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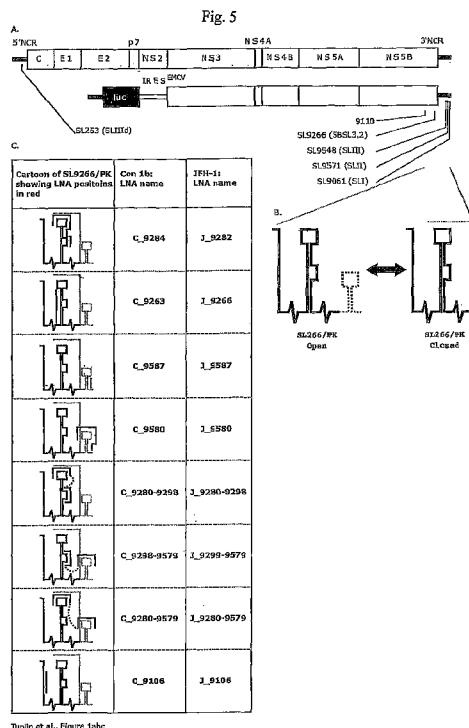
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(54) **Title:** INHIBITOR FOR TREATMENT OF HCV INFECTION



(57) **Abstract:** The invention relates to an inhibitor of hepatitis virus C (HCV) translation and/or replication, wherein the inhibitor interferes with the interaction between the stem-loop SL9266 and the stem-loop SL9571 in the HCV genome and uses thereof.

INHIBITOR FOR TREATMENT OF HCV INFECTION

Field of the Invention

The invention relates to an inhibitor of hepatitis virus C (HCV) translation and/or replication, wherein the inhibitor interferes with the interaction between the stem-loop SL9266 and the stem-loop SL9571 in the HCV genome and uses thereof.

Background to the Invention

HCV is a globally important viral pathogen infecting ~170 million individuals worldwide. If acute infection is not cleared the virus causes persistent liver disease leading to irreversible cirrhosis and is associated with over 100,000 cases of hepatocellular carcinoma per annum. In the US and Europe, HCV-induced liver disease is the major indication for liver transplantation.

With no current vaccines against HCV, and a level of virus variation that makes the prospect of an effective candidate unlikely, current treatment is restricted to a combination of Ribavirin and pegylated interferon- α . Novel therapies are urgently needed. Reverse genetic approaches to dissect the structure and function of the HCV genome have also been hampered by the limited number of *in vitro* replication systems available.

Summary of the Invention

The invention targets molecular interactions involving RNA secondary and higher order structures of the HCV virus in order to inhibit HCV translation and/or replication.

The inventors have surprisingly shown that a long-range molecular interaction involving the stem-loop SL9266 and the stem loop SL9571 forms a pseudoknot designated SL9266/PK. The “kissing interaction” between SL9266 and SL9571 within this pseudoknot has effects on HCV translation and replication. In the “kissing loop” interaction in SL9266/PK, the apical loop of the stem-loop SL9266 interacts with the apical loop of the stem-loop SL9571.

Accordingly, the invention provides an inhibitor of hepatitis virus C (HCV) translation and/or replication, wherein the inhibitor interferes with the interaction between the stem-loop SL9266 and the stem-loop SL9571 in the HCV genome and uses thereof. The inhibitor of the invention interferes with the formation of SL9266/PK.

The invention also provides:

- a pharmaceutical composition comprising an inhibitor of the invention and a pharmaceutically acceptable carrier or diluent;
- a method of inhibiting HCV translation and/or replication, comprising contacting the HCV with an effective amount of an inhibitor of HCV translation and/or replication of the invention or a pharmaceutical composition of the invention;
- a method of preventing or treating HCV infection in a subject, comprising administering to the subject an effective amount of an inhibitor of HCV translation and/or replication of the invention or a pharmaceutical composition of the invention;
- use of an inhibitor of HCV translation and/or replication according of the invention in the manufacture of a medicament for preventing or treating HCV infection; and
- an inhibitor of HCV translation and/or replication of the invention or a pharmaceutical composition of the invention for use in a method of preventing or treating HCV infection.

Brief Description of the Figures

Figure 1 shows analysis of the structure of SL9266/PK in two different *in vitro* HCV replication systems. (A) Con1b. (B) JFH-1. Key positions are prefixed ~ to indicate particular nucleotides, and adjacent stem-loops are shown in reverse video and the upstream and 'kissing loop' interactions labelled with grey shaded lozenges.

Figure 2 shows the dependency of the translation assay on an HCV IRES. The y-axis shows percentage change from untreated. The x-axis shows inhibitor concentration. The oligonucleotides were tested at three concentrations, namely 0nM, 30nM and 100nM (left to right on the x-axis).

Figure 3 shows the cell specificity of translational inhibition. The y-axis shows percentage change from untreated. The x-axis shows inhibitor concentration. The oligonucleotides were tested at three concentrations, namely 0nM, 30nM and 100nM (left to right on the x-axis).

Figure 4 shows the effect of oligonucleotides on wild type and GDD Con1b subgenomic replicons 24 hours post transfection. The y-axis shows percentage change in luciferase activity from untreated. The x-axis shows inhibitor concentration. The oligonucleotides were tested at three concentrations, namely 0nM, 100nM and 300nM (left to right on the y-axis).

Figure 5 shows nomenclature and binding sites of antisense-LNA oligonucleotides. (A) Schematic diagram of the genome of HCV JFH-1 (top) and Con1b-luc-rep (below)

indicating the location of RNA stem-loop structures (SL) using both standardised positional references and naming schemes from previous publications. (B) A cartoon representation of the dynamic SL9266/PK pseudoknot showing its open and closed conformations. A dashed line represents genome regions that switch between alternative conformations. (C) Table shows cartoon representations of SL9266/PK with antisense-LNA binding sites represented by lines, non-specific linkers are shown as dashed lines. LNA-oligonucleotides are named for the upstream nucleotide to which they are predicted to bind and are preceded by a C (for Con1b) or J (for JFH-1) - depending on which HCV isolate they were designed against.

Figure 6 shows the effect of SL9266/PK antisense-LNAs on replication and translation in Huh 7.5 cells of the Con1b-luc-rep sub-genomic replicon and replication of J6/JFH-1 virus. Results represent the average of three independent assays, and are expressed as a percentage of control transfections/infections lacking antisense-LNAs or scrambled LNA-oligonucleotides (error bars indicate the standard error from the mean). (A) Con1b-luc-rep: Relative luciferase levels (Firefly/Renilla) were measured 48 hours post RNA transfection. (B) J6/JFH-1 HCVcc: Replication was measured as focus forming units/ml (ffu/ml) 24 hours post infection in the presence of antisense-LNAs. (C) Relative luciferase levels (Firefly/Renilla) from transfected Con1b-luc-rep RNA compared to a replication defective mutant bearing a GDD>GND mutation within the active site of RdRp. Relative luciferase levels were measured 24 hours post transfection and expressed as a percentage of control transfections for either the wild type or GND mutant.

Figure 7 shows schematic diagrams of translation only reporter constructs. (i) represents a bicistronic construct with the 5'NCR from HCV upstream of the first 66 nucleotides of the HCV core coding region fused in frame to firefly luciferase. A downstream EMCV IRES initiates translation from the NS5B coding region, which is upstream of the complete HCV 3'NCR (in Δ NS5B versions NS5B is not translated due to a stop codon, represented by the black triangle, incorporated at the third codon position). Con1b and JFH-1 versions of this reporter were constructed and assayed. The Con1b reporter construct was further modified by replacing the 5'NCR with either an m⁷G cap (ii), a polio virus 5'NCR (iii) or by deleting the EMCV IRES and upstream portion of NS5B (an in frame stop codon was incorporated into the 3' truncated NS5B, represented again by a black filled triangle) (iv).

Figure 8 shows translation in Huh 7.5 cells from Con1b (Con1b_luc_trans: Δ NS5B) and JFH-1 (JFH-1_luc_trans: Δ NS5B) translation-only reporter construct RNA in the

presence of SL9266/PK antisense-LNAs or scrambled LNA-oligonucleotides. Relative luciferase levels (Firefly/Renilla) were measured 6 hours post-transfection. Results represent an average of three independent assays and are expressed as a percentage of control transfections lacking antisense-LNAs (error bars indicate the standard error from the mean). (A) Relative luciferase levels of Con1b_luc_trans:ΔNS5B RNA in the presence of antisense-LNAs. (B) Relative luciferase levels of JFH-1_luc_trans:ΔNS5B RNA in the presence of antisense-LNAs. (C) Relative luciferase levels of Con1b_luc_trans:ΔNS5B RNA in the presence of antisense-LNAs compared to variants in which the HCV 5'NCR was replaced with either a polio virus 5'NCR or m⁷G cap and a further mono-cistronic variant lacking the EMC IRES and upstream NS5B region.

Description of the Sequences

SEQ ID NO: 1 shows the nucleic acid sequence of Con1b SL9266.

SEQ ID NO: 2 shows the nucleic acid sequence of JFH-1 SL9266.

SEQ ID NO: 3 shows the nucleic acid sequence of Con1b and JFH-1 SL9571.

SEQ ID NO: 4 shows the nucleic acid sequence of the apical loop of Con1b SL9266. Nucleotides 5 to 11 of SEQ ID NO: 4 interact with (“kiss”) nucleotides 2 to 8 of SEQ ID NO: 6.

SEQ ID NO: 5 shows the nucleic acid sequence of the apical loop of JFH-1 SL9266. Nucleotides 5 to 11 of SEQ ID NO: 5 interact with (“kiss”) nucleotides 2 to 8 of SEQ ID NO: 6.

SEQ ID NO: 6 shows the nucleic acid sequence of the apical loop of SL9571. Nucleotides 2 to 8 of SEQ ID NO: 6 interact with (“kiss”) nucleotides 5 to 11 of SEQ ID NO: 4 or 5.

SEQ ID NO: 7 shows the nucleic acid sequence of the bulge loop of Con1b SL9266.

SEQ ID NO: 8 shows the nucleic acid sequence of the bulge loop of JFH-1 SL9266.

SEQ ID NO: 9 shows a preferred sequence which specifically hybridises to the apical loop of SL9266.

SEQ ID NO: 10 shows another preferred sequence which specifically hybridises to the apical loop of SL9266.

SEQ ID NO: 11 shows a preferred sequence which specifically hybridises to the apical loop of SL9571.

SEQ ID NO: 12 shows a preferred modified sequence which specifically hybridises to the apical loop of SL9571.

SEQ ID NO: 13 shows another preferred modified sequence which specifically hybridises to the apical loop of SL9266.

5 SEQ ID NO: 14 shows another preferred modified sequence which specifically hybridises to the apical loop of SL9266.

SEQ ID NO: 15 shows a preferred sequence which specifically hybridises to the bulge loop of SL9571.

10 SEQ ID NOs: 16 to 25 shows the oligonucleotides used in the Examples (see Table 1).

Detailed Description of the Invention

It is to be understood that different applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology
15 used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting. In addition as used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “an inhibitor” includes “inhibitors”, reference to “an oligonucleotide” includes two or more such oligonucleotides,
20 and the like. All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

Inhibitor of the invention

The invention provides an inhibitor which interferes with or inhibits the interaction
25 between SL9266 and SL9571 in the HCV genome, i.e. the “kissing” interaction. The inhibitor interferes with or inhibits the interaction between the apical loop of SL9266 and the apical loop of SL9571 in the HCV genome. This interaction is shown in Figure 1. The inhibitor interferes with or inhibits the formation of SL9266/PK.

The inhibitor of the invention may be provided isolated and/or purified from its
30 natural environment, in substantially pure or homogeneous form, or free or substantially free of other materials from its source or origin. Where used herein, the term “isolated” encompasses all of these possibilities. The inhibitor may optionally be labelled or conjugated to other compounds. Suitable labels include, but are not limited to, fluorescent molecules (such as Cy3 or AlexaFluor®555), radioisotopes, e.g. ¹²⁵I, ³⁵S, peptides,

proteins, enzymes, antibodies, antigens, oligonucleotides, polynucleotides and ligands such as biotin.

SL9266 and SL9571

5 SL9266/PK in the HCV genome comprises a core RNA stem-loop and sequences 5' and 3' to the stem-loop which form a region of extended RNA secondary structure, also described as a pseudoknot. The sequence of the SL9266/PK is derived from the 3' portion of the NS5B coding sequence and extends into the 3' non-coding region of the HCV genome.

10 The HCV 3' non-coding region is around 200 nucleotides in length and comprises three discrete stem-loops, sometimes known as SLI-III, numbered from the 3' end which forms a structure known as the X-tail. Of these SL-II is also designated SL9571. This structure is separated from the HCV coding region by a hypervariable domain and a pyrimidine-rich tract of variable length and sequence. The sequences 5' proximal to the
15 3'NCR encoding the NS5B polypeptide contain five additional phylogenetically conserved RNA stem-loop structures. These are designated, according to the convention described below as SL9033, SL9132, SL9217, SL9266 and SL9324. SL9266 is predicted to occupy the central position in a cruciform structure involving the adjacent SL9217 (5BSL3.1) and SL9324 (5BSL3.3) stem-loops.

20 As described by Tuplin *et al.* (Nucleic Acids Research, 2012; 40(14): 6908–6921), all stemloops are numbered according to their position in a reference sequence, namely H77 complete genome sequence (GenBank Accession #AF011753). This standardised naming scheme facilitates reference to structures in coding or non-coding regions, is independent of higher-order structures and can be logically extended as additional
25 structures are discovered. For this invention, stem-loop structures named 5BSL3.1–3.3, SLIV–VII or SL8828, SL8926, SL9011, SL9061 and SL9118 are designated SL9033, SL9132, SL9217, SL9266 and SL9324, respectively (as in Tuplin *et al. supra*). Likewise, the 5'NCR stem-loop IIIId is SL253 and the three structures that together form the X-tail [5'-SLIII, SLII and SLI-3'] are designated herein SL9548, SL9571 and SL9601 (as in
30 Tuplin *et al. supra*). The nomenclature of the HCV genome is usefully reviewed in Kuiken, C *et al* (Hepatology (2006) 44, pp 1355-1361) and Lemon *et al* (Fields virology 5th Ed. (2007), Hepatitis C virus p1253-1304).

The core RNA stem-loop termed SL9266 is also known in the art as 5BSL3.2 or SL-V. The sequences of Con1b and JFH-1 SL9266 are shown in SEQ ID NOs: 1 and 2

The SL9266 structure comprises an apical loop and two short base paired helices separated by a 3' subterminal bulge (see Figure 1). The apical loop and 3' subterminal bulge loop may be involved in upstream and downstream long range RNA-RNA interactions which create a region of extended RNA higher order structure. The long-range interactions occur with sequences around 200 nucleotides upstream and downstream of the SL9266 stem-loop. The structure of the SL9266/PK is reviewed in more detail in Diviney *et al* (J. Virol (2008) 82, pp 9008-9022) and Tuplin *et al. (supra)*.

