard siRNA were added to each sample. 0 hour samples were prepared by by mixing 100uL of 1mg/mL Cytosol with 25uL of 0.4mg/mL siRNA sample, 300uL of Phenomenex Lysis Loading Buffer, and 12.5uL of a 0.4mg/mL internal standard siRNA. siRNA was extracted from 24 hour samples and 0 hour samples using a Phenomenex Clarity OTX Starter Kit (Cat.# KSO-8494). After the samples were extracted they were transferred to a microcentrifuge tube and dried down using a Labconco CentriVap Concentrator (Cat.# 7810010). The samples were then resuspended with 500uL of nuclease free water. 50uL of each sample was run on an Agilent Technologies 1260 Infinity Binary LC with Agilent Technologies 6130 Quadrupole LC/MS. The Quaternary pump method was run for 12.20 minutes at 0.400mL/min with the following timetable:

Time Function	Parameter
0.20	5% Buffer A(16mM TEA 200mM HFIP), 95% Buffer B (100% Methanol)
2.50	5% Buffer A(16mM TEA 200mM HFIP), 95% Buffer B (100% Methanol)
3.00	100% Buffer A(16mM TEA 200mM HFIP)

The Binary Pump method was run for 12.20min at 0.700mL/min with the following timetable:

Time Function Parameter

Time Function	Parameter
0.00	100% Buffer A(16mM TEA 200mM HFIP)
0.40	100% Buffer A(16mM TEA 200mM HFIP)
10.00	60% Buffer A(16mM TEA 200mM HFIP), 40% Buffer B (100% ACN)
10.10	100% Buffer A(16mM TEA 200mM HFIP)
12.20	100% Buffer A(16mM TEA 200mM HFIP)

Both the left and right column was set at 75.00°C. The UV signal was measured at 260nm wavelength. The percent remaining of each strand was calculated using the following equation:

% Strand remaining =100*(Peak Area$_{\text{Strand 24h}}$/Peak Area$_{\text{Strand 0h}}$*(Peak Area$_{\text{Standard 24h}}$/Peak Area$_{\text{Standard 0h}}$)).

The results of the twenty-four hour tritosome stability assays are provided in Table 10 and the results of the twenty-four hour cytosol stability assays are provided in Table 11.

Table 10. Twenty-four hour tritosome stability assays.

% Antisense Remaining	% Sense Remaining	DuplexID
87.59	72.43	AD-65381
67.59	82.48	AD-65566
30.52	34.98	AD-63968
115.17	79.61	AD-65427
43.00	76.84	AD-65573
129.69	128.59	AD-64272
100.30	119.85	AD-65407
94.06	110.90	AD-64008
98.63	127.48	AD-65377
105.06	119.88	AD-65409
117.55	104.30	AD-65403

Table 11. Twenty-four hour cytosol stability assays.

% Antisense Remaining	% Sense Remaining	DuplexID
67.78	22.42	AD-65381
55.89	15.26	AD-65566
88.39	46.94	AD-63968
89.50	66.35	AD-65427
69.01	41.47	AD-65573
96.77	78.00	AD-64272
64.46	24.10	AD-65407
35.39	26.39	AD-64008
79.98	66.50	AD-65377
86.24	74.25	AD-65409
60.45	62.41	AD-65403

Example 4. Synthesis and Screening of Additional siRNA Duplexes

Additional iRNA molecules targeting the HBV genome were designed and synthesized as described above. A detailed list of the additional unmodified HBV sense and antisense strand sequences is shown in Table 12 and a detailed list of the modified HBV sense and antisense strand sequences is shown in Table 13.

Table 12. Unmodified Sense and Antisense Strand Sequences of HBV dsRNAs

Duplex ID	Sense ID	Sense Sequence Unmodified (5' to 3')	SEQ ID NO:	Antisense ID	Antisense Sequence Unmodified (5' to 3')	SEQ ID NO:
AD-65381	A-130366.9	UCGUGGUGGACUUCUCUCA	1025	A-131904.1	UGAGAGAAGUCCACCACGAUU	1036
AD-66019	A-130366.9	UCGUGGUGGACUUCUCUCA	1026	A-131904.1	UGAGAGAAGUCCACCACGAUU	1037
AD-65375	A-130366.9	UCGUGGUGGACUUCUCUCA	1027	A-130364.7	UGAGAGAAGUCCACCACGAUU	1038
AD-65427	A-130441.7	GUGCACUUCGCUUCACCUCUA	1028	A-131905.1	UAGAGGUGAAGCGAAGUGCACUU	1039
AD-66110	A-130441.7	GUGCACUUCGCUUCACCUCUA	1029	A-131905.1	UAGAGGUGAAGCGAAGUGCACUU	1040
AD-65421	A-130441.7	GUGCACUUCGCUUCACCUCUA	1030	A-130442.6	UAGAGGUGAAGCGAAGUGCACUU	1041
AD-65407	A-130371.12	CGUGGUGGACUUCUCUCAAUU	1031	A-130372.5	AAUUGAGAGAAGUCCACCAGCAG	1042
AD-65377	A-130384.4	CGUGGUGGUCTUCUCUAAAUU	1032	A-130748.3	AAUUGAGAGAAGUCCACCAGCUU	1043
AD-65409	A-130388.15	GGUGGACUUCUCUCAAUUUUA	1033	A-131906.1	UAAAAUUGAGAGAAGUCCACCAC	1044
AD-66111	A-130388.15	GGUGGACUUCUCUCAAUUUUA	1034	A-131906.1	UAAAAUUGAGAGAAGUCCACCAC	1045
AD-65403	A-130388.15	GGUGGACUUCUCUCAAUUUUA	1035	A-130389.4	UAAAAUUGAGAGAAGUCCACCAC	1046

Table 13. Modified Sense and Antisense Strand Sequences of HBV dsRNAs

Duplex ID	Sense ID	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense ID	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-65381	A-130366.9	uscsguGfgUfGfGfacuucucucaL96	1047	A-131904.1	PusGfsagaGfaAfGfuccaCfcAfcgasusu	1058
AD-66019	A-130366.9	uscsguGfgUfGfGfacuucucucaL96	1048	A-131904.1	VPusGfsagaGfaAfGfuccaCfcAfcgasusu	1059
AD-65375	A-130366.9	uscsguGfgUfGfGfacuucucucaL96	1049	A-130364.7	usGfsagaGfaAfGfuccaCfcAfcgasusu	1060
AD-65427	A-130441.7	gsusgcacUfuCfGfCfuucaccucuaL96	1050	A-131905.1	PusAfsgagGfugaagcgAfaGfugcacsusu	1061
AD-66110	A-130441.7	gsusgcacUfuCfGfCfuucaccucuaL96	1051	A-131905.1	VPusAfsgagGfugaagcgAfaGfugcacsusu	1062
AD-65421	A-130441.7	gsusgcacUfuCfGfCfuucaccucuaL96	1052	A-130442.6	usAfsgagGfugaagcgAfaGfugcacsusu	1063
AD-65407	A-130371.12	csgsugguGfgAfCfUfucucUfCfaauuL96	1053	A-130372.5	asAfsuugAfgAfgAfaguCfcAfccagcsasg	1064
AD-65377	A-130384.4	csgsuggudGgucdTucucuaaauuL96	1054	A-130748.3	asdAsuugagagdAagudCcaccagcsusu	1065
AD-65409	A-130388.15	gsgsuggaCfuUfCfUfcucaAfUfuuuaL96	1055	A-131906.1	PusAfsaaaUfuGfAfgagaAfgUfccaccsasc	1066
AD-66111	A-130388.15	gsgsuggaCfuUfCfUfcucaAfUfuuuaL96	1056	A-131906.1	VPusAfsaaaUfuGfAfgagaAfgUfccaccsasc	1067
AD-65403	A-130388.15	gsgsuggaCfuUfCfUfcucaAfUfuuuaL96	1057	A-130389.4	usAfsaaaUfuGfAfgagaAfgUfccaccsasc	1068

A primary single dose screen of these iRNA duplexes was performed using the Dual-Glo® Luciferase assay, as described above. The results of this screen in Cos7 cells transfected with the indicated HBV iRNAs are shown in Table 14. Data are expressed as percent of mRNA remaining relative to negative control at 24 hours.

