ANTIVIRAL COMBINATIONS COMPRISING LAMIVUDINE AND ABACAVIR

The present invention relates to therapeutic combinations comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one (lamivudine) and a second therapeutic agent (-)-(1S, 4R) -4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, (1592U89, abacavir). The present invention is also concerned with pharmaceutical compositions containing said combinations and their use in the treatment of HBV infections including infections with HBV mutants bearing resistance to nucleoside and/or non-nucleoside inhibitors of the replication of the hepatitis B virus.

Hepatitis B is a viral disease transmitted orally or parentally by contaminated material such as blood or blood products, contaminated needles, sexually, and vertically from infected or carrier mothers to their off-spring. In those areas of the world where the disease is common, vertical transmission at an early age results in a high proportion of infected individuals becoming chronic carriers of hepatitis B. An estimated 350 million people world-wide are chronically infected with hepatitis B and as many as 150 million may die from liver disease in the absence of intervention.

Currently, the only established approach to treatment of hepatitis B is repeated injections of interferon, which may be associated with unpleasant side effects, and produces a long lasting response in only one third or less of those treated. Interferon is an immune modulator designed to boost the disease fighting ability of the immune system.

Lamivudine has been reported to be effective against HBV in a two year study, showing that most patients showed substantially reduced levels of viral replication with 52% maintaining undetectable levels of virus thorough to the end of the second year.

Abacavir is described in EP 0434450 as having potent activity against HBV. It has been shown to effectively block the replication of HBV in vitro, *Antimicrobial Agents and Chemotherapy May 1997, P1082-1093.*

The use of the combinations of the present invention may give rise to equivalent antiviral effect with reduced toxicity, or an increase in drug efficacy because synergy between compounds occurs. Lower overall drug doses will also possibly reduce the frequency of occurrence of drug resistant variants of HBV.

We have now found that lamivudine exhibits unexpected advantages when used in combination with abacavir. In particular the combination shows a statistically significant synergistic anti-HBV effect as shown in Figure 1. It is a feature of this invention that the use of this drug combination will provide synergistic antiviral effects, more complete viral suppression, viral suppression over longer periods, limit the emergence of drug resistant HBV mutants and allow better management of drug related toxicities. The use of this drug combination may also result in a decrease of the number of, for example, tablets administered a day, therefore may increase patient compliance.

As will be appreciated by those skilled in the art, references herein to treatment extend to prophylaxis as well as to the treatment of established infections and symptoms.

As used herein "pharmaceutically acceptable derivative" includes any pharmaceutically acceptable salt, ester or salt of such ester, of lamivudine or abacavir or any other compound which, upon administration to the recipient, is capable of providing (directly or indirectly) such a compound or an antivirally active metabolite or residue thereof.

Pharmaceutically acceptable salts of lamivudine, and abacavir include those derived from pharmaceutically acceptable bases, inorganic and organic acids. Examples of suitable acids include hydrochloric, hydrobromic, sulphuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicylic, succinic, toluene- p-sulphonic, tartaric, acetic, citric, methanesulphonic, formic, benzoic, malonic, naphthalene-2-sulphonic and benzenesulphonic acids. Other acids

such as oxalic acid, while not in themselves pharmaceutically acceptable may be useful in the preparation of salts useful as intermediates in obtaining compounds of the invention and their pharmaceutically acceptable acid addition salts. Salts derived from appropriate bases include alkali metal (e.g. sodium), alkaline earth metal (e.g. magnesium), ammonium and NR_{4+} (where R is C_{1-4} alkyl) salts.

Preferred salts of abacavir are the sulphate salts particularly the hemi-sulphate salt.