The structure of the SL9266/PK in JFH-1, as shown in Figure 1, is characterised by the existence of interactions between two loop regions of the SL9266 stem-loop with upstream and downstream sequences. The apical loop of SL9266 interacts with the apical loop of stem-loop SL9571 located near the 3' terminus of the 3' noncoding region of the HCV genome in a "kissing loop" interaction. The sequence of SL9571 is shown in SEQ ID NO: 2. The 3' subterminal bulge loop of SL9266/PK interacts with an unstructured region centred on nucleotide 9110. The structures of SL9266/PK shown in Figure 1 represent two determined biochemically. Despite the structures being different, genetic evidence suggests that the 'kissing loop' interaction occurs in both and is necessary for successful completion of the virus replication cycle.

HCV genome

As discussed above, the SL9266 and SL9571 nomenclature references the HCV genotype 1a prototype strain H77 22, where the 5' nucleotide of the SL9266 core RNA stem-loop is at position 9266 and the 5' nucleotide of the SL9571 core RNA stem-loop is at position 9571. By definition, the Kuiken paper (*supra*) describes a numbering system that is universal for all HCV genotypes. Sequences that are aligned will always have the same structure in the same place assuming they are phylogenetically conserved. The SL9266 and SL9571 are phylogenetically conserved for all HCV genotypes. Accordingly, the skilled person may refer to the sequences referenced hereon in relation to the nucleotide positions given for sequences described herein and extrapolate to the identical position in other genomes and genotypes.

The invention may be used to inhibit the interaction between SL9266 and SL9571 and to inhibit the formation of SL9266/PK from any naturally derived genotype, serotype or isolate or clade of HCV. As is known to the skilled person, HCV viruses occurring in nature may be classified according to various biological systems. The skilled person can provide a sequence corresponding to the SL9266/PK, SL9266 and SL9571 from any

naturally derived genotype, serotype or isolate or clade of HCV based on their general knowledge.

HCV genotypes are typically referred to in terms of their genotype. HCV genotypes number from 1 to 11, each has a number of sub-types (a,b,c etc). Representative genotypes and accession numbers include: Genotype 1b (Con1 isolate) AJ238799, and Genotype 2a (JFH-1 isolate) AB047639.

HCV viruses may be referred to in terms of their serotype. A serotype corresponds to a variant subspecies of HCV which owing to its profile of expression of capsid surface antigens has a distinctive reactivity which can be used to distinguish it from other variant subspecies. Typically, a virus having a particular HCV serotype does not efficiently cross-react with neutralising antibodies specific for any other HCV serotype.

HCV viruses may also be referred to in terms of clades or clones. This refers to the phylogenetic relationship of naturally derived HCV viruses, and typically to a phylogenetic group of HCV viruses which can be traced back to a common ancestor, and includes all descendants thereof. Additionally, HCV viruses may be referred to in terms of a specific isolate, i.e. a genetic isolate of a specific HCV virus found in nature. The term genetic isolate describes a population of HCV viruses which has undergone limited genetic mixing with other naturally occurring HCV viruses, thereby defining a recognisably distinct population at a genetic level.

The skilled person can select an appropriate genotype, serotype, clade, clone or isolate of HCV for use in the invention on the basis of their common general knowledge. It should be understood that the invention also encompasses inhibiting the interaction between SL9266 and SL9571 and inhibiting the formation of SL9266/PK in the HCV genome of a genotype, serotype, clade, clone or isolate of HCV that may not yet have been identified or characterised.

In addition, the invention encompasses the inhibition of the interaction between SL9266 and SL9571 and inhibiting the formation of SL9266/PK from any known *in vitro* HCV replication systems. These include the sub-genomic replicon (SGR) generated from a HCV genotype 1b consensus sequence Con1b (Lohmann *et al*, (1999) Science 285, pp 110-113). Another suitable system is the full-length genotype 2a HCV described as JFH-1/HCVcc (Wakita *et al*, Nat Med (2005) 11, pp 791-796). A preferred system is described in International Application No. PCT/GB2012/052015 (published as WO 2013/027031).

Inhibition of HCV translation and/or replication

The inventors have surprisingly shown that the “kissing loop” interaction is important in HCV translation. Compounds which interfere or inhibit with this interaction inhibit, i.e. decrease or reduce, HCV translation and/or replication.

5 Levels of viral translation and/or replication may be inhibited, i.e. decreased or reduced, by any amount, such as at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or up to 100%.

 The ability of a compound to inhibit, i.e. decrease or reduce, HCV translation and/or replication can confirmed and measured as described in the Examples and as
10 described in International Application No. PCT/GB2012/052015 (published as WO 2013/027031). Any of the HCV genomes or *in vitro* HCV replication systems discussed above may be used.

Inhibitor

15 The inhibitor may be any compound which interferes with the interaction between SL9266 and SL9571 and interferes with the formation of SL9266PK in the HCV genome. The inhibitor(s) can be provided in any suitable form. Preferred forms are described below.

 The inhibitor may natural or synthetic. Inhibitors can be biomolecules including
20 peptides, peptide mimetics, polypeptides, proteins, oligonucleotides, polynucleotides, polymers, saccharides, fatty acids, steroids, purines, pyrimidines, interchelating agents, derivatives, structural analogs or combinations thereof. Inhibitors may be obtained from a wide variety of sources including libraries of synthetic or natural substances. Known pharmacological agents may be subjected to directed or random chemical modifications,
25 such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs. The inhibitor may be the product of a combinatorial library such as are now well known in the art (see e.g. Newton (1997) Expert Opinion Therapeutic Patents, 7(10): 1183-1194). Natural product libraries, such as display (e.g. phage display libraries), may also be used to derive the inhibitor.

30 Antibodies or antibody constructs are another class of suitable inhibitors. For example, inhibitors may be monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and humanised antibodies. The antibody may be an intact immunoglobulin molecule or a fragment thereof such as a Fab, F(ab')₂ or Fv fragment. Candidate inhibitor antibodies may be characterised and their binding regions

determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the relevant interaction.

Oligonucleotides are a preferred class of inhibitors of the invention. An oligonucleotide, such as a nucleic acid, is a polymer comprising two or more nucleotides.

5 The nucleotides can be naturally occurring or artificial. A nucleotide typically contains a nucleobase, a sugar and at least one linking group, such as a phosphate, 2'O-methyl, 2'methoxy-ethyl, phosphoramidate, methylphosphonate or phosphorothioate group. The nucleobase is typically heterocyclic. Nucleobases include, but are not limited to, purines and pyrimidines and more specifically adenine, guanine, thymine, uracil and cytosine. The
10 sugar is typically a pentose sugar. Nucleotide sugars include, but are not limited to, ribose and deoxyribose. The nucleotide is typically a ribonucleotide or deoxyribonucleotide. The nucleotide typically contains a monophosphate, diphosphate or triphosphate. Phosphates may be attached on the 5' or 3' side of a nucleotide.

Nucleotides include, but are not limited to, adenosine monophosphate (AMP),
15 guanosine monophosphate (GMP), thymidine monophosphate (TMP), uridine monophosphate (UMP), cytidine monophosphate (CMP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP) and deoxycytidine monophosphate
20 (dCMP). The nucleotides are preferably selected from AMP, TMP, GMP, CMP, UMP, dAMP, dTMP, dGMP or dCMP.

The oligonucleotide can be a nucleic acid, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The oligonucleotide may be any synthetic nucleic acid known in the art, such as peptide nucleic acid (PNA), glycerol nucleic acid (GNA), threose nucleic
25 acid (TNA), locked nucleic acid (LNA) or other synthetic polymers with nucleotide side chains. For example, LNA (Locked Nucleic Acids) oligonucleotides may be used in the invention (see http://en.wikipedia.org/wiki/Locked_nucleic_acid).

Typically, the oligonucleotide used in the invention is provided as a single-stranded nucleic acid having phosphodiester, 2'O-methyl, 2'methoxy-ethyl, phosphoramidate,
30 methylphosphonate, and/or phosphorothioate backbone chemistry. Typically, the oligonucleotide is provided as a DNA molecule, having modified chemistry at one or more positions to increase its stability. Typically, locked nucleic acids (LNA) may be used.

The oligonucleotide may be any length, but is typically less than 100 nucleotides. The inhibitor of the invention is preferably from 8 to 48 nucleotides in length, such as from 10 to 35 nucleotides in length or from 15 to 25 nucleotides in length.

Oligonucleotides can be made using standard methods in the art. Oligonucleotides, such as those comprising LNA (locked nucleic acids), are commercially available, for instance from Exiqon® or Invitrogen®.

Interfering with the interaction between SL9266 and SL9571

The inhibitor of the invention interferes with or inhibits the interaction between SL9266 and SL9571 in the HCV genome. The inhibitor interferes with or inhibits the formation of SL9266/PK in the HCV genome. The inhibitor can interfere in the interaction between SL9266 and SL9571 and the formation of SL9266/PK in any manner. For instance, the inhibitor may block the structural rearrangements that take place between SL9266 and SL9571. The inhibitor may lock SL9266 and/or SL9571 into positions that prevent(s) it/them from forming the kissing loop interaction.

The inhibitor typically interferes with the interaction by specifically binding to one or both of SL9266 and SL9571. The inhibitor typically interferes with the interaction by specifically binding to both SL9266 and SL9571. The inhibitor may bind to the apical loop and/or the bulge loop in SL9266. The inhibitor typically binds to the apical loop in SL9571.

The inhibitor preferably comprises (a) a portion that specifically binds to the apical loop of the SL9266 and/or the bulge loop of the SL9266 and (b) a portion that that specifically binds to the apical loop of SL9571. The inhibitor may comprise a portion in (a) that specifically binds to the apical loop of SL9266, the bulge loop of SL9266 or both the apical loop and the bulge loop of SL9266.

The portions in (a) and (b) are preferably antibodies or antibody constructs and/or oligonucleotides as discussed above. The portions in (a) and (b) may be the same or different. For instance, the portion in (a) and (b) may both be antibodies or antibody constructs or both be oligonucleotides. Alternatively, the portion in (a) may be an antibody or antibody construct and the portion in (b) may be an oligonucleotide or *vice versa*.

A portion “specifically binds” to a sequence when it binds with preferential or high affinity to that sequence but does not substantially bind, does not bind or binds with only low affinity to other sequences in the HCV genome. For instance, a portion “specifically binds” to the apical loop and/or the bulge loop of SL9266 when it binds with preferential

or high affinity to the apical loop and/or the bulge loop of SL9266 but does not substantially bind, does not bind or binds with only low affinity to other sequences in the HCV genome, including the apical loop of SL9571. Similarly, a portion “specifically binds” to the apical loop of SL9571 when it binds with preferential or high affinity to the apical loop of SL9571 but does not substantially bind, does not bind or binds with only low affinity to other sequences in the HCV genome, including the apical loop and/or the bulge loop of SL9266.

A portion binds with preferential or high affinity if it binds with a K_d of 1×10^{-7} M or less, more preferably 5×10^{-8} M or less, more preferably 1×10^{-8} M or less or more preferably 5×10^{-9} M or less. A portion binds with low affinity if it binds with a K_d of 1×10^{-6} M or more, more preferably 1×10^{-5} M or more, more preferably 1×10^{-4} M or more, more preferably 1×10^{-3} M or more, even more preferably 1×10^{-2} M or more. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of compounds, such as antibodies or antibody constructs and oligonucleotides are well known in the art (see for example Maddox *et al*, J. Exp. Med. 158, 1211-1226, 1993).

The inhibitor preferably comprises (a) an oligonucleotide that specifically hybridises to the apical loop of SL9266 and/or the bulge loop of SL9266 and (b) an oligonucleotide that specifically hybridises to the apical loop of SL9571. The inhibitor may comprise a portion in (a) that specifically hybridises to the apical loop of SL9266, the bulge loop of SL9266 or both the apical loop and the bulge loop of SL9266.

An oligonucleotide “specifically hybridises” to a sequence when it hybridises with preferential or high affinity to the sequence but does not substantially hybridise, does not hybridise or hybridises with only low affinity to other sequences in the HCV genome. For instance, an oligonucleotide “specifically hybridises” to the apical loop and/or the bulge loop of SL9266 when it hybridises with preferential or high affinity to the apical loop and/or the bulge loop of SL9266 but does not substantially hybridise, does not hybridise or hybridises with only low affinity to other sequences in the HCV genome, including the apical loop of SL9571. Alternatively, an oligonucleotide “specifically hybridises” to the apical loop of SL9571 when it hybridises with preferential or high affinity to the apical loop of SL9571 but does not substantially hybridise, does not hybridise or hybridises with only low affinity to other sequences in the HCV genome, including the apical loop and/or the bulge loop of SL9266.

A portion specifically hybridises to the apical and/or bulge loop of SL9266 or the apical loop of SL9571 if it hybridises to the target sequence with a melting temperature (T_m) that is at least 2 °C, such as at least 3 °C, at least 4 °C, at least 5 °C, at least 6 °C, at least 7 °C, at least 8 °C, at least 9 °C or at least 10 °C, greater than its T_m for other sequences in the HCV genome. More preferably, the portion hybridises to the target sequence with a T_m that is at least 2 °C, such as at least 3 °C, at least 4 °C, at least 5 °C, at least 6 °C, at least 7 °C, at least 8 °C, at least 9 °C, at least 10 °C, at least 20 °C, at least 30 °C or at least 40 °C, greater than its T_m for other nucleic acids. Preferably, the portion hybridises to the target sequence with a T_m that is at least 2 °C, such as at least 3 °C, at least 4 °C, at least 5 °C, at least 6 °C, at least 7 °C, at least 8 °C, at least 9 °C, at least 10 °C, at least 20 °C, at least 30 °C or at least 40 °C, greater than its T_m for a sequence which differs from the target sequence by one or more nucleotides, such as by 1, 2, 3, 4 or 5 or more nucleotides. The portion typically hybridises to the target sequence with a T_m of at least 90 °C, such as at least 92 °C or at least 95 °C. T_m can be measured experimentally using known techniques, including the use of DNA microarrays, or can be calculated using publicly-available T_m calculators, such as those available over the internet.

More preferably, the portions in (a) and (b) do not hybridise to other sequences in the HCV genome even under high stringency conditions. Most preferably, the portions in (a) and (b) do hybridise to any other nucleic acid even under high stringency conditions.

Conditions that permit the hybridisation are well-known in the art (for example, Sambrook *et al.*, 2001, Molecular Cloning: a laboratory manual, 3rd edition, Cold Spring Harbour Laboratory Press; and Current Protocols in Molecular Biology, Chapter 2, Ausubel *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995)). Hybridisation can be carried out under low stringency conditions, for example in the presence of a buffered solution of 30 to 35% formamide, 1 M NaCl and 1 % SDS (sodium dodecyl sulfate) at 37 °C followed by a wash in from 1X (0.1650 M Na⁺) to 2X (0.33 M Na⁺) SSC (standard sodium citrate) at 50 °C. Hybridisation can be carried out under moderate stringency conditions, for example in the presence of a buffer solution of 40 to 45% formamide, 1 M NaCl, and 1 % SDS at 37 °C, followed by a wash in from 0.5X (0.0825 M Na⁺) to 1X (0.1650 M Na⁺) SSC at 55 °C. Hybridisation can be carried out under high stringency conditions, for example in the presence of a buffered solution of 50% formamide, 1 M NaCl, 1% SDS at 37 °C, followed by a wash in 0.1X (0.0165 M Na⁺) SSC at 60 °C.

