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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF HCV INFECTIONS

(57) Abstract: The present invention relates to uses, methods and compositions for treating an infection by a member of the Flaviviridae family, such as HCV, in a subject in need thereof. More particularly, the prevention invention relates to an agent effective to inhibit the binding of a HCV envelope glycoprotein (E1 or E2) to CD229 protein present on the surface of hepatocytes or an inhibitor of CD229 gene expression for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.



METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF HCV INFECTIONS

FIELD OF THE INVENTION:

The present invention relates to uses, methods and compositions for treating an infection by a member of the *Flaviviridae* family, such as HCV, in a subject in need thereof.

BACKGROUND OF THE INVENTION:

HCV is a single-stranded positive RNA virus, which belongs to the family of *Flaviviridae*, genus *Hepacivirus*.

The genome of HCV comprises a single positive-stranded RNA that encodes a polyprotein of about 3010 amino acid residues, flanked at either end by noncoding regions (NCRs). The 5'-NCR and the first part of the region encoding the polyprotein fold into a complex structure of hairpin loops and unpaired regions that can act as an internal ribosome entry site. This means that translation of the virus genome is cap-independent with the initiation of translation directed to the AUG codon at the beginning of the polyprotein rather than the most 5'-terminal AUG. RNA secondary structures have also been described for the 3'-untranslated region, and it is thought that these might play a role in the replication of the virus genome, although there is at present no direct evidence for this. The N-terminus of the polyprotein is comprised of three structural proteins (core, E1 and E2) and release of these proteins from the polyprotein is dependent upon the signal peptidase associated with the cellular endoplasmic reticulum. Release of the six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) from the remainder of the polyprotein is mediated by the virus NS2-NS3 and NS3-NS4A proteases.

It is estimated that 170 million patients worldwide and about 1% of the population in developed countries are chronically infected with hepatitis C virus (HCV). The majority (70 to 80%) of acute HCV infections become chronic, some of which progress toward liver cirrhosis or hepatocellular carcinoma.

About 95% of individuals infected with HCV do not develop jaundice during the acute phase of infection, although symptoms may include nausea, anorexia and/or fatigue. Virus RNA can first be detected from 1 to 2 months after exposure, and this is usually accompanied or shortly followed by a rise in blood levels of alanine aminotransferase, a marker for liver damage. Seroconversion occurs from about 3 months after exposure, with reactivity first

detected to the NS3 and core antigens. Between 20 and 50% of acutely infected individuals are able to clear virus infection, and this outcome has been correlated with the presence of specific antibody responses to the HVR of the E2 protein and to a CD4+ response to part of the NS3 protein.

5 Chronic hepatitis C can be clinically silent for many years, although there will often be intermittent elevations in serum alanine aminotransferase levels. Histological examination of liver biopsies from chronically infected individuals usually reveals some signs of liver pathology, although these are often very mild. A typical pathological feature is the presence of lymphoid follicles within the portal tracts, together with periportal inflammation and
10 damage to the bile ducts. In the most extreme cases, the liver becomes cirrhotic with the accumulation of fibrous tissue between hepatocyte nodules eventually leading to liver failure. The extent of liver pathology generally increases with the duration of infection, albeit with a timescale of decades rather than years. Why the rate of progression to disease should vary between individuals is not fully understood. Immunodeficiency can lead to a relatively rapid
15 rate of progression, while there is conflicting evidence for an association between virus genotype and progression, perhaps because of underlying epidemiological associations between virus genotype, the age of acquisition of infection and the duration of infection. Some studies of liver transplant recipients suggest that more severe disease of the transplanted liver occurs in patients infected with HCV of genotype type 1b.

20 A strong association has been observed between the development of hepatocellular carcinoma and infection with HCV, although this progression is observed in only a small proportion of HCV-infected individuals, and occurs extremely slowly, usually following the development of cirrhosis. The mechanism of oncogenesis in vivo is not known, but the core protein of HCV has limited transforming activity in tissue culture cells and can repress
25 transcription of some cell cycle genes, while the NS5A can act as a transcriptional activator and repress PKR, a cellular protein kinase that is induced by interferon.

 A variety of nonhepatic disorders have been associated with HCV infection, including autoimmune and lymphoproliferative disorders, chronic fatigue, essential type II mixed cryoglobulinaemia, membranoproliferative glomerulonephritis and purpura cutanea tarda.
30 Although these manifestations have been interpreted by some as evidence for extrahepatic replication of HCV, several of these conditions could also be produced indirectly as consequences of chronic liver disease or because of the accumulation of HCV in immune complexes.

Current therapy consists in the association of pegylated interferon (IFN) alpha and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). However, the outcome of hepatitis C virus (HCV) infection varies among individuals and the likelihood of sustained response to antiviral treatment depends on viral and host characteristics. Naturally occurring variants of HCV are classified into 6 major genotypes. Viral genotype is one of the main factors associated to therapy response. Indeed, sustained virological response (SVR) is achieved in only 45% of the genotype 1 infected patients, whereas up to 80 % of the genotypes 2 or 3 infected patients reach a SVR. On the other side host factors associated to HCV outcome include factors such as age, race, body mass index.

Clearly there is a need to develop new drugs against HCV infections that will improve on the efficacy of currently available therapeutic tools. In this way, it has been suggested that drugs that impends the HCV entry in hepatocytes may be highly desirable.

HCV entry in hepatocytes represents a complex mechanism dependent on several environmental factors such as pH, temperature and other cellular factors. After an initial binding step involving molecules like GAGs and LDL-R facilitating initial attachment to the host cell, the particle₂, through interactions with glycoproteins-E1/E2 complex, interacts with SR-BI and CD81. CLDN1 and OCLDN act at a later stage and probably after a lateral migration of the virus-receptor complex to the tight junctions (TJ). Then, HCV is internalized by clathrin-mediated endocytosis, where fusion probably occurs in early endosomes. However, the entry process seems far from being fully understood as some human cell lines expressing these receptors remain resistant to HCV, suggesting that one or more human-specific HCV entry factor (s) remain (s) to be discovered.

SUMMARY OF THE INVENTION:

The present invention relates to uses, methods and compositions for treating an infection by a member of the *Flaviviridae* family, such as HCV, in a subject in need thereof.

More particularly, the prevention invention relates to an agent effective to inhibit the binding of a HCV envelope glycoprotein (E1 or E2) to CD229 protein present on the surface of hepatocytes for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma. The compound includes but is not limited to an antibody, a small molecule, and a polypeptide.

Another object of the invention is an inhibitor of CD229 gene expression for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.

The present invention also relates to a method of determining whether a compound is capable of inhibiting HCV infection of a cell which comprises the step consisting of determining whether a candidate compound is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes and ii) positively selecting the candidate compound that is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors show SLAM-CD229 gene expression on hepatocytes and its implication in HCV attachment and infection.

CD229 belongs to the Signaling Lymphocytic Activation Molecule (SLAM) family, which is constituted of nine cellular receptors known to be expressed on immune cells. All members of this family possess a similar structural organization, including an extracellular domain with either two or four Ig-like domains, a single transmembrane segment and an intracytoplasmic region with tyrosine-based motifs. CD229 is also known as hly9; mLY9; CD229; SLAMF3; LY9. A representative amino sequence of CD229 is the sequence SEQ ID NO:1. Unlike the other members of the SLAM family, CD229 has four rather than two extracellular Ig domains. Domains 1 and 3 are similar, as well as domains 2 and 4 (Engel, P., Eck, M. J., Terhorst, C. The SAP and SLAM families in immune responses and Xlinked lymphoproliferative disease. Nat Rev Immunol. 3, 813-21 (2003).; Sandrin, M. S., Henning, M. M., Lo, M. F., Baker, E., Sutherland, G. R., McKenzie, I. F. Isolation and characterization of cDNA clones for Humly9: the human homologue of mouse Ly9. Immunogenetics. 43, 13-9 (1996).). CD229 is known to be the only member able to be internalized by clathrin-mediated endocytosis (Del Valle, J.M., Engel, P., Martin, M. The cell surface expression of SAP-binding receptor CD229 is regulated via its interaction with clathrin-associated adaptor complex 2 (AP-2). J Biol chem. (2003).). In spite of its role in the immune system, CD229 is not well known and the exact functions of this receptor remain to be elucidated. The inventors described for the first time both the expression and functionality of CD299 in hepatocytes.

The analysis of CD229 mRNA and protein sequence is similar in lymphocytes as well as hepatocytes.

The inventors demonstrate that CD229 blockade by antibodies and down-modulation by siRNA decreases HCV-hepatocytes susceptibility. Conversely, CD229 overexpression in human hepatoma cells Huh-7 significantly increases HCV attachment and replication. Moreover, the ectopic expression of CD229 confers susceptibility to HCV in resistant humhepatocytest Hep-G2 and monkey kidney cells COS-7. The use of peptides mimicking each domain of CD229 shows that the first extracellular domain of CD229 is required for HCV attachment and entry.

The inventors have thus identified CD229 as novel key factor for HCV entry and a potential new target for antiviral drug development.

Accordingly, the present invention provides for uses, methods and compositions (such as pharmaceutical compositions) for treating an infection by a virus belonging to the *Flaviviridae* family in a subject in need thereof.

In one embodiment the member of the *Flaviviridae* family may be a member of the *Flavivirus* genus such as the Japanese encephalitis virus group, including Japanese encephalitis virus and West Nile Virus. Alternatively it may be a member of the Yellow fever virus group. Alternatively it may be a member of the Pestivirus genus, such as Bovine viral diarrhea virus (BVDV-1 and/or BVDV-2), Classical swine fever virus, Border disease virus. Alternatively, the *Flaviviridae* members may belong to the Hepacivirus genus such as the hepatitis G virus (HGV).

In a particular embodiment, the invention provides uses, methods and compositions for treating a hepatitis C virus (HCV) infection. More specifically the present invention provides for uses, methods and compositions for treating acute or chronic C hepatitis. Even more specifically the present invention provides for uses, methods and compositions for preventing diseases associated with a Hepatitis C virus infection such as autoimmune and lymphoproliferative disorders, chronic fatigue, essential type II mixed cryoglobulinaemia, membranoproliferative glomerulonephritis and purpura cutanea tardia. The present invention also encompasses uses, methods and composition for the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.