Table 14. HBV single dose primary screen In Cos7 cells using Dual-Glo Luciferase® Assay

	Dual luciferase primary screen				
	% Message remaining at 24 hr				DRC ED50
Duplex ID	at 50 nM	STDEV	at 1 nM	STDEV	(nM)
AD-65381	9.3	0.24	15.6	0.77	0.019
AD-66019	ND	ND	ND	ND	ND
AD-65375	24.2	0.36	71.4	0.69	No ED50
AD-65427	28.8	1.60	41.0	1.73	0.117
AD-66110	ND	ND	ND	ND	ND
AD-65421	47.6	3.49	85.5	4.76	No ED50
AD-65407	14.3	0.52	25.3	2.11	0.038
AD-65377	21.8	0.31	37.9	1.12	0.130
AD-65409	9.5	0.41	13.2	0.71	0.013
AD-66111	ND	ND	ND	ND	ND
AD-65403	12.6	0.50	37.2	2.31	0.069

ND – not done

These duplexes were also assayed for dose response for silencing viral RNA using the Dual-Glo® Luciferase assay, as described above. The doses of the duplexes used for these asaays were 50 nM, 8.333333333 nM, 1.388888889 nM, 0.231481481 nM, 0.038580247 nM, 0.006430041 nM, 0.001071674 nM, 0.000178612 nM, 2.97687 x 10^{-5} nM, 4.96145 x 10^{-6} nM, 8.26909 x 10^{-7} nM, and 1.37818E x 10^{-7} nM, which represent a 1 to 6 dilution of the duplexes starting at 50 nM over 12 doses. The results of this screen in Cos7 cells transfected with the indicated HBV iRNAs are shown in Table 15. Data are expressed as percent of mRNA remaining relative to negative control at 24 hours.

Table 15. Dose response screen In Cos7 cells using Dual-Glo Luciferase® Assay

Dual luciferase HBV reporter cells IC50 (nM) at 24 hr									
Duplex ID	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 6**	**Assay 7**	**Average[1]**	**Stdev**
AD-65381	0.019	ND	ND	ND	ND	ND	ND	0.019	
AD-66019	ND	0.021	0.021	0.016	0.026	0.019	0.031	0.022	0.005
AD-65375	UD	0.215	0.149	0.081	0.246	0.138	0.276	0.184	0.074
AD-65407	0.038	0.045	0.051	0.021	0.050	0.056	0.068	0.047	0.015
AD-65377	0.130	0.029	0.046	0.087	0.096	0.146	0.090	0.089	0.042
AD-65409	0.013	ND	ND	ND	ND	ND	ND	0.013	
AD-66111	ND	0.018	0.013	0.012	0.018	0.021	0.033	0.019	0.007
AD-65403	0.069	0.044	0.033	0.039	0.042	0.046	0.062	0.048	0.013
AD-65427	0.017	ND	ND	ND	ND	ND	ND	0.117	
AD-66110	ND	0.238	0.296	0.145	0.157	0.161	ND	0.199	0.065
AD-65421	UD	1.219	1.385	2.254	0.799	2.906	ND	1.713	0.852

[1]Averages from 5-7 biological replicates run in triplicate
ND – not done

The *in vitro* efficacy and potency of these duplexes were also assayed. In particular, the dose response of the duplexes for silencing viral RNA in transfected HepG2.2.15 and Hep3B cell lysates and for silencing HBsAg in HepG2.2.15 cell supernatants were determined. Cells were transfected with 12 separate doses of the duplexes ranging from 50 nM to 1 x 10^{-7} nM and at sevently-two hours after transfection, the level of viral RNA was

determined using primer/probe pairs to detect the P ORF and/or the S ORF. The level of HBsAg was determined using an ELISA assay.

The results of the P ORF viral RNA silencing in HepG2.2.15 cells using the indicated duplexes are provided in Table 16. The results of the S ORF viral RNA silencing in HepG2.2.15 cells using the indicated duplexes are provided in Table 17. The results of HBsAg silencing in HepG2.2.15 cells are provided in Table 18.

The results of the P ORF viral RNA silencing in Hep3B cells using the indicated duplexes are provided in Table 19.

Table 16. Dose response screen In HepG2.2.15 cells

Viral RNA silencing in HepG2.2.15 cells **P-ORF primer/probe set** **IC50 (nM) at 72 hr**								
Duplex ID	Assay Development					Optimized Assay		
						Assay 1	Assay 2	Assay 3
AD-65381	0.079	0.208	ND	ND		ND	ND	ND
AD-66019	ND	ND	0.265	0.010		0.022	0.032	0.023
AD-65375	12.3	UD	UD	UD		0.172	0.257	0.672
AD-65407	0.247	1.0	0.365	0.109		0.069	0.103	0.095
AD-65377	1.3	UD	4.9	UD		0.842	0.838	0.615
AD-65409	0.436	1.0	ND	ND		ND	ND	ND
AD-66111	ND	ND	0.456	0.030		50	0.294	ND
AD-65403	9.2	10.4	3.4	UD		0.114	0.384	1.0
AD-65427	0.007	0.018	ND	ND		ND	ND	ND
AD-66110	ND	ND	0.012	0.053		0.016	0.010	0.021
AD-65421	0.069	0.091	0.034	0.006		0.002	0.003	0.007

ND – not done

Table 17. Dose response screen In HepG2.2.15 cells

Viral RNA silencing in HepG2.2.15 cells **S-ORF primer/probe set** **IC50 (nM) at 72 hr**								
Duplex ID	Assay Development					Optimized Assay		
						Assay 1	Assay 2	Assay 3
AD-65381	0.252	0.215	ND	ND		ND	ND	ND
AD-66019	ND	ND	0.245	0.011		0.009	0.016	0.005
AD-65375	45	UD	UD	UD		0.124	0.048	0.056
AD-65407	0.232	0.645	0.577	0.015		0.021	0.023	0.016
AD-65377	1.4	8.6	UD	UD		0.575	0.483	0.117
AD-65409	0.433	0.242	ND	ND		ND	ND	ND
AD-66111	ND	ND	2.1	0.455		ND	0.416	ND
AD-65403	0.997	0.670	0.668	UD		0.074	0.270	1.1
AD-65427	0.008	0.018	ND	ND		ND	ND	ND
AD-66110	ND	ND	0.022	0.050		0.035	0.038	0.020
AD-65421	0.083	0.097	0.046	0.003		0.003	0.005	0.001

ND – not done

Table 18. Dose response screen In HepG2.2.15 cells

HBsAg ELISA **IC50 (nM)**	
Duplex ID	Assay 1
AD-65381	ND
AD-66019	0.105
AD-65375	1.2
AD-65407	0.102
AD-65377	2.9
AD-65409	ND
AD-66111	0.018
AD-65403	0.064
AD-65427	ND

AD-66110	0.002
AD-65421	0.008

ND – not done

Table 19. Dose response screen In Hep3B cells

	Hep3B cells screen		
	DRC ED50 P-ORF primer/probe set		
Duplex ID	**P-ORF run 1**	**P-ORF run 2**	**Combined**
AD-65381	0.239	0.110	0.194
AD-66019	ND	ND	ND
AD-65375	ND	ND	ND
AD-65427	0.023	0.006	0.018
AD-66110	ND	ND	ND
AD-65421	ND	ND	ND
AD-65407	0.140	1.383	0.527
AD-65377	No ED50	No ED50	No ED50
AD-65409	1.807	3.436	2.905
AD-66111	ND	ND	ND
AD-65403	0.511	18.036	5.013

ND – not done

These duplexes were also assayed for *in vitro* stability using two assays, a tritosome stability assay and a cytosol stability assay, as described above. The results of these assays are provided in Table 20.