The inhibitor of the invention can interfere with the interaction between SL9266 and SL9571 by specifically binding/hybridising to the apical loop and/or the bulge loop of SL9266. The oligonucleotide in (a) preferably comprises a sequence which is substantially complementary to part or all of the apical loop and/or the bulge loop of SL9266. The

5 oligonucleotide in (a) may comprise a sequence which is substantially complementary to part or all of the apical loop, the bulge loop or both the apical loop and the bulge loop of SL9266.

The apical loop of Con1b SL9266 is shown in SEQ ID NO: 4. The apical loop of JFH-1 SL9266 is shown in SEQ ID NO: 5. The oligonucleotide in (a) may comprise a

10 sequence which is substantially complementary to part or all of SEQ ID NO: 4 or 5.

Nucleotides 5 to 11 of SEQ ID NOs: 4 and 5 (which are identical in both sequences) interact with the apical loop of SL9571 (in particular nucleotides 2 to 8 of SEQ ID NO: 6). The oligonucleotide in (a) preferably comprises a sequence which is

substantially complementary to all or part of nucleotides 5 to 11 of SEQ ID NOs: 4 and 5.

The bulge loop of Con1b SL9266 is shown in SEQ ID NO: 7. The bulge loop of JFH-1 SL9266 is shown in SEQ ID NO: 8. The oligonucleotide in (a) may comprise a

15 sequence which is substantially complementary to part or all of SEQ ID NO: 7 or 8.

The inhibitor of the invention can interfere with the interaction between SL9266 and SL9571 by specifically binding/hybridising to the apical loop of SL9571. The

20 oligonucleotide in (b) preferably comprises a sequence which is substantially complementary to part or all of the apical loop of SL9571. The sequence of the apical loop of SL9571 is shown in SEQ ID NO: 6. Nucleotides 2 to 8 of SEQ ID NO: 6 interact with the apical loop of SL9266 (in particular nucleotides 5 to 11 of SEQ ID NO: 4 or 5). The oligonucleotide in (b) preferably comprises a sequence which is substantially

25 complementary to part or all of nucleotides 2 to 8 of SEQ ID NO: 6.

The oligonucleotides in (a) and (b) are substantially complementary to certain sequences. Typically, the oligonucleotides are 100% complementary. However, lower levels of complementarity may also be acceptable, such as 95%, 90%, 85%, 80%, 70%, 60% or even 50%. Complementarity below 100% is acceptable if the oligonucleotides are

30 modified as discussed below. An oligonucleotide may therefore have 1, 2, 3, 4 up to 5 mismatches across a region of 5, 10, 15, 20, 25 or 30 nucleotides. Preferably 100% complementarity is present at positions in part or all of the SL9266 or SL9571 that are conserved across HCV genotypes.

The oligonucleotides in (a) and/or (b) may be substantially complementary to part of certain sequences as described above. The part is typically at least 5 nucleotides in length, such as at least 6, at least 7, at least 8, at least 9 or at least 10 nucleotides in length. The part is typically 10 nucleotides in length. Preferred parts are nucleotides 5 to 11 of SEQ ID NOs: 4 and 5 and nucleotides 2 to 8 of SEQ ID NO: 6. These two sets of nucleotides interact in the “kissing loop” interaction. However, the oligonucleotide in (a) may be substantially complementary to part of nucleotides 5 to 11 of SEQ ID NOs: 4 and 5 and/or the oligonucleotide in (b) may be substantially complementary to part of nucleotides 2 to 8 of SEQ ID NO: 6.

Linkers

The two portions/oligonucleotides in (a) and (b) are typically linked to form the inhibitor of the invention. The portions/oligonucleotides may be linked using any method known in the art. The portions/oligonucleotides are typically linked using one or more linkers. The linkers may be flexible or rigid (i.e. inflexible). Flexible linkers allow the portions/oligonucleotides to orientate correctly so that they bind/hybridise with their target sequences and interfere with the interaction between SL9266 and SL9571 and the formation of SL9266/PK. If the linker is rigid or inflexible, it typically orientates the portions/oligonucleotides so that they can bind/hybridise with their target sequences and interfere with the interaction between SL9266 and SL9571 and the formation of SL9266/PK.

The portions/oligonucleotides may be linked using one or more chemical crosslinkers or one or more peptide linkers. The portions/oligonucleotides of (a) and (b) are preferably linked using one or more oligonucleotide linkers.

Suitable chemical crosslinkers are well-known in the art. Suitable chemical crosslinkers for peptide, polypeptide or protein portions (including antibodies and antibody constructs) include, but are not limited to, those including the following functional groups: maleimide, active esters, succinimide, azides, alkynes (such as dibenzocyclooctynol (DIBO or DBCO), difluoro cycloalkynes and linear alkynes), phosphines (such as those used in traceless and non-traceless Staudinger ligations), haloacetyls (such as iodoacetamide), phosgene type reagents, sulphonyl chloride reagents, isothiocyanates, acyl halides, hydrazines, disulphides, vinyl sulfones, aziridines and photoreactive reagents (such as aryl azides, diaziridines). Suitable chemical crosslinkers for oligonucleotides include, but are not limited to, those including the following functional groups: hydrazides

(which can be attached to RNA oligonucleotides oxidised by periodonate), amines (which can reacted with oligonucleotides activated with EDC (EDAC, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and imidazole), beta-cyanoethyl phosphoramidites (which react with the 5' terminus of oligonucleotides activated with 1H tetrazole) and phenylazide-based or psoralen-based photo reactive groups. A variety of chemical oligonucleotide crosslinkers are commercially available, for instance from Integrated DNA Technologies® (IDT®). Reactions between portions/oligonucleotides and functional groups in the crosslinkers may be spontaneous, such as cysteine/maleimide, or may require external reagents, such as EDC and imadizole for linking amines and oligonucleotides. Chemical crosslinkers can comprise any molecule that stretches across the distance required. Linkers can vary in length from one carbon (phosgene-type linkers) to many Angstroms. Examples of linear molecules, include but are not limited to, are polyethyleneglycols (PEGs), polypeptides, polysaccharides, oligonucleotides, nucleic acids, such as DNA, PNA, TNA, GNA and LNA, saturated and unsaturated hydrocarbons and polyamides. These linkers may be inert or reactive, in particular they may be chemically cleavable at a defined position, or may be themselves modified with a fluorophore or ligand.

Oligonucleotide linkers may be formed from any one of the oligonucleotides discussed above. Methods are known in the art for linking oligonucleotides to peptide, polypeptide or protein portions or for linking oligonucleotides to other oligonucleotides. Oligonucleotides may be linked together using enzymes, such as ligases, polymerases and terminal deoxynucleotidyl transferase, preferred oligonucleotide linkers are TTTT, TCTAA, TCT, TTT and TTTTT. Any of these may be used.

Suitable peptide linkers are also known in the art. The length, flexibility and hydrophilicity of the peptide linker may be designed such that it does not to disturb the functions of portions/oligonucleotides and orientates them correctly. Flexible peptide linkers include stretches of 2 to 20, such as 4, 6, 8, 10 or 16, serine and/or glycine amino acids. Other flexible linkers include (SG)1, (SG)2, (SG)3, (SG)4, (SG)5, (SG)8, (SG)10, (SG)15 or (SG)20 wherein S is serine and G is glycine. Rigid (or inflexible) linkers are stretches of 2 to 30, such as 4, 6, 8, 12, 16 or 24, proline amino acids. Peptide linkers may be linked to peptide or protein portions via peptide bonds using known methods. Methods are also know in the art for linking peptides to oligonucleotides.

Complementarity

The oligonucleotides in (a) and (b) may be complementary to their target sequences. The oligonucleotide in (a) preferably comprises a sequence which is complementary to part or all of the apical loop of SL9266, such as SEQ ID NO: 4 or 5.

- 5 The oligonucleotide in (a) more preferably comprises a sequence which is complementary to part or all of nucleotides 5 to 11 of SEQ ID NO: 4 or 5. The oligonucleotide in (a) more preferably comprises CTGTGATATA (SEQ ID NO: 9) or TGTGATATA (SEQ ID NO: 10).

- 10 The oligonucleotide in (b) preferably comprises a sequence which is complementary to part or all of the apical loop of SL9571, such as in SEQ ID NO: 6. The oligonucleotide in (b) preferably comprises a sequence which is complementary to part or all of the nucleotides 2 to 8 of SEQ ID NO: 6. The oligonucleotide in (b) more preferably comprises TTTCACAGCT (SEQ ID NO: 11).

- 15 The apical loops of SL9266 and SL9571 are complementary and so interact with each other. If the oligonucleotides in (a) and (b) comprise sequences which are complementary to the apical loops in SL9266 and SL9571, the oligonucleotides in (a) and (b) will themselves be complementary. This may interfere with the efficiency of the inhibitor of the invention because the complementary oligonucleotides in (a) and (b) might form dimers (i.e. two instances of the inhibitor bind together) or hairpin structures (i.e. oligonucleotides (a) and (b) in one instance of the inhibitor bind together).
- 20

- The oligonucleotides in (a) and (b) are preferably prevented from hybridising together. The oligonucleotides in (a) and (b) in different instances of the inhibitor may be prevented from hybridising together (i.e. may be prevented from forming a dimer) and/or the oligonucleotides in (a) and (b) in the same instance of the inhibitor may be prevented from hybridising together (i.e. may be prevented from forming a hairpin structure).
- 25

- The oligonucleotides in (a) and (b) are preferably prevented from hybridising together using a linker. The oligonucleotides in (a) and (b) in different instances of the inhibitor may be prevented from hybridising together (i.e. may be prevented from forming a dimer) using a bulky linker. The oligonucleotides in (a) and (b) in the same instance of the inhibitor may be prevented from hybridising together (i.e. may be prevented from forming a hairpin structure) using a rigid or inflexible linker. The oligonucleotides in (a) and (b) may be linked using a bulky and rigid (or inflexible) linker. The oligonucleotides in (a) and (b) must be orientated in the inhibitor such that they can hybridise to their target
- 30

sequences and interfere with the interaction between SL9266 and SL9571 and the formation of SL9266/PK. A skilled person is capable of designing such inhibitors.

Alternatively, one or both of the complementary oligonucleotides in (a) and (b) may be modified so that they are not complementary to one another. The oligonucleotide in (a) may be modified. The oligonucleotide in (b) may be modified. The oligonucleotides in (a) and (b) may be modified. The modified oligonucleotides in (a) and/or (b) still specifically hybridise to the apical loop of SL9266 and the apical loop of SL9571 and interfere with the interaction between SL9266 and SL9571. If the oligonucleotides in (a) and/or (b) comprise thymine (instead of uracil) as the nucleotide complementary to adenine in the HCV genome, the oligonucleotides in (a) and/or (b) are preferably modified by replacing one or more adenines with guanine. All or only some of the adenines in the oligonucleotides in (a) and/or (b) may be replaced with guanine. Any number of adenines may be replaced with guanine, such as 1, 2, 3, 4, 5 or more. In RNA, adenine is typically complementary to and interacts with uracil. However, guanine will also interact with uracil. Hence, replacement of one or more adenine with guanine in the oligonucleotides of (a) and/or (b) will result in modified oligonucleotides which will hybridise to the apical loop of SL9266 and the apical loop of SL9571 but are less likely to hybridise to each other. The oligonucleotides in (a) and/or (b) may be chemically modified such that they specifically hybridise to their respective targets in SL9266/PK but do not hybridise to each other. A person skilled in the art is capable of making such modifications.

The ability of the modified oligonucleotides in (a) and (b) to hybridise together may be reduced by any degree, such as by 10%, by 20%, by 30%, by 50%, by 60%, by 70%, by 80%, by 90%, by 95% or by 99%. The ability of the modified oligonucleotides in (a) and (b) to hybridise can be abolished. The ability of oligonucleotides to hybridise together can be measured as discussed above.

The inhibitor of the invention more preferably comprises:

- (i) an oligonucleotide in (a) which comprises CTGTGATATA (SEQ ID NO: 9) and/or an oligonucleotide in (b) which comprises TTTCGCGGCT (SEQ ID NO: 12);
- (ii) an oligonucleotide in (a) which comprises CTGTGGTGTG (SEQ ID NO: 13) and/or an oligonucleotide in (b) which comprises TTTCACAGCT (SEQ ID NO: 11);
- (iii) an oligonucleotide in (a) which comprises CTGTGGTGTG (SEQ ID NO: 13) and/or an oligonucleotide in (b) which comprises TTTCGCGGCT (SEQ ID NO: 12);
- (iv) an oligonucleotide in (a) which comprises CTGTGGGGGG (SEQ ID NO: 14) and/or an oligonucleotide in (b) which comprises TTTCACAGCT (SEQ ID NO: 11); or

(v) an oligonucleotide in (a) which comprises CTGTG**GGGGG** (SEQ ID NO: 14) and/or an oligonucleotide in (b) which comprises TTTC**GCGG**CT (SEQ ID NO: 12). Modified nucleotides are shown in bold and underlined.

The bulge loop of SL9266 is not complementary to the apical loop of SL9571. As a result, the oligonucleotide in (a) may be complementary to the bulge loop of SL9266 and the oligonucleotide in (b) may be complementary to the apical loop SL9571 and further modifications are not needed to prevent them from hybridising to each other. The oligonucleotide in (a) preferably comprises a sequence which is complementary to part or all of the bulge loop of SL9266, such as SEQ ID NO: 7 or 8, and/or the oligonucleotide in (b) preferably comprises a sequence which is substantially complementary to part or all of the apical loop of the stem-loop SL9571, such as SEQ ID NO: 6 or nucleotides 2 to 8 of SEQ ID NO: 6. The terms “part” and “substantially complementary” are discussed above.

The inhibitor of the invention more preferably comprises an oligonucleotide in (a) which comprises TCGGGCAC (SEQ ID NO: 15) and/or an oligonucleotide in (b) which comprises TTTCACAGCT (SEQ ID NO: 11).

Oligonucleotide inhibitors

The inhibitors of the invention are most preferably oligonucleotides in which the oligonucleotides in (a) and (b) are linked by oligonucleotide linkers. The oligonucleotides may be modified in any of the ways discussed above. Preferred inhibitors of the invention comprises SEQ ID NO: 17, 18, 19, 21, 23 or 24. SEQ ID NOs: 17, 18, 19, 23 and 24 interfere with the interaction between SL9266 and SL9571 by hybridising to the apical loop of SL9266 and the apical loop of SL9571. SEQ ID NO: 21 interferes with the interaction between SL9266 and SL9571 by hybridising to the bulge loop of SL9266 and the apical loop of SL9571.