Preferred esters of lamivudine and abacavir are independently selected from the following group: (1) carboxylic acid esters in which the non-carbonyl moiety of the carboxylic acid portion of the ester grouping is selected from straight or branched chain alkyl (for example, methyl, n-propyl, t-butyl, or n-butyl), cycloalkyl, alkoxyalkyl (for example, methoxymethyl), aralkyl (for example, benzyl), aryloxyalkyl (for example, phenoxymethyl), aryl (for example, phenyl optionally substituted by, for example, halogen, C_{1-4} alkyl, or C_{1-4} alkoxy), or amino; (2) sulphonate esters, such as alkyl- or aralkylsulphonyl (for example, methanesulphonyl); (3) amino acid esters (for example, L-valyl or L-isoleucyl); and (4) phosphonate esters. In such esters, unless otherwise specified, any alkyl moiety present advantageously contains from 1 to 18 carbon atoms, particularly from 1 to 6 carbon atoms, more particularly from 1 to 4 carbon atoms. Any cycloalkyl moiety present in such esters advantageously contains from 3 to 6 carbon atoms. Any aryl moiety present in such esters advantageously comprises a phenyl group. Any reference to any of the above compounds also includes a reference to a physiologically acceptable salt thereof.

Particularly preferred esters are the mono-, di-, and triphosphate esters of lamivudine and abacavir (which may be optionally blocked), or any other compound which upon administration to a human subject is capable of providing (directly or indirectly) said mono-, di-, or triphosphate ester.

Further preferred derivatives of abacavir are the mono-, di-, and tri-phosphate esters of (1R, 4S)-9-[4-(hydroxymethyl)-2-cyclopenten-1-yl]guanine (carbovir).

Thus according to one aspect, the present invention provides a combination comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one or a pharmaceutically acceptable derivative thereof and (-)-(1S, 4R) -4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol or a pharmaceutically acceptable derivative thereof.

Combinations as described above may herein after be referred to as combinations according to the invention.

The present invention further provides combinations according to the invention for use in therapy, particularly in the treatment of an HBV infection including infections resistant to nucleoside and/or non-nucleoside inhibitors of the replication of the hepatitis B virus.

According to another aspect, the present invention provides a method for the treatment of a mammal, including a human, suffering from an HBV infection comprising administration of a therapeutically effective amount of a combination according to the invention.

It will be appreciated that the compounds of the combination may be administered simultaneously, either in the same or different pharmaceutical composition, or sequentially. If there is sequential administration, the delay in administering the second active ingredient should not be such as to lose the benefit of a synergistic therapeutic effect of the combination of the active ingredients. It will also be understood that lamivudine, and abacavir or the pharmaceutically acceptable derivatives thereof whether presented simultaneously or sequentially, may be administered individually or in any combination thereof. Lamivudine, and abacavir are preferably administered simultaneously or sequentially in separate pharmaceutical formulations, most preferably simultaneously.

Preferably the combination according to the invention is administered as a single combined formulation.

The present invention also provides the use of lamivudine in the manufacture of a medicament for administration simultaneously or sequentially with abacavir for the treatment of HBV infections. The use of abacavir in the manufacture of a medicament for administration simultaneously or sequentially with lamivudine for the treatment of HBV infections, is a further aspect of the invention.

A further aspect of the invention is a combination according to the invention wherein the lamivudine and abacavir are present in a synergistic ratio.

The synergistic effects of the combination of lamivudine and abacavir or pharmaceutically acceptable derivatives thereof are seen over a ratio, for example, of 1:0.5 to 1:10 (by weight), preferably 1:1 to 1:8 (by weight).

Conveniently each compound will be employed in the combination in an amount at which it exhibits anti-HBV activity when used alone.

The amount of a combination of lamivudine and abacavir required to be effective as an anti-HBV agent will, of course, vary and is ultimately at the discretion of the medical practitioner. The factors to be considered include the route of administration and nature of the formulation, the animal's body weight, age and general condition and the nature and severity of the disease to be treated.

In general for lamivudine a suitable daily dose will be in the range of from about 0.1 to about 50 mg per kilogram body weight of the recipient per day, preferably in the range of 0.5 to 20 mg per kilogram body weight per day, most preferably in the range of 0.5 to 2 mg per kilogram body weight per day.

Preferably lamivudine is administered at a level in the range 25 to 150mg per day.

Preferably lamivudine is conveniently administered at a level of about 100 mg per day.

For abacavir a suitable daily dose will be in the range of from about 3 to about 120 mg per kilogram body weight of the recipient per day, preferably in the range of 1 to 90 mg per kilogram body weight per day, most preferably in the range of 5 to 60 mg per kilogram body weight per day.