Table 20. Twenty-four hour tritosome and cytosol stability assays.

	In vitro metabolic stability % parent remaining at 24hr incubation			
	Endo-lysosome		Cytosol	
Duplex ID	% AS	% SS	% AS	% SS
AD-65381	88	72	68	22
AD-66019	ND	ND	ND	ND
AD-65375	ND	ND	ND	ND
AD-65407	100	120	64	24
AD-65377	99	127	80	67
AD-65409	105	120	86	74
AD-66111	ND	ND	ND	ND
AD-65403	ND	ND	ND	ND
AD-65427	115	80	89	66
AD-66110	ND	ND	ND	ND
AD-65421	ND	ND	ND	ND

Dose response screens of various combinations of these duplexes were also performed in HepG2.215 cells. The doses of the duplexes used for these assays were 50 nM, 8.333333333 nM, 1.388888889 nM, 0.231481481 nM, 0.038580247 nM, 0.006430041 nM, 0.001071674 nM, 0.000178612 nM, 2.97687 x 10^{-5} nM, 4.96145 x 10^{-6} nM, 8.26909 x 10^{-7} nM, and 1.37818E x 10^{-7} nM, which represent a 1 to 6 dilution of the duplexes starting at 50 nM over 12 doses. At seventy-two hours after transfection of these duplexes, the level of viral RNA (P ORF and S ORF) and the level of secreted HBsAg were determined, as described above. The results of these assays are provided in Table 21.

Table 21. Seventy-two hour HBV single dose screen In HepG2.2.15 cells

DuplexID	S-ORF2 IC50_A (nM)	S-ORF2 IC50_B (nM)	S-ORF2 IC50_Combine (nM)	P-ORF1 IC50_A (nM)	P-ORF1 IC50_B (nM)	P-ORF1 IC50_Combine (nM)	S Ag ELISA ED50 (nM)
AD-66019/AD-66110	0.0091	0.0017	0.0038	0.0213	0.002	0.0076	0.007482
AD-66019/AD-65421	0.0438	0.2371	0.0131	0.0367	0.0106	0.0204	0.026398
AD-65375/AD-66110	0.0832	1.0896	0.193	0.0377	0.2348	0.2022	0.004174
AD-65375/AD-65421	0.084	0.0475	0.0708	0.0566	0.0388	0.0371	0.030822
AD-65407/AD-66110	0.0387	0.001	0.0083	0.0402	0.0018	0.0116	0.010172
AD-65407/AD-65421	0.0686	0.0062	0.0225	0.0711	0.0177	0.0396	0.066556
AD-65377/AD-66110	0.0634	0.8267	0.6269	0.0477	0.073	0.0618	0.01435
AD-65377/AD-65421	0.1461	0.0468	0.1372	0.1207	0.0088	0.0451	0.03419
AD-66111/AD-66110	0.0382	0.0094	0.0161	0.0292	0.0027	0.0088	0.013155
AD-66111/AD-65421	0.1628	0.0919	0.1579	0.1297	0.0396	0.0722	0.026889
AD-65403/AD-66110	0.0499	0.0094	0.0444	0.0383	0.0164	0.0348	0.003783
AD-65403/AD-65421	0.1011	0.0007	0.0208	0.1118	0.0031	0.0297	0.014569

Example 5. Synthesis and *In vitro* Screening of Additional siRNA Duplexes

Additional iRNA molecules targeting the X ORF of the HBV genome were designed and synthesized as described above. A detailed list of the additional unmodified HBV sense and antisense strand sequences is shown in Table 22. A detailed list of the additional modified HBV sense and antisense strand sequences is shown in Table 23.

Table 22. Unmodified Sense and Antisense Strand Sequences of HBV dsRNAs

DuplexID	Sense Oligo Name	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense OligoName	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-65776	A-131859.1	UGUGCACUUCGCUUCACCUCU	1069	A-131860.1	AGAGGUGAAGCGAAGUGCACACG	1115
AD-65782	A-131877.1	UGCACUUCGCUUCACCUCUGA	1070	A-131878.1	UCAGAGGUGAAGCGAAGUGCACA	1116
AD-65792	A-131865.1	GUGUGCACUUCGCUUCACCUA	1071	A-131866.1	UAGGUGAAGCGAAGUGCACACGG	1117
AD-65781	A-131861.1	CGUGUGCACUUCGCUUCACCU	1072	A-131862.1	AGGUGAAGCGAAGUGCACACGGU	1118
AD-64304	A-128443.6	GUGCACUUCGCUUCACCUCUA	1073	A-128444.5	UAGAGGUGAAGCGAAGUGCACAC	1119
AD-65771	A-131857.1	CCGUGUGCACUUCGCUUCACA	1074	A-131858.1	UGUGAAGCGAAGUGCACACGGUC	1120
AD-65758	A-131867.1	CACUUCGCUUCACCUCUGCAA	1075	A-131868.1	UUGCAGAGGUGAAGCGAAGUGCA	1121
AD-65777	A-131875.1	ACUUCGCUUCACCUCUGCACA	1076	A-131876.1	UGUGCAGAGGUGAAGCGAAGUGC	1122
AD-61567	A-123525.2	GGCUGUAGGCAUAAAUUGGUA	1077	A-123526.2	UACCAAUUUAUGCCUACAGCCUC	1123
AD-65772	A-131873.1	UUCGCUUCACCUCUGCACGUA	1078	A-131874.1	UACGUGCAGAGGUGAAGCGAAGU	1124
AD-65767	A-131871.1	UCGCUUCACCUCUGCACGUCA	1079	A-131872.1	UGACGUGCAGAGGUGAAGCGAAG	1125
AD-65763	A-131869.1	CUUCGCUUCACCUCUGCACGU	1080	A-131870.1	ACGUGCAGAGGUGAAGCGAAGUG	1126
AD-64281	A-128395.3	CCCCGUCUGUGCCUUCUCAUA	1081	A-128396.2	UAUGAGAAGGCACAGACGGGGAG	1127
AD-64311	A-128391.3	CCGUCUGUGCCUUCUCAUCUA	1082	A-128392.2	UAGAUGAGAAGGCACAGACGGGG	1128
AD-65790	A-131837.1	CCAGCACCAUGCAACUUUUUA	1083	A-131838.1	UAAAAAGUUGCAUGGUGCUGGUG	1129
AD-65761	A-131841.1	CACCAGCACCAUGCAACUUUU	1084	A-131842.1	AAAAGUUGCAUGGUGCUGGUGCG	1130
AD-65786	A-131849.1	CACCAUGCAACUUUUUCACCU	1085	A-131850.1	AGGUGAAAAAGUUGCAUGGUGCU	1131
AD-65785	A-131835.1	CAAUGUCAACGACCGACCUUA	1086	A-131836.1	UAAGGUCGGUCGUUGACAUUGCA	1132
AD-65787	A-131863.1	CGCUUCACCUCUGCACGUCGA	1087	A-131864.1	UCGACGUGCAGAGGUGAAGCGAA	1133
AD-65770	A-131845.1	ACCUUGAGGCAUACUUCAAAG	1088	A-131846.1	CUUUGAAGUAUGCCUCAAGGUCG	1134
AD-65766	A-131843.1	CCGACCUUGAGGCAUACUUCA	1089	A-131844.1	UGAAGUAUGCCUCAAGGUCGGUC	1135