Methods and medical use

The invention also provides a method of inhibiting HCV translation and/or replication, comprising contacting the HCV with an effective amount of an inhibitor of the invention. The method may be carried out *in vivo*, *in vitro* or *ex vivo*. The HCV is typically present in a population of cells when it is contacted with the inhibitor of the invention. The HCV may be present in any population of cells. The cells are preferably derived from a subject as discussed below. Alternatively, the HCV may be present in a cell line. An effective amount of the inhibitor of the invention is an amount which inhibits

HCV translation and/or replication. The amount may inhibit HCV translation and/or replication to any of the degrees discussed above.

The method is preferably for preventing or treating HCV infection in a subject. The invention also provides a method of preventing or treating HCV infection in a subject,
5 comprising administering to the subject an effective amount of a modulator of HCV translation and/or replication identified in accordance with the invention, or an oligonucleotide inhibitor as described above.

The invention also provides an inhibitor of the invention for use in a method of preventing or treating HCV infection. The invention further provides use of an inhibitor of
10 the invention in the manufacture of a medicament for preventing or treating HCV infection.

Typically, the individual is human, but alternatively it may be a chimpanzee. The subject is typically a patient.

The invention may be for treating HCV infection. In the case of treating, the
15 subject typically has an HCV infection, i.e. has been diagnosed as having an HCV infection, or is suspected as having an HCV infection, i.e. shows the symptoms of an HCV infection. The subject is typically symptomatic. As used herein, the term “treating” includes any of following: the prevention of an HCV infection or of one or more symptoms associated with an HCV infection; a reduction or prevention of the development or
20 progression of the HCV infection or symptoms; and the reduction or elimination of an existing HCV infection or symptoms. The inhibitor of the invention may be used to prevent liver disease caused by HCV infection or to prevent hepatocellular carcinoma.

The invention may be for preventing the HCV infection. In this embodiment, the subject can be asymptomatic. The subject may have a predisposition to infection by HCV,
25 for instance a genetic predisposition to infection by HCV. As used herein, the term “preventing” includes the prevention of the onset of an HCV infection or of one or more symptoms associated with an HCV infection.

Therapy and prevention includes, but is not limited to, preventing or eliciting an effective inhibition of HCV translation and/or replication, alleviating, reducing, curing or
30 at least partially arresting symptoms and/or complications resulting from or associated with an HCV infection. When provided therapeutically, the therapy is typically provided at or shortly after the onset of a symptom of an HCV infection. Such therapeutic administration is typically to prevent or ameliorate the progression of, or a symptom of the infection or to reduce the severity of such a symptom or infection. When provided prophylactically, the

treatment is typically provided before the onset of a symptom of an HCV infection. Such prophylactic administration is typically to prevent the onset of symptoms of the infection.

Specific routes, dosages and methods of administration of the inhibitor of the invention may be routinely determined by the medical practitioner. These are discussed in more detail below. Typically, a therapeutically effective or a prophylactically effective amount of the inhibitor of the invention is administered to the subject. A prophylactically effective amount is an amount which prevents the HCV infection and/or the onset of one or more symptoms of the HCV infection. A therapeutically effective amount of the inhibitor is an amount effective to ameliorate one or more symptoms of the HCV infection. A therapeutically effective amount of the inhibitor preferably abolishes one or more symptoms of the disease. Typically, such an amount reduces the HCV infection or viral titre in the subject.

The inhibitor of the invention may be used in combination with one or more other therapies intended to treat the same subject. By a combination is meant that the therapies may be administered simultaneously, in a combined or separate form, to a subject. The therapies may be administered separately or sequentially to a subject as part of the same therapeutic or prophylactic regimen. For example, the inhibitor of the invention may be used in combination with another therapy intended to inhibit HCV infection or manage a symptom thereof. The other therapy may be a general therapy aimed at treating or improving the condition of a subject with an HCV infection. For example, treatment with methotrexate, glucocorticoids, salicylates, nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, other DMARDs, aminosaliclates, corticosteroids, and/or immunomodulatory agents (e.g., 6-mercaptopurine and azathioprine) may be combined with an anti-TNF therapy.

The other therapy may be a specific treatment directed at the HCV infection suffered by the subject, or directed at a particular symptom of such an infection. For example, the treatment may comprise treatment with the inhibitor of the invention, and also treatment with pegylated α -interferon and ribavirin, telaprevir (marketed under the brand names Incivek® and Incivo®) or boceprevir (marketed under the name Victrelis®).

The inhibitor of the invention can be administered to the subject by any suitable means. The inhibitor of the invention can be administered by enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intravenous, intra-arterial, intramuscular, intraperitoneal, intraarticular, topical or other appropriate administration routes.

The formulation will depend upon factors such as the nature of the inhibitor of the invention. The inhibitor may be administered in a variety of dosage forms. It may be administered orally (e.g. as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules), parenterally, subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The inhibitor may also be administered as a suppository. A physician will be able to determine the required route of administration for each particular subject.

The inhibitor of the invention can be formulated into pharmaceutical compositions. These compositions may comprise, in addition to one of the above inhibitors, a pharmaceutically acceptable carrier or diluent. Such compositions may also comprise other excipients, buffers, stabilisers or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the inhibitor. The precise nature of the carrier or diluent may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the inhibitor, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tableting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the inhibitor, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures
5 containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders
10 and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the pharmaceutical composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. a suspension. Reconstitution is preferably effected in buffer.

Capsules, tablets and pills for oral administration to a subject may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate,
15 cellulose acetate phthalate or hydroxypropylmethyl cellulose.

Pharmaceutical compositions suitable for delivery by needleless injection, for example, transdermally, may also be used.

An effective amount, such as a therapeutically or prophylactically effective amount, of the inhibitor is administered. The dose may be determined according to various
20 parameters, especially according to the inhibitor used; the age, weight and condition of the subject to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular subject. A typical daily dose is from about 0.1 to 50mg per kg, preferably from about 0.1mg/kg to 10mg/kg of body weight, according to the activity of the specific
25 inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the infection and the frequency and route of administration. Preferably, daily dosage levels are from 5mg to 2g.

The inhibitor of the invention may be an oligonucleotide as discussed above. An oligonucleotide of the invention may be administered. Preferably, the oligonucleotide,
30 such as RNA, DNA or LNA, in particular LNA, is provided in the form of an expression vector, which may be expressed in the cells of the subject to be treated. The oligonucleotides maybe naked nucleotide sequences or be in combination with cationic lipids, polymers or targeting systems. The oligonucleotides may be delivered by any available technique. For example, the oligonucleotide may be introduced by needle

injection, preferably intradermally, subcutaneously or intramuscularly. Alternatively, the oligonucleotide may be delivered directly across the skin using a polynucleotide delivery device such as particle-mediated gene delivery. The oligonucleotide may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration.

Uptake of oligonucleotides may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate and DEAE-Dextran and lipofectants, for example, lipofectam and transfectam. The dosage of the oligonucleotide to be administered can be altered. Typically the polynucleotide is administered in the range of 1pg to 1mg, preferably to 1pg to 10µg nucleic acid for particle mediated gene delivery and 10µg to 1mg for other routes.

The inhibitor of the invention may also be provided as part of a kit for inhibiting HCV translation and/or replication or carrying out the method of the invention. The kit typically comprises the inhibitor of the invention, preferably an oligonucleotide inhibitor of the invention, and optionally instructions to enable the kit to be used in the method of the invention. The kit may further comprise means for expression of the oligonucleotide inhibitor, such as a plasmid.

The kit suitably further comprises components necessary for the process of transfection as described above.

The kit may additionally comprise one or more other reagents or instruments which enable any of the embodiments of the method mentioned above to be carried out. Such reagents or instruments include one or more of the following: suitable buffer(s) (aqueous solutions), antibodies conjugated to detection moieties, substrates for enzymatically active tags, means to obtain a sample from a subject (such as a vessel or an instrument comprising a needle) and/or expression or cell culture apparatus. Reagents may be present in the kit in a dry state such that a fluid sample resuspends the reagents.

The kit may also, optionally, comprise instructions to enable the kit to be used in the method of the invention.

The following Examples illustrate the invention.

Examples

1. Materials and methods

5 1.1 *Translation assay*

The translation assay uses the bicistronic reporter gene system. In brief, a firefly luciferase reporter gene was under the translational control of the HCV internal ribosome entry site (IRES) as part of the complete 5' untranslated region of HCV. The luciferase gene was followed by an IRES from an unrelated virus (for example encephalomyocarditis virus, EMCV, or cricket paralysis virus, CRPV) which drove expression of the RNA dependent RNA polymerase (RdRp) of HCV (the NS5B protein) which, in turn, was followed by the HCV 3' untranslated region (3' UTR).

In an alternative method, expression of the RNA dependent RNA polymerase (RdRp) was not required. All that was required was an 'authentic' HCV 3' end on the transcript, including sequences that contain the coding region of NS5B. Additional studies were carried out with a derivative of the bicistronic system that contained a STOP (termination) codon shortly after the initiation codon of NS5B. As a consequence, no NS5B was made.

All experiments were routinely conducted in human hepatoma cells – Huh-7.5. This is the cell line in which HCV replicates. However, we also investigated the bicistronic reporter system in HepG2, HeLa and murine cell lines, as well as in cell free translation systems supplemented with Huh-7.5 cell extracts. With the exception of murine cell lines we demonstrated translational control in all the cell lines tested, and in a cell free system.

The readout of the translation assay was luciferase activity. To control for differences in transfection we co-transfected a separate plasmid encoding renilla luciferase and normalise all firefly results to this.

The translation inhibition assay was typically conducted by co-transfecting RNA generated *in vitro* from the bicistronic reporter plasmid with oligonucleotides to be tested. Assays for luciferase were routinely conducted four hours post transfection. Alternatively, the oligonucleotides to be tested may be transfected before the bicistronic reporter plasmid is introduced.

1.2 Sub-genomic replicon assay

This assay used a sub-genomic replicon analogous to that described by Lohmann (Lohmann *et al.*, 1999). As above, we co-transfected an RNA encoding renilla firefly luciferase to allow normalization of transfection levels. In these assays we transfected the sub-genomic replicon (and renilla) RNA 24 hours before addition of the oligonucleotides. Alternatively, the oligonucleotides to be tested may be transfected before the bicistronic reporter plasmid is introduced.

1.3 Virus production assay

This assay used the HCVcc system that measures the production of infectious genotype 2a JFH-1 hepatitis C virus as described previously (Wakita *et al.*, 2005). In this assay oligonucleotides directed against RNA sequences or structures were transfected into Huh-7.5 cells and a known amount of virus added 4 hours later. After 24 hours incubation the cell sheet was fixed and virus replication quantified by immunofocal staining for virus antigen (in this instance the NS5A protein). Infected cells were counted using a fluorescence microscope.

1.4 Oligonucleotides

Synthetic oligonucleotides used in our studies were produced by Exiqon® and are LNA (locked nucleic acids). This means they have modified chemistry that renders them less easily degraded in the cell.

The following sequences and interactions contribute to the formation of the extended pseudoknot in HCV.

1. Sequences around nucleotide 9010 that are complementary with the sub-terminal bulge loop of SL9266 (a long-distance interaction).

2. Sequences that form SL9266 per se, composed of two duplex regions, a terminal loop (which forms the kissing loop interaction with the 3' untranslated region of HCV) and the sub-terminal bulge loop. The sequences that form the duplexes of SL9266 represent a short-distance interaction.

3. The kissing loop interaction between the terminal loop of SL9266 and the terminal loop of SL9571 (with the caveat that we do not believe that SL9571 forms a stemloop when these two regions interact). This is also a long distance interaction.

4. The single duplex region that forms the stem of SL9571 (a short distance interaction).

Ten oligonucleotides were designed to inhibit each of these four distinct interactions (see Table 1). In addition, we designed oligonucleotides that bridge the terminal loop and bulge loop of SL9266 with the intention of simultaneously inhibiting all long distance interactions of SL9266.

5 The naming scheme for these oligonucleotides (see Table 1) is based primarily upon the numbering of the sequences to which they are complementary. The prefix C_ or J_ indicates whether they are specific for Con1b or JFH-1 (JC_ indicates complementarity to both Con1b and JFH-1). All sequences were synthesised as antisense i.e. they will interact with the genome-sense RNA.

10 J_9280/9579 and C_9280/9579#3 (see Table 1) were designed to bind to two regions that are known to interact during the function of SL9266, namely the terminal loop of SL9266 and the terminal loop of SL9571 i.e. the kissing loop interaction.

By definition, an oligo directly complementary to these two already complementary regions will be self-complementary. Inevitably, the oligonucleotide will form stable dimers or hairpins and is therefore less likely to be effective in interacting with the target sequence.

We therefore introduced a small number of mismatches that exploit the ability of G to form basepairs with U nucleotides in RNA (by A-G substitutions in the oligo).

All inhibition figures for translation and replication are at 30nM concentration compared with untreated samples.

2. Results

2.1 New data on the relevance of the translation assay

25 We demonstrated that oligonucleotides directed against SL9266 that inhibit translation are dependent upon translation being initiated from an internal ribosome entry site (IRES). We generated transcripts with an HCV IRES or transcripts truncated at the 5' end and bearing a 5'-7mG 'cap' (the standard method by which cellular mRNAs are translated).

30 Anti-SL9266 Con1b oligonucleotides inhibited translation only when an HCV IRES was present (Figure 2). Control oligonucleotides directed against an unstructured region of the genome (designated 9006) had no inhibitory effect on translation, whether the transcript was capped or translated by an IRES.

2.2 *Cell specificity of translational inhibition*

Inhibition appears to be cell type and species independent. We demonstrated that anti-SL9266 oligonucleotides inhibit Con1b translation from the bicistronic reporter system in Huh7.5 (human hepatocytes), HepG2 (human liver carcinoma cells), HeLa (human epithelial cervical carcinoma), Vero (African Green Monkey kidney epithelial cells); Figure 3.