Preferably abacavir is administered at a level in the range 200 to 800 mg per day.

Preferably abacavir is administered at a level of about 600 mg per day. Conveniently this can be as 300 mg twice daily.

Unless otherwise indicated all weights of active ingredients are calculated in terms of the drug per se. In the case of a pharmaceutically acceptable derivatives of lamivudine and abacavir, or a solvate of any thereof the figures would be increased proportionately. The desired dose is preferably presented as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing from 1 to 1500 mg, preferably from 5 to 1000 mg, most preferably from 5 to 500 mg of active ingredient per unit dosage form. A sub-dose of abacavir may be administered independently of a dose of a combination of the invention. Alternatively, if the condition of the recipient so requires, the dose may be administered as a continuous infusion.

The components of the combination which may be referred to as active ingredients may be administered for therapy to an animal e.g. a mammal including a human in a conventional manner.

While it is possible for the active ingredients of the combination to be administered as the raw chemical it is preferable to present them as a pharmaceutical composition. Pharmaceutical compositions according to the present invention comprise a combination according to the invention in association with one or more pharmaceutically acceptable carriers or excipients and optionally other therapeutic agents. The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formula and not deleterious to the recipient thereof. When the individual components of the

combination are administered separately they are generally each presented as a pharmaceutical composition. The references hereinafter to compositions refer unless otherwise stated to compositions containing either the combination or a component thereof.

A combination of lamivudine and abacavir or pharmaceutically acceptable derivatives thereof may conveniently be presented as a pharmaceutical composition with one or more pharmaceutically acceptable carrier thereof in a unitary dosage form. A convenient unitary dosage formulation contains the active ingredients in amounts of from 1 mg to 2 g each, for example, 2 mg to 200 mg such as 25 to 150 mg of lamivudine and 200 to 800 mg of abacavir.

Pharmaceutical compositions may also be prescribed to the patient in "patient packs" containing the whole course of treatment in a single package, usually a blister pack. Patient packs have an advantage over traditional prescriptions, where a pharmacists divides a patients supply of a pharmaceutical from a bulk supply, in that the patient always has access to the package insert contained in the patient pack, normally missing in traditional prescriptions. The inclusion of a package insert has been shown to improve patient compliance with the physicians instructions.

It will be understood that the administration of the combination of the invention by means of a single patient pack, or patients packs of each composition, including a package insert directing the patient to the correct use of the invention is a desirable additional feature of this invention.

According to a further aspect of the invention there is provided a patient pack comprising at least one active ingredient, of the combination according to the invention and an information insert containing directions on the use of the combination of the invention.

According to another aspect the invention provides a double pack comprising in association for separate administration lamivudine and abacavir or pharmaceutically acceptable derivatives thereof.

Compositions include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods represent a further feature of the present invention and include the step of bringing into association the active ingredients with the carrier which constitutes one or more accessory ingredients. 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.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, caplets, cachets or tablets each containing a predetermined amount of the active ingredients; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g. sodium starch glycollate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding a mixture of the powdered compound moistened with an inert liquid diluent in a suitable machine. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredients therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Preferably the combinations according to the invention are administered orally.

Compositions suitable for topical administration in the mouth include lozenges comprising the active ingredients in a flavoured base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier. Compositions for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Topical administration may also be by means of a transdermal iontophoretic device.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Pharmaceutical formulations suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art. The suppositories may be conveniently formed by admixture of the active combination with the softened or melted carrier(s) followed by chilling and shaping in moulds.

Formulations suitable for parenteral administration include aqueous and nonaqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents; and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and

suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or daily sub-dose of the active ingredients, as herein before recited, or an appropriate fraction thereof.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include such further agents as sweeteners, thickeners and flavouring agents.

The compounds of the combination of the present invention may be obtained in a conventional manner.

Methods for the preparation of lamivudine are described in International Patent Application Numbers WO91/17159, and WO 95/29174 incorporated herein by reference.