AD-61555	A-123521.2	GACCUUGAGGCAUACUUCAAA	1090	A-123522.2	UUUGAAGUAUGCCUCAAGGUCGG	1136
AD-65762	A-131855.1	ACCGACCUUGAGGCAUACUUA	1091	A-131856.1	UAAGUAUGCCUCAAGGUCGGUCG	1137
AD-65755	A-131827.1	UCGCAUGGAGACCACCGUGAA	1092	A-131828.1	UUCACGGUGGUCUCCAUGCGACG	1138
AD-65788	A-131811.1	UUACAUAAGAGGACUCUUGGA	1093	A-131812.1	UCCAAGAGUCCUCUUAUGUAAGA	1139
AD-65768	A-131803.1	UCUUACAUAAGAGGACUCUUA	1094	A-131804.1	UAAGAGUCCUCUUAUGUAAGACC	1140
AD-61561	A-123523.2	ACUUCAAAGACUGUUUGUUUA	1095	A-123524.2	UAAACAAACAGUCUUUGAAGUAU	1141
AD-65764	A-131801.1	UACUUCAAAGACUGUUUGUUU	1096	A-131802.1	AAACAAACAGUCUUUGAAGUAUG	1142
AD-65753	A-131799.1	AUACUUCAAAGACUGUUUGUU	1097	A-131800.1	AACAAACAGUCUUUGAAGUAUGC	1143
AD-65765	A-131817.1	UUGUUUAAAGACUGGGAGGAA	1098	A-131818.1	UUCCUCCCAGUCUUUAAACAAAC	1144
AD-65769	A-131819.1	GCAUACUUCAAAGACUGUUUA	1099	A-131820.1	UAAACAGUCUUUGAAGUAUGCCU	1145
AD-65759	A-131815.1	CAAAGACUGUUUGUUUAAAGA	1100	A-131816.1	UCUUUAAACAAACAGUCUUUGAA	1146
AD-65774	A-131831.1	AGACUGUUUGUUUAAAGACUA	1101	A-131832.1	UAGUCUUUAAACAAACAGUCUUU	1147
AD-65778	A-131807.1	GUUUGUUUAAAGACUGGGAGA	1102	A-131808.1	UCUCCCAGUCUUUAAACAAACAG	1148
AD-65773	A-131805.1	GGGGGAGGAGAUUAGAUUAAA	1103	A-131806.1	UUUAAUCUAAUCUCCUCCCCCAA	1149
AD-65789	A-131825.1	GGGGAGGAGAUUAGAUUAAAG	1104	A-131826.1	CUUUAAUCUAAUCUCCUCCCCCA	1150
AD-65783	A-131809.1	GUUGGGGGAGGAGAUUAGAUU	1105	A-131810.1	AAUCUAAUCUCCUCCCCCAACUC	1151
AD-65754	A-131813.1	UUGGGGGAGGAGAUUAGAUUA	1106	A-131814.1	UAAUCUAAUCUCCUCCCCCAACU	1152
AD-65779	A-131821.1	GGGAGGAGAUUAGAUUAAAGA	1107	A-131822.1	UCUUUAAUCUAAUCUCCUCCCCC	1153
AD-65791	A-131851.1	UUAGAUUAAAGGUCUUUGUAA	1108	A-131852.1	UUACAAAGACCUUUAAUCUAAUC	1154
AD-65760	A-131829.1	UAGAUUAAAGGUCUUUGUACU	1109	A-131830.1	AGUACAAAGACCUUUAAUCUAAU	1155
AD-65784	A-131823.1	AUUAGAUUAAAGGUCUUUGUA	1110	A-131824.1	UACAAAGACCUUUAAUCUAAUCU	1156
AD-65757	A-131853.1	GAGGAGAUUAGAUUAAAGGUA	1111	A-131854.1	UACCUUUAAUCUAAUCUCCUCCC	1157
AD-65775	A-131847.1	GGACUCUUGGACUCUCUGCAA	1112	A-131848.1	UUGCAGAGAGUCCAAGAGUCCUC	1158
AD-65780	A-131833.1	ACUCUUGGACUCUCUGCAAUA	1113	A-131834.1	UAUUGCAGAGAGUCCAAGAGUCC	1159
AD-65756	A-131839.1	AGAUUAAAGGUCUUUGUACUA	1114	A-131840.1	UAGUACAAAGACCUUUAAUCUAA	1160

Table 23. Unmodified Sense and Antisense Strand Sequences of HBV dsRNAs

Duplex ID	Sense Oligo Name	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Oligo Name	Antisense Sequence (5' to 3')	SEQ ID NO:
AD-65776	A-131859.1	UfsgsUfgCfaCfuUfCfGfcUfuCfaCfcUfcUfL96	1161	A-131860.1	asGfsaGfgUfgAfaGfcgaAfgUfgCfaCfascsg	1207
AD-65782	A-131877.1	UfsgsCfaCfuUfcGfCfUfuCfaCfcUfcUfgAfL96	1162	A-131878.1	usCfsaGfaGfgUfgAfagcGfaAfgUfgCfascsa	1208
AD-65792	A-131865.1	GfsusGfuGfuGfcAfcAfUfUfCfgCfuUfcAfcCfuAfL96	1163	A-131866.1	usAfsgGfuGfuGfaAfgCfgaaGfuGfcAfcAfcsgsg	1209
AD-65781	A-131861.1	CfsgsUfgUfgCfaCfUfUfcGfcUfuCfaCfcUfL96	1164	A-131862.1	asGfsgUfgAfaGfcGfaagUfgCfaCfaCfgsgsu	1210
AD-64304	A-128443.6	GfsusGfcAfcUfuCfGfCfuUfcAfcCfuCfuAfL96	1165	A-128444.5	usAfsgAfgGfuGfaAfgcgAfaGfuGfcAfcsasc	1211
AD-65771	A-131857.1	CfscsGfuGfuGfcAfCfUfuCfgCfuUfcAfcAfL96	1166	A-131858.1	usGfsuGfaAfgCfgAfaguGfcAfcAfcGfgsusc	1212
AD-65758	A-131867.1	CfsasCfuUfcGfcUfUfUfCfaCfcUfgCfaAfL96	1167	A-131868.1	usUfsgCfaGfaGfgUfgaaGfcGfaAfgUfgscsa	1213
AD-65777	A-131875.1	AfscsUfuCfgCfuUfCfAfcCfuCfuGfcAfcAfL96	1168	A-131876.1	usGfsuGfcAfgAfgGfugaAfgCfgAfaGfusgsc	1214
AD-61567	A-123525.2	GfsgsCfuGfuAfgGfCfAfuAfaAfuUfgGfuAfL96	1169	A-123526.2	usAfscCfaAfuUfuAfugcCfuAfcAfgCfcsusc	1215
AD-65772	A-131873.1	UfsusCfgCfuUfcAfCfCfuCfuGfcAfcGfuAfL96	1170	A-131874.1	usAfscGfuGfcAfgAfgguGfaAfgCfgAfasgsu	1216
AD-65767	A-131871.1	UfscsGfcUfuCfaCfCfUfcUfgCfaCfgUfcAfL96	1171	A-131872.1	usGfsaCfgUfgCfaGfaggUfgAfaGfcGfasasg	1217
AD-65763	A-131869.1	CfsusUfcGfcUfuCfAfCfcUfcUfgCfaCfgUfL96	1172	A-131870.1	asCfsgUfgCfaGfaGfgugAfaGfcGfaAfgsusg	1218
AD-64281	A-128395.3	CfscsCfcGfuCfuGfUfGfcCfuUfcUfcAfuAfL96	1173	A-128396.2	usAfsuGfaGfaAfgGfcacAfgAfcGfgGfgsasg	1219
AD-64311	A-128391.3	CfscsGfuCfuGfuGfCfCfuUfcUfcAfuCfuAfL96	1174	A-128392.2	usAfsgAfuGfaGfaAfggcAfcAfgAfcGfgsgsg	1220
AD-65790	A-131837.1	CfscsAfgCfaCfcAfUfGfcAfaCfuUfuUfuAfL96	1175	A-131838.1	usAfsaAfaAfgUfuGfcauGfgUfgCfuGfgsusg	1221
AD-65761	A-131841.1	CfsasCfcAfgCfaCfCfAfuGfcAfaCfuUfuUfL96	1176	A-131842.1	asAfsaAfgUfuGfcAfuggUfgCfuGfgUfgscsg	1222
AD-65786	A-131849.1	CfsasCfcAfuGfcAfAfCfuUfuUfuCfaCfcUfL96	1177	A-131850.1	asGfsgUfgAfaAfaAfguuGfcAfuGfgUfgscsu	1223
AD-65785	A-131835.1	CfsasAfuGfuCfaAfCfCfGfaCfcGfaCfcUfuAfL96	1178	A-131836.1	usAfsaGfgUfcGfgUfcguUfgAfcAfuUfgscsa	1224
AD-65787	A-131863.1	CfsgsCfuUfcAfcCfUfCfuGfcAfcGfuCfgAfL96	1179	A-131864.1	usCfsgAfcGfuGfcAfgagGfuGfaAfgCfgsasa	1225
AD-65770	A-131845.1	AfscsCfuUfgAfgGfCfAfuAfcUfuCfaAfaGfL96	1180	A-131846.1	csUfsuUfgAfaGfuAfugcCfuCfaAfgGfuscsg	1226
AD-65766	A-131843.1	CfscsGfaCfcUfuGfAfGfgCfaUfaCfuUfcAfL96	1181	A-131844.1	usGfsaAfgUfaUfgCfcucAfaGfgUfcGfgsusc	1227

