Title: COMBINATIONS OF ANTI-HCV-ENTRY FACTOR ANTIBODIES AND INTERFERONS FOR THE TREATMENT AND THE PREVENTION OF HCV INFECTION

Abstract: The present invention provides combinations for use in the treatment or the prevention of HCV infection. In particular, combinations are provided that comprise at least one anti-HCV-entry factor antibody and at least one interferon, wherein the anti-HCV-entry factor antibody and interferon act in a highly synergistic manner to inhibit HCV infection of susceptible cells. Also provided are pharmaceutical compositions and kits comprising such combinations and methods of using these compositions and kits for treating or preventing HCV infection.
Combinations of Anti-HCV-Entry Factor Antibodies and Interferons for the Treatment and the Prevention of HCV Infection

Related Patent Application


Background of the Invention


Chronic HCV infection is the leading indication for liver transplantations (Seeff et al, Hepatology, 2002, 36: 1-2). Unfortunately, liver transplantation is not a cure for hepatitis C; viral recurrence being an invariable problem and the leading cause of graft loss (Brown, Nature, 2005, 436: 973-978; Watt et al, Am. J. Transplant, 2009, 9: 1707-1713). No vaccine protecting against HCV is yet available. Current therapies include administration of ribavirin and/or interferon-alpha (IFN-α), two non-specific anti-viral agents. Using a combination treatment of pegylated IFN-α and ribavirin, persistent clearance is achieved in about 50% of patients with genotype 1 chronic hepatitis C. However, a large number of patients have contraindications to one of the components of the combination; cannot tolerate the treatment; do not respond to
interferon therapy at all; or experience a relapse when administration is stopped. In


Cross-neutralizing antibodies inhibiting HCV entry have been shown to be associated with control of HCV infection and prevention of HCV re-infection in cohorts with self-limited acute infection (Osburn et al, Gastroenterology, 2009, 138: 315-324; Pestka et al, Proc. Natl. Acad. Sci. USA, 2007, 104: 6025-630). For example, monoclonal antibodies raised against native human SR-BI have been shown to inhibit HCV E2 binding to SR-BI and to efficiently block HCVcc infection of hepatoma cells in a dose-dependent manner (Catanese et al, J. Virol, 2007, 81: 8063-8071 ; WO 2006/005465). European patent application No. EP 1256 348 discloses substances, including antibodies, with antiviral effects that inhibit binding of HCV E2 and CD81. International patent application WO 2007/130646 describes in vitro and cell-based assays for identifying agents that interfere with HCV interactions with Claudin-1 thereby preventing HCV infection. The present Applicants have generated monoclonal antibodies that efficiently inhibit HCV infection by targeting host entry factor Claudin-1 (EP 08 305 597 and WO 2010/034812).

Recently, the present Applicants have demonstrated that blocking the activity of the newly discovered HCV entry co-factors, epidermal growth factor receptor (EGFR) and ephrin type-A receptor 2 (EphA2), using the approved kinase inhibitors, erlotinib...
and dasatinib respectively, broadly impaired infection by all major HCV genotypes and viral escape variants in vitro and in the human liver-chimeric Alb-uPA/SCID mouse model (Lupberger et al., Nature Medicine, 2011, 17: 589-595). Furthermore, the Applicants have shown that HCV entry is inhibited by antibodies directed against EGFR and EphA2.

Since the development of novel therapeutic approaches against HCV remains a high-priority goal, these studies are encouraging as they demonstrate that antibodies against receptor or co-receptors that affect HCV entry into susceptible cells may constitute an effective and safe alternative to current HCV therapies.

Summary of the Invention

The present invention relates to systems and improved strategies for the prevention and/or treatment of HCV infection and HCV-related diseases. More specifically, the present Applicants have demonstrated that interferon-alpha and an anti-claudin-1 antibody act in a highly synergistic manner on the inhibition of HCV infection (see Example 1). Similarly, they have shown that an anti-CD81 antibody or an anti-SRBI antibody in combination with interferon-alpha act in a highly synergistic manner to inhibit HCV infection (see Example 1). These results suggest that the combination of an interferon and an anti-HCV-entry factor antibody is an effective antiviral approach to prevent primary HCV infection, such as after liver transplantation, and might also restrain virus spread in chronically infected patients.

Consequently, in one aspect, the present invention provides a combination of at least one interferon and at least one anti-HCV-entry factor antibody for use in the treatment or prevention of HCV infection.

In preferred embodiments, the at least one interferon is a human interferon. The human interferon may be a natural human interferon, a recombinant human interferon, a synthetic version of a human interferon, or derivatives thereof.

In certain preferred embodiments, the at least one interferon of an inventive combination is selected from the group consisting of interferon-alpha (IFN-α), pegylated IFN-α, albumin-IFN-α, interferon-beta (IFN-β), pegylated IFN-β, albumin-IFN-β, interferon-omega (IFN-ω), pegylated IFN-ω, albumin-IFN-ω, interferon-gamma (IFN-γ), pegylated IFN-γ, albumin-IFN-γ, interferon-lambda (IFN-λ),...
pegylated IFN-λ, albumin-IFN-λ, equivalents thereof, analogs thereof, derivatives thereof and any combination thereof. In certain preferred embodiments, the at least one interferon is interferon-alpha (IFN-α), pegylated IFN-cc, or albumin-IFN-ct.

In certain preferred embodiments, the at least one interferon of an inventive combination is IFN-ct-2a or IFN-ct-2b.

The anti-HCV-entry factor antibody of a combination according to the invention may be an antibody against any HCV receptor, co-receptor, entry factor or entry co-factor known in the art or an antibody directed against any cell surface protein involved in the HCV infection process. The anti-HCV-entry factor antibody may be a polyclonal antibody or a monoclonal antibody. Preferably, the anti-HCV-entry factor antibody is a monoclonal antibody. In certain embodiments, the anti-HCV-entry factor antibody is an antibody against a receptor selected from the group consisting of heparan sulfate, the LDL receptor, the tetraspanin CD81, the scavenger receptor class B type I (SR-BI), Occludin, Claudin-1 (CLDN1), and Niemann-Pick C1-like 1 cholesterol absorption receptor. In certain preferred embodiments, the anti-HCV-entry factor antibody is an anti-CLDN1 antibody, in particular a monoclonal anti-CLDN1 antibody such as those developed by the present Applicants and described in EP 08 305 597 and WO 2010/034812. In other preferred embodiments, the anti-HCV-entry factor antibody is an anti-CD81 antibody or an anti-SRBI antibody.

The anti-HCV-entry factor antibody of a combination according to the present invention may be a full (complete) antibody, or a biologically active fragment of such antibody (i.e., any fragment or portion of such an antibody that retains the ability of the antibody to interfere with HCV-host cells interactions, and/or to specifically bind to a HCV receptor, and/or to inhibit or block HCV entry into HCV-susceptible cells, and/or to reduce or prevent HCV infection of susceptible cells). Antibodies or fragments thereof that are suitable for use in a combination according to the present invention also include chimeric antibodies, humanized antibodies, de-immunized antibodies and antibody-derived molecules comprising at least one complementary determining region (CDR) from either a heavy chain or light chain variable region of an anti-HCV-entry factor antibody, including molecules such as Fab fragments, F(ab')₂ fragments, Fd fragments, Sc antibodies (single chain antibodies), diabodies, individual antibody light single chains, individual antibody heavy chains, chimeric
fusions between antibody chains and other molecules, and antibody conjugates, such as antibodies conjugated to a therapeutic agent, so long as these antibody-related molecules retain at least one biologically relevant property of the antibody from which it is "derived". The biologically relevant property may be the ability to interfere with HCV-host cells interactions, to specifically bind to an HCV receptor protein, to inhibit or block HCV entry into HCV-susceptible cells, and/or to reduce or prevent HCV infection of susceptible cells.

In a combination according to the present invention, the interferon and the anti-HCV-entry factor antibody act in a highly synergistic manner to inhibit HCV infection. In certain embodiments, the interferon decreases the IC$_{50}$ for the inhibition of HCV infection by the anti-HCV-entry factor antibody by a factor of at least 2 fold, preferably at least 20 fold, and more preferably at least 50 fold or more than 50 fold. In other embodiments, the anti-HCV-entry factor antibody decreases the IC$_{50}$ for the inhibition of HCV infection by the interferon by a factor of at least 2 fold, preferably at least 20 fold, and more preferably at least 50 fold or more than 50 fold. The combination index (CI) of the at least one interferon and at least one anti-HCV-entry factor antibody is lower than 1, preferably lower than 0.90, more preferably lower than 0.75, and even more preferably lower than 0.60.

The combinations of the present invention can find application in a variety of prophylactic and therapeutic treatments. Thus, the combinations are provided for use in the prevention of HCV infection of a cell (e.g., a susceptible cell or a population of susceptible cells); for preventing or treating HCV infection or a HCV-related disease in a subject; for controlling chronic HCV infection; and for preventing HCV recurrence in a liver transplantation patient. HCV infection may be due to HCV of a genotype selected from the group consisting of genotype 1, genotype 2, genotype 3, genotype 4, genotype 5, genotype 6 and genotype 7, or more specifically of a subtype selected from the group consisting of subtype 1a, subtype 1b, subtype 2a, subtype 2b, subtype 2c, subtype 3a, subtype 4a-f, subtype 5a, and subtype 6a.

In a related aspect, the present invention provides a method of reducing the likelihood of a susceptible cell of becoming infected with HCV as a result of contact with HCV, which comprises contacting the susceptible cell with an effective amount of an inventive combination, or a pharmaceutical composition thereof. Also provided
is a method of reducing the likelihood of a subject's susceptible cells of becoming infected with HCV as a result of contact with HCV, which comprises administering to the subject an effective amount of an inventive combination, or a pharmaceutical composition thereof. The present invention also provides a method of treating or preventing HCV infection or a HCV-associated disease (e.g., a liver disease or pathology) in a subject in need thereof, which comprises administering to the subject an effective amount of an inventive combination, or a pharmaceutical composition thereof. The invention also provides a method for controlling chronic HCV infection in a subject in need thereof, which comprises administering to the subject an effective amount of an inventive combination, or a pharmaceutical composition thereof.

Also provided is a method of preventing HCV recurrence in a liver transplantation patient, which comprises administering to the patient an effective amount of an inventive combination, or a pharmaceutical composition thereof. Administration of an inventive combination, or pharmaceutical composition to a subject may be by any suitable route, including, for example, parenteral, aerosol, oral and topical routes. The inventive combination may be administered alone or in combination with a therapeutic agent, such as an anti-viral agent.

The inventive combinations may be administered per se or as pharmaceutical compositions. Accordingly, in another aspect, the present invention provides for the use of an inventive combination for the manufacture of medicaments, pharmaceutical compositions, or pharmaceutical kits for the treatment and/or prevention of HCV infection and HCV-associated diseases.

In a related aspect, the present invention provides a pharmaceutical composition comprising an effective amount of an inventive combination (i.e., at least one interferon and at least one anti-HCV-entry factor antibody as described herein) and at least one pharmaceutically acceptable carrier or excipient. In certain embodiments, the pharmaceutical composition is adapted for administration in combination with an additional therapeutic agent, such as an antiviral agent. In other embodiments, the pharmaceutical composition further comprises an additional therapeutic agent, such as an antiviral agent. Antiviral agents suitable for use in methods and pharmaceutical compositions of the present invention include, but are not limited to, ribavirin, anti-HCV (monoclonal or polyclonal) antibodies, RNA polymerase inhibitors, protease
inhibitors, IRES inhibitors, helicase inhibitors, antisense compounds, ribozymes, entry inhibitors, micro-RNA antagonists, cytokines, therapeutic vaccines, NS5A antagonists, cyclophilin A antagonists, polymerase inhibitors, and any combination thereof.