Abacavir may be prepared by the method described in European Specification EP 0434450, PCT application PCT/GB/4500225, PCT/GB95/02014, or U.S. Patent No. 5,034,394, which are incorporated herein by reference.

The following examples are intended for illustration only and are not intended to limit the scope of the invention in any way. "Active ingredient" denotes lamivudine or abacavir, multiples thereof or a physiologically functional derivative of any of the aforementioned compounds.

Example 1: Tablet Formulation

The following formulations A, B and C are prepared by wet granulation of the ingredients with a solution of povidone, followed by addition of magnesium stearate and compression.

Formulation A

	mg/tablet
Active Ingredient	250
Lactose B.P.	210
Povidone B.P.	15
Sodium Starch Glycollate	20
Magnesium Stearate	5
	500

Formulation B

	mg/tablet
Active Ingredient	250
Lactose B.P.	150
Avicel PH 101	60
Povidone B.P.	15
Sodium Starch Glycollate	20
Magnesium Stearate	5
	500

Formulation C

	mg/tablet
Active Ingredient	250

Lactose B.P.	200
Starch	50
Povidone	5
Magnesium Stearate	4
	359

The following formulations, D and E, are prepared by direct compression of the admixed ingredients. The lactose in formulation E is of the direct compression type (Dairy Crest-"Zeparox").

Formulation D

	mg/tablet
Active Ingredient	250
Pregelatinized Starch NF15	150
	400

Formulation E

	mg/tablet
Active Ingredient	250
Lactose B.P.	150
Avicel	100
	500

Formulation F (Controlled Release Formulation)

The formulation is prepared by wet granulation of the ingredients with a solution of povidone followed by the addition of magnesium stearate and compression.

	mg/tablet
Active Ingredient	500
Hydroxypropylmethylcellulose (Methocel K4M Premium)	112
Lactose B.P.	53
Povidone B.P.	28
Magnesium Stearate	7
	700

Drug release takes place over a period of about 6-8 hours and is complete after 12 hours.

Example 2: Capsule Formulations

Formulation A

A capsule formulation is prepared by admixing the ingredients of formulation D in Example 1 above and filling into a two-part hard gelatin capsule. Formulation B (infra) is prepared in a similar manner.

Formulation B

	mg/capsule
Active Ingredient	250
Lactose B.P.	143
Sodium Starch Glycollate	25
Magnesium Stearate	2

	420

Formulation C

	mg/capsule
Active Ingredient	250
Macrogel 4000 B.P.	350
	600

Capsules of formulation C are prepared by melting the Macrogel 4000 B.P., dispersing the active ingredient in the melt and filling the melt into a two-part hard gelatin capsule.

Formulation D

	mg/capsule
Active Ingredient	250
Lecithin	100
Arachis Oil	100
	450

Capsules of formulation D are prepared by dispersing the active ingredient in the lecithin and arachis oil and filling the dispersion into soft, elastic gelatin capsules.

Formulation E

	mg/capsule

Active Ingredient	150.0
Vitamin E TPGS	400.0
Polyethylene Glycol 400 NF	200.5
Propylene Glycol USP	39.5

Four (4) kilograms (kg) of Vitamin E TPGS (obtained from Eastman Chemical Co.) was heated at 50°C until liquefied. To the liquefied Vitamin E TPGS, 2.005 kg of polyethylene glycol 400 (PEG400) (low aldehyde, <10 ppm, obtained from Union Carbide or Dow Chemical Co.) heated to 50°C was added and mixed until a homogeneous solution was formed. The resultant solution was heated to 65°C. 1.5 kg of active ingredient was dissolved in the liquefied solution of Vitamin E TPGS and PEG 400. 0.395 kg of propylene glycol at room temperature was added and mixed until a homogenous solution was formed. The solution was cooled to 28-35°C. The solution was then de-gassed. The mixture was preferably encapsulated at 28-35°C at a fill weight equivalent to 150 mg of volatiles-free compound, into Size 12 oblong, white opaque soft gelatin capsules using a capsule filling machine. The capsule shells were dried to a constant fill moisture of 3-6% water and a shell hardness of 7-10 newtons, and placed in a suitable container.

