

DESCRIPTION**TITLE OF INVENTION: OLIGONUCLEOTIDES**

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FIELD OF THE INVENTION

[0001] The invention generally relates to the field of RNA splicing. In particular, the invention relates to splice-switching oligonucleotides (SSOs) capable of altering the splicing of a pre-mRNA encoding a variant of the *OTC* gene. The invention also relates to the use of SSOs as therapeutic candidates for treating a urea cycle disorder.

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BACKGROUND OF THE INVENTION

[0002] The urea cycle functions primarily in the liver, where enzymes and transporters work in tandem to convert ammonia, a product of protein metabolism, into urea for removal in the urine. Loss-of-function variants in any component of the urea cycle, as is the case for urea cycle disorders (UCDs), leads to an accumulation of ammonia in blood, a condition known as hyperammonemia, which can cause brain dysfunction and its associated neurological sequelae, inducing developmental delay and intellectual disabilities and even death. UCDs include but not limited to the known eight disorders, N-acetylglutamate synthase (NAGS) deficiency, carbamoyl-phosphate synthase 1 (CPS1) deficiency, ornithine transcarbamylase (OTC) deficiency, argininosuccinate synthase (ASS1) deficiency (also known as citrullinemia type I), citrin deficiency (also called citrullinemia type II), argininosuccinate lyase (ASL) deficiency, arginase (ARG1) deficiency, ornithine translocase deficiency (also known as hyperornithinemia-hyperammonemia-homocitrullinuria or HHH Syndrome).

[0003] Current management of UCDs involves dietary protein restriction and pharmacological interventions using nitrogen scavenging drugs and some nutritional supplements. For instance, OTC deficiency may be managed by a low protein diet with arginine and citrulline supplementation. Despite the currently available treatment options, metabolic decompensations, which can lead to further neurological damages, can occur due to reasons such as intercurrent infection or difficulties in adhering to treatment. Thus far, no curative management for OTC deficiency exists except for liver transplantation. However, liver transplantation is a major operation that comes with risks of medical complications such as bleeding, infections, biliary complications, immune rejection and transplanted liver failure.

[0004] There is thus a need for a new therapeutic strategy that corrects the underlying pathogenic genetic variant of the *OTC* gene that overcomes the drawbacks of the prior art. Furthermore, other desirable features and characteristics will become apparent from the

subsequent detailed description and the appended claims, taken in conjunction with the accompanying drawings and this background of the disclosure.

SUMMARY

- 5 [0005] In one aspect, the present invention provides a splice-switching oligonucleotide (SSO) that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, the target region comprising the sequence of SEQ ID NO: 31, wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.
- 10 [0006] In one embodiment, OTC-PE5 comprises the sequence of SEQ ID NO: 33.
[0007] In one embodiment, the target region comprises the sequence selected from the group consisting of SEQ ID NOs 37 and 39.
[0008] In various embodiments, the SSO as described herein comprises a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.
- 15 [0009] In one embodiment the SSO as described herein is between 17 and 34 nucleotides in length.
[0010] In one embodiment, at least one of the nucleotides of the SSO as described herein is chemically modified and the chemical modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.
- 20 [0011] In one embodiment, each nucleotide of the SSO as described herein comprises either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.
[0012] In one embodiment, the SSO as described herein comprises phosphorothioate linkages between all nucleotides of the SSO.
- 25 [0013] In another aspect, there is provided an SSO as described herein for use in treating a urea cycle disorder.
[0014] In one embodiment, the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.
- 30 [0015] In another aspect, there is provided a use of an SSO as described herein in the manufacture of a medicament for treating a urea cycle disorder.
[0016] In one embodiment, the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency,
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ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.

[0017] In another aspect, there is provided a method of treating a urea cycle disorder comprising administering to a subject a composition comprising an SSO as described herein.

[0018] In one embodiment, the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.

[0019] In another aspect, there is provided a pharmaceutical composition comprising (a) a therapeutically effective amount of an SSO as described herein and (b) one or more pharmaceutically acceptable carriers and/or diluents.

[0020] In one aspect, there is provided a method of exon-skipping comprising providing an SSO that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, wherein the target region comprises the sequence of SEQ ID NO: 31 and wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.

[0021] In one embodiment, the pre-mRNA transcript comprises a c.540+265G>A mutation.

[0022] In one embodiment, OTC-PE5 comprises the sequence of SEQ ID NO: 33.

[0023] In one embodiment, the target region comprises the sequence selected from the group consisting of SEQ ID NOs 37 and 39.

[0024] In one embodiment, the method as described herein comprises providing an SSO having a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.

[0025] In one embodiment, the method as described herein comprises providing an SSO that is between 17 and 34 nucleotides in length.

[0026] In one embodiment, the method as described herein comprises providing an SSO with at least one of the nucleotides of the SSO being chemically modified and wherein the chemical modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.

[0027] In one embodiment, the method as described herein comprises providing an SSO with each nucleotide having either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.

[0028] In one embodiment, the method as described herein comprises providing an SSO comprising phosphorothioate linkages between all nucleotides of the SSO.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The invention will be better understood with reference to the detailed description
5 when considered in conjunction with the non-limiting examples and the accompanying
drawings, in which:

[0030] **Fig. 1** shows the minigene system used for screening of the SSOs. Fig. 1A is a
schematic of a minigene that harbours the specific genomic mutation, c.540+265G>A, of the
OTC gene. The minigene includes the full sequence of exon 4 plus an addition nucleotide G
10 at the 5' end of exon 4 to maintain the reading frame, the first 652 bases (from exon 4 donor
splice site) and last 1,443 bases of intron 4 (or 1,443 bases before exon 5 acceptor site), the
complete sequence of exon 5, intron 5 (which includes OTC-PE5), and exon 6. The relative
loci of the G-to-A substitution is demarcated in the figure. Top panel of Fig 1B is a gel
electrophoresis image showing PCR products from three cell lines (HEK293T, HepG2 and
15 Huh7) transfected with 500ng of either pOTC (WT minigene construct) or pOTCmut (mutant
minigene construct containing the c.540+265G>A mutation). The mutant minigene
recapitulates the retention of the pseudoexon OTC-PE5 in mature mRNA. All three cell lines
showed correct splicing of exon 5 to exon 6 when transfected with pOTC, and all three cell
lines show incorporation of the 135bp pseudoexon, OTC_PE5, when transfected with
20 pOTCmut. Bottom panel of Fig. 1B shows the Sanger sequencing results of PCR bands
extracted from the gel corresponding to amplicons of transcripts expressed from pOTCmut.

[0031] **Fig. 2** shows that SSOs are capable of modulating splice-out of OTC-PE5 to restore
the correct splicing. Fig. 2A is a capillary electrophoresis image from screening of SSOs
against OTC-PE5. Huh7 were co-transfected with 500ng of pOTCmut and 25nM of SSOs,
25 after which PCR was performed on complementary DNA (cDNA) generated from RNA
extracted from the transfected cells. The data reveals efficacy of the SSOs in mediating splice
exclusion of OTC-PE5. Every sugar moiety in a SSO is modified with 2'-O-methyl that is linked
via phosphorothioate backbone. Fig. 2B is a graph showing SSO concentration response
curves of SSOs from the screen in Fig. 2A. Huh7 were co-transfected with 500ng of pOTCmut
30 and SSOs at the indicated amounts, after which PCR was performed on complementary DNA
generated from RNA extracted from the transfected cells. Capillary electrophoresis was used
on PCR products to quantify the amount of products with the pseudoexon and without the
pseudoexon. Efficacy is reflected as the percentage of PCR products without the pseudoexon
over the total amount of PCR products (% splicing correction). Fig. 2C is a gel electrophoresis
35 image whereby SSOs #2018 and #2021 were transfected into iHeps containing

c.540+265G>A variant (HOM1) at 100nM. The data shows that the SSOs are able to rescue the splicing aberration of endogenous *OTC* transcripts in the iHeps (top panel) back to wild type size. CAPN10 (bottom panel) serves as loading control.

[0032] **Fig. 3.** is a graph showing splicing correction efficacy of shortened SSOs #2042, #2043 and #2044 when (A) transfected at 2nM for 24 hours or (B) treated by free uptake in calcium-enriched medium (CEM) at 20nM for 72 hours on Huh7 stably expressing the mutant minigene. The use of CEM stimulates *in vitro* uptake of SSOs by cells and better reflects the *in vivo* efficacy when compared to transfection. #2042 is shortened from #2021 while #2043 and #2044 are shortened from #2018. #2018 and #2021 serve as efficacy controls. Each SSO is modified with 2'-O-methyl linked via phosphorothioate backbone.

[0033] **Fig. 4** shows screening of shortened mixmers. Fig. 4A is a graph depicting transfection of mixmers into Huh7 or HepG2 cells stably expressing the mutant minigene at 2nM or 10nM, respectively. Fig. 4B shows free uptake treatment in calcium-enriched medium (CEM) of Huh7 cells stably expressing the mutant minigene at 5nM of SSOs. The ribose in the mixmer SSOs (#2044.1 - #2044.22) are either modified with 2'-O-methyl or are substituted with locked nucleic acids, and are each linked via phosphorothioate backbone. The data shows that the shortened mixmers were able to consistently modulate splice-out of OTC-PE5. #2018 and #2044, both of which are entirely modified with 2'-O-methyl, serve as efficacy controls. Fig. 4C shows the dose response curves of the best-performing mixmer SSOs #2044.7 and #2044.18 when Huh7 cells stably expressing the mutant minigene are treated with different concentrations of the SSOs in CEM. EC₅₀ for each mixmer was subsequently inferred from the respective dose response curves.