As used herein, the term “treatment” refers to inhibiting the disease or condition, i.e. arresting its development; relieving the disease or condition, i.e. causing regression of the condition; or relieving the conditions caused by the disease, i.e. symptoms of the disease.

5 As used herein, the term “prevention” refers to preventing the disease or condition from occurring in a subject which has not yet been diagnosed as having it

More particularly the prevention invention relates to an agent effective to inhibit the binding of a flavivirus envelope glycoprotein to CD229 protein present on the surface of
10 hepatocytes for use in the treatment of a flavivirus infection or for use in the treatment of a disease associated with a flavivirus infection in a subject in need thereof.

More particularly the prevention invention relates to an agent effective to inhibit the binding of a HCV envelope glycoprotein (E1 or E2) to CD229 protein present on the surface
15 of hepatocytes for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma. The compound includes but is not limited to an antibody, a small molecule, and a polypeptide.

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In one embodiment, the agent is an antibody. The invention embraces antibodies or fragments of antibodies having the ability to block the interaction between HCV and CD229. The antibodies may have specificity to HCV or CD229. In one embodiment, the antibodies or fragment of antibodies are directed to all or a portion of the extracellular domain of CD229.
25 In one embodiment, the antibodies or fragment of antibodies are directed to the extracellular region of CD229 ranging from residue at position 1 to residue at position 448 in sequence SEQ ID NO:1. Even more particularly antibodies or fragments of antibodies are directed to a polypeptide comprises consecutive amino acids having a sequence which corresponds to the sequence of at least a portion of an extracellular domain of CD229, which portion binds to a
30 HCV envelope glycoprotein. In one embodiment, the antibodies or fragment of antibodies are directed to an extracellular domain of CD229. In one embodiment, the antibodies or fragment of antibodies are directed to the extracellular domain “domain 1” of CD229 ranging from residue at position 73 to residue at position 154 in sequence SEQ ID NO:1. In one embodiment the antibodies or fragment of antibodies are directed to the extracellular domain

comprises consecutive amino acids having a sequence as set forth in SEQ ID NO: 2 (peptide P1) or SEQ ID NO:3 (peptide P2). This invention also provides an antibody or portion thereof capable of inhibiting binding of CD229 to a HCV envelope glycoprotein, which antibody binds to an epitope located within a region of a HCV envelope glycoprotein, which region of a HCV envelope glycoprotein binds to CD229.

In one embodiment of the antibodies or portions thereof described herein, the antibody is a monoclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a polyclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a humanized antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a chimeric antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a light chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a heavy chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fab portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a F(ab')₂ portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fc portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fv portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a variable domain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises one or more CDR domains of the antibody.

As used herein, "antibody" includes both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or nonhuman antibody. A nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man.

Antibodies are prepared according to conventional methodology. Monoclonal antibodies may be generated using the method of Kohler and Milstein (Nature, 256:495, 1975). To prepare monoclonal antibodies useful in the invention, a mouse or other appropriate host animal is immunized at suitable intervals (e.g., twice-weekly, weekly, twice-monthly or

monthly) with antigenic forms of HCV, HCV envelope glycoproteins, or CD229. The animal may be administered a final "boost" of antigen within one week of sacrifice. It is often desirable to use an immunologic adjuvant during immunization. Suitable immunologic adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, alum, Ribi adjuvant, Hunter's Titermax, saponin adjuvants such as QS21 or Quil A, or CpG-containing immunostimulatory oligonucleotides. Other suitable adjuvants are well-known in the field. The animals may be immunized by subcutaneous, intraperitoneal, intramuscular, intravenous, intranasal or other routes. A given animal may be immunized with multiple forms of the antigen by multiple routes.

Briefly, the recombinant HCV envelope glycoproteins may be provided by surface expression on recombinant cell lines. CD229 may be provided in the form of human cells that express CD229. Recombinant forms of CD229 may be provided using any previously described method. Alternatively, the antigen may be provided as synthetic peptides corresponding to antigenic regions of interest (SEQ ID NO: 2 or SEQ ID NO:3). Following the immunization regimen, lymphocytes are isolated from the spleen, lymph node or other organ of the animal and fused with a suitable myeloma cell line using an agent such as polyethylene glycol to form a hybridoma. Following fusion, cells are placed in media permissive for growth of hybridomas but not the fusion partners using standard methods, as described (Coding, Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, 3rd edition, Academic Press, New York, 1996). Following culture of the hybridomas, cell supernatants are analyzed for the presence of antibodies of the desired specificity, i.e., that selectively bind the antigen. Suitable analytical techniques include ELISA, flow cytometry, immunoprecipitation, and western blotting. Other screening techniques are well-known in the field. Preferred techniques are those that confirm binding of antibodies to conformationally intact, natively folded antigen, such as non-denaturing ELISA, flow cytometry, and immunoprecipitation.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated

an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody.

This invention provides in certain embodiments compositions and methods that include humanized forms of antibodies. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid

rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid residue at the framework positions at which the amino acid is predicted to have a side chain atom within 3 Å of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies. One of ordinary skill in the art will be familiar with other methods for antibody humanization.

In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where 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 would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al., *J. Mol. Biol.* 294:151, 1999, the contents of which are incorporated herein by reference.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans.

In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and in vitro stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference.

5 Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂ Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by
10 homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

15 The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4.

In another embodiment, the antibody according to the invention is a single domain
20 antibody. The term "single domain antibody" (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called "nanobody®". According to the invention, sdAb can particularly be llama sdAb.

25 In one embodiment of the agents described herein, the agent is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random
30 sequence library. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. The invention embraces aptamers having the ability to block the interaction between HCV and CD229. The aptamers may have specificity to HCV or CD229. In one embodiment, the aptamers are directed to all or a portion of the extracellular

domain of CD229. In one embodiment, the aptamers are directed to the extracellular region of CD229 ranging from residue at position 1 to residue at position 448 in sequence SEQ ID NO:1. Even more particularly aptamers are directed to a polypeptide comprises consecutive amino acids having a sequence which corresponds to the sequence of at least a portion of an extracellular domain of CD229, which portion binds to a HCV envelope glycoprotein. In one embodiment, the aptamers are directed to an extracellular domain of CD229. In one embodiment, the aptamers are directed to the extracellular domain "domain 1" of CD229 ranging from residue at position 73 to residue at position 154 in sequence SEQ ID NO:1. In one embodiment the aptamers are directed to the extracellular domain comprises consecutive amino acids having a sequence as set forth in SEQ ID NO: 2 (peptide P1) or SEQ ID NO:3 (peptide P2). This invention also provides an aptamer capable of inhibiting binding of CD229 to a HCV envelope glycoprotein, which aptamer binds to an epitope located within a region of a HCV envelope glycoprotein, which region of a HCV envelope glycoprotein binds to CD229.

In one embodiment of the agents described herein, the agent is a polypeptide. In a particular embodiment the polypeptide is a functional equivalent of CD229. As used herein, a "functional equivalent of CD229 is a compound which is capable of binding to HCV, thereby preventing its interaction with CD229. The term "functional equivalent" includes fragments, mutants, and muteins of CD229. The term "functionally equivalent" thus includes any equivalent of CD229 obtained by altering the amino acid sequence, for example by one or more amino acid deletions, substitutions or additions such that the protein analogue retains the ability to bind to HCV, preferably the envelope of HCV. Amino acid substitutions may be made, for example, by point mutation of the DNA encoding the amino acid sequence.

Functional equivalents include molecules that bind HCV, preferably a HCV envelope glycoproteins, and comprise all or a portion of the extracellular domains of CD229.

The functional equivalents include soluble forms of the CD229 proteins. A suitable soluble form of these proteins, or functional equivalents thereof, might comprise, for example, a truncated form of the protein from which the transmembrane domain has been removed by chemical, proteolytic or recombinant methods.

In one embodiment, the functional equivalent comprises all or a portion of the extracellular domain of CD229. The extracellular region of CD229 ranges from residue at position 1 to residue at position 448 in sequence SEQ ID NO:1. In one embodiment, the functional equivalent comprises all or a portion of the extracellular domain "domain 1" of

CD229 ranging from residue at position 73 to residue at position 154 in sequence SEQ ID NO:1.

Preferably, the functional equivalent is at least 80% homologous to the corresponding protein. In a preferred embodiment, the functional equivalent is at least 90% homologous as assessed by any conventional analysis algorithm such as for example, the Pileup sequence analysis software (Program Manual for the Wisconsin Package, 1996)..

The term "a functionally equivalent fragment" as used herein also may mean any fragment or assembly of fragments of CD229 that binds to HCV, preferably that binds to a HCV envelope glycoproteins. Accordingly the present invention provides a polypeptide capable of inhibiting binding of CD229 to a HCV envelope glycoprotein, which polypeptide comprises consecutive amino acids having a sequence which corresponds to the sequence of at least a portion of an extracellular domain of CD229, which portion binds to a HCV envelope glycoprotein. In one embodiment, the polypeptide corresponds to an extracellular domain of CD229. In one embodiment of the polypeptide, the extracellular domain comprises consecutive amino acids having a sequence as set forth in SEQ ID NO:2 or SEQ ID NO:3.

Functionally equivalent fragments may belong to the same protein family as the human CD229 proteins identified herein. By "protein family" is meant a group of proteins that share a common function and exhibit common sequence homology. Homologous proteins may be derived from non-human species. Preferably, the homology between functionally equivalent protein sequences is at least 25% across the whole of amino acid sequence of the complete protein. More preferably, the homology is at least 50%, even more preferably 75% across the whole of amino acid sequence of the protein or protein fragment. More preferably, homology is greater than 80% across the whole of the sequence. More preferably, homology is greater than 90% across the whole of the sequence. More preferably, homology is greater than 95% across the whole of the sequence.

It is envisaged that such molecules will be useful in preventative therapy of HCV infection, because these molecules will bind specifically to the virus and will thus prevent entry of the virus into cells. As used herein, "binding specifically" means that the functionally equivalent analogue has high affinity for HCV or a HCV envelope glycoproteins but not for control proteins. Specific binding may be measured by a number of techniques such as ELISA, flow cytometry, western blotting, or immunoprecipitation. Preferably, the functionally equivalent analogue specifically binds to HCV or a HCV envelope glycoproteins at nanomolar or picomolar concentrations.