These and other objects, advantages and features of the present invention will become apparent to those of ordinary skill in the art having read the following detailed description of the preferred embodiments.

**Brief Description of the Drawing**

**Figure 1** is a set of three graphs illustrating the synergistic effect of an anti-CLDN1 antibody (A), an anti-CD81 antibody (B), or an anti-SRBI antibody (C) and interferon-alpha on the inhibition of HCVcc infection. Huh7.5.1 cells were pre-incubated for 1 hour with serial concentrations of monoclonal anti-CLDN1 antibody (A), anti-CD81 antibody (B), or anti-SRBI antibody (C) (shown in μg/mL) or IFN-CC (0.1 IU/ml) or a combination of IFN-CC with a fixe concentration of the anti-HCV-entry factor monoclonal antibody (0.01 μg/mL). Huh7.5.1 cells were then incubated with HCVcc Luc-Jcl in the presence of both compounds. HCVcc infection was analyzed as described in Example 1. Data were expressed relative to HCVcc infection without compound. A nonrelated monoclonal isotype antibody served as negative control.

**Figure 2** is a set of graphs showing the antiviral activity of IFN-CC and entry inhibitors in combination. Huh7.5.1 cells were pre-incubated for 1 hour with serial concentrations of (A) IFN-a2a or (B) IFN-a2b and 0.01 μg/ml of receptor-specific (anti-CD81, anti-SRBI, anti-CLDN1) mAbs before incubation with HCVcc Luc-Jcl in the presence of both compounds. HCVcc infection was analyzed by luciferase reporter gene expression. The CI for an IC$_{50}$ was calculated and is indicated in Table 3. Dotted lines at combination values of 0.9 and 1.1 indicate the boundaries of an additive interaction.

**Figure 3** is a set of graphs showing results obtained for the combination of IFN-cc-2a or IFN-cc-2b anti-CLDN1 mAb, which resulted in the highest shift in the IC$_{50}$ of IFN-cc-2a or IFN-cc-2b, respectively. (A-B) Means ± SEM from at least three independent experiments performed in triplicate are shown. (C-D) Synergy was
confirmed using the method of Prichard and Shipman. One representative experiment is shown.

Definitions

Throughout the specification, several terms are employed that are defined in the following paragraphs.

As used herein, the term "subject" refers to a human or another mammal (e.g., primate, dog, cat, goat, horse, pig, mouse, rat, rabbit, and the like), that can be the host of Hepatitis C virus (HCV), but may or may not be infected with the virus, and/or may or may not suffer from a HCV-related disease. Non-human subjects may be transgenic or otherwise genetically modified animals. In many embodiments of the present invention, the subject is a human being. In such embodiments, the subject is often referred to as an "individuaF. The term "individual" does not denote a particular age, and thus encompasses newborns, children, teenagers, and adults.

As used herein, the term "HCV" refers to any major HCV genotype, subtype, isolate and/or quasispecies. HCV genotypes include, but are not limited to, genotypes 1, 2, 3, 4, 5, 6 and 7; HCV subtypes include, but are not limited to, subtypes la, lb, 2a, 2b, 2c, 3a, 4a-f, 5a and 6a.

The terms "afflicted with HCV" or "infected with HCV" are used herein interchangeably. When used in reference to a subject, they refer to a subject that has at least one cell which is infected by HCV. The term "HCV infection" refers to the introduction of HCV genetic information into a target cell, such as by fusion of the target cell membrane with HCV or an HCV envelope glycoprotein-positive cell.

The terms "HCV-related disease" and "HCV-associated disease" are herein used interchangeably. They refer to any disease or disorder known or suspected to be associated with and/or caused, directly or indirectly, by HCV. HCV-related (or HCV-associated) diseases include, but are not limited to, a wide variety of liver diseases, such as subclinical carrier state of acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The term includes symptoms and side effects of any HCV infection, including latent, persistent and sub-clinical infections, whether or not the infection is clinically apparent.
The term "treatment" is used herein to characterize a method or process that is aimed at (1) delaying or preventing the onset of a disease or condition (e.g., HCV infection or HCV-related disease); (2) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease or condition; (3) bringing about amelioration of the symptoms of the disease or condition; or (4) curing the disease or condition. A treatment may be administered prior to the onset of the disease or condition, for a prophylactic or preventive action. Alternatively or additionally, a treatment may be administered after initiation of the disease or condition, for a therapeutic action.

A "pharmaceutical composition" is defined herein as comprising an effective amount of a combination of the invention, and at least one pharmaceutically acceptable carrier or excipient.

As used herein, the term "effective amount" refers to any amount of a compound, agent, antibody, combination or composition that is sufficient to fulfil its intended purpose(s), e.g., a desired biological or medicinal response in a cell, tissue, system or subject. For example, in certain embodiments of the present invention, the purpose(s) may be: to prevent HCV infection, to prevent the onset of a HCV-related disease, to slow down, alleviate or stop the progression, aggravation or deterioration of the symptoms of a HCV-related disease (e.g., chronic hepatitis C, cirrhosis, and the like); to bring about amelioration of the symptoms of the disease, or to cure the HCV-related disease.

The term "pharmaceutically acceptable carrier or excipient" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredient(s) and which is not significantly toxic to the host at the concentration at which it is administered. The term includes solvents, dispersion, media, coatings, antibacterial and antifungal agents, isotonic agents, and adsorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art (see for example "Remington's Pharmaceutical Sciences", E.W. Martin, 18th Ed., 1990, Mack Publishing Co.: Easton, PA, which is incorporated herein by reference in its entirety).

The term "antibody", as used herein, refers to any immunoglobulin (i.e., an intact immunoglobulin molecule, an active portion of an immunoglobulin molecule,
etc.) that binds to a specific epitope. The term encompasses monoclonal antibodies and polyclonal antibodies. All derivatives and fragments thereof, which maintain specific binding ability, are also included in the term. The term also encompasses any protein having a binding domain, which is homologous or largely homologous to an immunoglobulin-binding domain. These proteins may be derived from natural sources, or partly or wholly synthetically produced.

The term "**specific binding**", when used in reference to an antibody, refers to an antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity of at least $1 \times 10^7 \text{M}^{-1}$, and binds to the predetermined antigen with an affinity that is at least two-fold greater than the affinity for binding to a non-specific antigen *(e.g., BSA, casein)*.

The term "**human Claudin-1** or human **CLDN1**" refers to a protein having the sequence shown in NCBI Accession Number NP_066924, or any naturally occurring variants commonly found in HCV permissive human populations. The term "**extracellular domain**" or "**ectodomain**" of Claudin-1 refers to the region of the Claudin-1 sequence that extends into the extracellular space.

The term "**human SR-BI**" refers the scavenger receptor class B member 1, a protein having the sequence shown in NCBI Accession Number NP_005496.4, or any naturally occurring variants commonly found in HCV permissive human populations. The term "**extracellular domain**" or "**ectodomain**" of human SR-BI refers to the region of the SR-BI sequence that extends into the extracellular space *(i.e., the space outside a cell)*.

The term "**human CD81**" refers the Cluster of Differentiation 81), a protein having the sequence shown in NCBI Accession Number NP_004347.1.

The terms "**susceptible cell**" and "**HCV-susceptible cell**" are used interchangeably. They refer to any cell that may be infected with HCV. Susceptible cells include, but are not limited to, liver or hepatic cells, primary cells, hepatoma cells, CaCo2 cells, dendritic cells, placental cells, endometrial cells, lymph node cells, lymphoid cells (B and T cells), peripheral blood mononuclear cells, and monocytes/macrophages.
The term "preventing, inhibiting or blocking HCV infection" when used in reference to an inventive combination means reducing the amount of HCV genetic information introduced into a susceptible cell or susceptible cell population as compared to the amount of HCV genetic information that would be introduced in the absence of the combination.

The term “isolated”, as used herein in reference to a protein or polypeptide, means a protein or polypeptide, which by virtue of its origin or manipulation is separated from at least some of the components with which it is naturally associated or with which it is associated when initially obtained. By "isolated", it is alternatively or additionally meant that the protein or polypeptide of interest is produced or synthesized by the hand of man.

The terms "protein", "polypeptide", and "peptide" are used herein interchangeably, and refer to amino acid sequences of a variety of lengths, either in their neutral (uncharged) forms or as salts, and either unmodified or modified by glycosylation, side-chain oxidation, or phosphorylation. In certain embodiments, the amino acid sequence is a full-length native protein. In other embodiments, the amino acid sequence is a smaller fragment of the full-length protein. In still other embodiments, the amino acid sequence is modified by additional substituents attached to the amino acid side chains, such as glycosyl units, lipids, or inorganic ions such as phosphates, as well as modifications relating to chemical conversions of the chains such as oxidation of sulphydryl groups. Thus, the term "protein" (or its equivalent terms) is intended to include the amino acid sequence of the full-length native protein, or a fragment thereof, subject to those modifications that do not significantly change its specific properties. In particular, the term "protein" encompasses protein isoforms, i.e., variants that are encoded by the same gene, but that differ in their pi or MW, or both. Such isoforms can differ in their amino acid sequence (e.g., as a result of allelic variation, alternative splicing or limited proteolysis), or in the alternative, may arise from differential post-translational modification (e.g., glycosylation, acylation, phosphorylation).

The term "analog", as used herein in reference to a protein, refers to a polypeptide that possesses a similar or identical function as the protein but need not necessarily comprise an amino acid sequence that is similar or identical to the amino
acid sequence of the protein or a structure that is similar or identical to that of the protein. Preferably, in the context of the present invention, a protein analog has an amino acid sequence that is at least 30%, more preferably, at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the amino acid sequence of the protein.

The term "fragment" or the term "portion", as used herein in reference to a protein, refers to a polypeptide comprising an amino acid sequence of at least 5 consecutive amino acid residues (preferably, at least about: 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250 or more amino acid residues) of the amino acid sequence of a protein. The fragment of a protein may or may not possess a functional activity of the protein.

The term "biologically active", as used herein to characterize a protein variant, analog or fragment, refers to a molecule that shares sufficient amino acid sequence identity or homology with the protein to exhibit similar or identical properties to the protein. For example, in many embodiments of the present invention, a biologically active fragment of an inventive antibody is a fragment that retains the ability of the antibody to bind to a HCV receptor.

The term "homologous" (or "homology"), as used herein, is synonymous with the term "identity" and refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in both compared sequences is occupied by the same base or same amino acid residue, the respective molecules are then homologous at that position. The percentage of homology between two sequences corresponds to the number of matching or homologous positions shared by the two sequences divided by the number of positions compared and multiplied by 100. Generally, a comparison is made when two sequences are aligned to give maximum homology. Homologous amino acid sequences share identical or similar amino acid sequences. Similar residues are conservative substitutions for, or "allowed point mutations" of, corresponding amino acid residues in a reference sequence. "Conservative substitutions" of a residue in a reference sequence are substitutions that are physically or functionally similar to the corresponding reference residue, e.g. that have a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the

The terms "approximately" and "about", as used herein in reference to a number, generally include numbers that fall within a range of 10% in either direction of the number (greater than or less than the number) unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

Detailed Description of Certain Preferred Embodiments

As mentioned above, the present invention provides combinations and methods for the treatment and prevention of HCV infection.

I - Combinations

A combination according to the invention comprises at least one interferon and at least anti-HCV-entry factor antibody, and is intended for use in the treatment or the prevention of HCV infection.