2.3 *Oligonucleotides tested*

10 Table 1

Name	Sequence (<u>locked</u> nt. indicated with underline, linker sequences indicated in lowercase, <i>mismatched</i> positions indicated in bold) 5'-3'	Target description	Replication inhibition	Translation inhibition
C_9263 (SEQ ID NO: 16)	<u>TGTCTCCCCGCTGTAA</u>	Left hand (i.e. 5') lower and upper stem of SL9266	10% (Con1b)	19% (Con1b)
JC_9587 (SEQ ID NO: 17)	<u>GGCTCACGGACCTT</u>	Right hand (i.e. 3') stem of SL9571 in 3' untranslated region	33% (Con1b) N/D (JFH-1)	46% (Con1b) 32% (JFH-1)
C_9280/9579#1 (SEQ ID NO: 18)	<u>TTTCACAGCT</u> <u>TctttTGTGATAT</u> <u>A</u>	Bridges the terminal loop of SL9266 with the terminal loop of SL9571	41% (Con1b)	68% (Con1b)
C_9280/9579#2 (SEQ ID NO: 19)	<u>TTTCACAGCT</u> <u>TttCTGTGATAT</u> <u>A</u>	Bridges the terminal loop of SL9266 with the terminal loop of SL9571	ND (Con1b)	54% (Con1b)
C_9280/9579#3 (SEQ ID NO: 20)	<u>TTTCGCGGCT</u> <u>TttCTGTGATAT</u> <u>A</u>	Bridges the terminal loop of SL9266 with the terminal loop of SL9571 – mismatch prevents dimers	ND (Con1b)	ND (Con1b)

C_9280/9298 (SEQ ID NO: 21)	<u>TCGGGCAC</u> <u>TctaaGCTGTGATA</u> <u>TA</u>	Bridges the terminal loop and bulge loop of SL9266 – linker does not bind 3' upper duplex	76%	86%
C_9298/9579 (SEQ ID NO: 22)	<u>TTTCACAGCT</u> <u>tctTCGGGCAC</u>	Bridges the bulge loop fo SL9266 and the terminal loop of SL9571	75%	76%
C_9106 (SEQ ID NO: 23)	<u>GGACACTTCT</u> <u>TGGCCCGAT</u>	Binds to sequences around 9110	2% (Con1b)	20% (Con1b)
JC_9580 (SEQ ID NO: 24)	<u>TCAACGGACCT</u> <u>TTTCACAGC</u>	Binds to SL9571 of both Con1b and JFH-1	N/D (Con1b) 79% (JFH-1)	28% (Con1b) 37% (JFH-1)
J_9280/9579 (SEQ ID NO: 25)	<u>TTTCGCGGCT</u> <u>tttttCTGTGGGG</u> <u>GG</u>	Bridges the terminal loop of SL9266 with the terminal loop of SL9571 – mismatch prevents dimers	N/D (JFH-1)	N/D (JFH-1)

2.4 *Additional oligonucleotides tested*

Figure 4 indicates that Locked Nucleic Acid (LNA) oligonucleotides directed against SL9266/PK inhibit translation of a Con1b subgenomic replicon system. The prefix “w.t.” indicates that the genome was replication competent. The prefix “GDD” indicates that there was an active-site mutation in the virus RNA polymerase that rendered it incapable of replicating. Therefore, any luciferase activity generated must solely be due to translation of the input RNA of the GDD mutant.

The suffixes SL9266, BL-TL and 9006 indicate the LNA oligonucleotides used to ‘challenge’ the replicon. 9006 was a negative control. It was designed to bind an irrelevant and uninvolved region of the genome and simply demonstrates that the inhibition seen with the test oligonucleotides is not due to some sort of non-specific LNA-mediated degradation of the template.

BL-TL was an oligonucleotide that spans between the bulge loop of SL9266 and the terminal loop of SL9571 (i.e. an oligonucleotide of the invention). SL9266 was an LNA oligonucleotide that only interacts with the core stemloop SL9266.

5 **Further experimental details**

Materials and Methods

RNA stem-loop nomenclature

10 RNA stem-loops are designated by the position of the first 5' paired nucleotide in the structure aligned and referenced to the H77 complete genome sequence (GenBank Accession #AF011753) Tuplin *et al*, Nucleic Acids Research, 2012 40(14) 6908-21. Of relevance to this report, stem-loop structures named 5BSL3.1 – 3.3, SLIV – VII or SL8828, SL8926, SL9011, SL9061 and SL9118 are designated here SL9033, SL9132,
15 SL9217, SL9266 and SL9324 respectively. Likewise, the three structures that together form the X-tail (5'- SLIII, SLII and SLI -3';) are SL9548, SL9571 and SL9601. The higher order structure formed by the kissing loop interaction between the terminal loops of SL9266 and SL9571 has previously been designated SL9266/PK.

20 Design and nomenclature of locked nucleic acid (LNA) antisense oligonucleotides

Antisense-LNA oligonucleotides (hereafter antisense-LNAs) (Exiqon) were designed against a range of target sequences within the SL9266/PKs of Con1b and JFH-1 genomic RNA sequence (Fig. 5C). Individual antisense-LNA were either designed to be complementary to specific subunit motifs of SL9266/PK or against two structural motifs
25 within different subunits of the higher order structure – in which case antisense motifs were separated by non-specific linker sequences. Antisense-LNAs were numbered for the position of the 5' nucleotide of the target motif/s. They were designed with a minimum of four 5' and 3' terminal LNA nts, stretches of non-LNA nts were limited to four nts and they were predicted to have similar thermodynamic and sequence motif binding properties. The
30 relative binding efficiency of antisense-LNAs to their complementary RNA sequences was biochemically assayed (data not shown).

HCV cDNA plasmids, reporter construction and mutagenesis

The parental firefly luciferase-encoding Con1b replicon – designated pFKnt341-sp-P1-lucEI3420-9605/5.1 (for convenience designated here as Con1b-luc-rep) - was a generous gift from Ralf Bartenschlager and has been described previously, Friebe *et al*, J of Virology 2001, 75(24) 12047-57. The HCVcc - designated pFK-J6/JFH-1-C-846 (for convenience designated here as J6/JFH-1) - was generously provided by Takaji Wakita and NIH; full-length cDNA has previously been fully described, Lindenbach *et al*, Science 2005, 309, 623-6. Replication-incompetent derivatives of Con1b-luc-rep were generated by a GDD>GND substitution, within the active site of the NS5B polymerase as described previously Diviney et al, J Virol, 2008, 82(18) 9008-9022. Renilla luciferase RNA was generated from the cDNA plasmid pRL (Promega).

A translation-only reporter construct for genotype 1b (designated Con1b_luc_trans) was constructed by overlap extension PCR of the Con1b-luc-rep cDNA template. An upstream fragment spanning the complete 5'NCR to the end of firefly luciferase was amplified by PCR and joined to a downstream PCR amplification product spanning the complete NS5B and 3'NCR domains by overlap extension PCR. The final overlap PCR product incorporated in sequential order from the 5' end a unique Sac I restriction site, hammerhead ribozyme sequence (which cleaved transcribed RNA immediately upstream of the nucleotide 1 of the 5'NCR), complete Con1b 5'NCR and the first 48 nts of the core coding region in frame with firefly luciferase, an EMCV IRES immediately upstream of an AUG start codon, V5 peptide tag fused in frame to the Con1b NS5B coding region, complete 3'NCR and a unique Spe I restriction site.

An alternative version of this construct was made deficient in NS5B translation (designated Con1b_luc_trans:ΔNS5B). The third codon position of the V5 sequence was mutated to a stop codon (^A2683^T and ^G2685^A) to prevent translation of the NS5B encoding region. Mutations were also made to remove an in frame AUG start at codon 2 within the NS5B encoding region (^A2725^{G+T}2726^C). Blocking of NS5B translation was confirmed by western blotting after *in vitro* and *in vivo* translation (in Huh 7.5 cells and rabbit reticulate lysate respectively) and compared to active NS5B translation from a wild type construct (data not shown). The overlap PCR generated fragment was incorporated into pBluescript II KS (-) (Stratagene) downstream of a bacteriophage T7 transcription promoter sequence (T7 sequence) between unique Sac I and Spe I restriction sites.

Two plasmids for constructing the JFH-1 based translation-only reporter cDNA constructs were synthesised by GeneArt (Life technologies). In sequential order the

upstream plasmid encoded a unique Sac I restriction site, 5' hammerhead ribozyme sequence, complete JFH-1 5'NCR, the first 48 nucleotides (nts) of the core coding region and 63 nts of firefly luciferase. The downstream plasmid encoded the final 433 nts of EMCV, a V5 peptide tag sequence, complete NS5B encoding region, 3'NCR and a unique Spe I restriction site. The upstream and downstream regions were cloned into and replaced the equivalent regions in Con1b_luc_trans between Sac I to Xba I and Avr II to Xho I respectively (final construct designated JFH-1_luc_trans). As described earlier an alternative version was also constructed deficient in NS5B translation (designated JFH-1_luc_trans:ΔNS5B).

The HCV 5'NCR in Con1b_luc_trans:ΔNS5B was replaced with the complete 5'NCR sequence of Polio virus (Type 3:Leon), creating a construct designated Polio_luc_trans:ΔNS5B. A cDNA insert was generated by overlap PCR incorporating in sequential order a unique Sac I restriction site, the complete polio virus 5'NCR and the upstream 1159 nts of firefly luciferase. This fragment was cloned into and replaced the equivalent regions in Con1b_luc_trans:ΔNS5B between the Sac I and Xba I restriction sites; the downstream NS5B encoding region and 3'NCR from Con1b remained unchanged. A mono-cistronic translation reporter lacking the EMCV IRES, V5 peptide sequence tag and upstream 321 nts of the NS5B encoding region was constructed (designated Con1b_luc_trans: ΔEMCV), by deleting the fragment between unique restriction sites Eag I and Zra I. Con1b_luc_trans:Δ5'NCR+ΔNS5B cDNA was generated as a PCR product from a Con1b_luc_trans:ΔNS5B template from 5' nucleotide position of firefly luciferase to the 3' terminus of the 3'NCR. The forward primer encoded a T7 sequence immediately upstream of the firefly luciferase. Mutations were introduced using the Stratagene QuikChange™ system according to the manufacturers instructions, their presence confirmed by DNA sequencing, and rebuilt into the parental plasmid between unique restriction sites.

In vitro RNA transcription

1 µg of either J6/JFH-1 plasmid cDNA (which includes a 3' *cis*-acting ribozyme), Sca I linearized Con1b_luc_trans:ΔNS5B, Polio_luc_trans:ΔNS5B or Con1b_luc_trans:ΔEMCV+ΔNS5B, BspHI linearized JFH-1_luc_trans:ΔNS5B or Xba I linearized pRI cDNA was used as template for the production of RNA *in vitro* using a T7 MEGAscript kit (Life technologies), according to the manufacturers' instructions. PCR products amplified with a T7 sequence containing sense primer were generated as

templates for transcription of wild type and mutant NS5B-3'NCR RNA for *trans* supplementation assays. Con1b_luc_trans:Δ5'NCR RNA was generated from a PCR amplified template as described earlier, 250 ng was used as template for *in vitro* production of 5' [m⁷G(5')ppp(5')G] capped (m⁷G capped) RNA using a T7 mMessage mMachine kit (Life technologies) according to the manufactures instructions. Following transcription, DNA template was removed by DNase 1 (Life technologies) treatment and RNA purified with an RNeasy mini-kit column (Qiagen). RNA integrity was confirmed by denaturing agarose gel electrophoresis and quantified by NanoDrop spectroscopy.

10 Cell culture and transfections

Monolayers of the human hepatoma cell line Huh 7.5 (a generous gift from Charlie Rice) were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Life technologies), 1% non-essential amino acids, 2 mM L-glutamine and 100 U penicillin/100 µg streptomycin/ml (DMEM P/S). Cells were passaged after trypsin/EDTA treatment, seeded at dilutions of 1:3 to 1:5 and maintained at 37°C in 5 % CO₂.

Reporter transfection and analysis

Huh 7.5 cells were seeded in 24 well plates at $\sim 3 \times 10^5$ cells/well and maintained overnight in DMEM/PS before monolayers were transfected using Lipofectamine 2000 (Life technologies) transfection reagent. Briefly, monolayers at ~ 90 % confluence were washed twice in phosphate buffered saline (PBS) before adding 500 µl of DMEM supplemented with 1% non-essential amino acids and 2 mM L-glutamine before 100 µl of transfection medium was added in a drop wise manner. Transfection medium was prepared according to the manufacturers instructions with 2 µl Lipofectamine 2000, 0.32 pmoles of reporter RNA, 0.32 pmoles of renilla luciferase RNA and made up to 100 µl with Opti-Mem reduced serum media (Life technologies). In addition 40 nmoles of antisense-LNA or scrambled LNA oligonucleotide was included in the transfection medium of each steric hindrance assay and 0.32 pmoles or 3.20 pmoles of SL9266/PK RNA in *trans* complementation assays. After transfection monolayers were maintained for 6 hours before they were washed twice with PBS, lysed with 0.5 ml Glo-Lysis Buffer (Promega) and stored frozen prior to analysis using Dual-luciferase substrate (Promega) and a Turner TL-20 luminometer. In the case of Con1b-luc-rep media was changed after 4

hours, monolayers washed twice with PBS and replaced with 1 ml of DMEM/PS. They were maintained for 48 hours before harvesting and analysed as described earlier.

Virus analysis and quantification

5 Huh 7.5 cells were seeded in a 24 well plate at $\sim 3 \times 10^6$ cells/well and maintained overnight in DMEM P/S. The following day monolayers were washed twice with PBS and transfected with 300 nmoles of antisense-LNA using Lipofectamine 2000 as described earlier. 4 hours post transfection monolayers were washed twice with PBS and ones with DMEM P/S before incubating for 2 hours with 300 ul of filtered J6/JFH-1 virus
 10 supernatant (2×10^2 ffu/ml). Virus media was then removed, monolayers washed twice with PBS and replaced with 1 ml DMEM/PS. 24 hours after infection monolayers were washed twice with PBS, fixed with 1 ml 4 % paraformaldehyde for 20 min. and washed twice again in PBS before permeabilisation with 0.1% Triton PBS for 7 min with constant agitation. After a subsequent PBS wash infected cells were detected using a polyclonal
 15 sheep antibody to NS5A (α NS5A; generously supplied by Mark Harris) diluted 1:5000 in 10% foetal bovine serum (FBS). After incubation for 1 hr. the primary antibody was detected using an AlexaFluor594-conjugated secondary anti-sheep antibody (1:500 in 10% FBS; Invitrogen), washed in PBS and stored under PBS containing 0.1% VECTASHIELD DAPI (Vector Laboratories) before analysis by UV microscopy. Infected foci were
 20 counted and expressed in focus forming units per ml (ffu/ml).

Statistical analysis

Statistical analysis was carried out using two-tailed Student's *t*-tests for unpaired samples of equal variance. P values of ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) and ≤ 0.0001
 25 (****) were considered to represent degrees of significance.

Results

Steric hindrance of SL9266/PK: Replicon and HCVcc phenotypes

30 Using reverse genetic analysis we and others have previously shown that deletion or mutation of base-pairing components within SL9266/PK severely limit or block HCV virus replication. This affect has been observed for different genotypes and in different assay systems including sub-genomic replicons and full-length virus (HCVcc) in both cell culture and model animal systems. However, it has not been clear whether disruption of

SL9266/PK represses HCV replication via a direct effect on virus genome replication or an indirect effect via inhibition of virus translation - subsequently manifesting as suppression of HCV genome replication. In order to dissect whether SL9266/PK acts specifically on HCV virus genome translation or replication we designed antisense-LNAs complementary to different components of SL9266/PK and compared their effect on virus replication and translation (Fig. 5C). Alternative antisense-LNAs were designed to inhibit formation of the pseudoknot, simultaneously anneal to the upstream and downstream components of SL9266/PK - mimicking a locked closed conformation - and block different potential sequence specific signal motifs.