The Polypeptides of the invention may be produced by any suitable means, as will be apparent to those of skill in the art. In order to produce sufficient amounts of the CD229 or functional equivalents thereof for use in accordance with the present invention, expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the polypeptide of the invention. Preferably, the polypeptide is produced by recombinant means, by expression from an encoding nucleic acid molecule. Systems for cloning and expression of a polypeptide in a variety of different host cells are well known.

When expressed in recombinant form, the polypeptide is preferably generated by expression from an encoding nucleic acid in a host cell. Any host cell may be used, depending upon the individual requirements of a particular system. Suitable host cells include bacteria mammalian cells, plant cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells. HeLa cells, baby hamster kidney cells and many others. Bacteria are also preferred hosts for the production of recombinant protein, due to the ease with which bacteria may be manipulated and grown. A common, preferred bacterial host is *E coli*.

In specific embodiments, it is contemplated that polypeptides used in the therapeutic methods of the present invention may be modified in order to improve their therapeutic efficacy. Such modification of therapeutic compounds may be used to decrease toxicity, increase circulatory time, or modify biodistribution. For example, the toxicity of potentially important therapeutic compounds can be decreased significantly by combination with a variety of drug carrier vehicles that modify biodistribution. In example adding dipeptides can improve the penetration of a circulating agent in the eye through the blood retinal barrier by using endogenous transporters.

A strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers; and modify the rate of clearance from the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

Polyethylene glycol (PEG) has been widely used as a drug carrier, given its high degree of biocompatibility and ease of modification. Attachment to various drugs, proteins, and liposomes has been shown to improve residence time and decrease toxicity. PEG can be coupled to active agents through the hydroxyl groups at the ends of the chain and via other

chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids were explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would have the added advantage of numerous attachment points per molecule (providing greater drug loading), and which could be synthetically designed to suit a variety of applications.

Those of skill in the art are aware of PEGylation techniques for the effective modification of drugs. For example, drug delivery polymers that consist of alternating polymers of PEG and tri-functional monomers such as lysine have been used by VectraMed (Plainsboro, N.J.). The PEG chains (typically 2000 daltons or less) are linked to the α - and ϵ -amino groups of lysine through stable urethane linkages. Such copolymers retain the desirable properties of PEG, while providing reactive pendent groups (the carboxylic acid groups of lysine) at strictly controlled and predetermined intervals along the polymer chain. The reactive pendent groups can be used for derivatization, cross-linking, or conjugation with other molecules. These polymers are useful in producing stable, long-circulating pro-drugs by varying the molecular weight of the polymer, the molecular weight of the PEG segments, and the cleavable linkage between the drug and the polymer. The molecular weight of the PEG segments affects the spacing of the drug/linking group complex and the amount of drug per molecular weight of conjugate (smaller PEG segments provides greater drug loading). In general, increasing the overall molecular weight of the block co-polymer conjugate will increase the circulatory half-life of the conjugate. Nevertheless, the conjugate must either be readily degradable or have a molecular weight below the threshold-limiting glomerular filtration (e.g., less than 45 kDa).

In addition, to the polymer backbone being important in maintaining circulatory half-life, and biodistribution, linkers may be used to maintain the therapeutic agent in a pro-drug form until released from the backbone polymer by a specific trigger, typically enzyme activity in the targeted tissue. For example, this type of tissue activated drug delivery is particularly useful where delivery to a specific site of biodistribution is required and the therapeutic agent is released at or near the site of pathology. Linking group libraries for use in activated drug delivery are known to those of skill in the art and may be based on enzyme kinetics, prevalence of active enzyme, and cleavage specificity of the selected disease-specific enzymes. Such linkers may be used in modifying the protein or fragment of the protein described herein for therapeutic delivery.

Another object of the invention is an inhibitor of CD229 gene expression for use in the treatment of a flavivirus infection in a subject in need thereof.

Another object of the invention is an inhibitor of CD229 gene expression for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.

An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. Therefore, an “inhibitor of CD229 gene expression” denotes a natural or synthetic compound that has a biological effect to inhibit the expression of CD229 gene.

In a preferred embodiment of the invention, said inhibitor of CD229 gene expression is a siRNA, an antisense oligonucleotide or a ribozyme.

Inhibitors of CD229 gene expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of CD229 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of CD229 proteins, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding CD229 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of CD229 gene expression for use in the present invention. CD229 gene expression can be reduced by contacting the tumor, subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that CD229 gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO

99/32619, and WO 01/68836). Typically the siRNA according to the invention may those described in the EXAMPLE (SEQ ID NO:5-7).

Ribozymes can also function as inhibitors of CD229 gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of CD229 mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of CD229 gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing CD229. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but

are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following

5 viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

10 Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those

15 retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a

20 packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991).

25 Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse

30 lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in

tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively
5 described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the
10 viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific
15 fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold
20 particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

Another object of the invention relates to a method for use in the treatment of an HCV infection or a disease associated with an HCV infection, such as acute or chronic hepatitis C
25 or for the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma comprising the administration of a therapeutically effective amount of at least one agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression to a subject in need thereof.

30 By a "therapeutically effective amount" of the agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression as above described is meant a sufficient amount of the agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression to treat a

hepatitis C virus infection at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In a particular embodiment, the agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes is use in combination with an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD81 protein present on the surface of hepatocytes.

The term "CD81" as used herein means the human CD81 protein as defined by the protein sequence listed in the SWISSPROT database (Accession No. P18582) or the EMBLIGENBANK database (Accession No. M33690). CD81 has been shown to be involved in the entry of HCV in hepatocytes and the inventors demonstrates that the combination of such agents could synergize to inhibit the entry of HCV in hepatocytes.

Typically as described for CD229, the agent may be an antibody, a small organic molecules or a polypeptide. Typically, the agent may be a functional equivalent of CD81. A functional equivalent of CD81 is a compound which is capable of binding to HCV, preferably to the E2 envelope glycoprotein of HCV. The term "functional equivalent" thus includes an

analogue of CD81, a fragment of CD81, and CD81 mutants and muteins. One region of the human CD81 protein that is shown herein to be involved in binding to the E2 protein of HCV is the EC2" region comprising amino acids 113-201 of the full length human sequence of CD81. The invention encompasses proteins and protein fragments containing this region of human CD81, or containing functional equivalents of this region. Preferably, the functional equivalent is at least 80% homologous to the human CD81 sequence across the EC2 region of the protein, preferably at least 90% homologous as assessed by any conventional analysis algorithm such as for example, the Pileup sequence analysis software (Program Manual for the Wisconsin Package, 1996).

Agent effective to inhibit the binding of a HCV envelope glycoprotein to CD81 protein present on the surface of hepatocytes are well known in the art. Examples of functional equivalent of CD81 are described in US 2004/0258694. Example of small organic molecules that are agents effective to inhibit the binding of a HCV envelope glycoprotein to CD81 protein present on the surface of hepatocytes are described in US 2003/0219893 and JP2009215280. Examples of antibodies that are agents effective to inhibit the binding of a HCV envelope glycoprotein to CD81 protein present on the surface of hepatocytes are described; WO2010043650.

In one embodiment, the invention relates to the use of an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of a hepatocyte or inhibitor of CD229 gene expression as described above for use in the treatment of a subject undergoing a treatment with interferon-alpha.

In other embodiment, methods of the invention further comprises administering to the subject an effective amount of interferon alpha (IFN-alpha).

Any known IFN-alpha can be used in the instant invention. The term "interferon-alpha" or "IFN-alpha" as used herein refers to a family of related polypeptides that inhibit viral replication and cellular proliferation and modulate immune response. The term "IFN-alpha" includes naturally occurring IFN-alpha; synthetic IFN-alpha ; derivatized IFN-alpha (e.g. , PEGylated IFN-alpha, glycosylated IFN-alpha, and the like); and analogs of naturally occurring or synthetic IFN-alpha ; essentially any IFN-alpha that has antiviral properties, as described for naturally occurring IFN-alpha. Suitable interferons alpha include, but are not limited to, naturally-occurring IFN-alpha (including, but not limited to, naturally occurring IFN-alpha2a, IFN-alpha2b) ; recombinant interferon alpha-2b such as Intron-A interferon available from Schering Corporation, Kenilworth, N. J. ; recombinant interferon alpha-2a

such as Roferon interferon available from Hoffmann-La Roche, Nutley, N. J. ; recombinant interferon alpha-2C such as Berofer alpha 2 interferon available from Boehringer Ingelheim Pharmaceutical, Inc. , Ridgefield, Conn.; interferon alpha- n1, a purified blend of natural interferon alphas such as Sumiferon available from Sumitomo, Japan or as Wellferon
5 interferon alpha-n1 (INS) available from the Glaxo-Wellcome Ltd., London, Great Britain; and interferon alpha-n3 a mixture of natural interferon alphas made by Interferon Sciences and available from the Purdue Frederick Co. , Norwalk, Conn. , under the Alferon Tradename.

The term "IFN-alpha" also encompasses derivatives of IFN-alpha that are derivatized
10 (e. g. , are chemically modified) to alter certain properties such as serum half-life. As such, the term "IFN-alpha" includes glycosylated IFN-alpha ; IFN-alpha derivatized with polyethylene glycol ("PEGylated IFN-alpha"); and the like. PEGylated IFN-alpha, and methods for making same, is discussed in, e. g., U. S. Patent Nos. 5,382, 657; 5,981, 709; and 5,951, 974. PEGylated IFN-alpha encompasses conjugates of PEG and any of the above-
15 described IFN-alpha molecules, including, but not limited to, PEG conjugated to interferon alpha-2a (Roferon,Hoffman La-Roche, Nutley, N. J.), interferon alpha 2b (Intron, Schering-Plough, Madison, N. J.), interferon alpha-2c (Berofer Alpha, Boehringer Ingelheim, Ingelheim, Germany); and consensus interferon as defined by determination of a consensus sequence of naturally occurring interferons alpha (Infergen (InterMune, Inc. ,
20 Brisbane,Calif.).

In another embodiment, the invention relates to the use of an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression as described above for use in the treatment of a subject undergoing a treatment with a nucleoside analog.