[0034] **Fig. 5** shows *in vitro* characterization of #2044.7 and #2044.18. Fig. 5A is a graph showing MTT assay whereby Huh7 cells stably expressing the mutant minigene were treated by free uptake with different concentrations of SSOs for 72 hours. C2044, #2044.7 and C2044.18 are control sequences complementary to #2044, #2044.7 and #2044.18, with the position of the LNAs in C2044.7 and C2044.18 the same as that for #2044.7 and #2044.18, respectively. 2OM refers to 2'-O-methyl modifications, 2MOE refers to 2'-O-methoxyethyl modifications, while 2OML and 2MOL accordingly refer to 2OM and 2MOE with LNA (mixmer SSOs). The data suggests a lack of toxicity caused by the sequence and chemistry combinations for the mixmers. LNA32 and LNA41 are gapmers containing LNA in the flanking sequences previously shown to be respectively non-toxic and acutely hepatotoxic *in vivo* in mouse studies and correspondingly serve as negative and positive controls. Fig. 5B is a graph showing an immunogenic assay whereby BJAB cells were incubated with 2μM of SSOs for 24 hours, after which cells were harvested to check for markers of immunogenic response

CCL22, *FCRL3* and *TNF*. ODN2216 is a CpG-rich TLR9 agonist that serves as a positive control, while ISIS 104838 is a 2MOE-based gapmer previously shown to be minimally immunogenic *in vivo* and serves as a negative control.

[0035] **Fig. 6** shows the relative position of exemplary SSOs along the OTC-PE5 (indicated here as Exon 5*). SSO #2018, 2019, 2021, 2042, 2043 and 2044 lie entirely within OTC-PE5. SSO #2020 overlaps with the donor splice site of OTC-PE5. Figure is not drawn to scale.

[0036] **Fig. 7** shows the transgenic mouse model generated to carry the *OTC* c.540+265G>A mutation. The transgene, which called *PE5* and depicted in Fig. 7A, is an expression construct containing the complementary DNA sequence of human *OTC*, with parts of intron 4 and the whole of intron 5, including the c.540+265G>A variant, interspersed between the relevant exons. It was inserted into the *Rosa26* locus by CRISPR to create the transgenic strain, which was then crossed with a naturally occurring *Otc*-deficient mouse model, the *spf-ash* mice (doi: 10.1073/pnas.86.11.4142). Expression of OTC-PE5 containing transcript was confirmed in mice heterozygous for the *PE5* transgene in the *spf-ash* background with primers specific to human *OTC* (*PE5* Tg/+ *spf-ash*; Fig. 7B). there is no leaky No leaky expression of functional human OTC from the transgene was detected in *PE5* Tg/+ *spf-ash* mice (Fig. 7C), confirming that the *spf-ash* mice are indeed *Otc* deficient. Primary hepatocytes were isolated from the liver of mice homozygous for the *PE5* transgene under a wild-type (WT) non-*spf-ash* background. They were treated with N-acetylgalactosamine (GalNAc)-conjugated #2044.7 (GN-2044.7), resulting in the skipping of OTC-PE5 in human OTC transcripts (Fig. 7D) that translates to expression of functional OTC protein (Fig. 7E), and the subsequent rescue in the ability of *spf-ash* hepatocytes to produce urea (Fig. 7F) and clear ammonia (Fig. 7G).

[0037] **Fig. 8** shows further screening of 2OML SSOs with the 2044 sequence and with LNA positions designed as per good patterns for enhanced efficacy (SG Patent Application No. 10202403247X). The new SSOs were benchmarked to 2044.7 2OML, which was the best candidate in the earlier screen, upon CEM-mediated free uptake in HuH7 cells stably expressing minigene (Fig. 8A), and 2044.401 and 2044.501 2OML were identified as performing better than 2044.7 2OML. 2044.401 and 2044.501 showed no toxicity when tested by MTS assay (Fig. 8B) nor immunogenicity when tested by BJAB assay (Fig. 8C).

[0038] **Fig. 9** shows that GalNAc-conjugated 2044.401 (GN-2044.401) is able to rescue the OTC deficiency phenotype in a mouse model of OTC deficiency that is responsive to SSO (due to the *PE5* transgene). *Spf-ash* mice that were homozygous for the *PE5* transgene (*PE5* Tg/Tg *spf-ash*) were injected at 5 mg/kg (5mpk) or 25mg/kg (25mpk) once a week for four weeks, after which they were fed with high protein diet (51% calorie content from protein) to

exacerbate phenotype in the mice (Fig. 9A). Notably, there was a reduction in survival of mice injected with vehicle control (Veh) or GalNAc-conjugated non-targeting control (GN-NTC), while mice injected with either 5mpk or 25mpk of GN-2044.401 survived completely till the end of study (Fig. 9B). This corroborated with the extensive reduction in body weight of the *spf-*
 5 *ash* mice, which is commonly used as a read-out of health status, upon feeding of high protein diet, while mice injected with GN-2044.401 had a significantly milder reduction in body weight similar to that of WT mice (Fig. 9C-D). Hyperammonemia is a hallmark of OTC deficiency and other urea cycle disorders, and this leads to neurodegenerative symptoms that can manifest as motor deficits. Indeed, using a neuromotor pathology scoring chart developed by the ALS
 10 Therapeutic Development Institute, whereby higher scores indicate more severe neuromotor deficit, mice injected with Veh or GN-NTC had overall higher scores compared to those injected with GN-2044.401, which were scored at 0, indicating the absence of neuromotor pathology (Fig. 9E). This was further reflected by the reduction in plasma ammonia upon GN-2044.401 treatment both pre- and post-high protein diet feeding (Fig. 9F) and the increase in
 15 plasma urea (Fig. 9G), which taken together, suggests a recovery of the urea cycle in these mice.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0039] In one aspect, the present invention provides a splice-switching oligonucleotide
 20 (SSO) that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, the target region comprising the sequence of SEQ ID NO: 31, wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.

[0040] Alternatively, the target region comprises the sequence of SEQ ID NO: 35, spanning
 25 from part of intron 4 to part of intron 5.

[0041] By "oligonucleotide", it is meant to refer to any polynucleotide. A "polynucleotide" is an oligomer comprised of nucleotides. A polynucleotide may be comprised of DNA, RNA modified forms thereof, or a combination thereof. The term "nucleotide" or its plural as used
 30 herein is interchangeable with modified forms as discussed herein and otherwise known in the art. In certain instances, the art uses the term "nucleobase" which embraces naturally occurring nucleotides as well as modifications of nucleotides that can be polymerized. Thus, nucleotide or nucleobase means the naturally occurring nucleobases adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U) as well as non-naturally occurring nucleobases such as xanthine, diaminopurine, 8-oxo-N6-methyladenine, 7-deazaxanthine, 7-
 35 deazaguanine, N4,N4-ethanocytosin, N',N'-ethano-2,6- diaminopurine, 5-methylcytosine

(mC), 5-(C[3]- C6)-alkynyl-cytosine, 5-fluorouracil, 5- bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanine, inosine and non-naturally occurring nucleobases. The term "nucleobase" also includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. In various
 5 embodiments, polynucleotides also include one or more "nucleosidic bases" or "base units" which include compounds such as heterocyclic compounds that can serve like nucleobases, including certain "universal bases" that are not nucleosidic bases in the most classical sense but serve as nucleosidic bases. Universal bases include 3-nitropyrrole, optionally substituted indoles (e.g., 5-nitroindole), and optionally substituted hypoxanthine. Other desirable universal
 10 bases include pyrrole, and diazole or triazole derivatives, including those universal bases known in the art.

[0042] Polynucleotides may also include modified nucleobases. A "modified base" is understood in the art to be one that can pair with a natural base (e.g., adenine, guanine, cytosine, uracil, and/or thymine) and/or can pair with a non-naturally occurring base.
 15 Exemplary modified bases are described in EP 1 072 679 and WO 97/12896, the disclosures of which are incorporated herein by reference. Modified nucleobases include, without limitation, 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,
 20 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8- thioalkyl, 8-hydroxyl and 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, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.
 25 Further modified bases include tricyclic pyrimidines such as phenoxazine cytidine(IH-pyrimido[5,4-b] [1,4]benzoxazin-2(3H)- one), phenothiazine cytidine (IH-pyrimido[5,4-b] [1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b] [1,4]benzox- azin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridindole cytidine (H- pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified bases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Certain of these bases are useful for increasing the binding affinity of the polynucleotide and include 5-substituted pyrimidines, 6- azapyrimidines and N-
 30 2, N-6 and O-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.deg.C and are, in certain embodiments, combined with 2'-O-methoxyethyl sugar modifications. See, U. S. Pat. Nos. 3,687,808, U. S. Pat. Nos. 4,845,205; 5,130,302; 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; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,750,692 and 5,681,941, the disclosures of which are incorporated herein by reference.

[0043] As used herein, the term "splice switching oligonucleotides" (SSOs) or "splice switching oligomers" is meant to include synthetic antisense nucleic acids that base-pair with a pre-mRNA and disrupt the splicing process by sterically blocking the RNA-RNA base-pairing or protein-RNA binding interactions that occur between components of the splicing machinery and the pre-mRNA. The SSOs may also be known as "antisense nucleotides". In some embodiments, SSOs may be mixmers. The term "mixmer" includes an oligomer on which different types of chemical modifications are applied on its sugar moieties, or on its backbone linkages, or both. Examples of chemical modifications include phosphorothioate linkages, 2'-O-methyl (2OMe) RNA modifications, 2'-O-methoxyethyl (2MOE) RNA modifications and locked nucleic acid (LNA) substitutions. The terms "phosphorothioate bond" and "phosphorothioate linkage" are used interchangeably. The term "locked nucleic acid" (LNA) generally refers to a modified RNA nucleotide where the ribose ring is "locked" with a methylene bridge connecting the 2'-O atom with the 4'-C atom. Advantageously, the chemical modifications may increase the efficacy, selectivity and stability while manifesting superior toxicity profile of SSOs. In particular, the chemical modifications may result in increased target binding affinity and improved pharmacological properties.