The effect of SL9266/PK antisense-LNAs on HCV replication was assayed in Huh 7.5 cells using a Con1b sub-genomic replicon system 48 hrs post RNA transfection and expressed as a function of relative luciferase translation compared to untreated control assays (Fig. 6A). Scrambled LNA-oligonucleotides or antisense-LNAs targeting a non-structured domain within the HCV genome (C_9006), upstream of SL9266/PK, were also tested with no difference observed from untreated control assays. Antisense-LNAs specific to SL9266 domains known to be involved in pseudoknot formation (LNAs C_9284 and C_9298-9579) suppressed replication by between ~50% ($P=0.00032$) and ~75% ($P=0.00003$) respectively. Specifically targeting components of SL9571 (LNAs C_9587 and C_9580) resulted in smaller – but still statistically significant – repression in HCV replication. Antisense-LNAs simultaneously targeting the terminal or bulge loops SL9266 and the terminal loop of SL9571 (LNAs C_9280-9579 and C_9298-9579) inhibited replication by ~50% ($P=0.00021$) and 80% respectively ($P=0.00002$). Antisense-LNAs designed to only block SL9266 duplex-stem formation (LNA C_9263) or the bulge loop interaction with upstream position 9110 (LNA C_9110) had no statistically significant effect on replication.

The structural dynamics of SL9266/PK have previously been shown to differ between the predominant HCV model systems (Con1b/H77 and JFH-1). To investigate this, a subset of antisense LNAs were designed specific to JFH-1 SL9266/PK and their effect on full-length J6/JFH-1 virus replication in the HCVcc system measured (Fig. 6B). Targeting the pseudoknot with antisense-LNAs designed to the single stranded loops and upper duplex of SL9266 (J_9282) inhibited virus replication by ~65% ($P=0.0011$). Single antisense-LNAs simultaneously targeting either the terminal loop regions of SL9266 and SL9571 (J_9280-9579) or the bulge loop of SL9266 and terminal loop region of SL9571 (J_9299-9579) both inhibited virus replication by ~55 % ($P=0.0032$ and 0.0031)

respectively). Antisense-LNA J_9007, complementary to an unstructured region of the virus genome, had no effect in virus replication.

Having demonstrated that targeting SL9266/PK blocks HCV replication the specific impact on virus translation was assayed (Fig. 7). The effect of a subset of antisense-LNAs (earlier in the study shown to significantly inhibit replication of the Con1b sub-genomic replicon) on HCV IRES translation from a replication deficient replicon (mutant GDD> GND) was assayed and compared in parallel to that observed from wild-type replicons. 48 hrs post transfection relative differences in translation inhibition, between the GND mutant and wild type assays, was indistinguishable; suggesting that SL9266/PK antisense-LNAs were specifically inhibiting HCV translation rather than genome replication.

Steric hindrance of SL9266/PK: Translation reporter phenotype

In order to further investigate the specific role of SL9266/PK closed and open conformations in modulating translation initiation from the HCV IRES we developed a range of translation only reporter constructs (Fig. 7). A bicistronic construct was constructed with an upstream cistron encoding an HCV IRES initiating translation of firefly luciferase (Con1b_luc_trans). The down-stream cistron encoded an EMCV IRES upstream of the full length HCV NS5B encoding sequence and 3'NCR (which includes SL9266/PK). Different variants of this construct were produced based on either Con1b (Con1b_luc_trans) or JFH-1 (JFH-1_luc_trans) sequences, with alternative versions deficient in NS5B translation (:ΔNS5B). Changes in levels of HCV IRES driven translation, when challenged with SL9266/PK antisense-LNAs, was expressed as a function of relative luciferase expression 6 hrs post transfection compared to assays lacking LNA oligonucleotides (Fig. 8A). In initial assays we showed that NS5B expression had no observable effect on HCV IRES initiated translation, with relative luciferase expression levels indistinguishable between Con1b_luc_trans or JFH-1_luc_trans and their associated ΔNS5B versions. Similarly, NS5B expression (or lack thereof) had no effect on translation inhibition by SL9266/PK antisense-LNAs (data not shown). Consequently, the results presented here describe assays using translation reporter constructs deficient in NS5B translation (Con1b_luc_trans:ΔNS5B and JFH-1_luc_trans:ΔNS5B).

Non-specific scrambled LNA oligonucleotides and antisense-LNAs complementary to unstructured regions of the Con1b genome (LNA C_9006) had no effect on translation.

Antisense-LNAs specific to domains of SL9266 involved in pseudoknot formation - LNAs C_9284 and C_9280-9298 - inhibited HCV IRES driven translation by >60% (P= 0.00004) and >75% (P=>0.00000) respectively. Those complementary to SL9571 - LNAs C_9587 and C_9580 - had an inhibitory effect of >25% (P=0.00014) and >50% (P=0.00001)

5 respectively. Whilst those specific to multiple regions of SL9266/PK - either the terminal (LNA C_9280-9579) or bulge loop (LNA C_9298-9579) of SL9266 simultaneously with the terminal loop of SL9571 - resulted in suppression of translation from the HCV IRES of >60% (P=0.00001) and >70% (P=0.00001) respectively. Similar to the replication-based assay described earlier, antisense-LNAs targeting the up-stream duplex stem of SL9266 or
10 position 9110 had no statistically significant impact on HCV IRES driven translation.

Parallel assays across different mammalian cell types - using LNA_C9284 (complementary to the single stranded loops and upper duplex of SL9266) – revealed significant inhibitory effects on HCV IRES driven translation in non-hepatocyte (HeLa) and non-human primate (Vero) cells. Compared to Huh 7.5 cells (~65% inhibition) marginally smaller levels of
15 inhibition were observed (both ~50% inhibition). Differences in translation inhibition from those observed in Huh 7.5 cells were weakly significant (HeLa P=0.00844 and Vero P=0.01797).

Previously, we have shown that the equilibrium between SL9266/PK open and closed conformations in JFH-1 derived molecules favors formation of the closed structure
20 (rather than the open conformation favored in Con1b and H77). In order to investigate the effect of this dynamic difference on HCV IRES driven translation we compared levels of translation between JFH-1_luc_trans:ΔNS5B and Con1b_luc_trans:ΔNS5B and observed them to be statistically indistinguishable. JFH-1 IRES translation, after blocking SL9266/PK with antisense-LNAs, was assayed and compared to controls as described
25 earlier. In relation to complementarity to specific motifs within SL9266/PK, patterns of inhibition followed similar specificity to the Con1b based system. However, although still statistically significant relative inhibition levels were generally lower in the JFH-1 based system (Fig. 7B). Greatest inhibition of the HCV IRES (~60%) was observed with LNA J_9282 (P=0.0004) – complementary to the single stranded loops and upper down-stream
30 duplex of SL9266 – and was statistically indistinguishable from that observed when targeting the same domain features in Con1b_luc_trans:ΔNS5B.

In order to investigate whether inhibition of translation was specific to the HCV IRES we tested further translation reporters, in which the HCV IRES of Con1b_luc_trans:ΔNS5B was either deleted and replaced with an m⁷G cap or alternatively

a polio IRES (designated Con1b_luc_trans:Δ5'NCR and Polio-IRES_luc_trans respectively) (Fig. 7). The effect on relative luciferase translation from these RNA molecules, when the terminal and bulge loops of SL9266 were targeted with antisense-LNAs (LNA C_9280-9298), was assayed as described earlier (Fig. 8C). Translation from m⁷G 5' capped transcripts was inhibited by ~20% and from the Polio-IRES_luc trans by ~50% (compared to >80% inhibition from the Con1b IRES parallel assays). The >30% increase in translational repression observed from the HCV IRES compared to the Polio IRES was statistically significant (P=0.001).

It has previously been suggested that translation from an upstream HCV IRES in a bicistronic system is influenced by the downstream IRES Ito et al, J Virol, 72(11) 8789-96. To investigate if this was influencing the translational repression observed in the current study we constructed a monocistronic translation reporter (Fig. 7). This Con1b based construct – designated Con1b_luc_trans: ΔNS5B+ΔEMCV – included an upstream HCV IRES initiating firefly luciferase translation, lacked the EMCV IRES and upstream 321 nts of NS5B but incorporated the remainder of the NS5B encoding region and complete 3'NCR. HCV IRES translation levels from this construct were indistinguishable from the equivalent bicistronic construct and showed the same level of translational repression when SL9266/PK formation was blocked with LNA C_9280-9298 (>80%) (Fig. 8C).

CLAIMS

1. An inhibitor of hepatitis virus C (HCV) translation and/or replication, wherein the
5 inhibitor interferes with the interaction between the stem-loop SL9266 and the stem-loop
SL9571 in the HCV genome.
2. An inhibitor according to claim 1, wherein the inhibitor comprises:
 - (a) a portion that specifically binds to the apical loop of SL9266 and/or a
10 portion that specifically binds to the bulge loop of the SL9266; and
 - (b) a portion that that specifically binds to the apical loop of SL9571.
3. An inhibitor according to claim 1 or 2, wherein the inhibitor comprises:
 - (a) an oligonucleotide that specifically hybridises to the apical loop of SL9266
15 and/or the bulge loop of SL9266; and
 - (b) an oligonucleotide that specifically hybridises to the apical loop of SL9571.
4. An inhibitor according to claim 3, wherein the oligonucleotide in (a) is
substantially complementary to part or all of the apical loop and/or of the bulge loop of
20 SL9266 and/or wherein the oligonucleotide in (b) is substantially complementary to part or
all of the apical loop of SL9571.
5. An inhibitor according to claim 4, wherein the oligonucleotide in (a) comprises a
sequence which is complementary to part or all of the apical loop of SL9266 and/or
25 wherein the oligonucleotide in (b) comprises a sequence which is complementary to part or
all of the apical loop of SL9571.
6. An inhibitor according to claim 5, wherein the oligonucleotide in (a) comprises a
sequence which is complementary to part or all of SEQ ID NO: 4 or 5 and/or the
30 oligonucleotide in (b) comprises a sequence which is complementary to part or all of SEQ
ID NO: 6.
7. An inhibitor according to claim 5 or 6, wherein the oligonucleotide in (a)
comprises CTGTGATATA (SEQ ID NO: 9) or TGTGATATA (SEQ ID NO: 10) and/or

the oligonucleotide in (b) comprises TTTCACAGCT (SEQ ID NO: 11).

8. An inhibitor according to any one of claims 5 to 7, wherein the oligonucleotides in (a) and (b) are prevented from hybridising together.

5

9. An inhibitor according to claim 8, wherein the oligonucleotides in (a) and (b) are prevented from hybridising together by a linker.

10. An inhibitor according to any one of claims 5 to 8, wherein the one or both of the oligonucleotides in (a) and (b) are further modified so that they are not complementary to one another.

10

11. An inhibitor according to claim 10, wherein the oligonucleotides are modified by replacing one or more adenines with guanine.

15

12. An inhibitor according to claim 11, wherein:

(i) the oligonucleotide in (a) comprises CTGTGATATA (SEQ ID NO: 9) and/or the oligonucleotide in (b) comprises TTTCGCGGCT (SEQ ID NO: 12);

(ii) the oligonucleotide in (a) comprises CTGTGGTGTG (SEQ ID NO: 13)

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and/or the oligonucleotide in (b) comprises TTTCACAGCT (SEQ ID NO: 11); and

(iii) the oligonucleotide in (a) comprises CTGTGGTGTG (SEQ ID NO: 13)

and/or the oligonucleotide in (b) comprises TTTCGCGGCT (SEQ ID NO: 12);

(iv) the oligonucleotide in (a) comprises CTGTGGGGGG (SEQ ID NO: 14)

and/or the oligonucleotide in (b) comprises TTTCACAGCT (SEQ ID NO: 11); or

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(v) the oligonucleotide in (a) comprises CTGTGGGGGG (SEQ ID NO: 14)

and/or the oligonucleotide in (b) comprises TTTCGCGGCT (SEQ ID NO: 12).

13. An inhibitor according to claim 4, wherein the oligonucleotide in (a) comprises a sequence which is complementary to part or all of the bulge loop of SL9266 and/or wherein the oligonucleotide in (b) comprises a sequence which is substantially complementary to part or all of the apical loop SL9571.

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14. An inhibitor according to claim 13, wherein the oligonucleotide in (a) comprises TCGGGCAC (SEQ ID NO: 15) and/or the oligonucleotide in (b) comprises

TTTCACAGCT (SEQ ID NO: 11).

15. An inhibitor according to any one of the preceding claims, wherein the inhibitor is an oligonucleotide of from 8 to 48 nucleotides in length.

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16. An inhibitor according to any one of the preceding claims, wherein the inhibitor comprises one or more Locked Nucleic Acids.

17. A pharmaceutical composition comprising an inhibitor according to any one of the preceding claims and a pharmaceutically acceptable carrier or diluent.

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18. A method of inhibiting HCV translation and/or replication, comprising contacting the HCV with an effective amount of an inhibitor of HCV translation and/or replication according to any one of claims 1 to 16 or a pharmaceutical composition according to claim

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17.

19. A method according to claim 18, wherein the method is for preventing or treating HCV infection in a subject and the method comprises administering to the subject an effective amount of an inhibitor of HCV translation and/or replication according to any one of claims 1 to 16 or a pharmaceutical composition according to claim 17.

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20. Use of an inhibitor of HCV translation and/or replication according to any one of claims 1 to 16 in the manufacture of a medicament for preventing or treating HCV infection.

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21. An inhibitor of HCV translation and/or replication according to any one of claims 1 to 16 or a pharmaceutical composition according to claim 17 for use in a method of preventing or treating HCV infection.