25 In other embodiments, methods of the invention further comprises administering to the subject an effective amount of a nucleoside analog for achieving a sustained viral response. Said nucleoside analog may be ribavirin or derivatives thereof.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), is a nucleoside analog available from ICN Pharmaceuticals, Inc. , Costa Mesa,Calif., and is described in the Merck
30 Index, compound No. 8199, Eleventh Edition. Its manufacture and formulation is described in U. S. Pat. No. 4,211, 771.

The invention also contemplates use of derivatives of ribavirin as those described in US patent 6,277, 830, or International Patent Application WO2006067606. Other derivatives

include Levovirin which is the L-enantiomer of ribavirin, or Viramidine which is a 3-carboxamidine derivative of ribavirin.

In another embodiment, the invention relates to the use of an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression as described above for use in the treatment of a subject undergoing a treatment with interferon-alpha and a nucleoside analog.

In other embodiments, methods of the invention further comprises administering an effective amount of interferon alpha (IFN-alpha) and a nucleoside analog as those above described.

In another embodiment, the invention relates to the use of an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression as described above for use in the treatment of a subject undergoing a treatment with an inhibitor of HCV proteases and/or polymerases.

In other embodiments, methods of the invention further comprises administering to the subject an effective amount of an inhibitor of HCV polymerases. Such inhibitors include, but are not limited to a compound as disclosed in U. S. Patent No. 6,479, 508; a compound as disclosed in any of International Patent Application WO03010140; WO03007945, WO03010141, WO0147883, a dinucleotide analog as disclosed in Zhong et al. (2003); a benzothiadiazine compound as disclosed in Dhanak et al.(2002); an NS5B inhibitor as disclosed in WO02100846 or WO 0200851, WO0185172, WO 02098424, WO 0006529, WO 0206246, WO 03000254, or EP 1 256,628 A2.

In other embodiments, methods of the invention further comprises administering to the subject an effective amount of an inhibitor of HCV proteases. Inhibitors of NS3 protease include, but are not limited to, a compound as disclosed in International Patent Applications WO03066103 WO2004103996 or WO2004093915. HCV NS3 protease inhibitor peptide analogs include any compound disclosed in Patent Applications GB 2,337,262; JP10298151; JP 11126861; JP 11292840; JP 2001-103993; U.S. Pat. No. 6,159,938; U.S. Pat. No. 6,187,905; WO 97/43310; WO 98/17679; WO 98/22496; WO 98/46597; WO 98/46630; WO 99/38888; WO 99/50230; WO 99/64442; WO 99/07733; WO 99/07734; WO 00/09543; WO 00/09558; WO 00/20400; WO 00/59929; WO 00/31129; WO 01/02424; WO 01/07407; WO 01/16357; WO 01/32691; WO 01/40262; WO 01/58929; WO 01/64678; WO 01/74768; WO 01/77113; WO 01/81325; WO 02/08187; WO 02/08198; WO 02/08244; WO 02/08251; WO 02/08256; WO 02/18369; WO 02/60926 and WO 02/79234. Inhibitors of HCV NS3 protease have been also described in WO 03/064456, WO 03/064416 , WO 02/060926, WO

03/053349, WO 03/099316, WO 03/099274, WO 2004/032827, and. WO 2004/043339. Acyl sulfamide inhibitors of the HCV NS3 protease have also been described in WO 2005/046712 or WO2006000085. Inhibitors of NS3-NS4A protease are described in Patent Applications WO2005007681 WO2005035525 or WO2005028502.

5 In other embodiments, methods of the invention further comprises administering to the subject an effective amount of a statin compound. Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors may be potentially useful as anti-HCV reagents (Ikeda M. et al. 2006, Kapadia SB. et al. 2005, Ye J. et al. 2003).

10 In another embodiment, the invention relates to the use of an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression as described above for use in the treatment of a subject undergoing a treatment with interferon-alpha, a nucleoside analog and an inhibitor of HCV proteases and/or polymerases.

15 Another object of the present invention is a kit for use in the treatment of an infection by members of the *Flaviviridae* family or for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection, comprising a medicament comprising an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression
20 expression and at least a medicament selected from the group consisting of a medicament comprising interferon-alpha, a medicament comprising a nucleoside analog and a medicament comprising an inhibitor of HCV proteases and/or polymerases or medicament comprising an agent effective to inhibit the binding of a HCV envelope glycoprotein to CD81 protein present on the surface of hepatocytes.

25 The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

30 "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol ; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for

example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the

subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes or inhibitor of CD229 gene expression of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations ; time release capsules ; and any other form currently used.

The present invention also relates to a method of determining whether a compound is capable of inhibiting HCV infection of a cell which comprises the step consisting of determining whether a candidate compound is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes and ii) positively selecting the candidate compound that is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes.

In a particular embodiment the method comprises the step consisting of:

- a) immobilizing an HCV envelope glycoprotein on a solid support;
- b) contacting the immobilized HCV envelope glycoprotein with sufficient detectable CD229 protein to saturate all binding sites for the CD229 protein on the immobilized HCV envelope glycoprotein under conditions permitting binding of the CD229 protein to the immobilized HCV envelope glycoprotein so as to form a complex;
- c) removing unbound CD229 protein;
- d) contacting the complex with the compound; and
- e) determining whether any CD229 protein is displaced from the complex, wherein displacement of CD229 protein from the complex indicates that the compound binds to a HCV envelope glycoprotein, so as to thereby determine that the compound is one which is capable of inhibiting HCV infection of the cell.

In another particular embodiment, the method comprises the step consisting of:

- a) immobilizing a CD229 protein on a solid support;

b) contacting the immobilized CD229 protein with sufficient detectable HCV envelope glycoprotein to saturate all binding sites for a HCV envelope glycoprotein on the immobilized CD229 protein under conditions permitting binding of the immobilized CD229 protein to a HCV envelope glycoprotein so as to form a complex;

c) removing unbound HCV envelope glycoprotein;

d) contacting the complex with the compound;

e) determining whether any HCV envelope glycoprotein is displaced from the complex, wherein displacement of HCV envelope glycoprotein from the complex indicates that the compound binds to the CD229 protein, so as to thereby determine that the compound is one which is capable of inhibiting HCV infection of the cell.

In another particular embodiment, the method comprises the step consisting of:

(a) contacting an HCV envelope glycoprotein with sufficient detectable CD229 protein to saturate all binding sites for the CD229 protein on a HCV envelope glycoprotein under conditions permitting binding of the CD229 protein to a HCV envelope glycoprotein so as to form a complex;

(b) removing unbound CD229 protein;

(c) measuring the amount of CD229 protein which is bound to a HCV envelope glycoprotein in the complex;

(d) contacting the complex with the compound so as to displace CD229 protein from the complex;

(e) measuring the amount of CD229 protein which is bound to the compound in the presence of the compound; and

(f) comparing the amount of CD229 protein bound to a HCV envelope glycoprotein in step (e) with the amount measured in step (c), wherein a reduced amount measured in step (e) indicates that the compound binds to a HCV envelope glycoprotein, so as to thereby determine that the compound is one which is capable of inhibiting HCV infection of the cell.

In another particular embodiment, the method comprises the step consisting of:

(a) contacting an HCV envelope glycoprotein with the compound and detectable CD229 protein under conditions permitting binding of the CD229 protein to a HCV envelope glycoprotein in the absence of the compound so as to form a complex;

(b) removing unbound CD229 protein;
(c) comparing the amount of detectable CD229 protein which is bound to a HCV envelope glycoprotein in the complex in the presence of the compound with the amount of detectable CD229 protein which binds to the compound in the absence of the compound;
wherein a reduced amount of CD229 protein measured in presence of the compound indicates that the compound binds to a HCV envelope glycoprotein or CD229 protein so as to thereby determine that the compound is one which is capable of inhibiting HCV infection of the cell.

In the methods described herein, an entity may be made detectable by labeling it with a detectable marker. In one embodiment of the methods described herein, the detectable CD229 protein is labeled with a detectable marker. In one embodiment of the methods described herein, the detectable HCV envelope glycoprotein glycoprotein is labeled with a detectable marker. One skilled in the art would know various types of detectable markers. Such detectable markers include but are not limited to a radioactive, calorimetric, luminescent and fluorescent markers.

In one embodiment of the method described herein, the solid support is a microtiter plate well. In another embodiment, the solid support is a bead. In a further embodiment, the solid support is a surface plasmon resonance sensor chip. The surface plasmon resonance sensor chip can have pre-immobilized streptavidin. In one embodiment, the surface plasmon resonance sensor chip is a BIAcoreTM chip.

In one embodiment of the above methods, the detectable molecule is labeled with a detectable marker. In another embodiment of the above methods, the detectable molecule is detected by contacting it with another compound which is both capable of binding the detectable molecule and is detectable. The detectable markers include those described above.

As used herein, the term "candidate compound" includes both protein and non-protein moieties. In one embodiment, the candidate compound is a small molecule. In another embodiment, the candidate compound is a protein. The protein may be, by way of example, an antibody directed against a portion of an HCV envelope glycoprotein. The candidate compound may be derived from a library of low molecular weight compounds or a library of extracts from plants or other organisms. In an embodiment, the agent is known. In a separate embodiment, the candidate compound is not previously known. The agents/compounds of the subject invention include but are not limited to compounds or molecular entities such as peptides, polypeptides, and other organic or inorganic molecules and combinations thereof.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

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FIGURES:

Figure 1: The first extracellular domain of CD229 is involved in HCVcc attachment and entry. Huh-7 cells (a) and HHPH (b) were treated with antibodies prior to HCVcc infection as described in Methods. Cell-associated viral RNA was quantified 72 h p.i (mean of n=4; error bars, s.d). c, The diagram shows CD229 topology and matched CD229 peptides. CD229 and irrelevant peptides were incubated with HCVcc prior to Huh-7 cells infection (d, f) or HHPH (e) as described in Methods. Viral-cell attachment was evaluated 4 h p.i. (d, e), and cell-associated viral RNA was quantified at 72h p.i. (f). A neutralizing serum was used as control of infection inhibition (mean of n=4; error bars,s.d.). g, Huh-7 cells were treated with CD229 peptide and challenged with HCVcc. At 72 h p.i.intracellular viral protein E2 was determined by flow cytometry. Left panel: The white bar shows the irrelevant peptide, and the black bars show Peptides 1 and 2 (mean of n=3; error bars, s.d.). Right panel: The histogram shows intracellular E2-fluoresence intensity in cells previously treated with the irrelevant peptide (bold solid line) or Peptide 2 (dotted line).