[0044] In various embodiments, the modified polynucleotide backbone comprises at least one modified internucleotide linkage. The modified internucleotide linkage comprises a modified phosphate. More preferably, the modified phosphate is any one selected from the group comprising of a non-bridging oxygen atom substituting a sulfur atom, a phosphonate, a phosphorothioate, a phosphodiester, a phosphoromorpholidate, a phosphoropiperazide and a phosphoramidate. In various embodiments, the SSO comprises a backbone selected from the group comprising of ribonucleic acid, deoxyribonucleic acid, DNA phosphorothioate, RNA phosphorothioate, 2'-O-methyl-oligoribonucleotide and 2'-O-methyl-oligodeoxyribonucleotide, 2'-O-hydrocarbyl ribonucleic acid, 2'-O-hydrocarbyl DNA, 2'-O-hydrocarbyl RNA phosphorothioate, 2'-O-hydrocarbyl DNA phosphorothioate, 2'-F-phosphorothioate, 2'-F-phosphodiester, 2'-methoxyethyl phosphorothioate, 2-methoxyethyl phosphodiester, deoxy methylene(methylimino) (deoxy MMI), 2'-O-hydrocarby MMI, deoxy-methylphosphonate, 2'-

- O-hydrocarbyl methylphosphonate, morpholino, 4'-thio DNA, 4'-thio RNA, peptide nucleic acid, 3'-amidate, deoxy 3'-amidate, 2'-O- hydrocarbyl 3'-amidate, locked nucleic acid, cyclohexane nucleic acid, tricycle-DNA, 2'fluoro-arabino nucleic acid, N3'-P5' phosphoroamidate, carbamate linked, phosphotriester linked, a nylon backbone modification and mixtures of the aforementioned backbones.
- [0045] In various embodiments, the oligonucleotide is chemically linked to one or more conjugates that enhance the activity, cellular distribution, or cellular uptake of the SSO.
- [0046] The term "splicing" refers to an RNA processing mechanism in which a pre-mRNA is made into a mature mRNA. During splicing, introns are removed and exons are connected.
- 10 Splicing is catalysed by the spliceosome complex. As used herein, the term "alternative splicing" is meant to include a process by which a gene can encode for multiple mRNA and protein products by differentially selecting which exons are to be included in a mature mRNA transcript. For example, alternative splicing can take the form of one or more skipped exons, variable position of intron splicing, or intron retention.
- 15 [0047] As used herein, the term "intron" refers to a segment of non-coding nucleic acid sequence that is transcribed and is present in the pre-mRNA but is excised by the splicing machinery and therefore not present in the mature mRNA transcript.
- [0048] As used herein, the term "exon" refers to a segment of a nucleic acid sequence that is transcribed into mRNA and that is present in mature mRNA after splicing. The term "exon
- 20 skipping" is meant to include the process by which an entire exon, or a portion thereof, is removed from a given pre-mRNA and is thereby excluded from being present in the mature mRNA. For example, the portion of the protein that is otherwise encoded by the skipped exon is not present in the expressed form of the protein.
- [0049] The term "pseudoexon" refers to a potential exon, with defined 5' and 3' splice sites, that is not normally spliced into mature mRNA by the splicing machinery. The inclusion of a
- 25 pseudoexon in a mature mRNA, for example due to a mutation that either creates/activates or eliminates/diminishes a splicing motif or splice site, or dysregulation of the splicing machinery stemming from the absence or overproduction of one or more components of the spliceosome complex or specific RNA binding protein(s) acting as splicing enhancers or splicing silencers,
- 30 may cause a shift in the codon reading frame, an in-frame premature stop codon, or addition of novel amino acid residues, resulting in a loss of expression/function of the protein. For clarity, the genetic mutation(s) that effects the creation of a pseudoexon need not reside within the pseudoexon.
- [0050] In one embodiment, OTC-PE5 comprises the sequence of SEQ ID NO: 33. The
- 35 terms "OTC-PE5", "PE" and "Exon 5*" may be used interchangeably. In one embodiment,

OTC-PE5 (Chr X: 38401695-38401829 (hg38) with reference to NM_000531.6) refers to the pseudoexon caused by the c.540+265G>A mutation.

[0051] As used herein, the term "splice site" is meant to include specific nucleic acid sequences that can be recognized by the splicing machinery as being suitable for excision and/or ligation with the corresponding splice site. The splice site defines the precise exon-intron boundary that allows the excision of introns present in pre-mRNA transcripts. As used herein, the term "5' splice site" (also known as donor splice site) refers to a nucleic acid sequence surrounding the exon-intron boundary at the 5' end of an intron that marks the start of the intron and its boundary with the preceding exon sequence. The term "3' splice site" (also known as acceptor splice site) as used herein refers to a nucleic acid sequence surrounding the intron-exon boundary at the 3' end of an intron that marks the end of the intron and its boundary with the following exon sequence.

[0052] As used herein, the term "pre-mRNA" or "precursor mRNA" refers to a strand of messenger ribonucleic acid (mRNA), synthesized from a DNA template by transcription, prior to processing events such as splicing. Pre-mRNA is composed of exons, introns and untranslated sequences (before the first and after the last exons respectively). Generally, eukaryotic pre-mRNA exists only briefly before it is fully processed into mature mRNA.

[0053] The term "binding" as used in the context of an SSO is meant to include the hybridization of the SSO to a site within a target region on a pre-mRNA transcript. The term "hybridize" or "hybridization" may include the binding of a single-stranded nucleic acid or a locally single-stranded region of a double-stranded nucleic acid to another single-stranded nucleic acid or a locally single-stranded region of a double-stranded nucleic acid having a complementary sequence through the pairing of complementary nucleic acids. It is generally known to a person skilled in the art that binding or hybridization of one sequence to another does not require total complementarity of the sequences. For example, the sequence of the SSO may be fully complementary or partially complementary to the target region to which it binds.

[0054] The term "site" refers to a location that the SSO binds to, i.e. the binding site of the SSO. In the context of "A SSO that specifically binds to a site within a target region on a pre-mRNA transcript", the binding site of the SSO lies within the target region, and the target region forms a part of the pre-mRNA transcript. The binding sites of the SSOs of the present disclosure reside within the target region on the pre-mRNA transcript of the *OTC* gene.

[0055] By "variant" in the relevant gene, it is meant to include any variation or alteration in the sequences of said gene, such that the sequence differs from what is found naturally or in most people. Similarly, a "non-variant" may include any sequence of the gene that may be

- considered “wild-type”, i.e. a sequence that is deemed normal or typical for said gene. As such, a “variant” of the gene means any one or more alteration(s), i.e. a substitution, insertion, and/or deletion, at one or more (several) positions, of the polynucleotide of the gene. A substitution may include a replacement of one or more nucleotide(s) occupying a position with one or more different nucleotide(s); a deletion means removal of one or more nucleotide(s) occupying a position; and an insertion means adding one or more nucleotide(s) immediately adjacent to a nucleotide occupying a position. The term “variant” may also refer to any variation or alteration in the sequence of a gene that results in the loss of wild-type protein expression and/or function.
- 5 [0056] The pre-mRNA transcript of the present embodiments may be a pre-mRNA transcript of a variant of the *OTC* gene which comprises the c.540+265G>A mutation. The c.540+265G>A mutation (NC_000023.11:g.38401693G>A (GRCh38)) is a single DNA base mutation that, on the pre-mRNA transcript, causes retention of an out-of-frame exon, OTC-PE5. An “out-of-frame” exon is meant to refer to a pseudoexon which, when included in the mature mRNA, disrupts the reading frame and results in an altered amino acid sequence in the resulting protein. The binding of the SSOs as described herein to the pre-mRNA of the variant *OTC* gene induces the exclusion of OTC-PE5, thereby restoring the full reading frame of the mature mRNA of the variant *OTC* gene.
- 10 [0057] In various embodiments, the SSO as described herein comprises a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.
- 15 [0058] In one embodiment the SSO as described herein is between 17 and 34 nucleotides in length.
- [0059] In one embodiment, at least one of the nucleotides of the SSO as described herein is chemically modified and the chemical modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.
- 20 [0060] In one embodiment, each nucleotide of the SSO as described herein comprises either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.
- [0061] In one embodiment, the SSO as described herein comprises phosphorothioate linkages between all nucleotides of the SSO. In other words, all inter-nucleotide linkages of the SSOs are phosphorothioate linkages.
- 25 [0062] In another aspect, there is provided an SSO as described herein for use in treating a urea cycle disorder. As used herein, the term “treat” or “treating” in the context of treating a disease such as a urea cycle disorder is meant to include improving clinical outcomes of
- 30

patients having the disease. This includes improving the survival rates of patients having the disease. The term "treat" or "treatment" may refer to prophylactic and/or therapeutic treatment.

[0063] Urea cycle disorders can be caused by loss-of-function mutations in any one of the eight genes involved in the urea cycle. The eight genes refer to carbamoylphosphate synthase I (CPS1), ornithine transcarbamylase (OTC), argininosuccinate synthase (ASS1),
5 argininosuccinate lyase (ASL), arginase (ARG1), N-acetyl glutamate synthase (NAGS), ornithine translocase (ORNT1) and citrin (SLC25A13). Accumulation of ammonia can cause brain damage, learning disability and motor deficits, seizures, coma and death. There are limited treatment options for urea cycle disorders. While ammonia scavenger therapy is
10 available, this is prescribed together with dietary restriction and involves strict dosage regimes requiring multiple administrations daily. This has led to compliance issues with 75% of patients and caregivers reporting missing out on doses, thereby risking acute symptoms. While AAV delivery of OTC mRNA to the liver is in Phase 3 clinical trials, this treatment is limited to patients who are 12 years old and above. However, OTC deficiency typically manifests with
15 severe symptoms week to months after birth.

[0064] The SSOs of the present embodiments may be used in compositions that can be used for treatment, e.g. as a pharmaceutical composition comprising the SSOs of the present
embodiments and pharmaceutically acceptable carrier. The carrier is selected from the group
20 consisting of a nanoparticle, such as a polymeric nanoparticle; a liposome, such as pH-sensitive liposome, an antibody conjugated liposome; a viral vector, a cationic lipid, a polymer, and a cell penetrating peptide.

[0065] In one embodiment, the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin
25 deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.