Formulation F (Controlled Release Capsule)

The following controlled release capsule formulation is prepared by extruding ingredients a, b, and c using an extruder, followed by spheronization of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin capsule.

mg/capsule

(a)	Active Ingredient	250
(b)	Microcrystalline Cellulose	125
(c)	Lactose B.P.	125
(d)	Ethyl Cellulose	13
		513

Example 3: Injectable Formulation

Formulation A

	mg
Active Ingredient	200
Hydrochloric Acid Solution 0.1M or	4.0 to 7.0
Sodium Hydroxide Solution 0.1M q.s. to pH	
Sterile water q.s. to	10 ml

The active ingredient is dissolved in most of the water (35° - 40° C) and the pH adjusted to between 4.0 and 7.0 with the hydrochloric acid or the sodium hydroxide as appropriate. The batch is then made up to volume with water and filtered through a sterile micropore filter into a sterile 10 ml amber glass vial (type 1) and sealed with sterile closures and overseals.

Formulation B

Active Ingredient	125 mg
Sterile, Pyrogen-free, pH 7 Phosphate Buffer, q.s. to	25 ml

Example 4: Intramuscular Injection

Active Ingredient	200 mg
Benzyl Alcohol	0.10 g
Glycofurol 75	1.45 g
Water for injection q.s. to	3.00 ml

The active ingredient is dissolved in the glycofurol. The benzyl alcohol is then added and dissolved, and water added to 3 ml. The mixture is then filtered through a sterile micropore filter and sealed in sterile 3 ml amber glass vials (type 1).

Example 5: Syrup

Active Ingredient	250 mg
Sorbitol Solution	1.50 g
Glycerol	2.00 g
Sodium Benzoate	0.005 g
Flavor, Peach 17.42.3169	0.0125 ml
Purified Water q.s. to	5.00 ml

The active ingredient is dissolved in a mixture of the glycerol and most of the purified water. An aqueous solution of the sodium benzoate is then added to the solution, followed by addition of the sorbitol solution and finally the flavour. The volume is made up with purified water and mixed well.

Example 6: Suppository

mg/capsule suppository

Active Ingredient	250
Hard Fat, B.P. (Witepsol H15-Dynamit Nobel)	1770
	2020

One-fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45°C maximum. The active ingredient is sifted through a 200µm sieve and added to the molten base with mixing, using a Silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at 45° C, the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250µm stainless steel screen and, with continuous stirring, is allowed to cool to 45° C. At a temperature of 38° C to 40° C, 2.02 g of the mixture is filled into suitable, 2 ml plastic moulds. The suppositories are allowed to cool to room temperature.

Example 7: Pessaries

	mg/pessary
Active Ingredient	250
Anhydrate Dextrose	380
Potato Starch	363
Magnesium Stearate	7
	1000

The above ingredients are mixed directly and pessaries prepared by direct compression of the resulting mixture.

Biological Data

Example 8.

The human hepatoblastoma cell line (Hep-G2-2.2.15) which constitutively produces infectious HBV was seeded into 96 well microtiter plates at a density of 5 x 10^3 cells per well. These cells were treated with a combination of lamivudine and abacavir on triplicate plates. Culture media containing drugs was replenished every other day for 9 days, at which time supernatants were collected and analyzed for HBV content.

The lamivudine/abacavir combination was tested twice in triplicate in matrix fashion. Experiment 1 utilised a lamivudine range of 0.008 nM to 125 nM (5-fold dilutions in columns), and abacavir, at concentrations of 40 nM to 25 μM (5 fold dilutions in rows). Experiment 2 was performed with dilutions of lamivudine ranging from 0.14 nM to 100 nM (in 3-fold dilutions in columns), and a abacavir range of 250.7 nM to 25 μM (3.16 fold dilutions in rows). Experiment 3 was performed using dilutions of lamivudine ranging from 0.045 nM to 100 nM (in 3-fold dilutions and a abacavir range of 0.8 nM to 25 μM (3.16 fold dilutions in rows). Both drugs were diluted in a separate 96 well microtiter plate, and subsequently transferred onto plates containing the cell monolayers. Cells are grown in 150 μl RPMI 1640 supplemented with 2 mM L-Glutamine and 10% fetal bovine serum. Prior to transfer of drug, 120 μl of media was removed from the cells, leaving 30 μl on the monolayers to prevent drying. 90 μl of fresh media without drug was added, followed by the addition of 30 μl of 5X drug dilutions. Lamivudine and abacavir were each tested on their respective plates individually at the same concentrations. Data were normalised to values obtained with non-drug treated cells, and expressed as a percent of control for analysis.