AD-61555	A-123521.2	GfsasCfcUfuGfaGfGfCfaUfaCfuUfcAfaAfL96	1182	A-123522.2	usUfsuGfaAfgUfaUfgccUfcAfaGfgUfcsgsg	1228
AD-65762	A-131855.1	AfscsCfgAfcCfuUfGfAfgGfcAfuAfcUfuAfL96	1183	A-131856.1	usAfsaGfuAfuGfcCfucaAfgGfuCfgGfuscsg	1229
AD-65755	A-131827.1	UfscsGfcAfuGfgAfGfAfcCfaCfcGfuGfaAfL96	1184	A-131828.1	usUfscAfcGfgUfgGfucuCfcAfuGfcGfascsg	1230
AD-65788	A-131811.1	UfsusAfcAfuAfaGfAfGfgAfcUfcUfuGfgAfL96	1185	A-131812.1	usCfscAfaGfaGfuCfcucUfuAfuGfuAfasgsa	1231
AD-65768	A-131803.1	UfscsUfuAfcAfuAfAfGfaGfgAfcUfcUfuAfL96	1186	A-131804.1	usAfsaGfaGfuCfcUfcuuAfuGfuAfaGfascsc	1232
AD-61561	A-123523.2	AfscsUfuCfaAfaGfAfCfuGfuUfuGfuUfuAfL96	1187	A-123524.2	usAfsaAfcAfaAfcAfgucUfuUfgAfaGfusasu	1233
AD-65764	A-131801.1	UfsasCfuUfcAfaAfGfAfcUfgUfuUfgUfuUfL96	1188	A-131802.1	asAfsaCfaAfaCfaGfucuUfuGfaAfgUfasusg	1234
AD-65753	A-131799.1	AfsusAfcUfuCfaAfAfGfaCfuGfuUfuGfuUfL96	1189	A-131800.1	asAfscAfaAfcAfgUfcuuUfgAfaGfuAfusgsc	1235
AD-65765	A-131817.1	UfsusGfuUfuAfaAfGfAfcUfgGfgAfgGfaAfL96	1190	A-131818.1	usUfscCfuCfcCfaGfucuUfuAfaAfcAfasasc	1236
AD-65769	A-131819.1	GfscsAfuAfcUfuCfAfAfaGfaCfuGfuUfuAfL96	1191	A-131820.1	usAfsaAfcAfgUfcUfuugAfaGfuAfuGfcscsu	1237
AD-65759	A-131815.1	CfsasAfaGfaCfuGfUfUfuGfuUfuAfaAfgAfL96	1192	A-131816.1	usCfsuUfuAfaAfcAfaacAfgUfcUfuUfgsasa	1238
AD-65774	A-131831.1	AfsgsAfcUfgUfuUfGfUfuUfaAfaGfaCfuAfL96	1193	A-131832.1	usAfsgUfcUfuUfaAfacaAfaCfaGfuCfususu	1239
AD-65778	A-131807.1	GfsusUfuGfuUfuAfAfAfgAfcUfgGfgAfgAfL96	1194	A-131808.1	usCfsuCfcCfaGfuCfuuuAfaAfcAfaAfcsasg	1240
AD-65773	A-131805.1	GfsgsGfgGfaGfgAfGfAfuUfaGfaUfuAfaAfL96	1195	A-131806.1	usUfsuAfaUfcUfaAfucuCfcUfcCfcCfcsasa	1241
AD-65789	A-131825.1	GfsgsGfgAfgGfaGfAfUfuAfgAfuUfaAfaGfL96	1196	A-131826.1	csUfsuUfaAfuCfuAfaucUfcCfuCfcCfcscsa	1242
AD-65783	A-131809.1	GfsusUfgGfgGfgAfGfGfaGfaUfuAfgAfuUfL96	1197	A-131810.1	asAfsuCfuAfaUfcUfccuCfcCfcCfaAfcsusc	1243
AD-65754	A-131813.1	UfsusGfgGfgGfaGfGfAfgAfuUfaGfaUfuAfL96	1198	A-131814.1	usAfsaUfcUfaAfuCfuccUfcCfcCfcAfascsu	1244
AD-65779	A-131821.1	GfsgsGfaGfgAfgAfUfUfUfaGfaUfuAfaAfgAfL96	1199	A-131822.1	usCfsuUfuAfaUfcUfaauCfuCfcUfcCfcscsc	1245
AD-65791	A-131851.1	UfsusAfgAfuUfaAfAfGfgUfcUfuUfgUfaAfL96	1200	A-131852.1	usUfsaCfaAfaGfaCfcuuUfaAfuCfuAfasusc	1246
AD-65760	A-131829.1	UfsasGfaUfuAfaAfGfGfuCfuUfuGfuAfcUfL96	1201	A-131830.1	asGfsuAfcAfaAfgAfccuUfuAfaUfcUfasasu	1247
AD-65784	A-131823.1	AfsusUfaGfaUfuAfAfAfgGfuCfuUfuGfuAfL96	1202	A-131824.1	usAfscAfaAfgAfcCfuuuAfaUfcUfaAfuscsu	1248
AD-65757	A-131853.1	GfsasGfgAfgAfuUfAfGfaUfuAfaAfgGfuAfL96	1203	A-131854.1	usAfscCfuUfuAfaUfcuaAfuCfuCfcUfcscsc	1249
AD-65775	A-131847.1	GfsgsAfcUfcUfuGfGfAfcUfcUfcUfgCfaAfL96	1204	A-131848.1	usUfsgCfaGfaGfaGfuccAfaGfaGfuCfcsusc	1250
AD-65780	A-131833.1	AfscsUfcUfuGfgAfCfUfcUfcUfgCfaAfuAfL96	1205	A-131834.1	usAfsuUfgCfaGfaGfaguCfcAfaGfaGfuscsc	1251
AD-65756	A-131839.1	AfsgsAfuUfaAfaGfGfUfcUfuUfgUfaCfuAfL96	1206	A-131840.1	usAfsgUfaCfaAfaGfaccUfuUfaAfuCfusasa	1252

A single dose screen of these duplexes was performed in Cos7 cells at 1 nm and 50 nm using the Dual-Glo® Luciferase assay described above. The results of the assays are provided in Table 24.