[0066] In another aspect, there is provided a use of an SSO as described herein in the manufacture of a medicament for treating a urea cycle disorder.

[0067] In one embodiment, the urea cycle disorder is selected from the group consisting
30 of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.

[0068] In another aspect, there is provided a method of treating a urea cycle disorder
35 comprising administering to a subject a composition comprising an SSO as described herein.

[0069] In one embodiment, the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase
5 deficiency.

[0070] In another aspect, there is provided a pharmaceutical composition comprising (a) a therapeutically effective amount of an SSO as described herein and (b) one or more pharmaceutically acceptable carriers and/or diluents.

[0071] The term “therapeutically effective amount” refers to the amount of the SSO as
10 described herein that is required to confer the intended therapeutic effect in the subject, which amount will vary depending on the route of administration, status of disease, and possible inclusion of other therapeutics or excipients. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. A therapeutically effective amount may vary according to factors such as
15 the disease state, age, sex, and weight of the individual, and the ability of the therapeutic agent to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the SSO are outweighed by the therapeutically beneficial effects.

[0072] A “therapeutically effective amount” for therapy may also be measured by its ability
20 to stabilize the progression of disease. A therapeutically effective amount of a therapeutic agent may reduce or ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject’s size, the severity of the subject’s symptoms, and the particular composition or route of administration selected.

[0073] In the methods of the invention, therapy is used to provide a positive therapeutic
25 response with respect to a disease or condition. The term “positive therapeutic response” is intended to include an improvement in the disease or condition, and/or an improvement in the symptoms associated with the disease or condition, and/or prevent the worsening of symptoms associated with the disease or condition. Positive therapeutic responses in any given disease or condition can be determined by standardized response criteria specific to
30 that disease or condition. In addition to these positive therapeutic responses, the subject undergoing therapy may experience the beneficial effect of an improvement in the symptoms associated with the disease.

[0074] As used herein, the term “pharmaceutical composition” is meant to include any
35 pharmaceutical preparation or formulation which is suitable for administration to a subject in need thereof. The composition may be suitable for parenteral administration either naked or

complexed with a delivery agent to a patient. The carrier is selected from the group consisting of a nanoparticle, such as a polymeric nanoparticle; a liposome, such as pH-sensitive liposome, an antibody conjugated liposome; a viral vector, a cationic lipid, a polymer, and a cell penetrating peptide. It will be appreciated that pharmaceutical compositions provided

5 according to the disclosure may be administered by any means known in the art. The pharmaceutical composition may be administered orally, or rectal, or transmucosal, or intestinal, or intramuscular, or subcutaneous, or intramedullary, or intrathecal, or direct intraventricular, or intravenous, or intravitreal, or intraperitoneal, or intranasal, or intraocular.

[0075] A pharmaceutically acceptable carrier refers, generally, to materials that are

10 suitable for administration to a subject wherein the carrier is not biologically harmful, or otherwise, causes undesirable effects. Such carriers are typically inert ingredients of a medicament. Typically a carrier is administered to a subject along with an active ingredient without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of a pharmaceutical composition in which it is contained.

15 [0076] The pharmaceutical compositions or formulations of the disclosure, which may conveniently be presented in unit dosage form, may 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 bringing into association

20 the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0077] Combination therapy with an additional therapeutic agent may also contemplated by the disclosure. The term "combination" or "combination therapy" as used throughout the specification, is meant to encompass the administration of the referred therapeutic agents to

25 a subject suffering from a disease, disorder or pathological condition, in the same or separate pharmaceutical formulations, and at the same time or at different times. If the therapeutic agents are administered at different times, they should be administered sufficiently close in time to provide for the potentiating or synergistic response to occur. In such instances, it is contemplated that one would typically administer both therapeutic agents within about 12-24

30 hours of each other and, more preferably, within about 6-12 hours of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. In other situations, it might be desirable to reduce the time between administration, administering both therapeutic agents within seconds or minutes to

hours, preferably within about 6 hours from each other, more preferably within about 1 or 3 hours.

[0078] To practice the methods of this invention, the SSO as described herein may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally or via an implanted reservoir. The term "parenteral" as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, and intracranial injection or infusion techniques. A sterile injectable composition, e.g., a sterile injectable aqueous or oleaginous suspension, can be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as Tween 80) and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluents or solvent for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are mannitol, water, Ringer's Solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium (eg. Synthetic mono-or dyglycerides). Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions can also contain a long-chain alcohol diluents or dispersant, or carboxymethyl cellulose or similar dispersing agents. Other commonly used surfactants such as Tweens or Spans or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms can also be used for the purposes of formulation.

[0079] A composition for oral administration can be any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers that are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavouring, or colouring agents can be added. A nasal aerosol or inhalation composition can be prepared according to techniques well known in the art of pharmaceutical formulation. A SSO-containing composition can also be administered in the form of suppositories for rectal administration. The carrier in the pharmaceutical composition must be "acceptable" in the sense of being compatible with the

active ingredient of the formulation (and preferable, capable of stabilising it) and not deleterious to the subject to be treated. For example, one or more solubilising agents, which form more soluble complexes with the SSOs, or more solubilising agents, can be utilised as pharmaceutical carriers for delivery of the active compounds. Examples of other carriers
5 include colloidal silicon dioxide, magnesium stearate, sodium lauryl sulphate, and D&C Yellow #10.

[0080] In one aspect, there is provided a method of exon-skipping comprising providing an SSO that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, wherein the target region comprises the sequence of SEQ ID
10 NO: 31 and wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.

[0081] In one embodiment, the pre-mRNA transcript comprises a c.540+265G>A mutation.

[0082] In one embodiment, OTC-PE5 comprises the sequence of SEQ ID NO: 33.

[0083] In one embodiment, the method as described herein comprises providing an SSO
15 having a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.

[0084] In one embodiment, the method as described herein comprises providing an SSO that is between 17 and 34 nucleotides in length.

[0085] In one embodiment, the method as described herein comprises providing an SSO with at least one of the nucleotides of the SSO being chemically modified and wherein the
20 chemical modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.

[0086] In one embodiment, the method as described herein comprises providing an SSO with each nucleotide having either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.

25 [0087] In one embodiment, the method as described herein comprises providing an SSO comprising phosphorothioate linkages between all nucleotides of the SSO.

[0088] It should be noted that not all regions capable of being bound by SSOs can produce the desired effect of correcting the mis-splicing of OTC pre-mRNA by excluding OTC-PE5 from the mature mRNA. In particular, precise removal of OTC-PE5 is tricky because
30 c.540_265G>A occurs relatively near to the endogenous Exon 5's donor splice site and because the OTC-PE5 acceptor splice site is also relatively near to the endogenous Exon 5's donor splice site. Therefore, an attempt to remove OTC-PE5 may result in multiple undesirable possibilities such as (1) both Exon 5 and PE5 being removed in tandem; (2) the intronic sequence between Exon 5 and PE5 being retained, resulting in a new exon spanning from
35 Exon 5 to PE5; or (3) activation of other cryptic splice sites within the intronic sequence

between Exon 5 and PE5, where the use of any one of the cryptic splice sites will lead to changes in the donor site of Exon 5 and/or acceptor site of PE5. Advantageously, the SSOs of the present embodiments are able to achieve complete exclusion of OTC-PE5 without the undesirable embodiments mentioned above.

5 [0089] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the
10 use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be
15 resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0090] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or
20 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0091] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of
25 any individual member or subgroup of members of the Markush group.

[0092] **MATERIAL AND METHODS**

[0093] Cell culture

30 [0094] HEK293T, Huh7 and HepG2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose supplemented with 10% fetal bovine serum while BJAB cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal bovine serum. Cells were kept in a cell culture incubator set to 37°C and 5% carbon dioxide. Embryonic stem cells (ES cells) were maintained on ES cell-qualified Matrigel
35 (Corning) in mTeSR Plus medium (STEMCELL Technologies). For hepatocyte differentiation into induced hepatocytes (iHeps), ES cells seeded at 50% confluency were induced in

- definitive endoderm medium 1 (RPMI 1640 medium supplemented with B-27 supplement, 100ng/mL Activin A and 3uM CHIR99021) for two days, definitive endoderm medium 2 (RPMI 1640 medium supplemented with B-27 supplement) for three days, hepatic endoderm medium (definitive endoderm medium 2 with 20ng/mL BMP4 and 10ng/mL FGF2) for five days, and
- 5 Lonza Hepatocyte Culture Medium for fourteen days. CRISPR knock-in of the c.540+265G>A variant into ES cells was outsourced to the Duke-NUS Stem Cell and Gene Editing (SCAGE) Core Facility.
- [0095] Co-transfection of 500ng of minigene plasmid and differing amounts of SSOs into cells were performed using Lipofectamine 3000 reagent (Invitrogen) according to
- 10 manufacturer's instructions using Opti-MEM (Thermo Fisher). Cells were collected 24 hours post-transfection for RNA analysis.
- [0096] To establish Huh7 and HepG2 cells stably expressing the minigene, the minigene was sub-cloned into a Piggybac-based transfer vector and co-transfected with a Piggybac transposon-expressing plasmid at a 5:1 ratio for a total of 500ng using Lipofectamine 3000 as
- 15 above, after which cells were selected with puromycin at 1µg/mL for 2 weeks.
- [0097] Free uptake of SSOs by Huh7 was performed by treating cells with SSOs in calcium-enriched medium (cell culture media as above supplemented with 9mM calcium chloride). Cells were collected 72 hours post-treatment for RNA analysis.
- [0098] Splicing assay
- 20 [0099] RNA was extracted from cells using TRIzol Reagent (Invitrogen) and messenger RNA (mRNA) was converted to complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher). PCR was performed on cDNA and PCR products were separated using standard gel electrophoresis on agarose gel for qualitative analysis or using capillary electrophoresis on the Qsep100 Bio-Fragment Analyzer (BioOptic Inc) for quantitative
- 25 analysis. To analyze sequence of PCR products, specific PCR bands were excised and extracted from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) and Sanger sequencing was outsourced to Macrogen Inc. Primers used for PCR of minigene transcripts are: forward 5'-AAGGACGACGATGACAAG-3' (SEQ ID NO: 40), reverse 5'-ATCATGATGGAGTGCAGGAT-3' (SEQ ID NO: 41). Primers used for PCR of endogenous
- 30 *OTC* transcripts are: forward 5'-CGGCCCGTGTATTGTCTAGC-3' (SEQ ID NO: 42), reverse is same as for minigene (SEQ ID NO: 41).
- [00100] MTT assay
- [00101] Huh7 cells stably expressing the mutant minigene were treated with SSOs at 10nM, 50nM or 100nM by free uptake in calcium-enriched medium for 72 hours. MTT assay was then
- 35 performed using the MTT Assay Kit (Abcam) as per manufacturer's instructions. In brief, cells

were treated with MTT solution for 3 hours at 37°C, after which MTT solvent was added to the cells to release and dissolve the reduced formazan crystals. Signals were then measured using Tecan Spark 10M plate reader.