The method used for detection of HBV has been previously described *(Jansen RW, Johnson LC, Averett, DR. High-Capacity in vitro assessment of anti-hepatitis B virus compound selectivity by a virion-specific polymerase chain reaction assay. Antimicrob Agents Chem 1993; 37 (3): 441-447.)*. Briefly, HBV detection was performed by "capturing" virus from supernatants on Anti-HBsAg coated plates, washing, denaturing to release HBV DNA, performing PCR with biotinylated primers, streptavidin capture of biotinylated PCR products with concomitant probe hybridization, addition of substrate, and reading optical densities of the colorimetric reaction. Dilutions of a standardized HBV-containing supernatant were included on every plate, and HBV DNA concentrations of test wells were calculated from this HBV standard curve. The useful range of detection is at least .045 to 45 fg of HBV DNA, where 30 copies of the genome can be reliably detected. Samples were tested in conjunction with both positive (.448 fg/ul plasmid DNA) and negative (RPMI medium supplemented with 2 mM L-Glutamine and 10% Fetal calf serum) controls.

The average IC50 and standard error of the IC50s for the triplicate plates were calculated using SAS nonlinear regression to fit data to the Hill equation for each concentration response curve. When only a single determination of an IC50 for a particular dose combination could be made, the average of the standard errors from adjacent concentrations was used to estimate the standard error. Fractional inhibitory concentrations (FIC50) were calculated for each combination and plotted using the isobologram representation (Berenbaum, M.C. (1985) The Expected Effect of a Combination of Agents: the General Solution. J. Theor. Biol. 114, 413-431). To assess statistical significance of synergy or antagonism, an unpaired t-test was used to compare each sum of paired FIC50 values with the theoretical value of 1. P values less than 0.05 were considered statistically significant. Comparison of P values between experiments must be interpreted with great care, as the experiments utilized

different test concentration ranges (or ranges useable by the isobologram method). In some cases not all concentrations tested could support calculation of an IC50, since response was inhibited to a greater extent than 50 percent of control for all doses.

Figure 1 shows a single isobologram, produced by combining the data from the three experiments, showing statistically significant synergism.

Example 9.

The K_i for the active form of abacavir, (-) carbovir triphosphate, (-)CBV-tp, was determined against wild type and YMDD variant HBV polymerases in an *in vitro* assay consisting of HBV core particles isolated from transiently transfected or transduced HepG2 cells. HepG2 cells were transiently transfected with plasmid containing the HBV genome that had the wild type sequence or contained the following amino acid changes in the reverse transcriptase gene; M552I, M552V, L528M, L528MM552V, L528MM552I (*Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DLJ, Brown N for the Lamivudine Clinical Investigation Group, and Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine). HEPATOLOGY 1998; 27:1670-1677*). Transient transductions were performed using baculovirus containing the same constructs *(Condreay JP, Witherspoon SM, Clay WC, Kost TA. (1999) Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. PNAS, 96, 127-132)*. The core polymerase assay was a modification of a previously reported protocol (*Davis MG, Wilson, JE, VanDraanen NA, Miller WH, Freeman GA, Daluge SM, Boyd FL, Aulabaugh, AE, Painter, GR, Boone LR. (1996) DNA polymerase activity of hepatitis B virus particles: differential inhibition by L-enantiomers of nucleotide analogs. Antiviral Res 30, 133-145)*. HBV core particles were isolated