A. Anti-HCV-Entry Factor Antibodies

The term "anti-HCV-entry factor antibody", as used herein, refers to any antibody raised against a HCV receptor or co-receptor or HCV entry factor or cofactor (or a region of a HCV (co)-receptor or entry (co)-cofactor). The term also refers to any antibody directed against a cell surface protein involved in the HCV infection, in particular in HCV entry into susceptible cells. Examples of such HCV receptors or cell surface proteins include heparan sulfate, the LDL receptor (Agnello et al, Proc. Natl. Acad. Sci. USA, 1999, 96: 12766-12771; Molina et al, J. Hepatol, 2007, 46: 411-419), the tetraspanin CD81, the scavenger receptor class B type I (SR-BI), Occludin, Claudin-1 (CLDN1) or Niemann-Pick Cl-like 1 cholesterol absorption receptor.

Thus, anti-HCV-entry factor antibodies that are suitable for use in the practice of the present invention include antibodies against a HCV receptor selected from the group consisting of heparan sulfate, the LDL receptor, CD81, SR-BI, Occludin,
CLDN1, and specific regions thereof. In certain preferred embodiments, the anti-HCV-entry factor antibodies are antibodies against CD81, SR-BI or CLDN1 (or specific regions thereof).

Examples of anti-heparan sulfate antibodies that can be used in the practice of the present invention include, but are not limited to, the antibodies described or used in Kurup et al, J. Biol. Chem., 2007, 282: 21032-21042; Briani et al, J. Neurol. Sci., 2005, 229-230; U.S. Pat. Appln. No. 2009/0136964.

Examples of anti-LDL receptor antibodies that can be used in the practice of the present invention include, but are not limited to, the antibodies described or used in Agnello et al, Proc. Natl. Acad. Sci. USA, 1999, 96: 12766-12771; WO 01/68710; WO 2002/048388; U.S. Pat. Appln. No. US 2008/0213287, and antibodies commercially available, for example, from Amersham International (e.g., Clone C7).

Examples of anti-occludin antibodies that can be used in the practice of the present invention include, but are not limited to, the antibodies described or used in Tokunaga et al, J. Histochem. Cytochem., 2007, 55: 735-744.

Examples of anti-CD81 antibodies that can be used in the practice of the present invention include, but are not limited to, the antibodies described or used in Meuleman et al, Hepatology, 2008, 48: 1761-1769; Dijkstra et al, Exp. Neurol, 2006, 202: 57-66; Azorsa et al, J. Immunol. Methods, 1999, 229: 35-48.

Examples of anti-SR-BI antibodies that can be used in the practice of the present invention include, but are not limited to, the antibodies described or used in Haberstroh et al, Gastroenterology, 2008, 135: 1719-1728; Barth et al, J. Virol, 2008, 82: 3466-3479; Zeisel et al, Hepatology, 2007, 46: 1722-1731; Catanese et al, J. Virol, 2007, 81: 8063-8071; WO 2006/005465.

Examples of anti-CLDN1 antibodies that can be used in the practice of the present invention include, in particular, the polyclonal and monoclonal anti-CLDN1 antibodies that are disclosed in EP 08 305 597 and WO 2010/034812. As described in these documents, eight monoclonal antibodies have been produced by genetic immunization and shown to efficiently inhibit HCV infection by targeting the extracellular domain of CLDN1. Using an infectious HCV model system and primary human hepatocytes, these monoclonal anti-CLDN1 antibodies have been
demonstrated to efficiently inhibit HCV infection of all major genotypes as well as highly variable HCV quasispecies in individual patients. Furthermore, these antibodies efficiently blocked entry of highly infectious HCV escape variants that were resistant to neutralizing antibodies in six patients with HCV re-infection during liver transplantation. The monoclonal anti-CLDN1 antibodies are called OM-4A4-D4, OM-7C8-A8, OM-6D9-A6, OM-7D4-C1, OM-6E1-B5, OM-3E5-B6, OM-8A9-A3, and OM-7D3-B3. Other suitable anti-CLDN1 antibodies are monoclonal antibodies secreted by any one of the hybridoma cell lines deposited by the Applicants at the DSMZ (Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH, InhoffenstraBe 7 B, 38124 Braunschweig, Germany) on July 29, 2008 under Accession Numbers DSM ACC2931, DSM ACC2932, DSM ACC2933, DSM ACC2934, DSM ACC2935, DSM ACC2936, DSM ACC2937, and DSM ACC2938 (described in EP 08 305 597 and WO 2010/034812).

Other suitable anti-CLDN1 antibodies include those disclosed in European Pat. No. EP 1 167 389 and U.S. Pat. No. 6,627,439.

The anti-HCV-entry factor antibodies suitable for use in the present invention may be polyclonal antibodies or monoclonal antibodies. In certain preferred embodiments, the anti-HCV-entry factor antibody present in an inventive combination is a monoclonal antibody.

Anti-HCV-entry factor antibodies may be prepared by any suitable method known in the art. For example, an anti-HCV-entry factor monoclonal antibody may be prepared by recombinant DNA methods. These methods generally involve isolation of the genes encoding the desired antibody, transfer of the genes into a suitable vector, and bulk expression in a cell culture system. The genes or DNA encoding the desired monoclonal antibody may be readily isolated and sequenced using conventional procedures (e.g., using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). Hybridoma cell lines may serve as a preferred source of such DNA. Suitable host cells for recombinant production of antibodies include, but are not limited to, appropriate mammalian host cells, such as CHO, HeLa, or CV1. Suitable expression plasmids include, without limitation, pcDNA3.1 Zeo, pIND(SPl), pREP8 (all commercially available from Invitrogen, Carlsbad, CA, USA), and the like. The
antibody genes may be expressed via viral or retroviral vectors, including MLV-based vectors, vaccinia virus-based vectors, and the like. Cells may be grown using standard methods, in suitable culture media such as, for example, DMEM and RPMI-1640 medium. The anti-HCV-entry factor antibodies may be expressed as single chain antibodies. Isolation and purification of recombinantly produced antibodies may be performed by standard methods. For example, an anti-HCV-entry factor monoclonal antibody may be recovered and purified from cell cultures by protein A purification, ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, such as Protein A column, hydroxylapatite chromatography, lectin chromatography, or any suitable combination of these methods. High performance liquid chromatography (HPLC) can also be employed for purification.

Alternatively, an anti-HCV-entry factor antibody of a combination according to the present invention may be obtained from commercial sources.

In certain embodiments, an anti-HCV-entry factor antibody is used in its native form. In other embodiments, it may be truncated (e.g., via enzymatic cleavage or other suitable method) to provide immunoglobulin fragments or portions, in particular, fragments or portions that are biologically active. Biologically active fragments or portions of an anti-HCV-entry factor antibody include fragments or portions that retain the ability of the antibody to interfere with HCV-host cells interactions, and/or to specifically bind to the HCV-receptor, and/or to inhibit or block HCV entry into susceptible cells, and/or to reduce or prevent HCV infection of susceptible cells.

A biologically active fragment or portion of an anti-HCV-entry factor antibody may be an Fab fragment or portion, an F(\(ab\))\(_2\) fragment or portion, a variable domain, or one or more CDRs (complementary determining regions) of the antibody. Alternatively, a biologically active fragment or portion of an anti-HCV-entry factor antibody may be derived from the carboxyl portion or terminus of the antibody protein and may comprise an Fc fragment, an Fd fragment or an Fv fragment.

Antibody fragments of the present invention may be produced by any suitable method known in the art including, but not limited to, enzymatic cleavage
(e.g., proteolytic digestion of intact antibodies) or by synthetic or recombinant techniques. F(ab')2, Fab, Fv and ScFv (single chain Fv) antibody fragments can, for example, be expressed in and secreted from mammalian host cells or from E. coli. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

Anti-HCV-entry factor antibodies (or fragments thereof) suitable for use in a combination according to the present invention may be produced in a modified form, such as a fusion protein (i.e., an immunoglobulin molecule or portion linked to a polypeptide entity). Preferably, the fusion protein retains the biological property of the antibody. A polypeptide entity to be fused to an anti-HCV-entry factor antibody, or a fragment thereof, may be selected to confer any of a number of advantageous properties to the resulting fusion protein. For example, the polypeptide entity may be selected to provide increased expression of the recombinant fusion protein. Alternatively or additionally, the polypeptide entity may facilitate purification of the fusion protein, for example, by acting as a ligand in affinity purification. A proteolytic cleavage site may be added to the recombinant protein so that the desired sequence can ultimately be separated from the polypeptide entity after purification. The polypeptide entity may also be selected to confer an improved stability to the fusion protein, when stability is a goal. Examples of suitable polypeptide entities include, for example, polyhistidine tags, that allow for the easy purification of the resulting fusion protein on a nickel chelating column. Glutathione-S-transferase (GST), maltose B binding protein, or protein A are other examples of suitable polypeptide entities.

Depending on the use intended, an anti-HCV-entry factor antibody of a combination of the invention may be re-engineered so as to optimize stability, solubility, in vivo half-life, or ability to bind additional targets. Genetic engineering approaches as well as chemical modifications to accomplish any or all of these changes in properties are well known in the art. For example, the addition, removal, and/or modification of the constant regions of an antibody are known to play a particularly important role in the bioavailability, distribution, and half-life of
therapeutically administered antibodies. The antibody class and subclass, determined by the Fc or constant region of the antibody (which mediates effector functions), when present, imparts important additional properties.

Additional fusion proteins of the invention may be generated through the techniques of DNA shuffling well known in the art (see, for example, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458).

Anti-HCV-entry factor antibodies suitable for use in a combination according to the present invention may also be "humanized": sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site-directed mutagenesis of individual residues or by grafting of entire regions or by chemical synthesis. Humanized antibodies can also be produced using recombinant methods. In the humanized form of the antibody, some, most or all of the amino acids outside the CDR regions are replaced with amino acids from human immunoglobulin molecules, while some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they do not significantly modify the biological activity of the resulting antibody. Suitable human "replacement" immunoglobulin molecules include IgGl, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgA, IgM, IgD or IgE molecules, and fragments thereof.

Alternatively, the T-cell epitopes present in rodent antibodies can be modified by mutation (de-immunization) to generate non-immunogenic rodent antibodies that can be applied for therapeutic purposes in humans (see www.accurobio.com).

Anti-HCV-entry factor antibodies (or biologically active variants or fragments thereof) suitable for use in a combination according to the invention may be functionally linked (e.g., by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities. Methods for the preparation of such modified antibodies (or conjugated antibodies) are known in the art (see, for example, "Affinity Techniques. Enzyme Purification: Part B", Methods in Enzymol., 1974, Vol. 34, Jakoby and Wilneck (Eds.), Academic Press: New York, NY; and Wilchek and Bayer, Anal. Biochem., 1988, 171: 1-32). Preferably, molecular entities are attached at positions on the antibody molecule that do not
interfere with the binding properties of the resulting conjugate, e.g., positions that do not participate in the specific binding of the antibody to its target.

The antibody molecule and molecular entity may be covalently, directly linked to each other. Or, alternatively, the antibody molecule and molecular entity may be covalently linked to each other through a linker group. This can be accomplished by using any of a wide variety of stable bifunctional agents well known in the art, including homofunctional and heterofunctional linkers.