Table 24. HBV single dose screen using Dual-Glo Luciferase® Assay

DuplexID	50 nM	STDEV	1 nM	STDEV
AD-65776	20.11	4.21	40.79	1.89
AD-65782	26.31	3.10	61.07	9.16
AD-65792	43.31	5.24	61.09	6.02
AD-65781	25.77	3.66	39.63	2.87
AD-64304	18.87	1.26	29.72	3.37
AD-65771	17.16	1.78	37.55	2.20
AD-65758	31.74	8.26	65.77	11.05
AD-65777	59.76	11.15	77.63	5.14
AD-61567	17.69	5.29	26.45	5.66
AD-65772	58.07	9.67	75.66	4.92
AD-65767	29.65	1.60	39.64	4.36
AD-65763	25.10	5.77	47.78	9.99
AD-64281	39.07	6.80	51.46	4.19
AD-64311	20.51	1.96	37.80	3.53
AD-65790	50.41	7.00	70.30	1.95
AD-65761	13.30	4.38	21.14	3.49
AD-65786	12.45	3.51	22.62	0.33
AD-65785	36.87	6.04	51.49	4.18
AD-65787	27.97	5.73	48.18	7.65
AD-65770	22.67	5.39	41.48	8.52
AD-65766	31.44	3.35	50.25	0.45
AD-61555	18.43	10.83	22.61	0.57
AD-65762	18.87	4.86	34.94	4.81
AD-65755	47.03	9.38	83.19	9.68
AD-65788	35.85	10.13	58.07	4.78
AD-65768	24.02	2.49	28.55	2.53
AD-61561	8.11	1.29	14.26	2.27
AD-65764	16.89	3.99	29.10	1.03
AD-65753	19.10	2.87	29.79	5.26
AD-65765	55.40	10.72	76.93	8.79
AD-65769	19.24	4.47	23.18	2.54
AD-65759	48.86	4.81	87.31	13.75
AD-65774	102.27	12.33	100.79	3.24
AD-65778	64.39	2.60	80.67	2.59
AD-65773	72.64	7.87	80.80	4.83

AD-65789	73.59	4.35	94.72	3.32
AD-65783	54.41	7.15	84.46	4.32
AD-65754	62.51	4.12	102.63	21.42
AD-65779	47.40	7.51	76.20	2.05
AD-65791	12.09	0.70	19.19	3.46
AD-65760	13.50	4.84	25.37	2.09
AD-65784	19.84	1.27	31.04	3.49
AD-65757	22.66	3.97	24.50	5.81
AD-65775	47.78	3.30	58.81	3.05
AD-65780	29.10	2.87	42.85	2.73
AD-65756	10.49	1.62	19.95	2.58

Based on these assays, RNAi agents targeting five sites in the HBV X ORF (nucleotides 1551, 1577, 1580, 1806, and 1812 of GenBank Accession No. NC_003977.1 were selected for lead optimization and additional agents were designed and synthesized. These additional agents are evaluated in *in vitro* assays as described above. A detailed list of the additional unmodified sense and antisense strand sequences targeting the HBV X ORF is shown in Table 25. A detailed list of the additional modified sense and antisense strand sequences targeting the HBV X ORF is shown in Table 26.

These iRNA agents were also assessed for *in vivo* efficacy using an AAV-HBV mouse model (see, *e.g.,* Yang, *et al.* (2014) *Cell and Mol Immunol* 11:71). This mouse model exhibits sustained HBV viremia after infection with a recombinant adeno-associated virus (AAV) carrying a replicable HBV genome. Liver expression of the HBV gene in these mice mimics HBV infection in humans and these mice exhibit significant liver inflammation and liver damage, manifested by increased ALT levels, fibrosis and steatosis.

These AAV-HBV mice were subcutaneously administered a single 3 mg/kg dose of AD-66808, AD-66809, AD-66810, AD-66811, AD-66812, AD-66813, AD-66814, AD-66815, AD-66816, and AD-66817 and the level of HBsAg was determined in the serum of the animals pre-dose, and at day 14/15 post-dose. The results of these experiments are provided in Figure 2 and Table 27 and demonstrate that serum levels of HBsAg are decrease following a single administration of these agents. Table 27 also provides the results of a single dose screen in Cos7 cells transfected with the indicated HBV iRNAs using the Dual-Glo® Luciferase assay, as described above, for the same RNAi agents. Data are expressed as percent of mRNA remaining relative to negative control at 24 hours.

Table 25. Unmodified HBV X ORF Sense and Antisense Sequences.

DuplexID	Sense Sequence Unmodified (5' to 3')	SEQ ID NO:	Antisense Sequence Umodified (5' to 3')	SEQ ID NO:
AD-66808	GUCUGUGCCUUCUCAUCUA	1253	UAGAUGAGAAGGCACAGACUU	1263
AD-66809	GUCUGUGCCUUCUCAUCUA	1254	UAGAUGAGAAGGCACAGACUU	1264
AD-66810	GUGUGCACUUCGCUUCACA	1255	UGUGAAGCGAAGUGCACACUU	1265
AD-66811	GUGUGCACUUCGCUUCACA	1256	UGUGAAGCGAAGUGCACACUU	1266
AD-66812	UGUGCACUUCGCUUCACCUCU	1257	AGAGGUGAAGCGAAGUGCACAUU	1267
AD-66813	UGUGCACUUCGCUUCACCUCU	1258	AGAGGUGAAGCGAAGUGCACAUU	1268
AD-66814	CACCAGCACCAUGCAACUUUU	1259	AAAAGUUGCAUGGUGCUGGUGUU	1269
AD-66815	CACCAGCACCAUGCAACUUUU	1260	AAAAGUUGCAUGGUGCUGGUGUU	1270
AD-66816	CACCAUGCAACUUUUUCACCU	1261	AGGUGAAAAAGUUGCAUGGUGUU	1271
AD-66817	CACCAUGCAACUUUUUCACCU	1262	AGGUGAAAAAGUUGCAUGGUGUU	1272

Table 26. Modified HBV X ORF Sense and Antisense Sequences.