20

EXAMPLE:

Methods:

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Cell culture grown HCV (HCVcc). The HCV strain J6/JFH virus was generated as previously described ¹⁷. Briefly, RNA was transcribed *in vitro* from full-length genomes using the Megascript T7 kit (Ambion, USA) and electroporated into Huh-7 cells. HCVcc was collected from supernatants 72 h after transfection. High-titre stocks were generated by serial passage through naive Huh-7 cells.

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Hepatic CD229 blockade. Huh-7 and HHPC were treated with monoclonal antibody directed against CD81 (JS-81), or CD229 (3H or ZY) and IgG1 isotype-control (5 µg/mL) for 1 h prior to addition HCVcc for 72 h. Peptides derived from CD229 were used in order to

check which CD229 domains are required for HCV entry. HCVcc was incubated with peptides (0.25 μ M) for 1h and the mix was then added to Huh-7 for 3 h. After washes in PBS 1X, cells were either lysed for virus-cell attachment analysis or incubated during supplementary 72 h.

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HCVcc infectivity. For the adsorbed and internalized virus, Viral RNA was extracted using the RNeasy Qiagen Kit following customers instructions (Qiagen). In some experiments, the collected supernatants were used to infect naive Huh-7 during 72 h. For viral load quantification, viral RNA from supernatants was extracted with the QiAmp® Viral RNA Kit (Qiagen). Viral RNA quantification was performed by the 7900 HT Sequence Detection System, with the TaqMan Gene Expression protocol (Applied Biosystems). GAPDH was used to normalize the quantified viral mRNA.

Viral entry and replication were checked by flow cytometry while positive foci forming units (FFU) were determined by fluorescence microscopy. To do so, intracellular viral E2 protein staining was performed with the BD Cytotfix/Cytoperm™ Plus kit (BD Biosciences) and cells were analyzed by FACSCanto II (Becton Dickinson). Results were then processed with FACSDiva™ (Becton Dickinson) and FlowJo software. Data were expressed in percentage of E2-infected cells and E2-fluorescence intensity per cell (MFI).

20 **Supplementary Information:**

CD229 down-modulation by siRNA. To knock down *CD229 gene expression* in Huh-7, three specific siRNA were predesigned by Ambion®. The following siRNA were used: ID114219 (Ly9-1): 5'-CCAAGUGGAGUUACUCCCUtt-3' (SEQ ID NO:5), ID212885 (Ly9-2): 5'-CGUCCCAAAGAAAAUGUAAtt-3' (SEQ ID NO:6) , ID106775 (Ly9-3): 5'-GGAAUUCACCCUGUUCGUCtt-3' (SEQ ID NO:7). siRNA were introduced in Huh-7 cells using the *Silencer®* siRNA Transfection II Kit (Applied Biosystems, Ambion®) according to the manufacture's instructions. Briefly, 2 x10⁵ cells were seeded in 6-well plates and were incubated with siRNA (60 nM) in the presence of NeoFX solution (5 μ L) in 100 μ L of final reaction volume in OptiMEM medium, for 50 h at 37 °C. The kit includes positive and negative siRNA controls. The positive siRNA control targeted the GAPDH gene and the negative control siRNA was a scrambled sequence that bears no homology to the human, mouse, or rat genomes. To evaluate the siRNA effect on gene expression, CD229 mRNA was

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analyzed before and after transfection. The siRNA effect on protein expression was evaluated by Western Blot and flow cytometry using a monoclonal anti-CD229.

Plasmid constructions and transfections. For CD229 over-expression, CD229 from Huh-7 was cloned in pBud vector. The cloned CD229 in pCIneo (a gift from Pablo Engel, Barcelona, Spain) was used in some experiments. The primers used for PCR were: forward 5'-GATCATCATGGTGGCACCAAAGAGTCACA-3' (SEQ ID NO: 8) and reverse 5'-GCAGCAGCTGCTTTTCCTTTCAGGTGAA-3' (SEQ ID NO: 9). After a step of purification using the QIAquick gel extraction kit (Qiagen), PCR products were cloned using the Topo TA cloning (Invitrogen Life Technologies), sequenced and subcloned into the pBud CE4.1 vector using the Kpn-1 and Xho-1 sites. For CD229 over-expression in Huh-7, Hep-G2 and COS-7, cells (0.3×10^6) were seeded into 6-well dishes and 24h later were transfected with 0.8 μ g of DNA using the FuGENE HD Transfection Reagent Kit (Roche) according to the manufacturer's instructions. Cells were incubated for 24 h at 37 °C before expression analysis by CD229 mRNA quantification, flow cytometry and Western Blot.

Cells, antibodies and peptides. Huh-7, Hep-G2, and Cos-7 cell lines were from American Type Culture Collection (ATCC). Hep-G2 CD81-positive cells (Hep-G2 CD81pos) were obtained from Dr. Jean Dubuisson (Institut de Biologie de Lille, France). All the cell lines were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal calf serum (PAA), 100 UI/mL penicillin, 100 μ g/mL streptomycin and 2 mM L glutamine. Healthy human primary hepatocytes (Lonza, Basel, Switzerland) were maintained in phenol and serum-free HBC™ Basal Medium. HBC™SingleQuots®Kit containing 500 μ L hEGF, 500 μ L transferrin, 500 μ L hydrocortisone, 10 mL BSA, 500 μ L Ascorbic Acid, 500 μ L GA-1000 and 500 μ L insulin (Lonza) was added to basal medium. Mouse anti-human monoclonal antibody directed against CD229 (clone HLy 9.1.25) conjugated to Fluorescein Isothiocyanate (FITC), unconjugated anti-CD229 (clone HLy 9.1.25) and mouse IgG1 negative control were from AbD serotec (Colmar, France). Mouse monoclonal anti-CD229 (clone ZY-263), mouse monoclonal anti-CD229 (clone 3H, 1998), anti-actin (clone C-11), rabbit polyclonal anti-SR-B1 (clone H-180) were from Santa Cruz Biotechnology. Mouse anti-human monoclonal antibody directed against CD81 (clone JS-81) unconjugated and conjugated to Phycoerythrin (PE) and FITC-mouse IgG1 κ isotype control (clone MOPC-21) were from BD Pharmingen. PE-anti-mouse IgG1 was from Beckman Coulter. FITC-antimouse Occludin (clone OC-3F10) was from Invitrogen. FITC-anti-goat

IgG and FITC-antirabbit IgG were from Sigma-Aldrich. Rabbit-anti-Claudin 1 was from Cell Signaling. Unconjugated Rat-anti-E2 was a gift from Dr. Debuissou (Lille, France). PE-anti-rat antibody was purchased from Jackson Immuno Research Laboratories.

5 CD229 peptides were synthesized by Genosphere. The HCV serum was from HCVseropositive patients, a gift from Dr. Sandrine Castelain, (Laboratoire de Virologie, Amiens).

Flow cytometry and immunofluorescence analysis. For membrane staining, cells were treated with non-enzymatic cell dissociation solution (Sigma-Aldrich) for 10 min at 10 37°C before washing in cold PBS/0.02% sodium azide/0.5% BSA. Cells (0.5x10⁶ cells per assay) were incubated with conjugated monoclonal or isotype-matched antibodies (5µg/ml) for 20 min at 4°C. For indirect staining, cells were incubated for an additional 20 min at 4°C with secondary antibodies (1/100). After a wash in PBS/0.02% sodium azide/0.5% BSA, cells were fixed with paraformaldehyde 1% (PFA) and stored in the dark at 4°C before analysis.

15 The BD Cytotfix/Cytoperm™ Plus kit (BD Biosciences) was used for intracellular E2 staining. After two washes in cold PBS, cells were incubated in 100 µL fixation/permeabilization solution during 20 min at 4 °C, and washed with BD Perm/Wash™ Buffer 1X containing Fetal Bovine Serum (FBS) and saponin. Then, cells were incubated with the primary anti-E2 antibody in Perm/Wash™ Buffer during 30 min at 4 °C. After a 20 wash, cells were then incubated with the secondary anti-rat-Pe antibody during 20 min.

For Huh-7 immunofluorescence staining, cells (10⁶ cells/ml) were cultured on slides, washed in cold PBS and fixed in PFA 4 % for 10 min at room temperature. Cells were washed and incubated with primary antibodies (1:10) for 18h at 4 °C in humid room. Cells were washed in PBS-BSA-Tween, and then incubated with secondary antibody (1:100) in 25 PBS-BSATween buffer for 30 min at room temperature. Following extensive washes in (PBS/0.1% Tween 20), Slides were mounted with Vectashield® and DAPI for nuclei staining (Vector laboratories Burlingame, CA). The staining was visualized by confocal microscopy (Zeiss LSM710) and analyzed by ImageJ software.

30 **Primers.** The following primers were used for QPCR: HCV-AS1 primer 5'-TCCAAGAAAGGACCCGGT-C-3' (SEQ ID NO: 10) , HCV-AS2 primer 5'-TTCAAGAAAGGACCCAGTC-3' (SEQ ID NO: 11), HCV-S 1 5'-TCCCGGGAGAGCCATAGTG-3'(SEQ ID NO: 12) and MGB probe with 6-FAM (5'-TCTGCGGAACCGGTG-3') (SEQ ID NO: 13) from Eurogentec. For CD229 transcripts

quantification: forward 5'-TGGGACTAAGAGCCTCTGGAAA-3' (SEQ ID NO: 14), reverse 5'-CCAGATGACGTTCTCAATCTC-3' (SEQ ID NO: 15) and MGB probe with 6-FAM (5'-CCCCAACAGTGGTGTC-3') (SEQ ID NO: 16). The expression of GAPDH gene was used as housekeeping endogen control mRNA with Human GAPDH VIC-MGB from Applied Biosystems and the level of GAPDH was used to normalize the quantified mRNA.

Statistics. Results are expressed as mean $\pm$ standard deviation (s.d.) of the mean. Statistical analysis were performed using the Mann-whitney rank-Sum test (ex. Prism 4.0 (GraphPad, San Diego, CA) with a $p < 0.05$ being considered statistically significant.