In certain embodiments, an anti-HCV-entry factor antibody (or a biologically active fragment thereof) of a combination of the present invention is conjugated to a therapeutic moiety. Any of a wide variety of therapeutic moieties may be suitable for use in the practice of the present invention including, without limitation, cytotoxins (e.g., cytostatic or cytocidal agents), therapeutic agents, and radioactive metal ions (e.g., alpha-emitters and alpha-emitters attached to macrocyclic chelators such as DOTA). Cytotoxins or cytotoxic agents include any agent that is detrimental to cells. Examples include, but are not limited to, paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, thymidine kinase, endonuclease, RNAse, and puromycin and fragments, variants or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioea chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin and doxorubicin), antibiotics (e.g., dactinomycin, bleomycin, mithramycin, and anthramycin), and anti-mitotic agents (e.g., vincristine and vinblastine). Combinations comprising the resulting antibody conjugate may find application in the treatment of liver cancer associated with HCV infection (see below).

Other therapeutic moieties include proteins or polypeptides possessing a desired biological activity. Such proteins include, but are not limited to, toxins (e.g., abrin, ricin A, alpha toxin, pseudomonas exotoxin, diphtheria toxin, saporin, momordin, ...
gelonin, pokeweed antiviral protein, alpha-sarcin and cholera toxin); proteins such as tumor necrosis factor, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; apoptotic agents (e.g., TNF-α, TNF-β) or, biological response modifiers (e.g., lymphokines, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), or other growth factors).

Thus, an inventive combination of the present invention may comprise anti-HCV-entry factor antibodies under the form of full length antibodies, biologically active variants or fragments thereof, chimeric antibodies, humanized antibodies, and antibody-derived molecules comprising at least one complementary determining region (CDR) from either a heavy chain or light chain variable region of an anti-HCV-entry factor antibody, including molecules such as Fab fragments, (Fab')₂ fragments, Fd fragments, Fabc fragments, Sc antibodies (single chain antibodies), diabodies, individual antibody light single chains, individual antibody heavy chains, chimeric fusions between antibody chains and other molecules, and antibody conjugates, such as antibodies conjugated to a therapeutic agent.

B. Interferons

The combinations of the present invention comprise at least one anti-HCV-entry factor and at least one interferon. The terms "interferon", "IFN" and "interferon molecule" are used herein interchangeably. They refer to any interferon or interferon derivative (e.g., pegylated interferon) that can be used in the prevention or treatment of HCV infection and/or in the prevention or treatment of HCV-related diseases, in particular cirrhosis and liver cancer.

Interferons are a family of cytokines produced by eukaryotic cells in response to viral infection and other antigenic stimuli, which display broad-spectrum antiviral, antiproliferative and immunomodulatory effects. Recombinant forms of interferons have been widely applied in the treatment of various conditions and diseases, such as viral infections (e.g., HCV, HBV and HIV), inflammatory disorders and diseases (e.g., multiple sclerosis, arthritis, cystic fibrosis), and tumors (e.g., liver cancer, lymphomas, myelomas, etc…).

Interferons are classified as Type I, Type II and Type III, depending on the cell receptor to which they bind. Type I interferons bind to a specific cell surface receptor
complex known as the IFN-a receptor (IFNAR) that consists of two chains (IFNAR1 and IFNAR2). The type I interferons present in humans are interferon-alpha (IFN-α), interferon-beta (IFN-β) and interferon-omega (IFN-ω). Treatments based on the use of IFN-α or pegylated IFN-α remain the cornerstone of therapy for chronic HCV infection. A new form of IFN-α with an extended in vivo half-life, albumin-interferon or albinterferon (a recombinant formulation of IFN-α genetically fused to the human blood protein albumin), has been developed. Albumin-interferon has been shown to exhibit high antiviral activity and to offer safety/tolerability comparable to the current standard of care and fared well in phase III clinical trials in patients with chronic HCV infection (Zeuzem et al, Gastroenterology, 2010, 139: 1257-1266; Nelson et al, Gastroenterology, 2010, 139: 1267-1276). Interferon-β has also been demonstrated to display antiviral activity against HCV and to be useful in the treatment of HCV infection, alone or in combination with ribavirin (Fukutomi et al, J. Hepatology, 2001, 34: 100-107; Sang Hoon Ahn et al, Gut and Liver, 2009, 3: 20-25). Pegylated IFN-β is currently undergoing clinical testing in Japan for HCV patients who do not respond well to the conventional combination therapy of ribavirin and IFN-α (Toray Industries Inc., Press Release, Feb. 2009). Interferon-co also exhibits antiviral properties (Buckwold et al, Antiviral Res., 2007, 73: 118-125) and has been tested for anti-HCV effect using an implantable infusion pump for the continuous delivery and consistent dose of drug for 3 to 12 months (Rohloff et al, J. Diabetes Sci. Technol, 2008, 2: 461-467).

Type II interferons bind to the interferon-gamma receptor (IFNGR). The only type II interferon is interferon-gamma (IFN-γ). IFN-γ does have anti-viral and anti-tumor effects, however these effects are weaker when compared to IFN-α.

Type III interferons signal through a receptor complex consisting of the interferon-lambda receptor (IFNLR1 or CRF2-12) and the interleukin 10 receptor 2 (IL10R2 or CRF2-4). In humans, type III interferons include three interferon lambda (IFN-λ) proteins referred to as IFN-λ1, IPN-λ2 and IPN-λ3 also known as interleukin 29 (IL29), interleukin 28A (IL28A) and interleukin 28B (IL28B), respectively. It has recently been reported (European Association for the Study of Liver, 46th Annual Meeting, April 2011) that pegylated IFN-λ showed virological responses superior to
the standard of care, when tested in patients infected with HCV genotypes 1 or 4 and was better tolerated and safer.

Therefore, in certain embodiments, the at least one interferon molecule present in a combination according to the invention is selected from the group consisting of IFN-cc, IFN-β, IFN-co, IFN-γ, IFN-λ, analogs thereof and derivatives thereof. In certain preferred embodiments, the interferon present in the combination is selected from the group consisting of IFN-cc, analogs thereof and derivatives thereof. In other preferred embodiments, the interferon present in the combination is selected from the group consisting of IFN-CO, analogs thereof and derivatives thereof. In yet other preferred embodiments, the interferon present in the combination is selected from the group consisting of IFN-λ, analogs thereof and derivatives thereof.

As used herein, the terms "interferon", "IFN", and "IFN molecule" more specifically refer to a peptide or protein having an amino acid substantially identical (e.g., at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% identical) to all or a portion of the sequence of an interferon (e.g., a human interferon), such as IFN-cc, IFN-β, IFN-CO, IFN-γ, and IFN-λ that are known in the art. Interferons suitable for use in the present invention include, but are not limited to, natural human interferons produced using human cells, recombinant human interferons produced from mammalian cells, E. coli-produced recombinant human interferons, synthetic versions of human interferons and equivalents thereof. Other suitable interferons include consensus interferons which are a type of synthetic interferons having an amino acid sequence that is a rough average of the sequence of all the known human IFN subtypes (for example, all the known IFN-cc subtypes, or all the known IFN-β subtypes, or all the known IFN-CO subtypes, or all the known IFN-γ subtypes, or all the known IFN-λ subtypes. In certain embodiments, interferons present in combinations according to the invention have been approved for human use. In other embodiments, interferons present in combinations according to the present are undergoing human clinical trials.

The terms "interferon", "IFN", and "IFN molecule" also include interferon derivatives, i.e., molecules of interferon (as described above) that have been modified or transformed. A suitable transformation may be any modification that imparts a desirable property to the interferon molecule. Examples of desirable properties
include, but are not limited to, prolongation of *in vivo* half-life, improvement of therapeutic efficacy, decrease of dosing frequency, increase of solubility/water solubility, increase of resistance against proteolysis, facilitation of controlled release, and the like. As mentioned above, pegylated interferons have been produced (*e.g.*, pegylated IFN-a) and are currently used to treat hepatitis. Pegylated interferons exhibit longer half-lives, which allows for less frequent administration of the drug. Pegylating an interferon molecule involves covalently binding the interferon to polyethylene glycol (PEG), an inert, non-toxic and biodegradable organic polymer. Therefore, in certain embodiments, the at least one interferon present in a combination according to the invention is a pegylated interferon. Interferons have also been produced as fusion proteins with human albumin (*e.g.*, albumin-IFN-Cc). The albumin-fusion platform takes advantage of the long half-life of human albumin to provide a treatment that allows the dosing frequency of IFN to be reduced in individuals with chronic hepatitis C. Therefore, in certain embodiments, the at least one interferon present in a combination according to the invention is an albumin-interferon fusion protein.

The terms "*alpha interferon*", "*interferon-alpha*", "*interferon-a*" and "IFN-a" are used herein interchangeably and refer to the family of highly homologous species-specific proteins (*i.e.*, glycoproteins) that are known in the art and inhibit viral replication and cellular proliferation, and modulate immune response. Typical IFN-a molecules suitable for use in the present invention include, but are not limited to, recombinant IFN-a-2b (such as INTRON-A® interferon available from Schering Corporation); recombinant IFN-a-2a (such as ROFERON® interferon available from Hoffman-La Roche); recombinant IFN-a-2C (such as BEROFOR® alpha 2 interferon available from Boehringer Ingelheim Pharmaceutical, Inc); IFN-a-nl, a purified blend of natural alpha interferons (such as SUMERIFERON® available from Sumitomo, Japan or WELLFERON® interferon alpha-nl (INS) available from Glaxo-Wellcome Ltd); IFN-a-n3, a mixture of natural alpha interferons (such as ALFERON® made by Interferon Sciences); human leukocyte interferon-a obtained from the leukocyte fraction of human blood following induction with Sendai virus (such as MULTIFERON®, available from Swedish Orphan Biovitrium, which contains several naturally occurring IFN-a subtypes); a consensus IFN-a (such as INFERGEN®,

interferon alfacon-1, available from Three Rivers Pharmaceuticals, LLC, and those described in U.S. Pat. Nos. 4,897,471; and equivalents thereof.

Other suitable interferon alpha molecules include IFN-CC derivatives, including, but not limited to, pegylated IFN-ct-2a (such as PEGASYS ® available from Hoffman-La Roche); pegylated IFN-ct-2b (such as PEGINTRON ® available from Schering Corporation); albumin IFN-ct-2b also known as albinterferon (such as ALBUFERON ® available from Human Genome Sciences), and equivalents thereof.

The terms "beta interferon", "interferon-beta", "interferon-β" and "IFN-β" are used herein interchangeably and refer to the family of highly homologous species-specific proteins (i.e., glycoproteins) that are known in the art and have the ability to induce resistance to viral antigens. Typical IFN-β molecules suitable for use in the present invention include, but are not limited to, recombinant IFN-β-la (such as, REBIF ® available from Pfizer or AVONEX ® available from Biogen Idec), recombinant IFN-p-lb (such as BETAFERON ®/BETASERON ® available from Bayer HealthCare or EXTAVIA ®, the generic form of BETAFERON, available from Novartis, or ZIFERON ®, an interferon-β 1b biosimilar, available from Zistdaru Danesh Ltd), IFN-β molecules described in U.S. Pat. Nos. 4,820,638 and 5,795,779 and equivalents thereof.

Other suitable interferon beta molecules include IFN-β derivatives, including, but not limited to, pegylated INF-β (such as TRK-560 being developed by Toray Industries, Inc.), pegylated IPN-β-la (such as BUBO17 being developed by Biogen Idec); pegylated IPN-β-lb (such as NU100 and NU400 being developed by Nuron Biotech); albumin- IFN-β fusion proteins such as those described in U.S. Pat. No. 7,572,437, and equivalents thereof.