[00102] BJAB assay and quantitative real-time PCR (qPCR)

- 5 [00103] 0.5 million BJAB cells were seeded in 50µL of media per well of a 96-well plate, after which SSOs were added to a final concentration of 2µM in a final incubation volume of 100µL. After 24 hours, cells were harvested and RNA was extracted and processed to cDNA as above, after which qPCR was performed on the cDNA using PowerUp SYBR Green Master Mix (Applied Biosystems) and detected using the CFX Touch Real-Time PCR Detection
- 10 System (Bio-Rad). Primers used are: *CCL22* forward 5'-CGCGTGGTGAAACACTTCTA-3' (SEQ ID NO: 43), reverse 5'-GATCGGCACAGATCTCCTTATC-3' (SEQ ID NO: 44); *FCRL3* forward 5'-CTGCTGCTCTGCTGCATTAC-3' (SEQ ID NO: 45), reverse 5'-TTGCTGTACATTGGCTCCAG-3' (SEQ ID NO: 46); *TNF* forward 5'-CCTCTCTCTAATCAGCCCTCTG-3' (SEQ ID NO: 47), reverse 5'-
- 15 GAGGACCTGGGAGTAGATGAG-3' (SEQ ID NO: 48).

[00104] Primary hepatocyte isolation

- [00105] Liver isolated from mice were diced into small pieces with a scalpel, after which the liver pieces were dissociated by incubation with 5mL of dissociation media (125µg/mL collagenase, 0.5U/mL dispase II, 100µg/mL DNase I in PBS) at 37°C for 30 minutes with
- 20 rocking at 75rpm. After incubation, samples were further physically dissociated using an 18G syringe on a 5mL needle. Debris was then removed using a 70µm cell strainer, and filtrates were topped up with cold DMEM before centrifugation at 300g for 3 minutes. Supernatant was then removed, and the pellet was resuspended in ACK lysing buffer (A1049201, Gibco) and incubated at room temperature for 5 minutes to allow lysis of red blood cells to occur. Samples
- 25 were then topped up with cold PBS and centrifuged at 150g for 5 minutes. Supernatant was discarded, while the cell pellet was resuspended in Lonza Hepatocyte Culture Medium and seeded onto 12-well plates coated with 0.5mg/mL Collagen I (A1048301, Gibco). Media was refreshed after 4 hours to remove additional debris. Primary hepatocytes were then treated with SSOs 48 hours post-seeding by free uptake for 72 hours.

30 [00106] Animal work

- [00107] *PE5* transgenic mice was generated by Cyagen Biosciences using CRISPR, whereby the *PE5* expression cassette was inserted into the *Rosa26* locus in a C57BL/6 background. *Spf-ash* mice were obtained from Jackson Laboratory (strain #001811). The mice were maintained on standard chow *ad libitum* under a 12-hour light/dark cycle at the A*STAR
- 35 Biological Resource Centre. *PE5* and *spf-ash* mice were cross-bred and backcrossed for at

least 3 generations. GalNAc-conjugated SSOs were diluted in saline and injected at 25mg/kg subcutaneously. For collection of blood, mice were bled retro-orbitally under anesthesia and blood was collected in K2 EDTA-coated tubes (16.444.100, Sarstedt). For isolation of plasma, the blood was centrifuged at 2,000g for 10 minutes after which the supernatant layer was
 5 extracted and subjected to biochemical assay. For high protein diet, mice were fed with diet containing 51% caloric contribution from protein (modified AIN-93G, TestDiet) *ab libitum* for 3 days.

[00108] Neurological scoring was performed using holding mice up by the tail to assess the hind legs and determining the scoring based on the scoring criteria developed by the ALS
 10 Therapeutic Development Institute. Briefly the scoring scheme is as follows:

- Score of 0: Full extension of hind legs away from lateral midline, and mouse can hold this for two seconds, suspended two to three times.
- Score of 1: Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension.
- 15 - Score of 2: Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom.
- Score of 3: Rigid paralysis or minimal joint movement, food not being used for generating forward motion.
- Score of 4: Mouse cannot right itself within 30 seconds after being placed on either
 20 side.

[00109] For survival analysis, mice are considered at humane endpoint if unable to right itself within 15 seconds after being placed on the side and counted as non-survival.

[00110] All animal experiments were performed under a protocol approved by the A*STAR Institutional Animal Care and Use Committee (#221703) in accordance with the National
 25 Advisory Committee for Laboratory Animal Research Guidelines.

[00111] Protein extraction and immunoblot

[00112] Proteins were extracted from tissue using Radioimmunoprecipitation buffer (RIPA buffer; 50mM Tris-HCl pH8.0, 50mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, supplemented with protease inhibitor) by homogenization using lysing matrix
 30 tubes containing ceramic beads (MP Biomedical) and the Bertin Precellys homogenizer. For cell culture, pellets were resuspended in RIPA buffer and sonicated for 3 cycles (30 sec ON/OFF per cycle) at high power on the Bioruptor sonicator (Diagenode). Protein was then quantified with the Quick Start Bradford Dye Reagent (5000205, Bio-Rad), after which lysates were resolved by polyacrylamide gel electrophoresis and transferred onto polyvinylidene
 35 difluoride membrane with the Bio-Rad Mini Protean System. Membrane was then probed with

primary antibodies overnight, washed with 0.1% PBS-Tween, probed with the relevant horseradish peroxidase-conjugated secondary antibodies, washed again with 0.1% PBS-Tween, and visualized for chemiluminescence with the iBright CL1500 Imaging System (Invitrogen). Primary antibodies used for immunoblotting are anti-SLC25A13/Citrin (26470-1-AP, ProteinTech) and anti-Hsp90 (610418, BD Bioscience).

[00113] Biochemical assays

[00114] Ammonia in the medium or plasma was measured using the EnzyChrom Ammonia/Ammonium Assay Kit according to the provided manual. Ammonia clearance in media was then calculated by subtracting the amount of ammonia measured from that of a control well with no cells. Urea in the medium or plasma was measured using the QuantiChrom Urea Assay Kit following the protocol for low urea samples. MTS assay was performed using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay. Absorbance for the biochemical assays were detected using the Tecan Spark 10M Plate Reader.

[00115] **EXAMPLES**

[00116] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

[00117] Example 1

[00118] To facilitate the validation of the rationally designed SSOs, a minigene system was constructed that harbours the specific genomic mutation, c.540+265G>A of the *OTC* gene. As shown in Figure 1A, the minigene includes the sequence of partial exon 4, both the first 652 bp and last 1,443 bp of intron 4, exon 5, intron 5 (where the genetic mutation of interest resides), and exon 6; the relative loci of the G>A substitution is demarcated in the figure. The inclusion of the 135 bp pseudoexon (Exon 5* or OTC-PE5) at intron 5 was confirmed when the minigene was expressed in several human cell lines (Figure 1B).

[00119] Example 2

[00120] As a therapeutic strategy to restore the expression of the wildtype protein, 4 SSOs were designed to induce the exclusion of the pseudoexon. As shown in Figure 2A, each of the SSO were highly efficient in inducing OTC-PE5 exon skipping at 25 nM. Titration experiments were performed to obtain their respective dose responses (Figure 2B), for the purpose to further differentiate the best performing SSO(s). Each of the SSO is observed to achieve complete exclusion of the pseudoexon at a concentration lower than 10 nM that is, $IC_{100} < 10$ nM. SSOs #2021 is the best performing SSO whose $IC_{50} < 0.5$ nM and $IC_{75} < 1$ nM.

[00121] Example 3

[00122] To verify that the SSOs can modulate splice out of OTC-PE5 from endogenous transcripts, SSOs #2018 and #2021 were transfected into hepatocytes differentiated from ES cells (iHeps) which were modified by CRISPR to harbour the c.540+265G>A variant (Figure 2C). HOM1 is iHeps containing c.540+265G>A variant and CAPN10 serves as a loading control. NC is a non-targeting SSO that serves as a negative control. As expected, there was an observed splice-switch from the higher band containing OTC-PE5 to the lower wild type band.

[00123] Example 4

[00124] The best performing SSOs, SSO #2018 and #2021, were shortened to clinically relevant length (typically 17 to 22 nucleotide long), giving SSO #2042 (shortened from SSO #2021), #2043 and #2044 (both shortened from SSO #2018). The shortened sequences were screened in Huh7 cells stably expressing the minigene and SSO #2044 was identified to be the most efficient (Figure 3). Based on this, mixmer SSOs with the same sequence as SSO #2044 were generated with each ribose either modified with 2'-O-methyl or substituted with locked nucleic acid at different combinations of positions (SSO #2044.1 to SSO #2044.22). The % splicing correction efficiency represents the percentage of PCR products without the pseudoexon over the total amount of PCR products.

[00125] Example 5

[00126] A third screening of the mixmer SSOs in two different cell lines (Figure 4A) and using two different methods of SSO treatment (transfection or free uptake treatment in calcium-enriched medium) on Huh7 cells stably expressing the minigene led to the identification of the optimized SSO leads (Figure 4B), with the two best-performing mixmer SSOs, #2044.7 and #2044.18, having EC50 of <5nM upon free uptake treatment (Figure 4C).