DuplexID	**Sense Sequence Modified (5' to 3')**	SEQ ID NO:	Antisense Sequence Modified (5' to 3')	SEQ ID NO:
AD-66808	gsuscuGfuGfCfCfuucucaucuaL96	1273	usAfsgauGfaGfAfaggcAfcAfgacsusu	1283
AD-66809	gsuscuGfuGfCfCfuucucaucuaL96	1274	UfsAfsgauGfaGfAfaggcAfcAfgacsusu	1284
AD-66810	gsusguGfcAfCfUfucgcuucacaL96	1275	usGfsugaAfgCfGfaaguGfcAfcacsusu	1285
AD-66811	gsusguGfcAfCfUfucgcuucacaL96	1276	UfsGfsugaAfgCfGfaaguGfcAfcacsusu	1286
AD-66812	usgsugcaCfuUfCfGfcuucaccucuL96	1277	asGfsaggUfgAfAfgcgaAfgUfgcacasusu	1287
AD-66813	usgsugcaCfuUfCfGfcuucaccucuL96	1278	AfsGfsaggUfgAfAfgcgaAfgUfgcacasusu	1288
AD-66814	csasccagCfaCfCfAfugcaacuuuuL96	1279	asAfsaagUfuGfCfauggUfgCfuggugsusu	1289
AD-66815	csasccagCfaCfCfAfugcaacuuuuL96	1280	AfsAfsaagUfuGfCfauggUfgCfuggugsusu	1290
AD-66816	csasccauGfcAfAfCfuuuuucaccuL96	1281	asGfsgugAfaAfAfaguuGfcAfuggugsusu	1291
AD-66817	csasccauGfcAfAfCfuuuuucaccuL96	1282	AfsGfsgugAfaAfAfaguuGfcAfuggugsusu	1292

Table 27.

Site (# vRNA[1])	Duplex ID	In vitro IC_{50} Luc HBV (nM)	Log_{10} HBsAg KD In Vivo @3 mg/kg
1551 (4)	AD-66808	**0.187**	**2.4**
	AD-66809	**0.014**	**1.46**
1577 (4)	AD-66810	**0.290**	**1.7**
	AD-66811	**0.029**	**1.3**
1580 (4)	AD-66812	**0.795**	**2.19**
	AD-66813	**0.074**	**>>1.14**
1806 (4)	AD-66814	**0.0002**	**1.5**
	AD-66815	**0.0001**	**>>1.56**
1812 (4)	AD-66816	**0.047**	**1.61**
	AD-66817	**0.0001**	**1.60**

[1] Number of viral RNAs silenced

Example 6. *In vivo* Screening of siRNA Duplexes

A subset of lead iRNA agents was assessed for *in vivo* efficacy using the AAV-HBV mouse model described above. AAV-HBV mice were administered a single 3 mg/kg dose of AD-66019, AD-65375, AD-65047, AD-65377, AD-66111, AD-65421, or AD-66110 and the level of HBsAg was determined in the serum of the animals pre-dose, and at days 5 and 10 post-dose. As a control, AAV-HBV mice were administered a 3 mg/kg dose of a dsRNA targeting mouse/rat transtherytin (mrTTR). The results of these experiments are depicted in Figure 3 and demonstrate that serum levels of HBsAg are decreased following a single administration of these agents.

Figure 4 is a graph depicting the percent of pre-dose HBsAg remaining at days 5 and 10 in these animals following administration of a single 3 mg/kg dose was also determined. The results of these experiments are depicted in Figure 4. Figure 4 also depicts the percent of HBsAG remaining at day 10 post-dose relative to the percent of HBsAG remaining at day 10 post-dose in an animal administered 3 mg/kg of a control dsRNA targeting mouse/rat transtherytin (mrTTR).

Based, at least in part, on the results of the *in vitro* and *in vivo* assays described above, AD-65403, which silences 3 HBV RNAs, and AD-66810, which silences the X gene, were selected as drug candidates (DC) for use in a monotherapy or in a combination therapy.

Figure 5 demonstrates that, in the AAV-HBV mouse model of HBV infection, a single 3 mg/kg dose of AD-65403 achieves potent and specific knockdown of HBsAg. In particular, a single 3 mg/kg subcutaneous dose of AD-65403 achieves up to a 3.9 $\log_{10}$ reduction in HBsAg levels, with a mean HBsAg reduction of 1.8 $\log_{10}$ 5-10 days after a single dose.

Figures 6A and 6B demonstrate that, in the AAV-HBV mouse model of HBV infection, a single 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 9 mg/kg subcutaneous dose of AD-66810 achieves potent and specific knockdown of HBsAg, especially at the higher doses of AD-66810. The percent decrease of HBsAg in serum is shown on a standard scale in Figure 6A and on a $\log_{10}$ scale in Figure 6B. Figure 7 demonstrates that, in the AAV-HBV mouse model of HBV infection, AD-66810 administered in three weekly subcutaneous 3 mg/kg doses, achieves potent and specific knockdown of HBsAg for a period of greater than 4 months.

Example 7. Treatment of HBV infection with a combination of agents targeting HBV

A subset of iRNA agents of the invention are assessed for *in vivo* efficacy using the AAV-HBV mouse model described above. AAV-HBV mice are administered one or more doses of AD-65403 and AD-66810, either alone or in combination with each other. Exemplary dosing regimens include a single 3 mg/kg total iRNA dose of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810 (*i.e.*, 1.5 mg/kg of each iRNA agent for

a total of 3 mg/kg of iRNA administered as an admixture or as two separate doses); or a single dose of 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 9 mg/kg total iRNA agent dose of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810. Exemplary multi-dose regimens include, for example, three weekly doses, one per week using any of the dosage levels provided in the exemplary single dose regimens. An appropriate control iRNA agent is also administered as a control as is routine in the art.

The level of HBsAg is determined in the serum of the animals pre-dose, and at predetermined intervals post-dose, *e.g.*, every five days post-dose until the HBsAg level returns to baseline for all animals. Administration of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810 results in sustained and specific knockdown of serum HBsAg.

Example 8. Treatment of HDV infection with iRNA agents targeting Hepatitis B virus

Hepatitis Delta virus (HDV) is a defective RNA virus which requires the help of HBV for its replication and assembly of new virions. Therefore, HDV is only infectious in the presence of active HBV infection. The HDV genome contains only one actively transcribed open reading frame which encodes two isoforms of hepatitis delta antigen. Post-translational modifications of small and large delta antigens (S-HDAg and L-HDAg) involving phosphorylation and isoprenylation respectively confer these antigens their specific properties. Effective treatment of HBV will also ameliorate HDV infection.

A chimpanzee model of HDV is known. A subset of iRNA agents of the invention are assessed for *in vivo* efficacy using the chimpanzee HDV model or other appropriate model of HDV. HDV infected chimpanzees are administered one or more doses of AD-65403 and AD-66810, either alone or in combination with each other. Exemplary dosing regimens include a single 3 mg/kg total iRNA agent dose of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810 (*i.e.*, 1.5 mg/kg of each iRNA agent for a total of 3 mg/kg of iRNA agent administered as an admixture or as two separate doses); or a single dose of 0.3 mg/kg, 1 mg/kg, 3 mg/kg, or 9 mg/kg total iRNA agent dose of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810. Exemplary multi-dose regimens include, for example, three weekly doses, one per week using any of the dosage levels provided in the exemplary single dose regimens. An appropriate control iRNA is also administered as a control as is routine in the art.