The terms "omega interferon", "interferon-omega", "interferon-ω" and "IFN-ω" are used herein interchangeably and refer to the family of highly homologous species-specific proteins (i.e., glycoproteins) that are known in the art and have the ability to inhibit viral replication and cellular proliferation and modulate immune response. Typical IFN-CO molecules suitable for use in the present invention include, but are not limited to, IFN-CO described in European patent No. EPO 170 204, ITCA being developed by Intarcia Therapeutics, Inc., and equivalents thereof.
Other suitable interferon omega molecules include IFN-CO derivatives, including, but not limited to, pegylated INF-CO that can be obtained using a method described in U.S. Pat. Nos. 5,612,460; 5,711,944; 5,951,974 or 5,951,974; albumin-IFN-CO fusion proteins such as those described in U.S. Pat. No. 7,572,437, and equivalents thereof. The terms "gamma interferon", "interferon-gamma", "interferon-\(\gamma\)" and "IFN-\(\gamma\)" are used herein interchangeably and refer to the family of highly homologous species-specific proteins (\(i.e.,\) glycoproteins) that are known in the art and have the ability to induce resistance to certain viral antigens. Typical IFN-\(\gamma\) molecules suitable for use in the present invention include, but are not limited to, IFN-\(\gamma\) described in U.S. Pat. Nos. 4,727,138, 4,762,791, 4,845,196, 4,929,554, 5,574,137, and 5,690,925; interferon gamma 1b (such as ACTIMMUNE \(\text{®}\) available from InterMune, Inc.), and equivalents thereof.

Other suitable interferon omega molecules include IFN-\(\gamma\) derivatives, including, but not limited to, pegylated INF-\(\gamma\) that can be obtained using a method described in U.S. Pat. Nos. 5,612,460; 5,711,944; 5,951,974 or 5,951,974 albumin-IFN-CO fusion proteins such as those described in U.S. Pat. No. 7,572,437, and equivalents thereof. The terms "lambda interferon", "interferon-lambda", "interferon-\(\lambda\)" and "IFN-\(\lambda\)" are used herein interchangeably and refer to the family of highly homologous species-specific proteins (\(i.e.,\) glycoproteins) that are known in the art and have antiviral properties. Typical IFN-\(\lambda\) molecules suitable for use in the present invention include, but are not limited to, IFN-\(\lambda\)1, IPN-\(\lambda\)2 and IPN-\(\lambda\)3 molecules described in international patent applications number WO02/086087, WO2004/037995 and WO/2005/023862 and equivalents thereof.

Other suitable interferon omega molecules include IFN-\(\gamma\) derivatives including, but not limited to, pegylated IPN-\(\lambda\)-la (such as BMS-914143 being developed by Bristol-Myers Squibb), albumin-IFN-CO fusion proteins such as those described in U.S. Pat. No. 7,572,437, and equivalents thereof. The terms "interferon", "IFN", and "IFN molecule" also include interferon-like molecules, \(i.e.,\) molecules that have functional and/or structural features exhibited by or similar to known interferons or interferon analogs, such as those described above.
C. Properties of the Combinations

A combination according to the present invention is such that (1) it is intended for use in the treatment or the prevention of HCV infection and (2) the at least one anti-HCV-entry factor antibody and at least one interferon act in a highly synergistic manner on the inhibition of HCV infection.

In certain embodiments, the interferon decreases the IC₅₀ for the inhibition of HCV infection by the anti-HCV-entry factor antibody by a factor of at least 2 fold, preferably at least 20 fold, and more preferably at least 50 fold or more than 50 fold. In other words, in the presence of the interferon, the concentration of anti-HCV-entry factor antibody necessary to obtain a 50% inhibition of HCV entry is at least 2 times, preferably at least 20 times, more preferably at least 50 times or more than 50 times lower than the concentration of anti-HCV-entry factor antibody that would be necessary to obtain the same HCV entry inhibition in the absence of the interferon.

In other embodiments, the anti-HCV-entry factor antibody decreases the IC₅₀ for the inhibition of HCV infection by the interferon by a factor of at least 2 fold, preferably at least 20 fold, and more preferably at least 50 fold or more than 50 fold. In other words, in the presence of the anti-HCV-entry factor antibody, the concentration of interferon necessary to obtain a 50% inhibition of HCV entry is at least 2 times, preferably at least 20 times, more preferably at least 50 times or more than 50 times lower than the concentration of interferon that would be necessary to obtain the same HCV entry inhibition in the absence of anti-HCV-entry factor antibody.

In certain embodiments, a combination of the present invention is characterized by a combination index (CI) that is lower than 1 (which is defined as a marked synergy). A combination of the present invention is preferably characterized by a CI lower than 0.9, more preferably by a CI lower than 0.75, and even more preferably by a CI lower than 0.60.

II - Treatment or Prevention of HCV infection and HCV-associated Diseases

A. Indications

The combinations according to the present invention may be used in therapeutic and prophylactic methods to treat and/or prevent HCV infection, or to treat and/or
prevent a liver disease or a pathological condition affecting HCV-susceptible cells, such as liver cells, lymphoid cells, or monocytes/macrophages.

Methods of treatment of the present invention may be accomplished using an inventive combination or a pharmaceutical composition comprising an inventive combination (see below). These methods generally comprise administration of an effective amount of at least one anti-HCV-entry factor antibody and at least one interferon, or a pharmaceutical composition thereof, to a subject in need thereof. The anti-HCV-entry factor antibody and interferon may be administered concurrently (i.e., together or separately but at about the same time, e.g., within 5 minutes, 15 minutes or 30 minutes of each other), or alternatively, they may be administered sequentially (i.e., separately and at different times, e.g., different times of the same day or different times of the same week or different times of the same month, etc.).

Administration may be performed using any of the methods known to one skilled in the art. In particular, the combination of anti-HCV-entry factor antibody and interferon or a pharmaceutical composition thereof may be administered by various routes including, but not limited to, aerosol, parenteral, oral or topical route.

In general, the combination or pharmaceutical composition thereof will be administered in an effective amount, i.e. an amount that is sufficient to fulfill its intended purpose. The exact amount of the combination or pharmaceutical composition to be administered will vary from subject to subject, depending on the age, sex, weight and general health condition of the subject to be treated, the desired biological or medical response (e.g., prevention of HCV infection or treatment of HCV-associated liver disease), and the like. In many embodiments, an effective amount is one that inhibits or prevents HCV from entering into a subject's susceptible cells and/or infecting a subject's cells, so as to prevent HCV infection, treat or prevent liver disease or another HCV-associated pathology in the subject.

Combinations and pharmaceutical compositions of the present invention may be used in a variety of therapeutic or prophylactic methods. In particular, the present invention provides a method for treating or preventing a liver disease or pathology in a subject, which comprises administering to the subject an effective amount of at least one anti-HCV-entry factor antibody and at least one interferon (as defined above) (or pharmaceutical composition thereof) which inhibits HCV from entering or infecting
the subject's cells, so as to treat or prevent the liver disease or pathology in the subject. The liver disease or pathology may be inflammation of the liver, liver fibrosis, cirrhosis, and/or hepatocellular carcinoma (i.e., liver cancer) associated with HCV infection.

The present invention also provides a method for treating or preventing a HCV-associated disease or condition (including a liver disease) in a subject, which comprises administering to the subject an effective amount of at least one anti-HCV-entry factor antibody and at least one interferon (as defined above) (or pharmaceutical composition thereof) which inhibits HCV from entering or infecting the subject's cells, so as to treat or prevent the HCV-associated disease or condition in the subject. In certain embodiments of the present invention, the combination (or pharmaceutical composition thereof) is administered to a subject diagnosed with acute hepatitis C. In other embodiments of the invention, the combination (or pharmaceutical composition thereof) is administered to a subject diagnosed with chronic hepatitis C.

Administration of an inventive combination, or pharmaceutical composition, according to such methods may result in amelioration of at least one of the symptoms experienced by the individual including, but not limited to, symptoms of acute hepatitis C such as decreased appetite, fatigue, abdominal pain, jaundice, itching, and flu-like symptoms; symptoms of chronic hepatitis C such as fatigue, marked weight loss, flu-like symptoms, muscle pain, joint pain, intermittent low-grade fevers, itching, sleep disturbances, abdominal pain, appetite changes, nausea, diarrhea, dyspepsia, cognitive changes, depression, headaches, and mood swings; symptoms of cirrhosis such as ascites, bruising and bleeding tendency, bone pain, varices (especially in the stomach and esophagus), steatorrhea, jaundice and hepatic encephalopathy; and symptoms of extrahepatic manifestations associated with HCV such as thyroiditis, porphyria cutanea tarda, cryoglobulinemia, glomerulonephritis, sicca syndrome, thrombocytopenia, lichen planus, diabetes mellitus and B-cell lymphoproliferative disorders.

Alternatively or additionally, administration of a combination or pharmaceutical composition thereof according to such methods may slow down, reduce, stop or alleviate the progression of HCV infection or an HCV-associated disease, or reverse the progression to the point of eliminating the infection or disease. Administration of
a combination or pharmaceutical composition of the present invention according to such methods may also result in a reduction in the number of viral infections, reduction in the number of infectious viral particles, and/or reduction in the number of virally infected cells.

The effects of a treatment according to the invention may be monitored using any of the assays known in the art for the diagnosis of HCV infection and/or liver disease. Such assays include, but are not limited to, serological blood tests, liver function tests to measure one or more of albumin, alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST), and gamma glutamyl transpeptidase (GGT), and molecular nucleic acid tests using different techniques such as polymerase chain reaction (PCR), transcription mediated amplification (TMA), or branched DNA (bDNA).

Combinations and pharmaceutical compositions of the present invention may also be used in immunization therapies. Accordingly, the present invention provides a method of reducing the likelihood of susceptible cells of becoming infected with HCV as a result of contact with HCV. The method comprises contacting the susceptible cells with an effective amount of at least one anti-HCV-entry factor antibody and at least one interferon (as defined above) or a pharmaceutical composition thereof which inhibits HCV from entering or infecting the susceptible cells, so as to reduce the likelihood of the cells to become infected with HCV as a result of contact with HCV. The present invention also provides a method of reducing the likelihood of a subject's susceptible cells of becoming infected with HCV as a result of contact with HCV. In this method, contacting the susceptible cells with the combination or pharmaceutical composition may be performed by administrating the combination or a pharmaceutical composition thereof to the subject.

Reducing the likelihood of susceptible cells or of a subject of becoming infected with HCV means decreasing the probability of susceptible cells or a subject to become infected with HCV as a result of contact with HCV. The decrease may be of any significant amount, e.g., at least a 2-fold decrease, more than a 2-fold decrease, at least a 10-fold decrease, more than a 10-fold decrease, at least a 100-fold decrease, or more than a 100-fold decrease.
In certain embodiments, the subject is infected with HCV prior to administration of the inventive composition. In other embodiments, the subject is not infected with HCV prior to administration of the inventive composition. In yet other embodiments, the subject is not infected with, but has been exposed to, HCV. In certain embodiments, the subject may be infected with HIV or HBV.

For example, the methods of the present invention may be used to reduce the likelihood of a subject's susceptible cells of becoming infected with HCV as a result of liver transplant. As already mentioned above, when a diseased liver is removed from a HCV-infected patient, serum viral levels plummet. However, after receiving a healthy liver transplant, virus levels rebound and can surpass pre-transplant levels within a few days (Powers et al., Liver Transpl, 2006, 12: 207-216). Liver transplant patients may benefit from administration of a combination according the invention. Administration may be performed prior to liver transplant, during liver transplant, and/or following liver transplant.