[00127] Example 6

[00128] Figure 5 shows there is neither cellular toxicity (Figure 5A, measured by a MTT assay) nor immunogenic response (Figure 5B, measured by expression of immunogenic markers CCL22, FCRL3 and TNF) caused by the sequence and chemistry combinations for the mixmers.

[00129] Example 7

[00130] To create a mouse model that is responsive to SSOs targeting *OTC* c.540+265G>A, a transgenic strain was generated in which the transgene is an expression construct containing the complementary DNA sequence of human *OTC*, with parts of intron 4 and the whole of intron 5, including the c.540+265G>A variant, interspersed between the relevant exons (Figure 7A). The transgene, which called *PE5*, was inserted into the *Rosa26* locus by CRISPR. This transgenic strain was then crossed with a naturally occurring *Otc*-deficient

mouse model, the *spf-ash* mice (doi: 10.1073/pnas.86.11.4142). Using primers specific for human *OTC*, expression of *OTC* transcripts containing *OTC-PE5* was confirmed in mice heterozygous for the *PE5* transgene in the *spf-ash* background (*PE5 Tg/+ spf-ash*; Figure 7B). The lack of *OTC* expression was also verified in *PE5 Tg/+ spf-ash* mice (Figure 7C), confirming

5 that the *spf-ash* mice is indeed *Otc* deficient, and that there is no leaky expression of functional human *OTC* from the transgene. Using hepatocytes isolated from the liver of mice homozygous for the *PE5* transgene under a wild-type (WT) non-*spf-ash* background, it was also verified that transcripts expressed from the transgenes are responsive to treatment with N-acetylgalactosamine (GalNAc)-conjugated SSOs (GN-2044.7; Figure 7D). This translates

10 to expression of functional *OTC* protein (Figure 7E), leading to a rescue in the ability of *spf-ash* hepatocytes to produce urea (Figure 7F) and clear ammonia (Figure 7G).

[00131] Having validated the mouse model, the SSOs targeting *OTC* c.540+265G>A were then further improved upon. Implementing the mixed chemistry patterns for improved efficacy (Singapore Patent Application No: 10202403247X), additional chemical modifications of

15 #2044 were identified that performed mildly better than 2044.7 (Figure 8A) through calcium-enriched medium mediated free uptake treatment of HuH7 cells stably expressing minigene (for minigene construct, see Figure 1A). Using MTS assay and BJAB assay, respectively, it was shown that both 2044.401 and 2044.501 are neither toxic (Figure 8B) nor immunogenic (Figure 8C). It was then settled with 2044.401 for the *in vivo* study as it contains a lower

20 number of locked nucleic acid compared to 2044.501.

[00132] Mice that are homozygous for the *PE5* transgene in the *spf-ash* background (*PE5 Tg/Tg spf-ash*) were subcutaneously injected with vehicle (Veh), 25mg/kg of GalNAc-conjugated non-targeting control (GN-NTC), or 25mg/kg of GalNAc-conjugated 2044.401 (GN-2044.401) weekly for 4 weeks (Figure 9A). On day 22, the mice were fed with high protein

25 diet *ab libitum* for 3 days to worsen the urea cycle disorder phenotype till the end point at day 25. At the end point of the experiment at day 25, mortality was observed in *PE5 Tg/Tg spf-ash* mice injected with vehicle or GN-NTC, while those injected with GN-2044.401 had all survived (Figure 9B). When body weight, which is a good measure of health, was monitored, there was an apparent loss of body weight in the control mice, while the body weight of those injected

30 with GN-2044.401 was similar to WT mice (Figure 9C and Figure 9D) and likely a result of metabolic adaptation to increased protein intake. Prior to euthanasia, the mice were scored using a neurological scoring system developed by the ALS Therapeutic Development Institute (ALSTDI) to determine neurological symptoms, which are typically manifested in *OTC* deficiency patients as a result of hyperammonemia. Notably, GN-2044.401 rescued

35 neurological symptoms in *PE5 Tg/Tg spf-ash* mice (Figure 9E), which was likely a result of

reduced plasma ammonia (Figure 9F) that was being converted and disposed as urea (Figure 9G).

[00133] Example 8

[00134] Table 1 shows the sequences of the present embodiments.

5

SEQ ID NO	Description of sequence	Sequence from 5' to 3'
1	SSO 2018	UUU GUA UGC UCC AGU GUU CUC UAU UCA UUC AUU
2	SSO 2019	UGU CCA UUU UGU AUG CUC CAG UGU UCU C
3	SSO 2020	CCU CUU CCA GAG UGA CUU AGU AAC UAA CCU UU
4	SSO 2021	GAU GUC UAU AGA AUC ACA UAU GGC UUG UCC AUU U
5	SSO 2042	AUA UGG CUU GUC CAU UU
6	SSO 2043	UUC UCU AUU CAU UCA UU
7	SSO 2044	UUU GUA UGC UCC AGU GUU CUC
8	SSO 2044.1	UUU+GUAUGCUGCA+GUGUUCUC
9	SSO 2044.2	UUUGUAUG+CUCCAGUGUUCU+C
10	SSO 2044.3	UUUGU+AUGCUGC+AGUGUUCUC
11	SSO 2044.4	UUUG+TAUGCUGCAGUGUUC+TC
12	SSO 2044.5	UUUG+TAUGC+TCCAG+TGUUCUC
13	SSO 2044.6	+T+TUGUAUGCUGCAGUGUUCUC
14	SSO 2044.7	U+T+TGUAUGCUGCAGUGUUCUC
15	SSO 2044.8	UU+T+GUAUGCUGCAGUGUUCUC
16	SSO 2044.9	+TU+TGUAUGCUGCAGUGUUCUC
17	SSO 2044.10	+TUU+GUAUGCUGCAGUGUUCUC
18	SSO 2044.11	UUUGUAUGCUGCAGUGUUC+T+C
19	SSO 2044.12	UUUGUAUGCUGCAGUGUU+C+TC
20	SSO 2044.13	UUUGUAUGCUGCAGUGU+T+CUC
21	SSO 2044.14	UUUGUAUGCUGCAGUGUU+CU+C
22	SSO 2044.15	UUUGUAUGCUGCAGUGU+TCU+C
23	SSO 2044.16	+TUUGUAUGCUGCAGUGUUCU+C
24	SSO 2044.17	+TUUGUAUGCUGCAGUGUUC+TC
25	SSO 2044.18	+TUUGUAUGCUGCAGUGUU+CUC
26	SSO 2044.19	+TUUGUAUGCUGCAGUGU+TCUC
27	SSO 2044.20	U+TUGUAUGCUGCAGUGUUCU+C

28	SSO 2044.21	UU+TGUAUGCUCAGUGUUCU+C
29	SSO 2044.22	UUU+GUAUGCUCAGUGUUCU+C
30	DNA sequence of target region	AATGAATGAATAGAGAACACTGGAGCATACAAAATGGACAA GCCATATGTGATTCTATAGACATCAGGTCTGTGGCTTGGGA GTGAAGACTGGTGGAGTGTGCTTTGAATATTCAAAAAGGTTA GTTACTAAGTCACTCTGGAAGAGG
31	RNA sequence of target region	AAUGAAUGAAUAGAGAACACUGGAGCAUACAAAUGGACA AGCCAU AUGUGAUUCU AUAGACAUCAGGUCUGUGGCUUG GGAGUGAAGACUGGUGGAGUGUGCUUUGAAUUAUUCAAAA GGUUAGUUACUAAGUCACUCUGGAAGAGG
32	DNA sequence of OTC-PE5	TGCCATATTTAACACAATGAATGAATAGAGAACACTGGAGCA TACAAAATGGACAAGCCATATGTGATTCTATAGACATCAGGT CTGTGGCTTGGGAGTGAAGACTGGTGGAGTGTGCTTTGAAT ATTCAAAAAG
33	RNA sequence of OTC-PE5	UGCCAUUUUAACACAUGAAUGAAUAGAGAACACUGGAG CAUACAAAUGGACAAGCCAU AUGUGAUUCU AUAGACAUC AGGUCUGUGGCUUGGGAGUGAAGACUGGUGGAGUGUGCU UUGAAUUAUUCAAAAAG
34	DNA sequence of intron 4 to intron 5	TGCTCTTAATTTGGTAAATAAAGAGTTGCAAATCTTATGTTGA TCTCCACATATTTTTGAAATCTTGCTGGTTCATGAAATTTGA AAATCTGGGAGCCATGGGCATGATAAATCCATTAAAAATTAG AAAAATCTGATCTTTAAGTCTAGAAAAATATTGTTATAGGAA TATAGTTTTTAATCTTTGATTCTCTAATCCCTGGGGCCAAGC AGTCCTTGAATTTTGTAAGCACCAGAGTTTATTGCTTTCTGT GTCAGTTCAGGTCCACTAAGCAGATTTCAAGAAGGAATTAG ACTTGCAAGAGATTTATTGAGGGAACCAAGAAACATAAA GAGAGAGGCAGCAGGAGTGGGCAAGAGGGCCTTCAGACAG GGAGGCAGGTCTGACCCCTGTGGGAAAGAAGGATGCGTGG GTAGGGAGAGAGCCTCAGCCAGTAGCGCAGCTCTGAGAAA GTCTCAGCCAGGCTGGTGGGAGCCCCAGAGCACAGACTG TCTATCAGAGAGGTCCCTGTCACGCAGGAATGGCCCAGCG CTATTCTCCCCACTGTGTTCTGTCATCAGGTTGGAACAGCCT GGGGAGGGTGGCCTTGGCAGGAATGCTGCAGGGCATCCGA AGGTGTAGCAGCTAGAGTGTGAGCCAATGGCTCTCCTCACA GCAAGTTCTCTTCTGTAGGGCGATCTGTGTGTGCACTTCCA