in the following manner. Briefly, 5 days post-transfection or transduction, the cells were harvested and pelleted. The cell pellet was resuspended in a hypotonic solution and the cells lysed with NP-40 at a final concentration of 0.5%. The nuclei were pelleted and the cell supernatant was digested with RNase and DNase. The treated lysate was pelleted through a sucrose step gradient. The resulting pellet, which contained HBV cores, was resuspended by sonication in a buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM $MgCl_2$. This material was stored at –80° C until used in polymerase assays. Antibody-capture assay plates were prepared using the following protocol: Costar #3632(Corning, Corning, NY), were sequentially coated with 1) Neutravidin (Pierce, Rockford, IL) in 50 mM sodium carbonate buffer (Sigma, St. Louis, MO), pH 9.6, 0.10 mg/ml, 0.15 ml per well for 2 hours at room temperature, then washed with 3 x 0.25 ml/well with wash buffer ,WB,(150 mM NaCl, 100 mM Sodium Phosphate, pH 7.2, 0.02%Tween-20 (Pierce, Rockford, IL), 0.02% Sodium Azide, 0.01% Bovine Serum Albumin (Sigma, St. Louis, MO)): 2) Biotinylated goat anti-rabbit IgG (Pierce, Rockford, IL) diluted to 0.0015mg/ml in WB at 0.15 ml per well for 1 hour at room temperature, then washed 3 x 0.25 ml/well with WB; and 3) Rabbit anti-HBV core antigen antibody (Zymed, San Francisco, CA) diluted 1:300 in WB at 0.15 ml per well for 1 hour at room temperature. The plates were washed 2 x 0.25 ml/well with WB and WB at 0.25 ml per well added at the end of the wash. Plates were stored in a humidified chamber at 4° C. The core polymerase assay was a modification of a previously reported protocol (*Davis MG, Wilson, JE, VanDraanen NA, Miller WH, Freeman GA, Daluge SM, Boyd FL, Aulabaugh, AE, Painter, GR, Boone LR. (1996) DNA polymerase activity of hepatitis B virus particles: differential inhibition by L-enantiomers of nucleotide analogs. Antiviral Res 30, 133-145).* Core particle preparations were thawed on ice and resuspended by sonication prior to each assay. Core particle assays contained the following: Core particles (usually 10^7 to 10^8 / 10 ul reaction) in 230 mM KCl, 33 mM Tris-HCl, pH 7.4, 8

mM $MgCl_2$, 0.2 % NP-40, 5 mM DTT, 7 units/ml pyruvate kinase buffer (Sigma, St. Louis, MO), 2 mM phosphoenol pyruvate buffer (Sigma, St. Louis, MO), 25 mM unlabeled dNTP's (Amersham Pharmacia Biotech, Piscataway, NJ), ^{33}P labelled dNTP (NEN™ Life Science Products, Boston, MA, 2000 Ci/mmole) at 0 to 4 μM. Assays of 10 ul were incubated at 37° C for 45 to 90 minutes in a 96 well polypropylene plate (USA Scientific, Ocala, FL), then stopped with 90 ul of the following buffer: 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.05% Tween-20, 2 mM sodium pyrophosphate, 0.02% sodium azide. The entire 100 ul-stopped assay was transferred to a 96-well antibody capture plate and incubated at room temperature with shaking for two hours. After antibody capture, the wells were washed 5 times with the following buffer: 150 mM NaCl, 100 mM sodium phosphate, pH 7.2, 0.05% Tween-20, 2 mM sodium pyrophosphate, 0.02% sodium azide. Excess buffer was removed by tapping the plates on absorbent paper. 170 ul of Wallac Optiphase "Supermix" (Wallac Inc, Gaithersburg, MD) was added to each well. The plates were counted on a Wallac 1450 Microbeta Plus Liquid Scintillation Counter (Wallac Inc, Gaithersburg, MD). Inhibition assays were essentially the same as a core polymerase assays except that the concentration of $\alpha^{33}P$-dGTP was at K_m for each polymerase assayed. The concentration of (-)CBV-tp ranged from 0.001 μM to 312 mM. K_i's were calculated using the following formula: $K_i=IC_{50}/(1+[S]/K_m)$. The IC_{50} is the inhibitory concentration of compound at which polymerase activity is 50%. [S] is the concentration of competing substrate (for (-)CBV-tp it is ^{33}P labelled dGTP). All IC_{50}'s were determined at the K_m for dCTP for each polymerase. Concentration response curves generated were fit to the Hill equation y=(Vmax*x^n)/(K^n+x^n) using non-linear regression to estimate the IC_{50} of (-)CBV-tp. Calculations were performed using the RoboSage Add-in for Microsoft EXCEL.