Other subjects that may benefit from administration of a combination of anti-HCV-entry factor antibody and interferon according to the present invention include, but are not limited to, babies born to HCV-infected mothers, in particular if the mother is also HIV-positive; health-care workers who have been in contact with HCV-contaminated blood or blood contaminated medical instruments; drug users who have been exposed to HCV by sharing equipments for injecting or otherwise administering drugs; and people who have been exposed to HCV through tattooing, ear/body piercing and acupuncture with poor infection control procedures.

Other subjects that may benefit from administration of a combination according to the invention include, but are not limited to, subjects that exhibit one or more factors that are known to increase the rate of HCV disease progression. Such factors include, in particular, age, gender (males generally exhibit more rapid disease progression than females), alcohol consumption, HIV co-infection (associated with a markedly increased rate of disease progression), and fatty liver.

Still other subjects that may benefit from administration of a combination according to the invention include patients with HCV infections that are resistant to the standard of care or to other combinations of antivirals - antiviral resistance being a

In certain embodiments, a combination of an anti-HCV-entry factor antibody and an interferon or a composition thereof is administered alone according to a method of treatment of the present invention. In other embodiments, a combination of an anti-HCV-entry factor antibody and an interferon or a pharmaceutical composition thereof is administered in combination with at least one additional therapeutic agent. The combination or composition may be administered prior to administration of the therapeutic agent, concurrently with the therapeutic agent, and/or following administration of the therapeutic agent.

Therapeutic agents that may be administered in combination with an inventive combination or pharmaceutical composition may be selected among a large variety of biologically active compounds that are known to have a beneficial effect in the treatment or prevention of HCV infection, or a HCV-associated disease or condition. Such agents include, in particular, antiviral agents including, but not limited to, ribavirin, anti-HCV (monoclonal or polyclonal) antibodies, RNA polymerase inhibitors, protease inhibitors, IRES inhibitors, helicase inhibitors, antisense compounds, ribozymes, micro-RNA antagonists, cytokines, therapeutic vaccines, NS5A antagonists, polymerase inhibitors, cyclophilin A antagonists, and any combination thereof.

**B. Administration**

An inventive combination (optionally after formulation with one or more appropriate pharmaceutically acceptable carriers or excipients), in a desired dosage can be administered to a subject in need thereof by any suitable route. Various delivery systems are known and can be used to administer combinations of the present invention, including tablets, capsules, injectable solutions, encapsulation in liposomes, microparticles, microcapsules, etc. Methods of administration include, but are not limited to, dermal, intradermal, intramuscular, intraperitoneal, intralesional, intravenous, subcutaneous, intranasal, pulmonary, epidural, ocular, and oral routes. An inventive combination or composition may be administered by any convenient or other appropriate route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral, mucosa, rectal and intestinal
mucosa, etc). Administration can be systemic or local. Parenteral administration may be preferentially directed to the patient's liver, such as by catheterization to hepatic arteries or into a bile duct. As will be appreciated by those of ordinary skill in the art, in embodiments where the anti-HCV-entry factor antibody and interferon are administered sequentially (i.e., at different times or separately but at substantially the same time), the anti-HCV-entry factor antibody and interferon may be administered by the same route (e.g., intravenously) or by different routes (e.g., orally and intravenously). Similarly, in embodiments where an inventive combination is administered along with an additional therapeutic agent, the combination and therapeutic agent may be administered by the same route or different routes.

C. Dosage

Administration of an inventive combination (or a composition thereof) of the present invention will be in a dosage such that the amount delivered is effective for the intended purpose. The route of administration, formulation and dosage administered will depend upon the therapeutic effect desired, the severity of the HCV-related condition to be treated if already present, the presence of any infection, the age, sex, weight, and general health condition of the patient as well as upon the potency, bioavailability, and in vivo half-life of the anti-HCV-entry factor antibody and interferon used, the use (or not) of concomitant therapies, and other clinical factors. These factors are readily determinable by the attending physician in the course of the therapy. Alternatively or additionally, the dosage to be administered can be determined from studies using animal models (e.g., chimpanzee or mice). Adjusting the dose to achieve maximal efficacy based on these or other methods are well known in the art and are within the capabilities of trained physicians. As studies are conducted using the inventive combination of an anti-HCV-entry factor antibody and an interferon, further information will emerge regarding the appropriate dosage levels and duration of treatment.

A treatment according to the present invention may consist of a single dose or multiple doses. Thus, administration of an inventive combination, or composition thereof, may be constant for a certain period of time or periodic and at specific intervals, e.g., hourly, daily, weekly (or at some other multiple day interval), monthly, yearly (e.g., in a time release form). Alternatively, the delivery may occur at multiple
times during a given time period, e.g., two or more times per week; two or more times per month, and the like. The delivery may be continuous delivery for a period of time, e.g., intravenous delivery.

In general, the amount of combination administered will preferably be in the range of about 1 ng/kg to about 100 mg/kg body weight of the subject, for example, between about 100 ng/kg and about 50 mg/kg body weight of the subject; or between about 1 µg/kg and about 10 mg/kg body weight of the subject, or between about 100 µg/kg and about 1 mg/kg body weight of the subject.

III - Pharmaceutical Compositions

As mentioned above, a combination of the invention may be administered per se or as a pharmaceutical composition. Accordingly, the present invention provides pharmaceutical compositions comprising an effective amount of at least one anti-HCV-entry factor antibody and at least one interferon as described herein and at least one pharmaceutically acceptable carrier or excipient. In some embodiments, the composition further comprises one or more additional biologically active agents.

The combinations and pharmaceutical compositions thereof may be administered in any amount and using any route of administration effective for achieving the desired prophylactic and/or therapeutic effect. The optimal pharmaceutical formulation can be varied depending upon the route of administration and desired dosage. Such formulations may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the administered active ingredient.

The pharmaceutical compositions of the present invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "unit dosage form", as used herein, refers to a physically discrete unit of an anti-HCV-entry factor antibody or of an interferon or of both an anti-HCV-entry factor antibody and an interferon for the patient to be treated. It will be understood, however, that the total daily dosage of the compositions will be decided by the attending physician within the scope of sound medical judgement.

A. Formulation

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing
or wetting agents, and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 2,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solution or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid may also be used in the preparation of injectable formulations. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration.

Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use. Liquid pharmaceutical compositions which are sterile solutions or suspensions can be administered by, for example, intravenous, intramuscular, intraperitoneal or subcutaneous injection. Injection may be via single push or by gradual infusion. Where necessary or desired, the composition may include a local anesthetic to ease pain at the site of injection.

In order to prolong the effect of an active ingredient (i.e., a combination of an anti-HCV-entry factor antibody and an interferon), it is often desirable to slow the absorption of the ingredient from subcutaneous or intramuscular injection. Delaying absorption of a parenterally administered active ingredient may be accomplished by dissolving or suspending the ingredient in an oil vehicle. Injectable depot forms are made by forming micro-encapsulated matrices of the active ingredient in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of active ingredient to polymer and the nature of the particular polymer employed, the rate of ingredient release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations can also be prepared by entrapping the active ingredient in liposomes or microemulsions which are compatible with body tissues.

Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions,
syrups, elixirs, and pressurized compositions. In addition to the active principles, the liquid dosage form may contain inert diluents commonly used in the art such as, for example, water or other solvent, solubilising agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butyylene glycol, dimethylformamide, oils (in particular, cotton seed, ground nut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, and fatty acid esters of sorbitan and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, suspending agents, preservatives, sweetening, flavouring, and perfuming agents, thickening agents, colors, viscosity regulators, stabilizes or osmo-regulators. Examples of suitable liquid carriers for oral administration include water (potentially containing additives as above, e.g., cellulose derivatives, such as sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols such as glycols) and their derivatives, and oils (e.g., fractionated coconut oil and arachis oil). For pressurized compositions, the liquid carrier can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Solid dosage forms for oral administration include, for example, capsules, tablets, pills, powders, and granules. In such solid dosage forms, an inventive combination may be mixed with at least one inert, physiologically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and one or more of: (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; (b) binders such as, for example, carboxymethylcellulose, alginates, gelatine, polyvinylpyrrolidone, sucrose, and acacia; (c) humectants such as glycerol; (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (e) solution retarding agents such as paraffin; absorption accelerators such as quaternary ammonium compounds; (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate; (h) absorbents such as kaolin and bentonite clay; and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulphate, and mixtures thereof. Other excipients suitable for solid formulations include surface modifying agents such as non-ionic and anionic surface modifying agents. Representative examples of surface modifying agents include, but are not limited to,
poloxamer 188, benzalkonium chloride, calcium stearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, magnesium aluminum silicate, and triethanolamine. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatine capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition such that they release the active ingredient(s) only, or preferably, in a certain part of the intestinal tract, optionally, in a delaying manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

In certain embodiments, it may be desirable to administer an inventive composition locally to an area in need of treatment (e.g., the liver). This may be achieved, for example, and not by way of limitation, by local infusion during surgery (e.g., liver transplant), topical application, by injection, by means of a catheter, by means of suppository, or by means of a skin patch or stent or other implant.

For topical administration, the composition is preferably formulated as a gel, an ointment, a lotion, or a cream which can include carriers such as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oil. Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylenemonolaurat (5%) in water, or sodium lauryl sulphate (5%) in water. Other materials such as antioxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In addition, in certain instances, it is expected that the inventive compositions may be disposed within transdermal devices placed upon, in, or under the skin. Such devices include patches, implants, and injections which release the active ingredient by either passive or active release mechanisms. Transdermal administrations include all administration across the surface of the body and the inner linings of bodily
passage including epithelial and mucosal tissues. Such administrations may be carried out using the present compositions in lotions, creams, foams, patches, suspensions, solutions, and suppositories (rectal and vaginal).

Transdermal administration may be accomplished through the use of a transdermal patch containing an active ingredient \( i.e., \) a combination of an anti-HCV-entry factor antibody and of an interferon and a carrier that is non-toxic to the skin, and allows the delivery of the ingredient for systemic absorption into the bloodstream \textit{via} the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may be suitable. A variety of occlusive devices may be used to release the active ingredient into the bloodstream such as a semi-permeable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient.

Suppository formulations may be made from traditional materials, including cocoa butter, with or without the addition of waxes to alter the suppository's melting point, and glycerine. Water soluble suppository bases, such as polyethylene glycols of various molecular weights, may also be used.

When a pharmaceutical composition of the present invention is used as "vaccine" to prevent HCV-susceptible cells from becoming infected with HCV, the pharmaceutical composition may further comprise vaccine carriers known in the art such as, for example, thyroglobulin, albumin, tetanus toxoid, and polyamino acids such as polymers of D-lysine and D-glutamate. The vaccine may also include any of a variety of well known adjuvants such as, for example, incomplete Freund's adjuvant, alum, aluminium phosphate, aluminium hydroxide, monophosphoryl lipid A (MPL, GlaxoSmithKline), a saponin, CpG oligonucleotides, montanide, vitamin A and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol, Quil A, Ribi Detox, CRL-1005, L-121 and combinations thereof.

Materials and methods for producing various formulations are known in the art and may be adapted for practicing the subject invention. Suitable formulations for the
delivery of antibodies can be found, for example, in “Remington’s Pharmaceutical Sciences”, E.W. Martin, 18th Ed., 1990, Mack Publishing Co.: Easton, PA.