The level of one or more of S-HDAg, L-HDAg, and HDV RNA, optionally in combination with HBsAg, is determined in the serum of the animals pre-dose and at predetermined intervals post-dose, *e.g.*, every five days to monitor antigen or RNA levels. Administration of AD-65403, AD-66810, or a combination of AD-65403 and AD-66810 results in sustained and specific knockdown of serum HBsAg resulting in amelioration of HDV as demonstrated by, for example, a statistically significant decrease in one or more of

S-HDAg, L-HDAg, and HDV RNA. These results demonstrate that administration of one or both of AD-65403 and AD-66810 is effective in the treatment of HDV.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

We claim:

1. A double stranded RNAi agent for inhibiting expression of hepatitis B virus (HBV) in a cell, wherein said double stranded RNAi agent comprises a sense strand and an antisense strand forming a double-stranded region,

wherein said sense strand comprises 5'- GUGUGCACUUCGCUUCACA -3' (SEQ IDNO:39), and said antisense strand comprises 5'- UGUGAAGCGAAGUGCACACUU -3' (SEQ ID NO:40),

wherein substantially all of the nucleotides of said sense strand and substantially all of the nucleotides of said antisense strand are modified nucleotides,

wherein said sense strand is conjugated to a ligand attached at the 3'-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

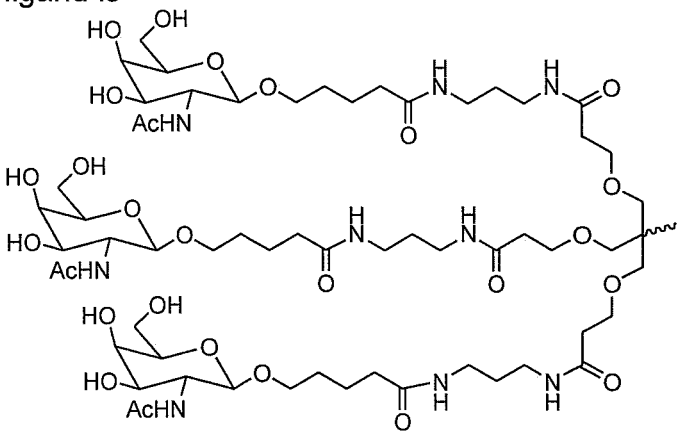
2. The double stranded RNAi agent of claim 1, wherein all of the nucleotides of said sense strand and all of the nucleotides of said antisense strand comprise a modification.

3. The double stranded RNAi agent of claim 1, wherein at least one of said modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

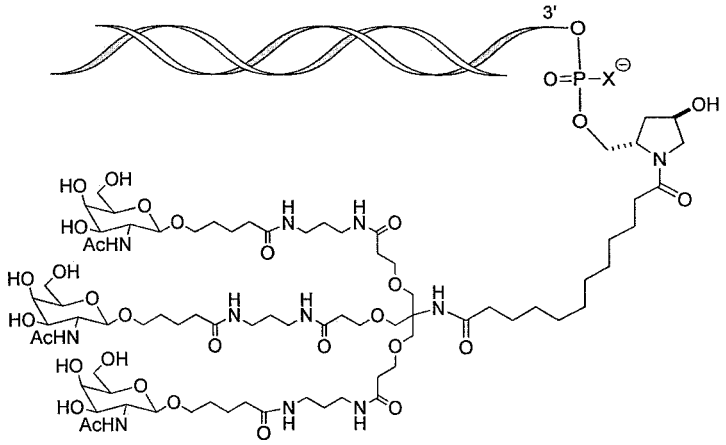
4. The double stranded RNAi agent of claim 1, wherein the sense strand comprises 5'- gsusguGfcAfCfUfucgcuucaca -3' (SEQ ID NO:41) and the antisense strand comprises 5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:42),

wherein a, g, c, and u are 2'-O-methyl (2'-OMe) A, 2'-OMe G, 2'-OMe C, or 2'-OMe G, respectively; Af, Cf, Gf or Uf are 2'-fluoro A,2'-fluoro C, 2'-fluoro G, or 2'-fluoro U, respectively; and s is a phosphorothioate linkage.

5. The double stranded RNAi agent of any one of claims 1-4, wherein the ligand is



preferably, wherein the RNAi agent is conjugated to the ligand as shown in the following schematic



wherein X is O or S.

6. The double stranded RNAi agent of claim 1, wherein the sense strand comprises 5'- gsusguGfcAfCfUfucgcuucacaL96 -3' (SEQ ID NO:1275) and the antisense strand comprises 5'- usGfsugaAfgCfGfaaguGfcAfcacsusu -3' (SEQ ID NO:1285), wherein a, g, c, and u are 2'-O-methyl (2'-OMe) A, 2'-OMe G, 2'-OMe C, and 2'-OMe U, respectively; Af, Cf, Gf, and Uf are 2'-fluoro A, 2'-fluoro C, 2'-fluoro G, and 2'-fluoro U, respectively; s is a phosphorothioate linkage; and L96 is N-[tris(GalNAc-alkyl)-amidodecanoyl]-4-hydroxyprolinol Hyp-(GalNAc-alkyl)3.

7. A pharmaceutical composition comprising the double stranded RNAi agent of claim 1 or 6.

8. The use of a double stranded RNAi agent of claim 1 or 6, or a pharmaceutical composition of claim 7, in the manufacture of a medicament for inhibiting Hepatitis B virus (HBV) gene expression or replication of HBV in a cell, wherein:

(a) the agent or the composition is to be contacted with the the cell; and

(b) the cell produced in step (a) is to be maintained for a time sufficient to obtain degradation of the mRNA transcript of an HBV gene, thereby expression of the HBV gene in the cell is inhibited, wherein the HBV gene is optionally selected from the group consisting of C, X, P, S, and a combination thereof.

9. The use of claim 8, wherein the HBV gene expression or replication is to be inhibited by at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100%.

10. The use of a therapeutically effective amount of the double stranded RNAi agent of claim 1 or 6, or the composition of claim 7, in the manufacture of a medicament for reducing the viral load of Hepatitis B virus (HBV) in a subject infected with HBV, wherein the agent or the composition is to be administered to the subject, thereby the viral load of HBV in the subject is reduced.

11. The use of a therapeutically effective amount of the double stranded RNAi agent of claim 1 or 6, or the pharmaceutical composition of claim 7, in the manufacture of a medicament for treating a subject having a Hepatitis B virus (HBV) infection, wherein the agent or the composition is to be administered to the subject.

12. The use of claim 10 or 11, wherein the double stranded RNAi agent is to be administered at a dose of 0.01 mg/kg to 10 mg/kg, 0.5 mg/kg to 50 mg/kg, 10 mg/kg to 30 mg/kg, 3 mg/kg, 10 mg/kg, or at a fixed dose of 50 mg to 200 mg.

13. The use of any one of claims 10-12, wherein the double stranded RNAi agent is to be administered subcutaneously, or intravenously.

14. The use of any one of claims 10-12, wherein an additional therapeutic agent is to be further administered to the subject, optionally the additional therapeutic agent is selected from the group consisting of an antiviral agent, a reverse transcriptase inhibitor (e.g., Tenofovir disoproxil fumarate (TDF), Tenofovir alafenamide, Lamivudine, Adefovir dipivoxil, Entecavir (ETV), Telbivudine, or AGX-

1009), an immune stimulator (e.g., pegylated interferon alfa 2a (PEG-IFN-α2a), Interferon alfa-2b, a recombinant human interleukin-7, and a Toll-like receptor 7 (TLR7) agonist), a therapeutic vaccine, a viral entry inhibitor, an oligonucleotide that inhibits the secretion or release of HbsAg, a capsid inhibitor, a covalently closed circular (ccc) HBV DNA inhibitor, and a combination of any of the foregoing.

15. The use of a therapeutically effective amount of the double stranded RNAi agent of claim 1 or 6, or the pharmaceutical composition of claim 7, in the manufacture of a medicament for reducing the level of a Hepatitis D virus (HDV) antigen or reducing the viral load of HDV in a subject infected with HDV, wherein the agent or the composition is to be administered to the subject, thereby the level of the HDV antigen in the subject is reduced, wherein the HDV antigen is optionally S-HDAg or L-HDAg.