		TGGCTGCCACACTGGGACTTGCAATTCCACAGTGAAAATGT ATCTTTTTCTGTTGGATGATGTGAAAGAAAATTAGCCATAT GGCAGGTCATTAATGTTTCTCCGTATATCTTGCTTTTGGTCT TGTCCTTTTAACCAATGGCCATTTATTAATTATAACTCATCA TTATGTCTGCTGGGCCCACAATTATCTCCATAAAAAATGTAA AATCGCTGGTCACTGGATAAGTCGTGGGAGGTAGAACATCT GGCTTATGTAATGAATACATGTTTAATTATTACCTTTTAAACCT GTAATGTTGGTAGGTTTTCATGTAGTAAAAATTCTATTTATTA GTATTTCTTGTTATTATTTCTGATCTTATATTAAATAAGGCATT ATTAAGCATAATTATCTTAGATTATCTTTTTCTTGTTTGCCA CAGTGTATTGTCTAGCATGGCAGATGCAGTATTGGCTCGAG TGTATAACAATCAGATTTGGACACCCTGGCTAAAGAAGCAT CCATCCCAATTATCAATGGGCTGTCAGATTTGTACCATCCTA TCCAGATCCTGGCTGATTACCTCACGCTCCAGGTTGGTTTAT TTATTTGTCTTACAAAAGAGCAAAATCAAATAATTCCTGACTT GCTTTAAGTGAAACAGTTTACTTAAATCATGGTATTGGTGTTT TATTTTCAAATACCAGCTTGACAAAGAATTACAGCTTGTGTG TGCATTTTCTGGGGAAAACAGGACATTGTTAAAAACAGACTC TAAGATCGCTCTGCACTCAACCCTATTCCCAAACCCTTTTAG CCCATATCTCATCTTATGTTCTGTCAATCCCTTTGATTTTCA GTGCCATATTTAACACAATGAATGAATAGAGAACACTGGAGC ATACAAAATGGACAAGCCATATGTGATTCTATAGACATCAGG TCTGTGGCTTGGGAGTGAAGACTGGTGGAGTGTGCTTTGAA TATTCAAAAGGTTAGTTACTAAGTCACTCTGGAAGAGGGG GACACTTTCTATCATTTTAATAAACTGGTTATTTAAAGTAGGG TTAAGAATATGCAGAATTTATAGAATTGGGGCTCAAATTTAG CCATTCTAGTCCTCCCAAATTAGGAACTTGAATAACTTTGAA AGATTCCTCTTGTTTGGTTTGCCACAGCCAATCTACATTATA TTGCCCCTGGAAATCCTAACAGCCATGGTTTCTATGTCTATG GATAACTCTGAGGAGACAAGATAAACTGGATAGGCCCATCT TCTTCCACATTTGAATAAACTACACACATTTAAATGTGAGGTT AAGCTCAAAATAAAACACTTTGGACATGAGGATGAGATCCAT GCAGAGAAGATGATATAATTATGTCAGATACCAAAGTGGCT GTCTGTGGGTGTGGCTTTACATATACACCAGATAATTTAATG CTTCCCCCTGGGGCTTTATCATTACTAGATTATAAAATTCATT
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		GAGGGAAAATGAGCTGTCCTATTCATCATAATGTCCCCTGC AGGACCTTGTGCAATGCCTTCATATAGGAGACACTTAAAGTA GGAGCTGAATGAATAAATAGCAGCTTGTTAAGAGCATGGAA TCTAAATCAGATGGATCTGGAATTAATTCTAGCTTAGCCAA CTAGTAGTTCTGTGATCTCCAAATTGCTTAGCCTTTCTCTTTT TTAATCTTTTTAAATATATAAAATATATGAAACCTACAGACAC TTCAAGAAAATAAAACAAACATTCATGTGTTACACAGTTTTG TCAATTCTTAACATGATGTTGATACTTTATAATTCTTTGCTGT TTTTCAATAAAACATTAAAGATAGAGTTGAAGCCCACCATGT AACTCTGCCTAATCTTATTTTCTTCCTTTCCACAAATATGTTA TTATCCTGATGTTAGTATCTTTACACATATCTATAGCCATAAA CAACCAACAGTATTATTATTATTATTTTTGTTATTATTATTATT TGAGACAGAGTCTCACTCTGTCACCCAGACTGGAGTGCAGT GGCATGATCTCAACTCACTGCAACCTCCGCCTCCGGGTTTC AAGCGATTCTTGTGCCTCAGCCTCCGGAGTAGCTGGGATTA CAAGTGTGTGCCACGCCTGGCTAATTTTTGTGTTTTTAGTAG ACATGGGGTTTCATCATGTTGACCAGGCTGGTCTCGAACTT CTGACCTCAGGTGATCCGCCCACCTTGGTCTCCCAAAGTGC TGGGATTACAGGAATGAGCCACTGCACCTGGCCAACATAACA GTATTATTGAGCATCTTTGTCAACTTCGTATAATGGGAACAT GCTCTGAGCCACTATGCTACACTGTTCCAGGTATTCTGTATT TCCCCTTTCTCATACCAAAGAATTTAGCTCCATAAAAGCAG TGATTTTTGTCTCCTTTGTTTATTGTATCTCCAGGGCCAAGG ACAGTGTCTCAGTGTCTGGATGTTAATGGTGCTCATTTGTAT CCAATGAGTGAATACAACATAAGACTCAGATTTGAAATGTATG AACTCT
35	RNA sequence of intron 4 to intron 5	UGCUCUUAUUUUGGUAAAUAAGAGUUGCAAAUCUUAUGU UGAUCUCCACAUAUUUUUGGAAAUCUUGCUGGUUCAUGAA AUUUGAAAAUCUGGGAGCCAUGGGCAUGAUAAUCCAUA AAAAUJAGAAAAAUCUGAUCUUUAAGUCUAGAAAAUAUU GUUAUAGGAUAUAGUUUUUAUCUUUGAUUCUCUAAUCC CUGGGGCCAAGCAGUCCUUGAAUUUUGUAAGCACCAGAG UUUAUUGCUUUCUGUGUCAGUUCAGGUCCACUAAGCAGAU UUCAAGAAGGAUUAGACUUGCAAGAGAUUUUUGAGGGA ACCACCAAGAAACAUAAGAGAGAGGCAGCAGGAGUGGGC