B. Additional Biologically Active Agents

In certain embodiments, an inventive combination (i.e., at least one anti-HCV-entry factor and at least one interferon) is the only active ingredient in a pharmaceutical composition of the present invention. In other embodiments, the pharmaceutical composition further comprises one or more biologically active agents. Examples of suitable biologically active agents include, but are not limited to, vaccine adjuvants and therapeutic agents such as anti-viral agents (as described above), anti-inflammatory agents, immunomodulatory agents, analgesics, antimicrobial agents, antibacterial agents, antibiotics, antioxidants, antiseptic agents, and combinations thereof.

In such pharmaceutical compositions, the anti-HCV-entry factor antibody, interferon and additional therapeutic agent(s) may be combined in one or more preparations for simultaneous, separate or sequential administration of the different components. More specifically, an inventive composition may be formulated in such a way that the anti-HCV-entry factor antibody, interferon and therapeutic agent(s) can be administered together or independently from one another. For example, an anti-HCV-entry factor antibody, an interferon and a therapeutic agent can be formulated together in a single composition. Alternatively, they may be maintained (e.g., in different compositions and/or containers) and administered separately.

C. Pharmaceutical Packs of Kits

In another aspect, the present invention provides a pharmaceutical pack or kit comprising one or more containers (e.g., vials, ampoules, test tubes, flasks or bottles) containing one or more ingredients of an inventive pharmaceutical composition, allowing administration of a combination of the present invention.

Different ingredients of a pharmaceutical pack or kit may be supplied in a solid (e.g., lyophilized) or liquid form. Each ingredient will generally be suitable as aliquoted in its respective container or provided in a concentrated form. Pharmaceutical packs or kits may include media for the reconstitution of lyophilized
ingredients. Individual containers of the kits will preferably be maintained in close confinement for commercial sale.

In certain embodiments, a pharmaceutical pack or kit includes one or more additional therapeutic agent(s) (*e.g.*, one or more anti-viral agents, as described above). Optionally associated with the container(s) can be a notice or package insert in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The notice of package insert may contain instructions for use of a pharmaceutical composition according to methods of treatment disclosed herein.

An identifier, *e.g.*, a bar code, radio frequency, ID tags, etc., may be present in or on the kit. The identifier can be used, for example, to uniquely identify the kit for purposes of quality control, inventory control, tracking movement between workstations, etc.

**Examples**

The following example describes some of the preferred modes of making and practicing the present invention. However, it should be understood that the examples are for illustrative purposes only and are not meant to limit the scope of the invention. Furthermore, unless the description in an Example is presented in the past tense, the text, like the rest of the specification, is not intended to suggest that experiments were actually performed or data are actually obtained.

**Example 1: Inhibition of HCVcc Infection Using an Inventive Combination**

**Materials and Methods**

**Cell Lines.** Cultures of Huh7.5.1 cells which have previously been described (Zhong et al., Proc. Natl. Acad. Sci. USA, 2005, 102: 9294-2929), were used in this study.

**Production of Anti-CLDN1 mAbs.** Anti-CLDN1 mAbs were raised by genetic immunization of Wistar rats using a eukaryotic expression vector encoding the full-length human CLDN1 cDNA as described in EP 08 305 597 and WO 2010/034812. Following completion of immunization, antibodies were selected by flow cytometry
for their ability to bind to human CLDN1 expressed on the cell surface of non-permeabilized HEK293T-BOSC23 cells and CHO cells which had been transfected with pCMV-SPORT6/CLDN1. In the present invention, anti-CLDN1 mAb OM-7D3-B3 was used.

Production of Anti-CD81 mAb and Anti-SRBI mAb. The Anti-human SRBI and anti-human CD81 antibodies were produced, as described for anti-CLDN1 antibodies (Fofana et al, Gastroenterology, 2010, 139: 953-964).

Interferon-alpha. Interferon-alpha-2a (Lupberger et al, Nature Medicine, 2011, 17: 589-595) was obtained from Roche.

HCVcc Production and Infection. Cell-culture derived HCVcc (Luc-Jcl) were generated as previously described (Koutsoudakis et al, J. Virol, 2006, 80: 5308-5320; Zeisel et al, Hepatology, 2007, 46: 1722-1731). For infection experiments, Huh7.5.1 cells were incubated with HCVcc infected as described previously (Fofana et al, Gastroenterology, 2010, 139: 953-964; Lupberger et al, Nature Medicine, 2011, 17: 589-595).

Combination Experiments. The anti-CLDN1 antibody, anti-CD81 antibody, anti-SRBI antibody and interferon-alpha were tested individually and in combination. More specifically, Huh7.5.1 cells were pre-incubated with the anti-HCV-entry factor antibody and with interferon-alpha for 1 hour. Huh7.5.1 cells were then infected with HCVcc in the presence of compounds. HCVcc infection was analyzed two days later by luciferase reporter gene expression as previously described (Krieger et al, Hepatology, 2010, 54: 1144-1157; Fofana et al, Gastroenterology, 2010, 139: 953-964; Koutsoudakis et al, J. Virol, 2006, 80: 5308-5320). The Combination Index (CI) was calculated as described (Zhao et al, Clin. Cancer Res., 2004, 10: 7994-8004). A CI of less than 1 indicates synergy; a CI equal to 1 indicates additivity; and a CI of more than 1 indicates antagonism.

Statistical Analysis. Results are expressed as means ± standard deviation (SD). Statistical analyses were performed using Student's t test with a P value of <0.05 being considered statistically significant.

Results and Discussion

The results obtained are presented on Figure 1 and in tables 1 and 2 below.
Table 1. IC₅₀ and CI for the combination studied on HCVcc infection.

<table>
<thead>
<tr>
<th>Interferon</th>
<th>anti-HCV-entry factor antibody</th>
<th>IC₅₀ (µg/mL)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>anti-CLDN1</td>
<td>0.28±0.09</td>
<td></td>
</tr>
<tr>
<td>Interferon-alpha (0.1 IU/mL)</td>
<td>anti-CLDN1</td>
<td>0.005±0.001</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>control</td>
<td>anti-CD81</td>
<td>0.02±0.003</td>
<td></td>
</tr>
<tr>
<td>Interferon-alpha (0.1 IU/mL)</td>
<td>anti-CD81</td>
<td>0.0055±0.0007</td>
<td>0.60±0.04</td>
</tr>
<tr>
<td>control</td>
<td>anti-SRBI</td>
<td>1.5±0.3</td>
<td></td>
</tr>
<tr>
<td>Interferon-alpha (0.1 IU/mL)</td>
<td>anti-SRBI</td>
<td>0.09±0.009</td>
<td>0.40±0.03</td>
</tr>
</tbody>
</table>

Table 2. IC₅₀ and CI for the combination studied on HCVcc infection.

<table>
<thead>
<tr>
<th>anti-HCV-entry factor antibody</th>
<th>Interferon</th>
<th>IC₅₀ (IU/mL)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>Interferon-alpha</td>
<td>0.3±0.16</td>
<td></td>
</tr>
<tr>
<td>anti-CLDN1 (0.01 µg/mL)</td>
<td>Interferon-alpha</td>
<td>0.03±0.01</td>
<td>0.14±0.05</td>
</tr>
<tr>
<td>anti-CD81 (0.01 µg/mL)</td>
<td>Interferon-alpha</td>
<td>0.03±0.001</td>
<td>0.60±0.05</td>
</tr>
<tr>
<td>anti-SRBI (0.01 µg/mL)</td>
<td>Interferon-alpha</td>
<td>0.08±0.04</td>
<td>0.30±0.03</td>
</tr>
</tbody>
</table>

As can be concluded from Figure 1 and Table 1, the combination of anti-HCV-entry factor antibody and interferon-alpha resulted in a synergistic activity in the inhibition of HCVcc infection, with a CI lower than 0.60. Furthermore, the presence of interferon-alpha in the combination decreased the IC₅₀ of the anti-CLDN1 antibody from 0.28 µg/mL to 0.005 µg/mL (i.e., by a factor of 56); the IC₅₀ of the anti-CD81 antibody from 0.02 µg/mL to 0.0055 µg/mL (i.e., by a factor of 3.6); and the IC₅₀ of the anti-SRBI antibody from 1.5 µg/mL to 0.09 µg/mL (i.e., by a factor of 16).

As can be concluded from Figure 2 and Table 2, the combination interferon-alpha and anti-HCV-entry factor antibody resulted in a synergistic activity in the inhibition of HCVcc infection, with a CI lower than 0.60. Furthermore, the presence of an anti-entry factor antibody in the combination decreased the IC₅₀ of interferon-alpha from 0.3 IU/mL to 0.03 IU/mL for anti-CLDN1 antibody (i.e., by a factor of 10), 0.03 IU/mL for anti-CD81 antibody (i.e., by a factor of 10) and 0.08 IU/mL for anti-SRBI antibody (i.e., by a factor of 3.75).

These results demonstrate that anti-HCV-entry factor antibodies in combination with interferons could provide a valuable alternative to current standard of care.
Example 2: Further Characterization in *in vitro* Models for HCV Infection

Following screening of combinations including other interferons using different HCV genotypes and model systems, combinations according to the invention will be further characterized by comparative analysis of neutralization in state-of-the-art *in vitro* models (Krieger *et al*., Hepatology, 2010, 51: 1144-1 157; Fofana *et al*., 2010, 139: 953-964).

Example 3: Characterization in an *in vivo* Model for HCV Infection

As a first step to evaluate the combinations according to the present invention, and establish the essential parameters for protection and treatment of HCV infection, the human liver-chimeric SCID/Alb-uPA mouse model will be used in a preclinical study. This model is a well characterized preclinical model for the *in vivo* assessment of antivirals. Pharmacokinetic and toxicity of selected combinations in uPA/SCID mice will be examined as previously described (Law *et al*., Nat. Med., 2008, 14: 25-27; Vanwolleghem *et al*., Hepatology, 2008, 47: 1846-1855). Briefly, transplanted SCID/Alb-uPA mice will be infected with HCV-infected human serum intravenously and the effect of combinations of the invention on viral load will be assessed. Treatment outcome will be evaluated clinically (toxicity), virologically (viral load), and morphologically (histopathology of transplanted hepatocytes and other tissues) as described recently (Vanwolleghem *et al*., Gastroenterology, 2007, 133: 1144-1 155). The safety profile will be further assessed in non human primates.

Example 4: Phase I/IIa Clinical Trials

Following completion of the studies in the uPA-SCID mouse model as well as toxicity studies in non human primates, clinical phase I/IIa trials will be initiated in HCV infected humans resistant or not eligible to standard of care using a longstanding collaboration of Inserm U748-University of Strasbourg with the Strasbourg Center for Clinical Investigation (CIC) at Strasbourg. Two study designs are required to assess safety and efficacy for prevention and treatment of HCV infection:

**Prevention of HCV infection in subjects undergoing liver transplantation.** The combinations will be evaluated for their ability to prevent the universal re-infection of the liver graft following liver transplantation by achieving a reduction in viral load (as
measured quantitatively by HCV RT-PCR) post-transplant by ≥ 1 log_{10} from the baseline value.

*Treatment of HCV infection in subjects chronically infected patients.* The combinations will be evaluated for their ability to achieve reduction in viral load by ≥ 1 log_{10} from the baseline value.

**Example 5: Further Investigations**

**Materials and Methods**

**Cell Lines.** Cultures of Huh7.5.1 (Zhong *et al.*, Proc. Natl. Acad. Sci. USA, 2005, 102: 9294-2929) and HEK293T (Pestka *et al.*, Proc. Natl. Acad. Sci. USA, 2007, 104: 6025-6030) cells, which have previously been described, were used in this study.