Fig. 1

Fig. 1B

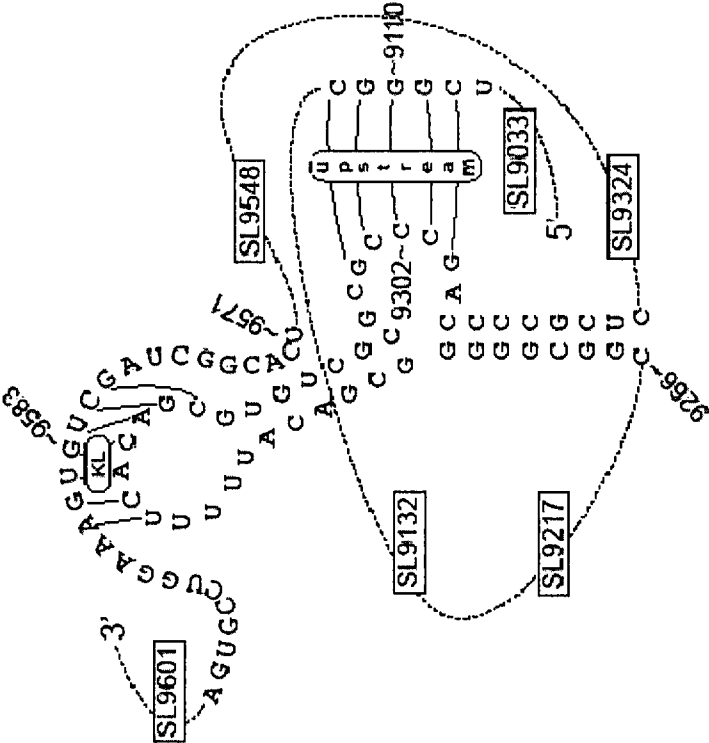


Fig. 1A

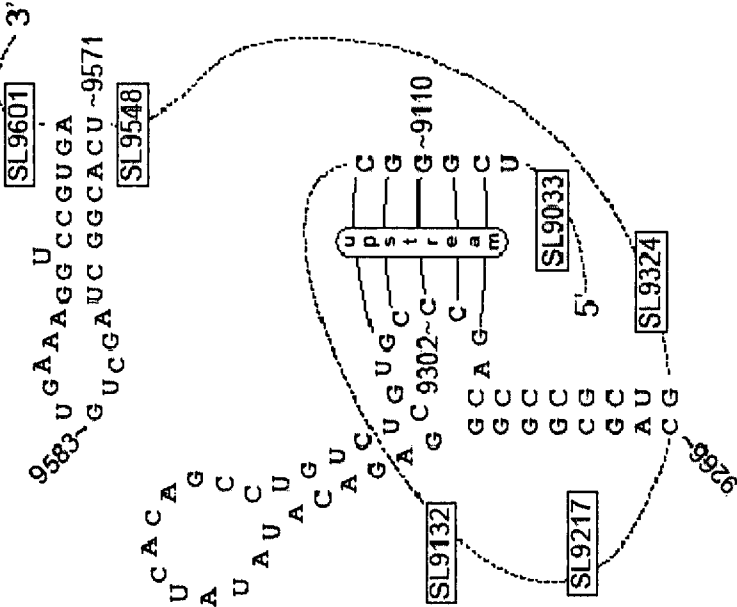


Fig. 2

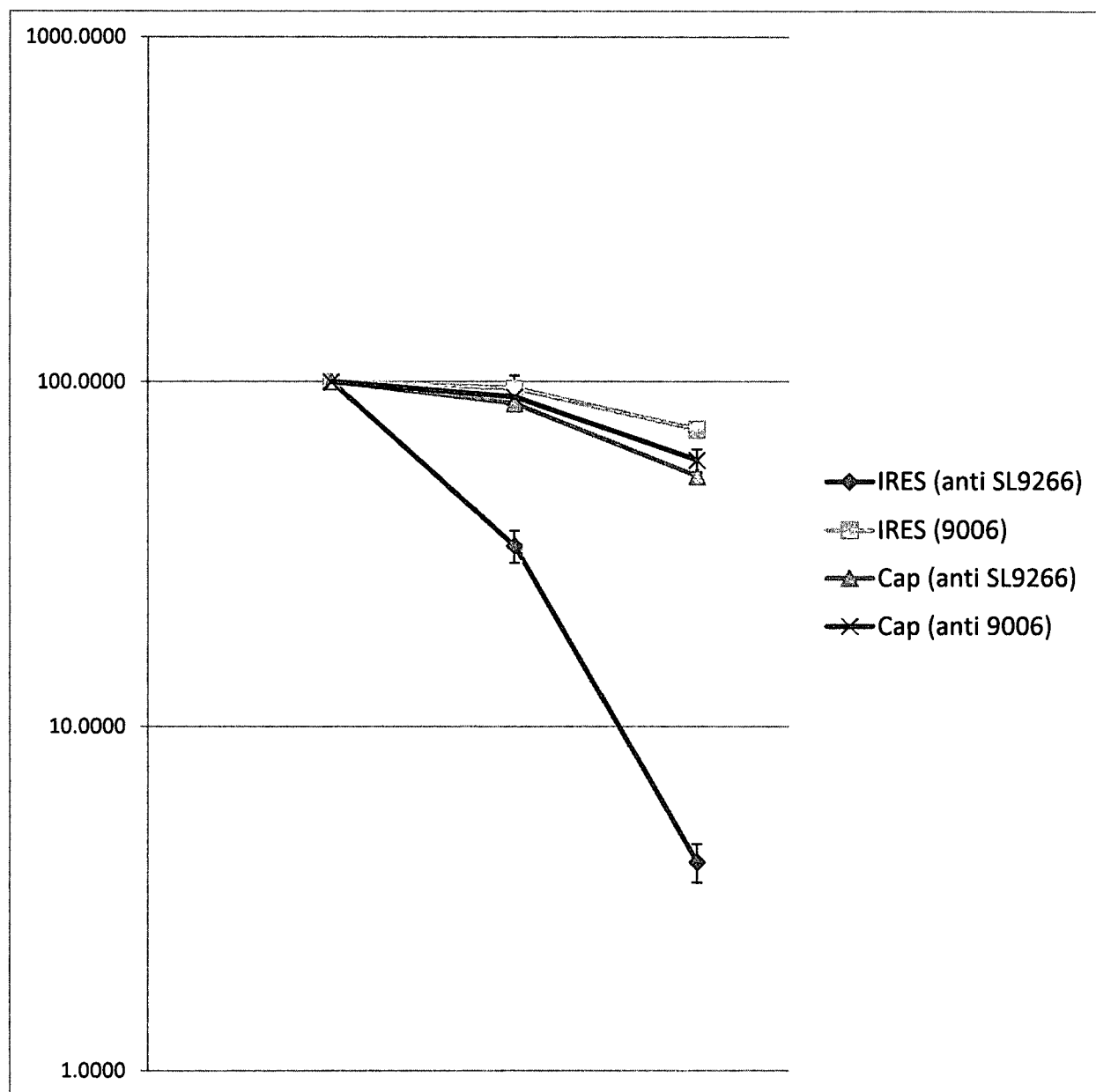


Fig. 3

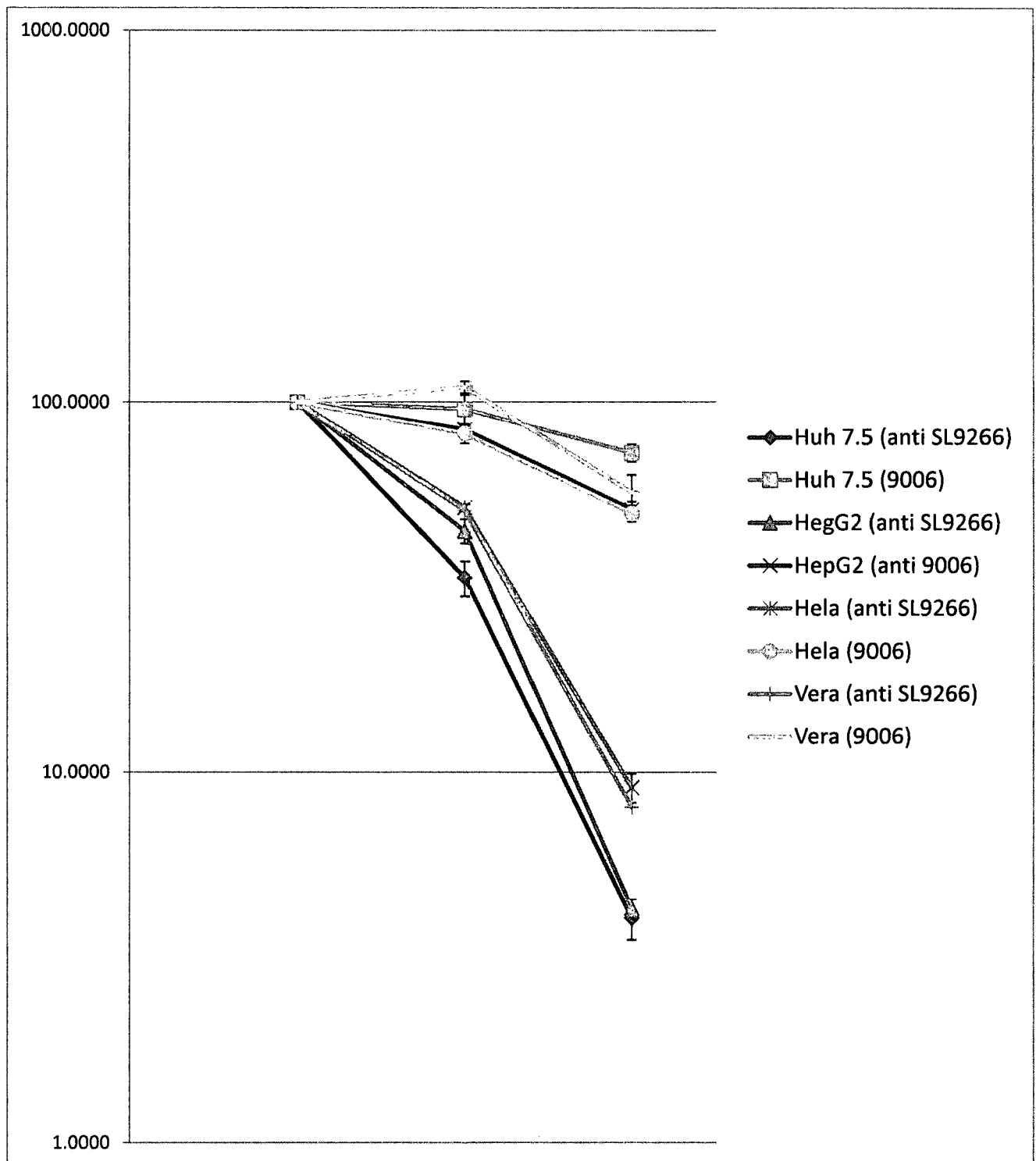


Fig. 4

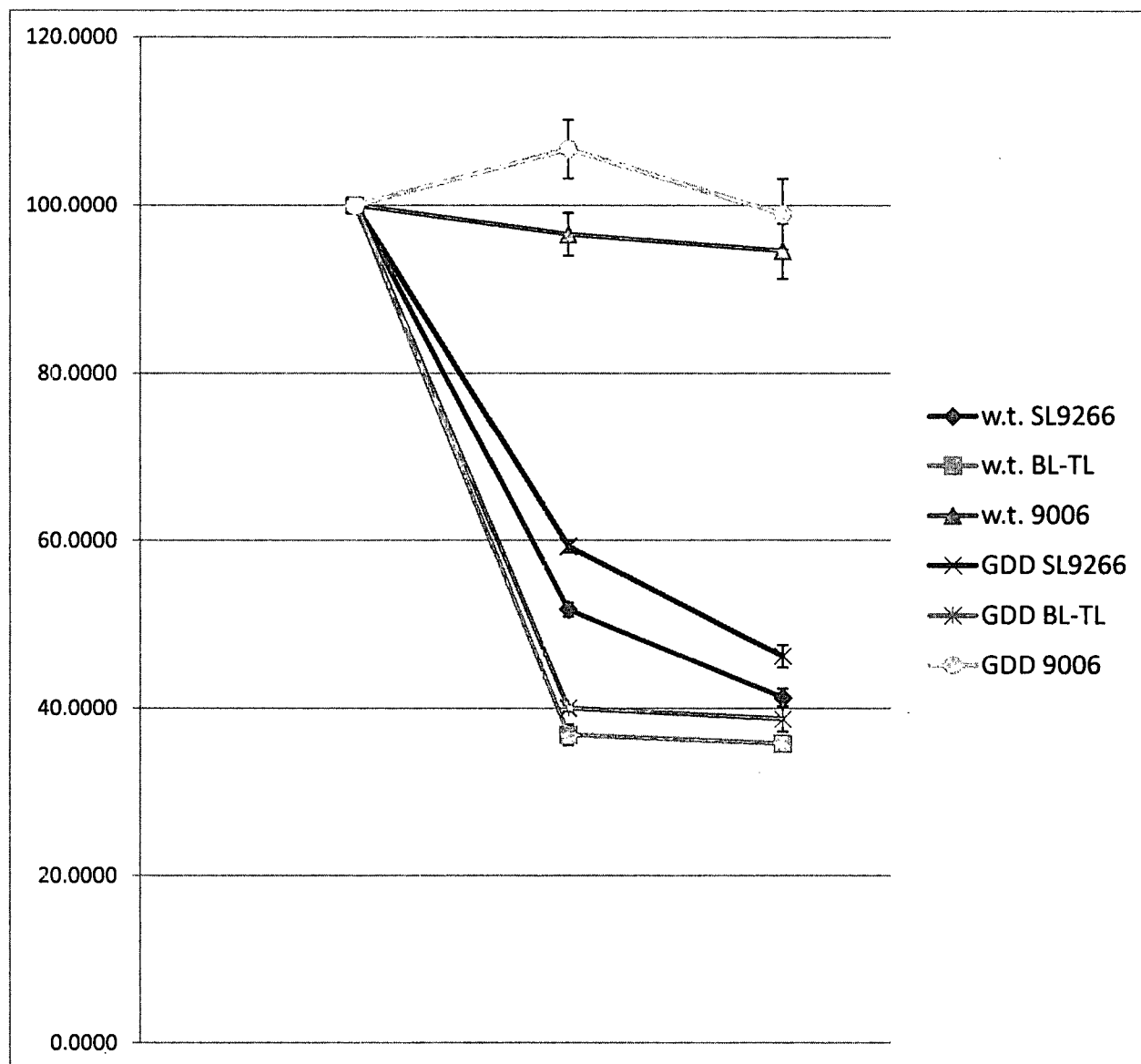
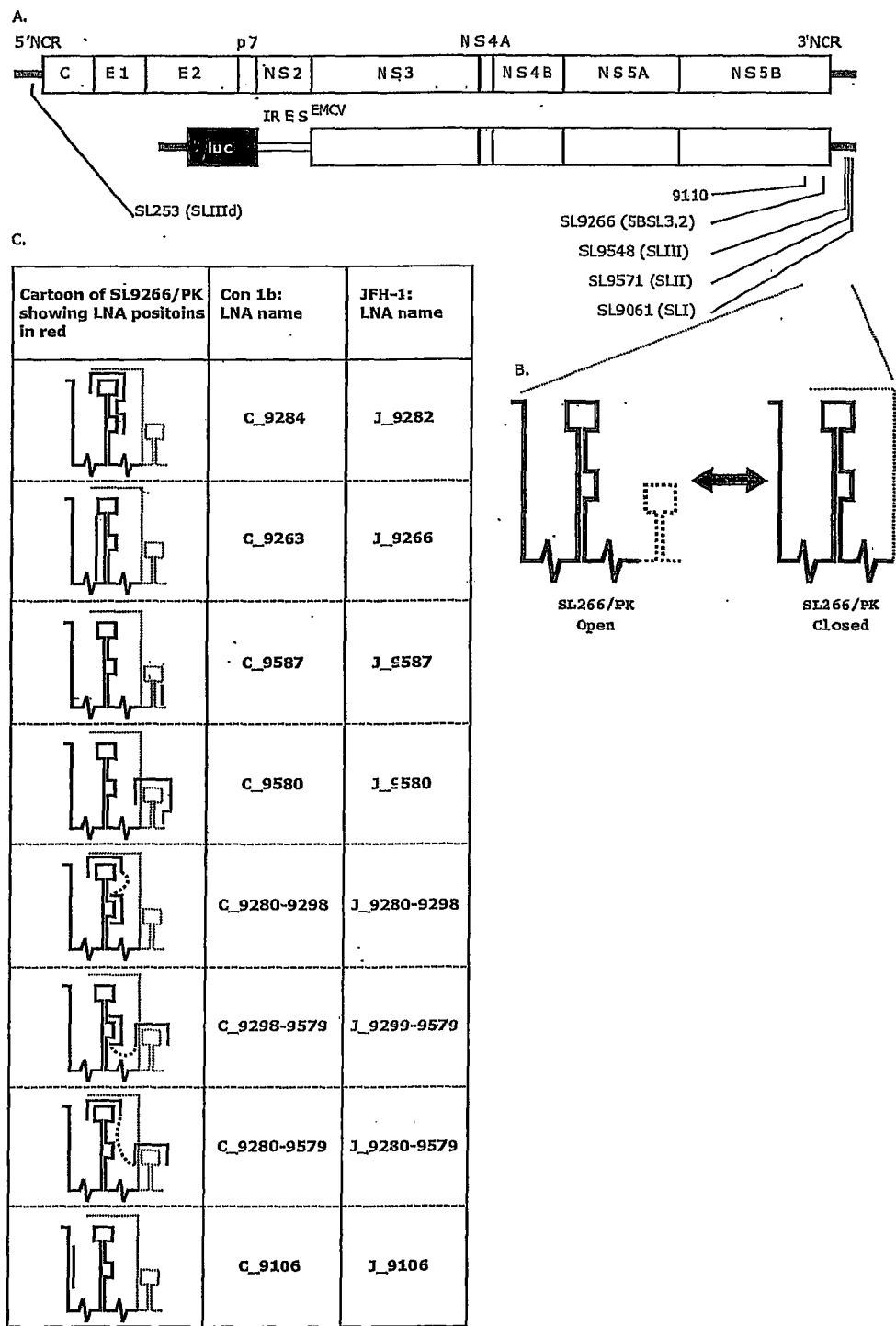