Results

HCV entry in hepatocytes represents a complex mechanism dependent on several environmental factors such as pH, temperature and other cellular factors¹. After an initial binding step involving molecules like GAGs and LDL-R facilitating initial attachment to the host cell, the particle²⁻⁴, through interactions with glycoproteins-E1/E2 complex, interacts with SR-BI and CD81⁵⁻⁷. CLDN1 and OCLDN act at a later stage and probably after a lateral migration of the virus-receptor complex to the tight junctions (TJ)⁸. Then, HCV is internalized by clathrin-mediated endocytosis, where fusion probably occurs in early endosomes^{9,10}. However, the entry process seems far from being fully understood as some human cell lines expressing these receptors remain resistant to HCV¹¹, suggesting that one or more human-specific HCV entry factor (s) remain (s) to be discovered.

In this study, we have identified the CD229 molecule as a new potential HCV entry factor in hepatocytes. CD229 belongs to the Signaling Lymphocytic Activation Molecule (SLAM) family, which is constituted of nine cellular receptors known to be expressed on immune cells. All members of this family possess a similar structural organization, including an extracellular domain with either two or four Ig-like domains, a single transmembrane segment and an intracytoplasmic region with tyrosine-based motifs. Unlike the other members of the SLAM family, CD229 has four rather than two extracellular Ig domains. Domains 1 and 3 are similar, as well as domains 2 and 4^{12,13}. CD229 is known to be the only member able to be internalized by clathrin-mediated endocytosis¹⁴. In spite of its role in the immune system, CD229 is not well known and the exact functions of this receptor remain to be elucidated. We described for the first time both the expression and functionality of CD229 in

hepatocytes. The analysis of CD229 mRNA and protein sequence is similar in lymphocytes as well as hepatocytes.

SLAM-CD150 was described as co-receptor for the measles virus and other morbilliviruses^{15,16}. The analysis of SLAM molecules expression revealed that only CD229 is expressed on hepatocytes. We analyzed HCV receptors expression on healthy human primary hepatocytes (HHPH), human hepatoma Huh-7 and hepatocarcinoma Hep-G2 cell lines. HHPH express CD229 between 25 and 55%, CD81 at 60%, CLDN1 and OCLDN at about 30% and few SR-B1 (5%). Huh-7 and Hep-G2 cells express CD229 at 20% and less than 5% respectively. CLDN1 is localized at TJ areas. By confocal microscopy analysis, no or few co-localizations between CD229 and CLDN1 were observed, suggesting a major membrane and sub-membrane localization of CD229.

To study the hepatic CD229 involvement in HCV-entry process, we performed infection experiments on the human Huh-7 cell line, which is the one mostly used as *in vitro* model for HCV infection. We used the HCV 2a genotype, an authentic JFH-1 viral strain growing in Huh-7 cell cultures (HCVcc)¹⁷. First, we sought to determine if CD229-expression level in Huh-7 cells correlated with HCV-susceptibility. To do so, we analyzed the infectivity of two Huh-7 sub-populations: CD229^{neg}CD81^{pos} and CD229^{pos}CD81^{pos}. CD229 and CD81 expression were checked by mRNA quantification and flow cytometry. CD81 expression remained unchanged in both sub-populations. At 4 and 24 h post infection (p.i.), the amount of cell-associated virus was twice as high in CD229^{pos}CD81^{pos} as in CD229^{neg}CD81^{pos} cells. At 72 h p.i. the viral load released from CD229^{pos}CD81^{pos} cells was significantly 1.5 times as high as CD229^{neg}CD81^{pos} cells, thus showing the high HCV-susceptibility of the CD229^{pos}CD81^{pos} sub-population.

In order to check whether endogenous CD229 was required for HCV entry in permissive liver cells, we next transfected Huh-7 cells with short interfering RNAs (siRNAs) targeting CD229. The optimal effect of siRNAs was reached between 50 and 54 h after transfection, and they decreased CD229 gene expression by 98% at the most. CD229 siRNAs had no effect on CD81, SR-BI and CLDN-1 expression. The knockdown of CD229 by siRNA Ly9.2 and Ly9.3 resulted in a 50% inhibition of HCVcc entry.

Conversely, CD229 over-expression was resorted to in three cell lines to study the link between high CD229 gene expression and HCV susceptibility. In addition to the reference HCV-permissive Huh-7 line, we used the human HCV-resistant Hep-G2, which does not express CD81, and the monkey kidney COS-7 cell line, which expresses CD81 at 80%, CLDN1 and OCLDN at about 15% and very few SR-B1. Cells were transfected with hepatic

pBud-CD229 (Ly9) or the free vector control pBud (mock) (Supplementary Methods). The ectopic CD229 introduction strongly increased its expression in Huh-7, Hep-G2 and COS-7 and CD81 expression remained unchanged. At 4 h p.i., CD229 over-expression in Huh-7 tripled virus-cell attachment. CD229 gene expression in Hep-G2 and Cos-7 enabled them to fix HCVcc. No significant intracellular E2-fluorescence was detected at 4 h p.i. by flow cytometry. At 72 h p.i., the percentage of infected E2-cells as well as the E2-fluorescence intensity per cell was twice as high in Ly9-Huh-7 as in mock Huh-7 suggesting that CD229 over-expression increased HCV-Huh-7 permissiveness. At 72 h p.i., the intracellular viral E2 protein was detected in 10% of Ly9-Hep-G2 and Ly9-Cos-7 cells. Hence, the introduction of CD229 in these cells made them permissive to HCVcc.

Being resistant to HCV infection, the Hep-G2 cell line is the model used the most because these cells naturally do not express CD81. We focused on CD81-transfected Hep-G2 cells (Hep-G2 CD81_{pos}). These cells express CD81 at 50%. We compared HCVcc infection in parental, mock, Ly9-Hep-G2, Hep-G2 CD81_{pos} and Ly9-Hep-G2 CD81_{pos} cells. We showed that the addition of CD229 increased the infection of HepG-2 CD81_{pos} cells by 30%. In another experiment, mock and Ly9-Huh-7 as well as mock and Ly9-Hep-G2 CD81_{pos} were infected for 72 h, and the collected supernatants from both conditions were added to naive Huh-7 for an additional 72 h. We confirmed that the high level of CD229 gene expression amplified HCV-hepatocytes permissiveness. Ly9-Hep-G2 CD81_{pos} supernatant was less infectious than Ly9-Huh-7 one, as it infected half as many naive Huh-7 cells, at 72 h p.i.

After experiments targeting the endogen CD229 gene expression, we focused on the blockade of the receptor by monoclonal antibodies directed against CD81 and CD229 on HHPH and Huh-7. The amount of cell-associated virus at 72 h p.i. showed that infection decreased by 90%, 70% and 75% in the presence of JS-81, ZY, 3H, respectively (Fig. 1a) in Huh-7. The cell-associated virus was inhibited by JS-81 and 3H by 70% and 65%, respectively in HHPH (Fig. 1b). To check precisely which domain of CD229 was required for HCV attachment and entry in Huh-7, we tested three peptides derived from each domain of CD229 (Fig. 1c). Peptides were incubated with HCVcc before infection (Supplementary Methods). The 3 peptides seemed to be involved in viral attachment as they inhibited viral attachment at more than 50% at 4 h p.i. in Huh-7 cells and HHPH. (Fig. 1d-e). Only the treatment by peptide 2 (P2) was able to inhibit 50% of the infection (Fig 1f-g) suggesting that the first extracellular domain of CD229 is potentially involved in HCV attachment and entry.

In our experiments we used HCVcc that generate infectious particles in cell culture and made an important contribution to the study of the fully HCV life cycle¹⁸. We

demonstrate the correlation between CD229-expression level and HCVcc-hepatocytes permissiveness, in addition to a productive pathway, as shown by the high infectious power of Ly9-Huh-7. We succeeded in infecting the HCVresistant human Hep-G2 and non-human COS-7 cells, but contrary to naturally permissive Huh-7 we failed to obtain a productive pathway as we did not succeed in infecting naive Huh-7 with their supernatants. The introduction of CD81 induced a small up-regulation of the other HCV receptors in Hep-G2 CD81_{pos}. Moreover CD229 over-expression in these cells allowed to increase HCV-Hep-G2 CD81_{pos} permissiveness, but here again the infectious power of these cells was lower than that of Ly9-Huh-7.

CD229 knockdown and blocking result in an incomplete inhibition of HCVcc entry in Huh-7, suggesting that the CD81-dependent entry was not affected by CD229 down-modulation. Thus the entry pathway of HCV involving CD229 may be CD81-independent.

Hepatocytes polarity is relevant to HCV entry as growing evidence suggests that interplay between HCV and TJ proteins CLDN1 and OCLDN₁₉₋₂₁ is essential for viral uptake.

We show that the hepatic CD229 was not expressed at TJ areas contrary to CLND-1 and OCLDN. By immunofluorescence, the virus fixation on hepatocytes did not induce CD229 co-localization with CD81/CLDN-1/OCLDN. Taken together, we envisage CD229 could play a role at two essential steps of the viral cycle. First, we have shown its requirement in cell-virus attachment. Like GAG and LDL-R it could concentrate HCV at the cell surface allowing more effective HCVinteraction with CD81 and other late co-receptors. Then, we know CD229 is able to internalize, which makes us think it could play a role in HCV entry independently of the other receptors. Even if the exact role of CD229 in HCV entry is only partially elucidated, we have precisely identified the first extracellular domain of the receptor as a crucial element for attachment and virus entry and the use of peptides could contribute to the breakthrough of new preventive therapies.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. An agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a
5 subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.
2. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 1 which is
10 selected from the group consisting of antibodies, small molecules, aptamers, or polypeptides.
3. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 2 which is an antibody directed to all or a portion of the extracellular domain of CD229.
- 15 4. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 3 which is an antibody directed to the extracellular domain "domain 1" of CD229 ranging from residue at position 73 to residue at position 154 in sequence SEQ ID NO:1.
- 20 5. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 4 which is an antibody directed to the extracellular domain comprises consecutive amino acids having a sequence as set forth in SEQ ID NO: 2 (peptide P1) or SEQ ID NO:3 (peptide P2).
- 25 6. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to any of claims 3 to 5 which is a chimeric, humanized or human antibody.
7. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 2 which is a polypeptide.

8. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 7 which is a polypeptide comprising all or a portion of the extracellular domain of CD229.
- 5 9. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 7 which is a polypeptide consisting of the extracellular region of CD229 ranging from residue at position 1 to residue at position 448 in sequence SEQ ID NO:1
- 10 10. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 7 which is a polypeptide comprising all or a portion of the extracellular domain "domain 1" of CD229 ranging from residue at position 73 to residue at position 154 in sequence SEQ ID NO:1.
- 15 11. The agent effective to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes for use according to claim 10 which is a polypeptide having a sequence as set forth in SEQ ID NO:2 or SEQ ID NO:3.
- 20 12. An inhibitor of CD229 gene expression for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma.
- 25 13. The inhibitor of CD229 gene expression for use in the treatment of an HCV infection or for use according to claim 13 which is a siRNA, an antisense oligonucleotide or a ribozyme.
14. A pharmaceutical composition for use in the treatment of an HCV infection or for use in the treatment of a disease associated with an HCV infection in a subject in need thereof, such as acute or chronic hepatitis C or for use to prevent a subject from contracting HCV or for use in the prevention of liver diseases, such as liver fibrosis, liver cirrhosis or hepatocellular carcinoma comprising an agent according to any of claims 1 to 11 or an inhibitor according to claim 12 or 13.

15. A method of determining whether a compound is capable of inhibiting HCV infection of a cell which comprises the step consisting of determining whether a candidate compound is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes and ii) positively selecting the candidate compound that is efficient to inhibit the binding of a HCV envelope glycoprotein to CD229 protein present on the surface of hepatocytes.

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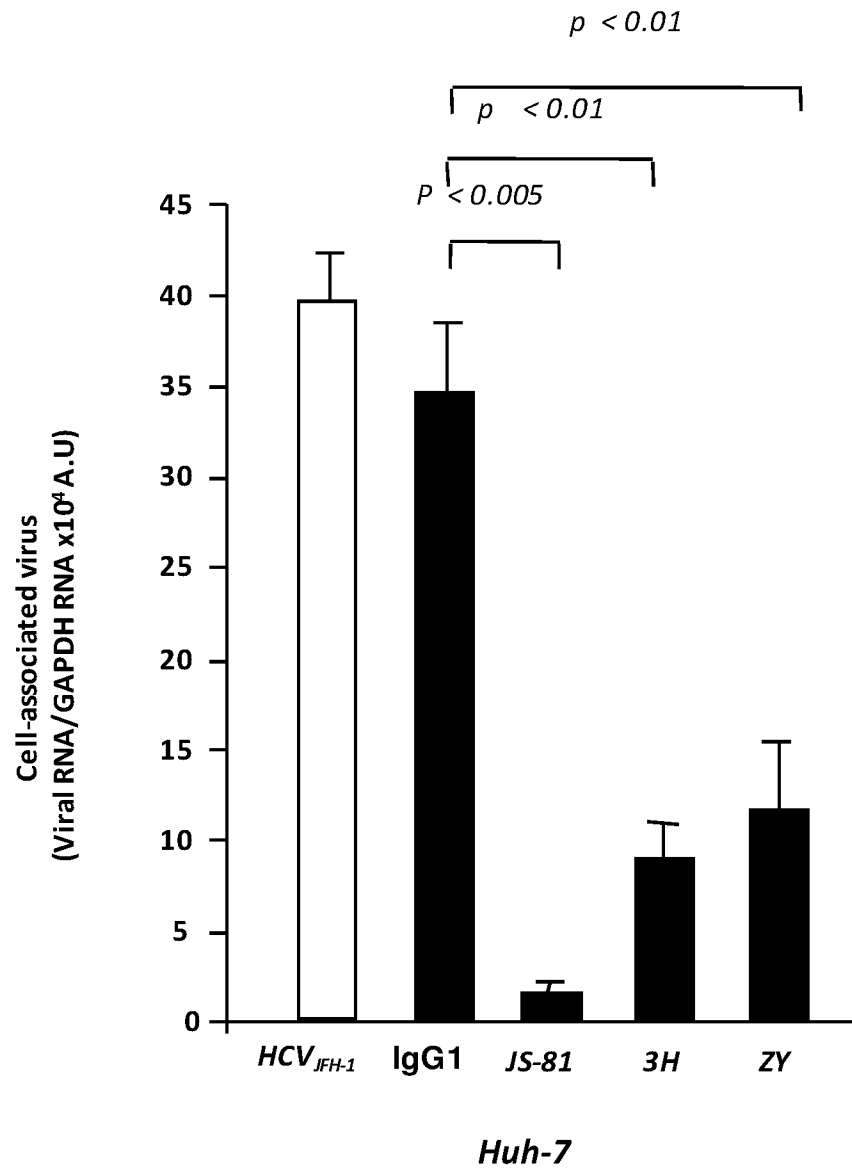


Figure 1a

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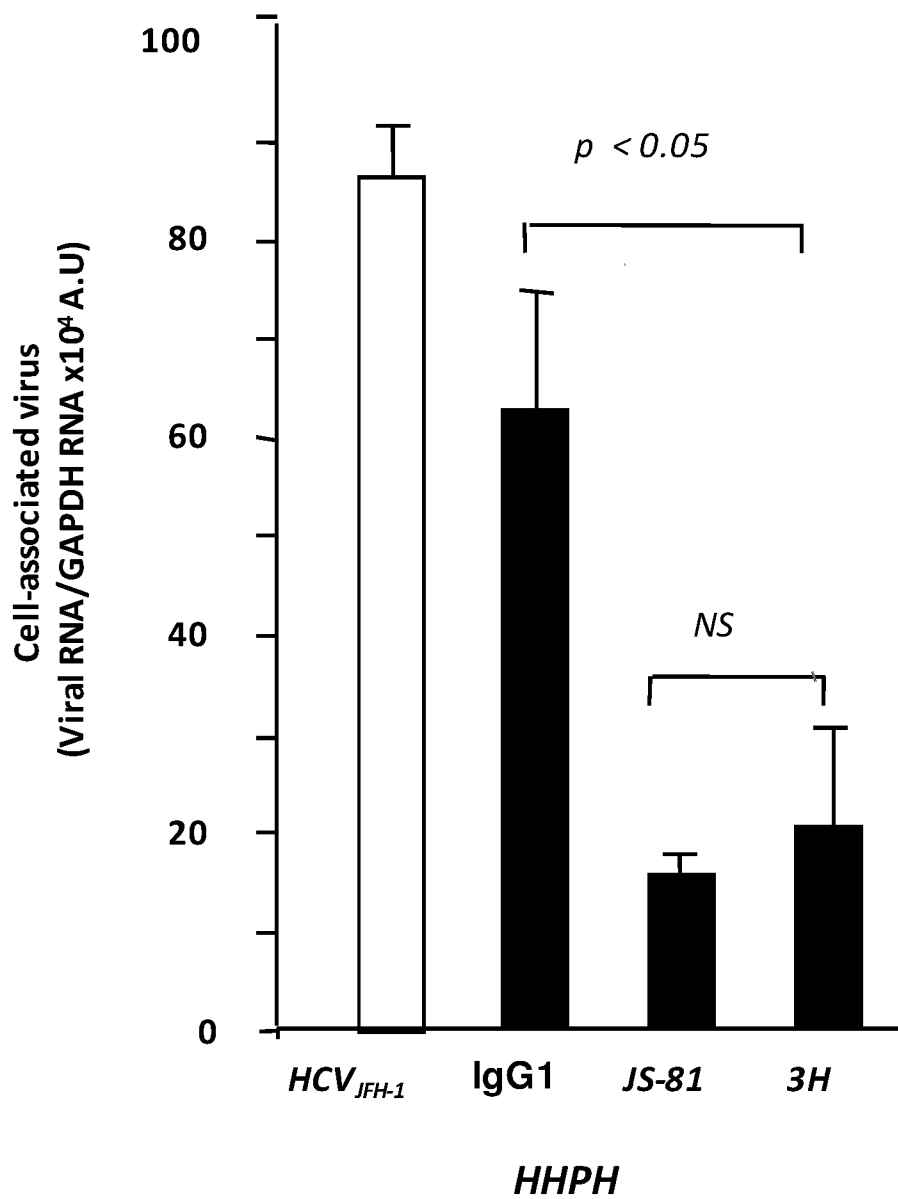


Figure 1b

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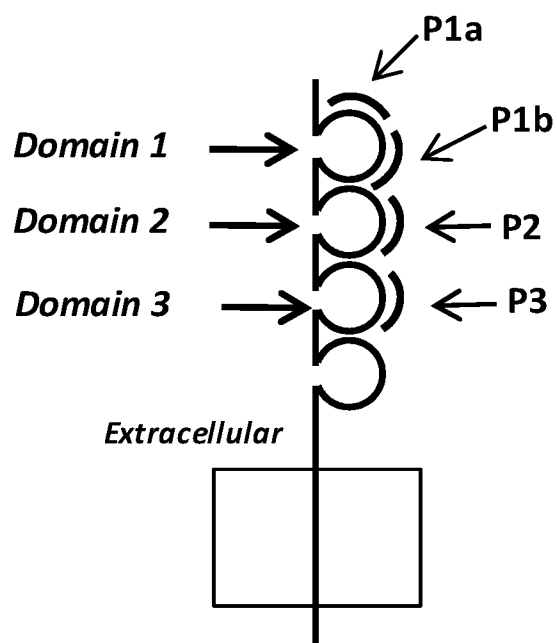