		AAGAGGGCCUUCAGACAGGGAGGCAGGUCUGACCCCUGU GGGAAAGAAGGAUGCGUGGGUAGGGAGAGAGCCUCAGCC AGUAGCGCAGCUCUGAGAAAGUCUCAGCCAGGCUGGUGG GGAGCCCCAGAGCACAGACUGUCUAUCAGAGAGGUCCCU GUCACGCAGGAUUGGCCAGCGCUAUUCUCCCCACUGUG UUCUGUCAUCAGGUUGGAACAGCCUGGGGAGGGUGGCCU UGGCAGGAUUGCUGCAGGGCAUCCGAAGGUGUAGCAGCU AGAGUGUCAGCCAAUGGCUCUCCUCACAGCAAGUUCUCUU CUGUAGGGCGAUCUGUGUGUGCACUCCAUGGCUGCCAC ACUGGGACUUGCAAUCCACAGUGAAAAUGUAUCUUUUUU CUGUUGGAUGAUGUGAAAGAAAAUUAGCCAUUAGGCAGGU CAUUAUUGUUUCUCCGUUAUCUUGCUUUUGGUCUUGUC CCUUUUAACCAUGGCCAUUUUAUUUAUUUAACUCAUCAU UAUGUCUGCUGGGCCCACAAUAUCUCCAUAUUUUUGUA AAUUCGUGGUCACUGGAUAAGUCGUGGGAGGUAGAACA UCUGGCUUAUGUAAUGAAUACAUGUUAAUUAUJACCUUU AAACCUGUAUUGUUGGUAGGUUUUCAUGUAGUAAAAUUC UAUUUAUUAGUAUUUCUUGUUAUUUAUUUCUGAUCUUAU UAAUAAGGCAUUAUUAAGCAUAAUUAUCUUAAGAUUAUCU UUUUCUUGGUUUGCCACAGUGUAUUGUCUAGCAUGGCAG AUGCAGUAUUGGCUCGAGUGUAUAAACAAUCAGAUUUGGA CACCUGGCUAAAGAAGCAUCCAUCCCAAUUUAUCAAUGGG CUGUCAGAUUUGUACCAUCCUAUCCAGAUCCUGGCUGAUU ACCUCACGCUCCAGGUUGGUUUUAUUUAUUUGUCUUACAAA AGAGCAAAAUCAAUAAUUCUGACUUGCUUUAAGUGAAA CAGUUUACUUAUUAUUGGUUUGGUGUUUUUAUUUUCAA UACCAGCUUGACAAAGAAUACAGCUUGUGUGUGCAUUUU CUGGGGAAAACAGGACAUUGUUAUAAACAGACUCUAAGAU CGCUCUGCACUCAACCCUAUCCCAAACCCUUUUAGCCCA UAUCUCAUCUUAUGUUCUGUCAAUCCCUUUGAUUUUUUCAG UGCCAUAUUUAACACAAUGAAUGAAUAGAGAACACUGGAG CAUACAAAUGGACAAGCCAUAUGUGAUUCUAUAGACAUC AGGUCUGUGGCUUGGGAGUGAAGACUGGUGGAGUGUGCU UUGAAUAUCAAAGGUUAGUUACUAAGUCACUCUGGAA GAGGGGGACACUUUCUAUCAUUUUAAUAAACUGGUUAUUU
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		AAAGUAGGGUUAAGAAUAUGCAGAAUUUAUAGAAUUGGGG CUCAAAUUUAGCCAUUCUAGUCCUCCCAAUUAGGAACUU GAAUAACUUUGAAAGAUUCCUCUUGUUUGGUUUGCCACAG CCAUCUACAUUAUUAUUGCCCCUGGAAUCCUACAGCCA UGGUUUUCUUAUGUCUAUGGAUAACUCUGAGGAGACAAGAU AACUGGAUAGGCCCAUCUUCUCCACAUUUGAAUAAACUA CACACAUUUAAAUGUGAGGUUAAGCUCAAAAUAAAACACUU UGGACAUGAGGAUGAGAUCCAUGCAGAGAAGAUGAUAUAA UUUUGUCAGAUACCAAAGUGGCUGUCUGUGGGUGUGGCU UUACAUUAACACCAGAUAAUUUAAUGCUUCCCCUGGGGC UUUAUCAUUACUAGAUUAUAAAUUCAUUGAGGGAAAAUGA GCUGUCCUAUUCAUCAUAAUGUCCCCUGCAGGACCUUGU GCAUUGCCUUCAUUAUAGGAGACACUUAAGUAGGAGCUGA AUGAAUAAAUAGCAGCUUGUUAAGAGCAUGGAAUCUAAAU CAGAUUGAUCUGGAAUUAAAUUCUAGCUUAGCCAACUAGU AGUUCUGUGAUCUCCAAAUUGCUUAGCCUUUCUCUUUUUU AAUCUUUUUAAAUAUAUAAAAUAUUGAAACCUACAGACAC UUCAAGAAAAUAAAACAAACAUUCAUGUGUUCACACAGUUU UGUCAAUUCUUAACAUGAUGUUGAUACUUUAUAAUUCUUU GCUGUUUUUCAAUAAAACAUUAAAGAUAGAGUUGAAGCCC ACCAUGUAACUCUGCCUAAUCUUAUUUUUCUCCUUCAC AAUAUGUUUAUUAUCCUGAUGUUAGUAUCUUUACACAUAU CUAUAGCCAUAAACAACCAACAGUAUUUAUUUAUUUUUU UGUUUAUUUAUUUAUUUGAGACAGAGUCUCACUCUGUCAC CCAGACUGGAGUGCAGUGGCAUGAUCUCAACUCACUGCAA CCUCCGCCUCCCGGUUCAAGCGAUUCUUGUGCCUCAGC CUCCGAGUAGCUGGGAUUACAAGUGUGUGCCACGCCUG GCUAAUUUUUGUGUUUUUAGUAGACAUGGGGUUUCAUCA UGUUGACCAGGCUGGUCUCGAACUUCUGACCUCAGGUGA UCCGCCACCUUGGUCUCCCAAAGUGCUGGGAUUACAGG AAUGAGCCACUGCACCUGGCCAACUAACAGUAUUUAUUGAG CAUCUUUGUCAACUUCGUAAAUUGGAACAUGCUCUGAGC CACUAUGCUACACUGUCCAGGUAAUUCUGUAUUUCCCCUU UCUCAUACCAAAGAAUUUCAGCUCCAUAUAAAGCAGUGAUU UUUGUCUCCUUUGUUUAUUGUAUCUCCAGGGCCAAGGAC
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		AGUGUCUCAGUGUCUGGAUGUUAUGGUGCUCAUUUGUA UCCAAUGAGUGAAUACAACUAAGACUCAGAUUUGAAAUGU AUGAAACUCU
36	DNA sequence of region spanning binding site of SSO #2018 to #2021	AATGAATGAATAGAGAACTGGAGCATACAAAATGGACAA GCCATATGTGATTCTATAGACATC
37	RNA sequence of region spanning binding site of SSO #2018 to #2021	AAUGAAUGAAUAGAGAACACUGGAGCAUACAAAUGGACA AGCCAU AUGUGAUUCUAUAGACAUC
38	DNA sequence of region spanning binding site of SSO #2020	AAAGGTTAGTTACTAAGTCACTCTGGAAGAGG
39	RNA sequence of region spanning binding site of SSO #2020	AAAGGUUAGUUACUAAGUCACUCUGGAAGAGG
40	Forward primer (minigene) splicing PCR	AAGGACGACGATGACAAG
41	Reverse primer (minigene) splicing PCR	ATCATGATGGAGTGCAGGAT
42	Forward primer (endogenous) splicing PCR	CGGCCCGTGTATTGTCTAGC

43	CCL22 forward primer	CGCGTGGTGAAACACTTCTA
44	CCL22 reverse primer	GATCGGCACAGATCTCCTTATC
45	FCRL3 forward primer	CTGCTGCTCTGCTGCATTAC
46	FCRL3 reverse primer	TTGCTGTACATTGGCTCCAG
47	TNF forward primer	CCTCTCTCTAATCAGCCCTCTG
48	TNF reverse primer	GAGGACCTGGGAGTAGATGAG
49	SSO 2044.301	+T+T+TGUAUGCuccAGUGUUCUC
50	SSO 2044.302	+T+TUGUAUGCuccAGUGUUCU+C
51	SSO 2044.303	+TUUGUAUGCuccAGUGUUC+T+C
52	SSO 2044.401	+T+T+TGUAUGCuccAGUGUUCU+C
53	SSO 2044.402	+T+TUGUAUGCuccAGUGUUC+T+C
54	SSO 2044.501	+T+T+TGUAUGCuccAGUGUUC+T+C
55	SSO 2044.601	+T+T+TGUAUGCuccAGUGUU+C+T+C
56	SSO 2044.411	U+T+T+GUAUGCuccAGUGUUCU+C
57	SSO 2044.312	U+T+TGUAUGCuccAGUGUUCU+C
58	SSO 2044.511	U+T+T+GUAUGCuccAGUGUUC+T+C
59	SSO 2044.412	U+T+TGUAUGCuccAGUGUUC+T+C
60	SSO 2044.118	U+TUGUAUGCuccAGUGUU+CUC

Table 1.

[00135] In the embodiments shown in Table 1, bases modified as locked nucleic acids are prefixed with "+". For SEQ ID NOs 1-29, all other bases are either 2'-O-methyl (2OMe) or 2'-O-methoxyethyl (2MOE) RNA. In various embodiments, the bases that are not locked nucleic acids can be entirely 2OMe, entirely 2MOE or a mix of both modifications. For SEQ ID NOs 49-60, which show SSOs implementing mixed chemistry with enhanced efficacy, +A, +G, +T,

+C denote locked nucleic acid residues, and all other bases are either 2'-O-methyl (2OMe) or 2'-O-methoxyethyl (2MOE) RNA.

[00136] While embodiments of the invention have been particularly shown and described with reference to specific embodiments, it should be understood by those skilled in the art that
5 various changes in form and detail may be made therein without departing from the scope of the invention as defined by the appended claims. The scope of the invention is thus indicated by the appended claims and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced.

CLAIMS

1. A splice-switching oligonucleotide (SSO) that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, the target region comprising the sequence of SEQ ID NO: 31, wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.
2. The SSO of claim 1, wherein OTC-PE5 comprises the sequence of SEQ ID NO: 33.
3. The SSO of claims 1 or 2, wherein the target region comprises the sequence selected from the group consisting of SEQ ID NOs 37 and 39.
4. The SSO of any one of claims 1 to 3, comprising a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.
5. The SSO of any one of claims 1 to 4, wherein the SSO is between 17 and 34 nucleotides in length.
6. The SSO of any one of claims 1 to 5, wherein at least one of the nucleotides of the SSO is chemically modified and wherein the chemical modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.
7. The SSO of any one of claims 1 to 6, wherein each nucleotide of the SSO comprises either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.
8. The SSO of any one of claims 1 to 7, wherein the SSO comprises phosphorothioate linkages between all nucleotides of the SSO.
9. An SSO of any one of claims 1 to 8 for use in treating a urea cycle disorder.
10. The SSO of claim 9, wherein the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.

11. Use of an SSO of any one of claims 1 to 8 in the manufacture of a medicament for treating a urea cycle disorder.
- 5 12. The use of claim 11, wherein the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.
- 10 13. A method of treating a urea cycle disorder comprising administering to a subject a composition comprising an SSO according to any one of claims 1 to 8.
- 15 14. The method of claim 13, wherein the urea cycle disorder is selected from the group consisting of N-acetylglutamate synthase deficiency, carbamoyl-phosphate synthase 1 deficiency, ornithine transcarbamylase deficiency, argininosuccinate synthase deficiency, citrin deficiency, argininosuccinate lyase deficiency, arginase deficiency and ornithine translocase deficiency.
- 20 15. A pharmaceutical composition comprising (a) a therapeutically effective amount of an SSO according to any one of claims 1 to 8 and (b) one or more pharmaceutically acceptable carriers and/or diluents.
- 25 16. A method of exon-skipping comprising providing an SSO that specifically binds to a site within a target region present on a pre-mRNA transcript of a variant of the *OTC* gene, wherein the target region comprises the sequence of SEQ ID NO: 31 and wherein binding of the SSO induces the exclusion of OTC-PE5 from a mature mRNA transcript of the variant of the *OTC* gene.
- 30 17. The method of claim 16, wherein the pre-mRNA transcript comprises a c.540+265G>A mutation.
- 35 18. The method of claims 16 or 17, wherein OTC-PE5 comprises the sequence of SEQ ID NO: 33.
19. The method of any one of claims 16 to 18, wherein the target region comprises the sequence selected from the group consisting of SEQ ID NOs 37 and 39.

20. The method of any one of claims 16 to 19, comprising providing an SSO having a sequence selected from the group consisting of SEQ ID NOs 1 to 29 and 49 to 60.
- 5 21. The method of any one of claims 16 to 20, comprising providing an SSO that is between 17 and 34 nucleotides in length.
22. The method of any one of claims 16 to 21, comprising providing an SSO with at least one of the nucleotides of the SSO being chemically modified and wherein the chemical
10 modification is 2'-O-methyl RNA modification, 2'-O-methoxyethyl RNA modification, locked nucleic acid substitution or phosphorothioate linkage.
23. The method of any one of claims 16 to 22, comprising providing an SSO with each
15 nucleotide having either a 2'-O-methyl RNA modification, a 2'-O-methoxyethyl RNA modification or a locked nucleic acid substitution.
24. The method of any one of claims 16 to 23, comprising providing an SSO comprising phosphorothioate linkages between all nucleotides of the SSO.