Fig. 5



Tuplin et al., Figure 1abc

Fig. 6

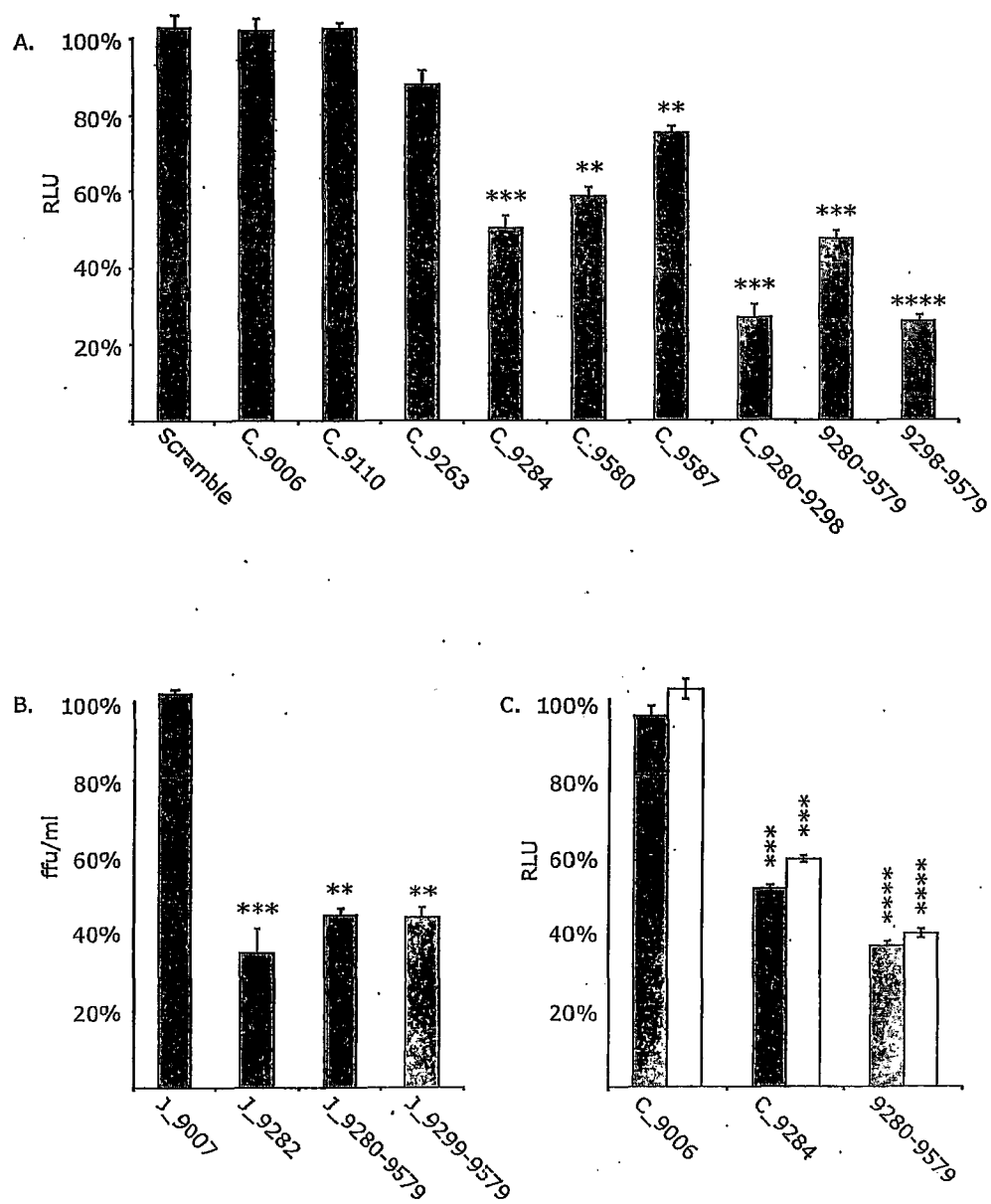


Fig. 7

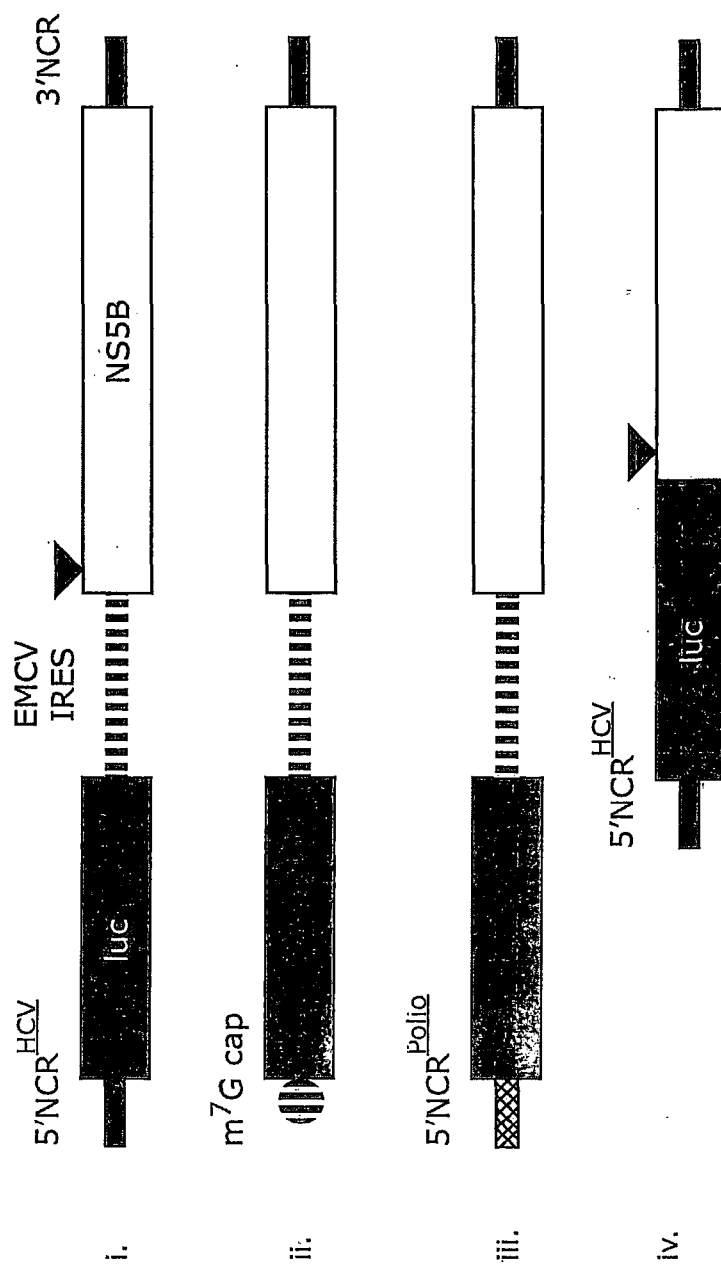
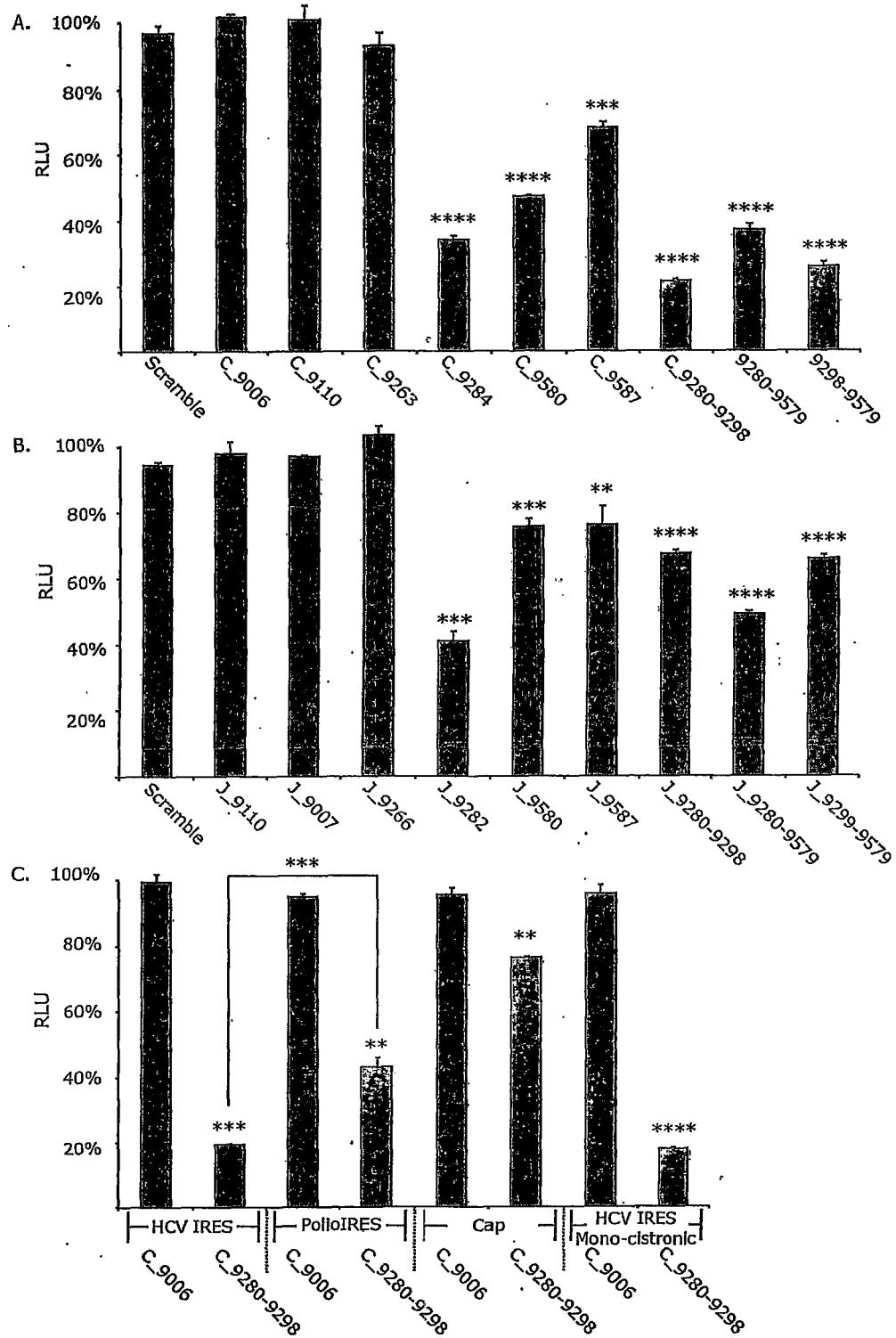


Fig. 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/050943

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/113 A61P31/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. MARTON ET AL: "RNA aptamer-mediated interference of HCV replication by targeting the CRE-5BSL3.2 domain", JOURNAL OF VIRAL HEPATITIS, vol. 20, no. 2, 17 July 2012 (2012-07-17), pages 103-112, XP055124416, ISSN: 1352-0504, DOI: 10.1111/j.1365-2893.2012.01629.x the whole document	1-21
X	WO 2013/027031 A1 (UNIV WARWICK [GB]; UNIV EDINBURGH [GB]; EVANS DAVID J [GB]; SIMMONDS P) 28 February 2013 (2013-02-28) cited in the application the whole document ----- -/-	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

26 June 2014

Date of mailing of the international search report

03/07/2014

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Piret, Bernard

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/050943

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. FRIEBE ET AL: "Kissing-Loop Interaction in the 3' End of the Hepatitis C Virus Genome Essential for RNA Replication", JOURNAL OF VIROLOGY, vol. 79, no. 1, 13 December 2004 (2004-12-13), pages 380-392, XP055125408, ISSN: 0022-538X, DOI: 10.1128/JVI.79.1.380-392.2005 the whole document	1-14, 16-21
A	----- SOLEDAD MARTON ET AL: "Anti-HCV RNA Aptamers Targeting the Genomic cis-Acting Replication Element", PHARMACEUTICALS, vol. 5, no. 12, 28 December 2011 (2011-12-28), pages 49-60, XP055124399, DOI: 10.3390/ph5010049	1-21
A	----- A. TUPLIN ET AL: "A twist in the tail: SHAPE mapping of long-range interactions and structural rearrangements of RNA elements involved in HCV replication", NUCLEIC ACIDS RESEARCH, vol. 40, no. 14, 4 May 2012 (2012-05-04), pages 6908-6921, XP055043436, ISSN: 0305-1048, DOI: 10.1093/nar/gks370 cited in the application	1-21
A	----- S. SHETTY ET AL: "Hepatitis C virus RNA: molecular switches mediated by long-range RNA-RNA interactions?", NUCLEIC ACIDS RESEARCH, vol. 41, no. 4, 28 December 2012 (2012-12-28), pages 2526-2540, XP055124415, ISSN: 0305-1048, DOI: 10.1093/nar/gks1318 -----	1-21