Table 1 (-)CBV-tp: IC50, K_i, and Fold Change in K_i*

HBV pol	(-)CBV-tp IC_{50} (μM)	(-)CBV-tp K_i (μM)	(-)CBV-tp K_i Fold Change
wt	0.21	0.10	1
LMMV	2.69	1.34	13
MI	8.70	4.35	42
LMMI	2.54	1.27	12
LM	0.67	0.33	3
MV	4.64	2.32	22

*Average of two independent assays

LMMV- HBV genome with L528MM552V amino acid mutation.

MI - HBV genome with M552I amino acid mutation.

LMMI - HBV genome with L528MM552I amino acid mutation.

LM - HBV genome with L528M amino acid mutation.

MV - HBV genome with M552V amino acid mutation.

Claims

1. A combination comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one or a pharmaceutically acceptable derivative thereof and (-)-(1S, 4R) -4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol or a pharmaceutically acceptable derivative thereof .

2. A combination according to claim 1 wherein the compounds are present in a synergistic ratio.

3. A combination according to claim 2 wherein the ratio is in the range 1:0.5 to 1:10 by weight of active ingredients.

4. A combination according to claim 3 wherein the ratio of 1:1 to 1:8

5. A combination according to any one of claims 1 to 4 for use in medicine.

6. A pharmaceutical formulation comprising a combination according to any one of claims 1 to 4 in association with one or more pharmaceutically acceptable carriers therefor.

7. A formulation according to claim 6 in unit dosage form.

8. A formulation according to any one of claims 6 to 7 suitable for oral administration.

9. A formulation according to any one of claims 6 to 8 comprising between 25 to 150 mg of lamivudine and 200 to 800 mg abacavir.

10. A formulation according to claim 9 comprising 100 mg of lamivudine and 300 mg abacavir.

11. A method for the treatment of a mammal, including a human, with an HBV infection comprising administration of a therapeutically effective amount of a combination comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one or a pharmaceutically acceptable derivative thereof and a (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol or a pharmaceutically acceptable derivative thereof.

12. A method as claimed in claim 11 wherein the combination is as claimed in any of claims 1 to 4.

13. A method according to claim 11 or claim 12 wherein the combination is administered simultaneously.

14. A method according to claim 11 or claim 12 wherein the combination is administered sequentially.

15. A method according to claim 11 or claim 12 wherein the combination is administered as a single combined formulation.

16. A method as claimed in any one of claims 11 to 15 for the treatment of an HBV infection resistant to nucleoside and/or non-nucleoside inhibitors of the replication of the hepatitis B virus.

17. Use of (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one in the manufacture of a medicament for administration either simultaneously or sequentially with (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, for the treatment of an HBV infection.

18. Use of (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol in the manufacture of a medicament for administration either simultaneously or sequentially with (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one for the treatment of an HBV infection.

19. Use of a combination comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one or a pharmaceutically acceptable derivative

thereof and a second therapeutic agent (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol or a pharmaceutically acceptable derivative thereof for the treatment of an HBV infection.

20. Use of a combination as claimed in any one of claims 1 to 4 for the treatment of an HBV infection.

21. Use of a combination comprising (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one or a pharmaceutically acceptable derivative thereof and a second therapeutic agent (-)-(1S, 4R) -4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol or a pharmaceutically acceptable derivative thereof, for the treatment of an HBV infection resistant to nucleoside and/or nonnucleoside inhibitor.

22. Use of a combination as claimed in any one of claims 1 to 4 for the treatment of an HBV infection resistant to nucleoside and/or nonnucleoside inhibitor of the replication of the hepatitis B virus.

23. A patient pack comprising of at least one active ingredient selected from (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-pyrimidin-2-one, or (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol and an information insert containing directions on the use of both active ingredients together in combination.