**Production of Monoclonal Antibodies.** Anti-CLDN1 mAbs (OM-7D3-B3) was prepared as described in Example 1. Anti-SR-BI and anti-CD81 mAbs were produced by DNA-immunization as described for anti-CLDN1 mAbs (Fofana *et al.*, Gastroenterology, 2010, 139: 953-964, 964.el-4).

**Interferons.** IFN-a-2a and IFN-a-2b were obtained from Roche and Merck, respectively.

**Analysis of Antiviral Activity of Compounds and Combinations on HCV Infection.** The *in vitro* antiviral activity of each compound was tested individually and in combination with a second compound using the HCVcc Huh7.5.1 cell culture described in Example 1. For combination of entry inhibitors (anti-CLDN1, anti-SR-BI, and anti-CD81) with IFN-ccs, Huh7.5.1 cells (cultured in 96-well-plates) were pre-incubated with IFN-ct-2a or IFN-α-2b and the entry inhibitor for 1 hour at 37°C before incubation for 4 hours at 37°C with HCVcc in the presence of both compounds.

For combinations of entry inhibitors with entry inhibitors, Huh7.5.1 cells were pre-incubated with both entry inhibitors or control reagent for 1 hour at 37°C. The mix was removed and Huh7.5.1 cells were incubated for 4 hours at 37°C with HCVcc in the presence of both compounds. Viral infection was analyzed by assessing luciferase activity as described in Example 1.

**Analysis of Synergy.** Synergy was assessed by two independent methods: the combination index as described in Example 1 and the method of Prichard and
Shipman (Zhao et al, Clin. Cancer Res., 2004, 10: 7994-8004; Prichard et al, Antiviral Res., 1990, 14: 181-205). A CI of less than 0.9 indicates synergy; a CI equal to 0.9-1.1 indicates additivity; and a CI of more than 1.1 indicates antagonism (Zhao et al, Clin. Cancer Res., 2004, 10: 7994-8004; Zhu et al, J. Infect. Dis., 2012, 205: 656-662). The method of Prichard and Shipman was applied as described (Prichard et al, Antiviral Res., 1990, 14: 181-205). Surface amplitudes > 20% above the zero plane indicate a synergistic effect, while surface amplitudes < 20% below the zero plane indicate antagonism. The validity of the assay and methods were confirmed by comparative analyses of combinations showing a non-synergistic effect.

Toxicity Assays. Huh7.5.1 cells and primary human hepatocytes isolated and cultured as described were incubated with the compounds for 48 hours (Krieger et al, Hepatology, 2010, 51: 1144-1157). Cytotoxic effects were analysed by the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described (Lupberger et al, Nat. Med., 2011, 17: 589-595). An anti-Fas antibody (10 µg/m) was used as a positive control.

Results

The results are presented on Figures 2-3 and Table 3.

Since IFN-a is the key component of standard-of-care, the Applicants investigated whether entry inhibitors could potentiate the antiviral activity of IFN-a. Thus, they investigated the effect of combining an entry inhibitor with IFN-a2a or IFN-a2b on inhibition of HCVcc infection. The antiviral effect of each molecule was tested alone or in combination to determine the combination index (CI) (Figure 2A-B). Combination of IFN-a2a or IFN-a2b with a sub-IC₅₀ concentration of CD81-, SRRB1 or CLDN1-specific mAbs - which exerts only minimal inhibitory effect on HCV infection - resulted in a synergistic activity in inhibition of HCVcc infection (CIs of 0.14 to 0.67) (Figure 2A-B and Table 3). In contrast, combination of IFN-a2a or IFN-a2b with sorafenib, a different kinase inhibitor, resulted in an antagonistic effect (CI of 1.32±0.02 and 1.34±0.07, respectively), demonstrating that the observed synergies are specific for the combinations and not related to technical issues of the model system.
Table 3. Synergy of entry inhibitors and IFN-a2a or IFN-a2b on inhibition of HCV infection. Huh7.5.1 cells were pre-incubated with serial concentrations of IFN-a2a or IFN-a2b and 0.01 µg/ml of receptor-specific (anti-CD81, anti-SRBI or anti-CLDN1) or respective isotype control mAbs for 1 hour at 37°C. The mix was removed and Huh7.5.1 cells were incubated for 4 hour at 37°C with HCVcc Luc-Jcl in the presence of both compounds. HCVcc infection was analyzed by luciferase reporter gene expression. The Combination Index (CI) was calculated. Means ± SD from at least three independent experiments performed in triplicate are shown. IC50 of receptor-specific mAbs: anti-CD81, 0.015±0.014 µg/ml; anti-SR-BI, 1.3±0.4 µg/ml; anti-CLDN1, 0.18±0.03 µg/ml.

<table>
<thead>
<tr>
<th>Compound 1</th>
<th>IC50 (UI/ml)</th>
<th>Compound 2</th>
<th>IC50 (UI/ml) for combination</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-a2a</td>
<td>0.3±0.16</td>
<td>anti-CD81</td>
<td>0.03±0.001</td>
<td>0.60±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-SR-BI</td>
<td>0.08±0.04</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-CLDN1</td>
<td>0.03±0.01</td>
<td>0.14±0.05</td>
</tr>
<tr>
<td>IFN-a2b</td>
<td>0.2±0.05</td>
<td>anti-CD81</td>
<td>0.035±0.01</td>
<td>0.67±0.05</td>
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<tr>
<td></td>
<td></td>
<td>anti-SR-BI</td>
<td>0.08±0.04</td>
<td>0.56±0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-CLDN1</td>
<td>0.02±0.05</td>
<td>0.16±0.05</td>
</tr>
</tbody>
</table>

The most potent combination was observed using CLDN1-specific mAb. Indeed the addition of a very low dose of CLDN1-specific antibody to IFN-a2a or IFN-a2b decreased their IC50s up to 10 fold for [from 0.3±0.16 to 0.03±0.01 IU/ml for IFN-a2a; and from 0.2±0.05 to 0.02±0.05 IU/ml for IFN-a2b] (Figure 3A-B). Synergy of low doses between IFN-a and CLDN1-specific mAbs was further confirmed using the method of Prichard and Shipman (Figure 3C-D).

Noteworthy, none of the combinations resulted in detectable toxic effects at the highest concentrations used in combination whereas pre-incubation of cells and anti-Fas antibody resulted in readily detectable toxicity (Table 4).

Taken together, these data demonstrate that entry inhibitors enhance the antiviral activity of IFN-a, and provide the perspective for improved efficacy of IFN-based therapies for HCV infection.
Table 4. Absence of toxicity of combinations of compounds in primary human hepatocytes (PHH). Cytotoxic effects on PHH using the highest concentrations of each compound used in combination (IFN-CC, 10 IU/ml and receptor-specific mAbs, 10 µg/ml) were assessed by analyzing the ability to metabolize MTT. Anti-Fas antibody (10 µg/ml) was used as a positive control of toxicity. Toxicity analyses of the most efficient combinations are shown. Data are presented as relative cell viability compared to PHH cultured in the absence of compounds or solvent (=100%). Means ± SD from one representative experiment performed in triplicate is shown.

<table>
<thead>
<tr>
<th>Compound 1</th>
<th>Concentration</th>
<th>Compound 2</th>
<th>Concentration</th>
<th>Relative cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-a2a</td>
<td>10 IU/ml</td>
<td>anti-CLDN 1</td>
<td>10 µg/ml</td>
<td>101±2</td>
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<tr>
<td>IFN-a2b</td>
<td>10 IU/ml</td>
<td>anti-CLDN 1</td>
<td>10 µg/ml</td>
<td>97±3</td>
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<tr>
<td>anti-Fas</td>
<td>10 µg/ml</td>
<td></td>
<td></td>
<td>16±2</td>
</tr>
</tbody>
</table>

Other Embodiments

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of the specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope of the invention being indicated by the following claims.
Claims

What is claimed is:

1. A combination comprising at least one anti-HCV-entry factor antibody and at least one interferon for use in the treatment or the prevention of HCV infection, wherein the at least anti-HCV-entry factor antibody and at least one interferon act in synergy to inhibit HCV infection.

2. The combination according to claim 1, wherein the at least one anti-HCV-entry factor antibody is a monoclonal antibody or a biologically active fragment thereof.

3. The combination according to claim 1 or claim 2, wherein the at least one anti-HCV-entry factor antibody is an antibody against a HCV receptor selected from the group consisting of heparan sulfate, LDL receptor, CD81, SR-BI, Occludin, Claudin-1, and Niemann-Pick CI-like 1 cholesterol absorption receptor or against a region of such a HCV receptor that is involved in HCV entry into susceptible cells.

4. The combination according to claim 3, wherein the at least one anti-HCV-entry factor antibody is an anti-Claudin 1 antibody that binds to the extracellular domain of Claudin 1 and is preferably a monoclonal antibody selected from the group consisting of OM-4A4-D4, OM-7C8-A8, OM-6D9-A6, OM-7D4-C1, OM-6E1-B5, OM-3E5-B6, OM-8A9-A3, OM-7D3-B3 and any biologically active fragment thereof that binds Claudin 1 extracellular domain.

5. The combination according to any one of claims 2 to 4, wherein the monoclonal antibody is humanized, de-immunized or chimeric.

6. The combination according to any one of claims 1 to 5, wherein the at least one interferon is a human interferon.

7. The combination according to claim 6, wherein the at least one interferon is a human interferon selected from the group consisting of interferon-alpha (IFN-α), pegylated IFN-α, albumin-IFN-α, interferon-beta (IFN-β), pegylated IFN-β...
β, albumin-INF-β, interferon-omega (IFN-ω), pegylated IFN-ω, albumin-INF-ω, interferon-gamma (IFN-γ), pegylated IFN-γ, albumin-INF-γ, interferon-lambda (IFN-λ), pegylated IFN-λ, albumin-INF-λ, equivalents thereof, and combinations thereof.

8. The combination according to claim 7, wherein the at least one interferon is IFNcc-2a or IFNcc-2b.

9. The combination according any of claims 1 to 8, wherein the combination index (CI) of the combination is lower than 1, preferably lower than 0.90, more preferably lower than 0.75, and even more preferably lower than 0.60.

10. The combination according any one of claims 1 to 9, wherein the combination is used for the treatment of HCV infection or a HCV-related disease in a subject, or for the control of chronic HCV infection in a subject.

11. The combination according any one of claims 1 to 10, wherein the combination is used for preventing HCV re-infection and recurrence in a liver transplantation patient.

12. A pharmaceutical composition comprising a combination according to any one of claims 1 to 11 and at least one pharmaceutically acceptable carrier or excipient.

13. The pharmaceutical composition according to claim 12 further comprising at least one anti-viral agent.

14. The pharmaceutical composition according to claim 13, wherein the anti-viral agent is selected from the group consisting of rabivirin, anti-hepatitis C virus monoclonal antibodies, anti-hepatitis C virus polyclonal antibodies, RNA polymerase inhibitors, protease inhibitors, IRES inhibitors, helicase inhibitors, antisense compounds, ribozymes, micro-RNA antagonists, cytokines, therapeutic vaccines, NS5A antagonists, polymerase inhibitors, cyclophilin A antagonists, and any combination thereof.
15. A kit comprising at least one anti-HCV-entry factor antibody and at least one interferon for simultaneous or sequential use in the treatment or the prevention of HCV infection, wherein the at least one anti-HCV-entry factor antibody and at least one interferon act in synergy to inhibit HCV infection.

16. The kit according to claim 15, wherein the at least one interferon is as defined in any one of claims 6 to 8 and the at least one anti-HCV-entry factor antibody is as defined in any one of claims 2 to 5.
Figure 1

A

B

C

Figure 1
Figure 2
Figure 3