Figure 1c

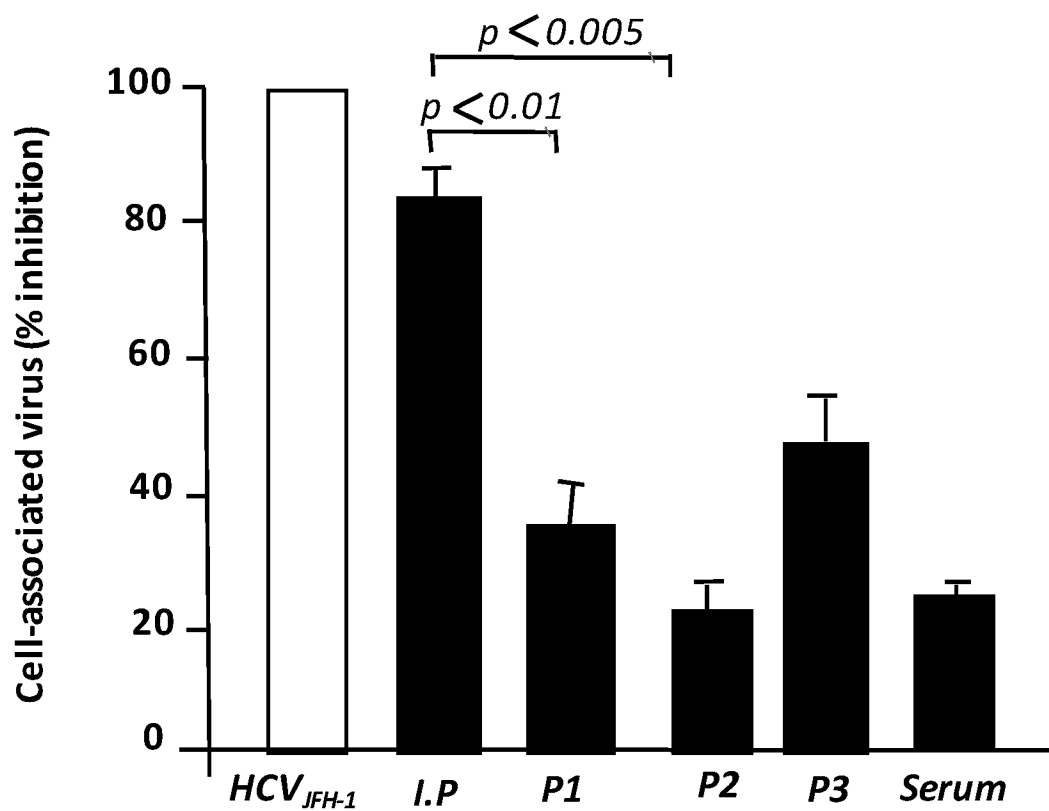


Figure 1d

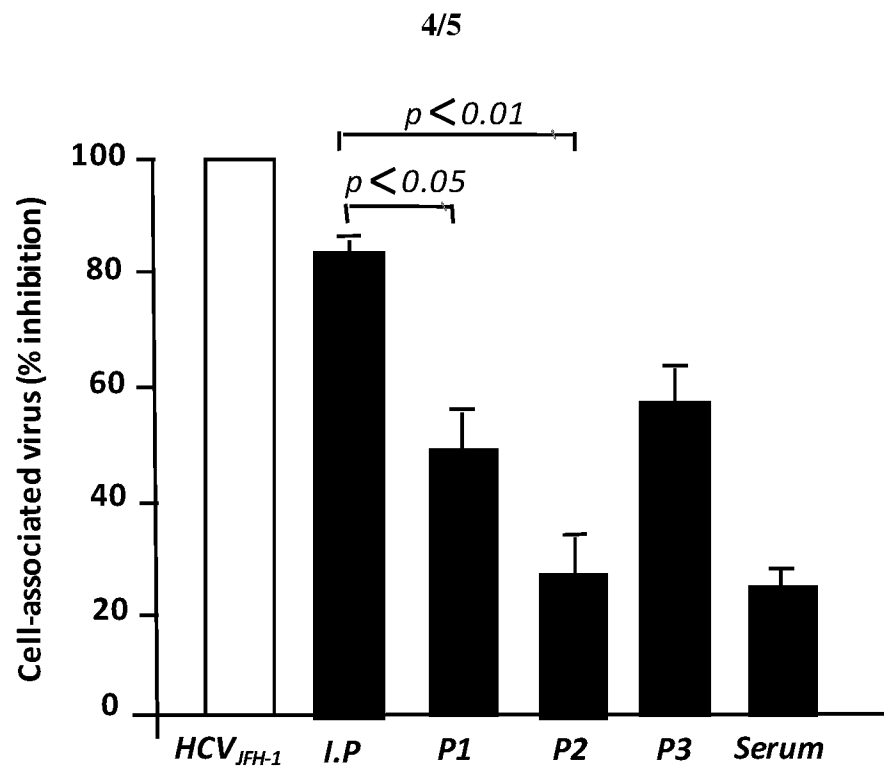


Figure 1e

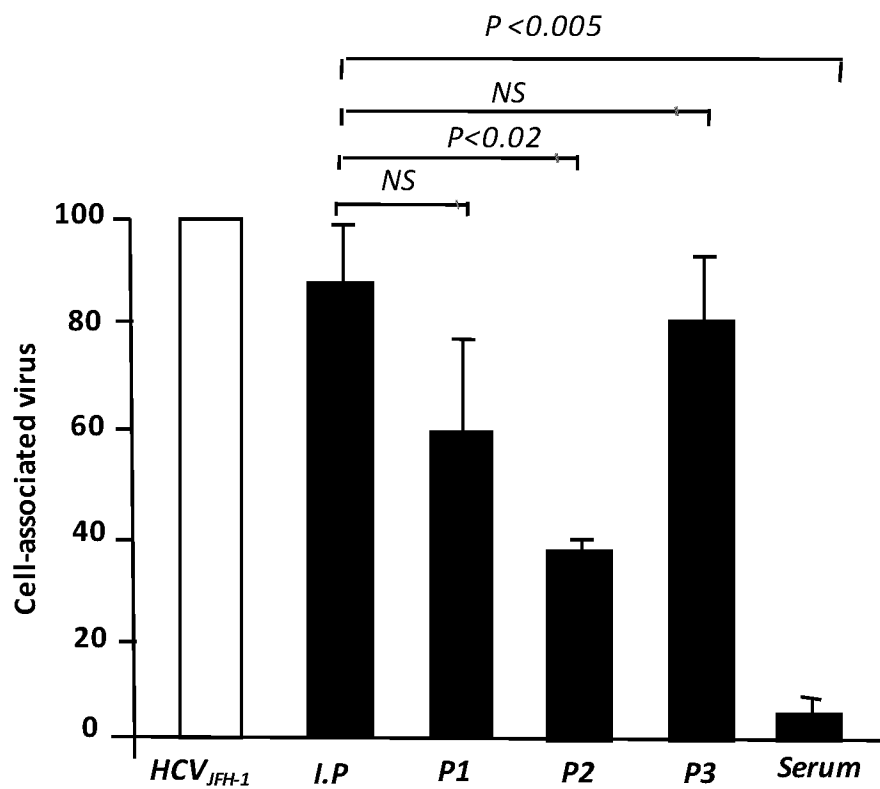


Figure 1f

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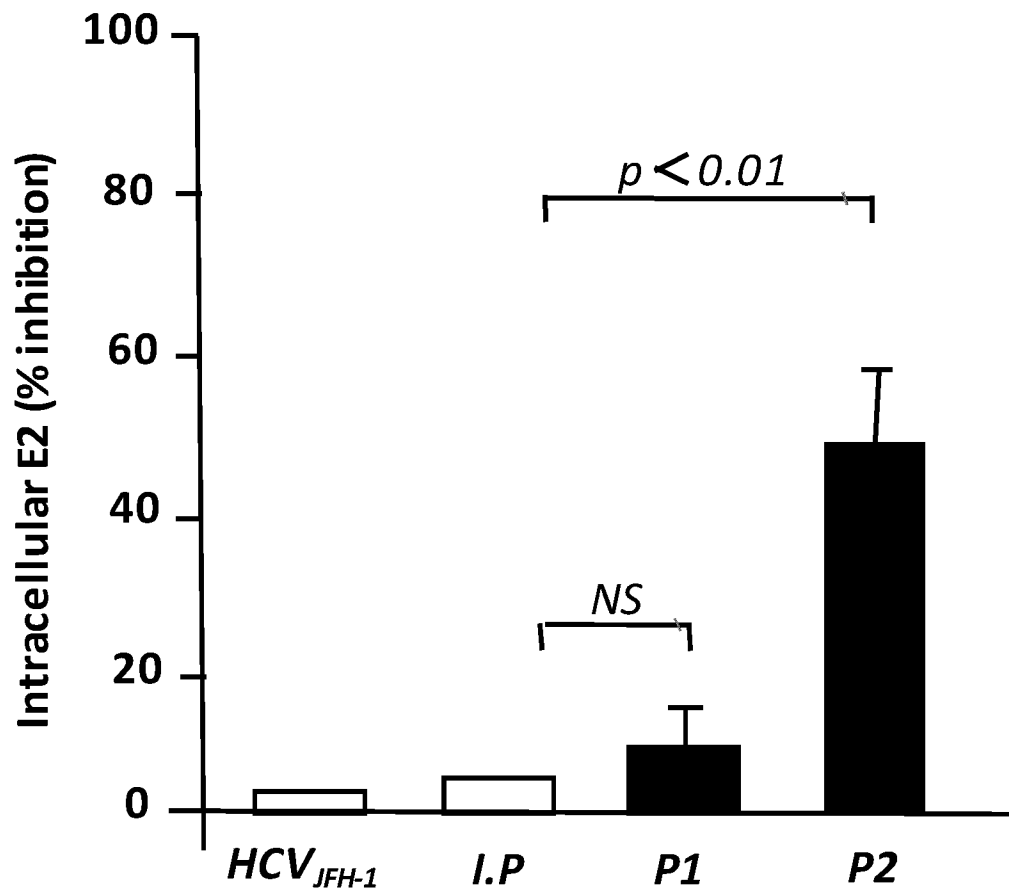


Figure 1g

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/052398

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K16/28 C12N15/113 A61P35/00 A61P31/14 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/035292 A1 (NATIMAB THERAPEUTICS S R L [IT]; BURIONI ROBERTO [IT]; CLEMENTI MASSIM) 1 April 2010 (2010-04-01) page 1, paragraph 1 example 2 page 19 - page 20	1-4,6,7, 14
X	WO 2010/039154 A1 (UNIV LELAND STANFORD JUNIOR [US]; FOUNG STEVEN [US]) 8 April 2010 (2010-04-08) claims 1,36; examples 1,2 ----- -/-	1,2,7,14
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 23 April 2012		Date of mailing of the international search report 03/05/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Le Flao, Katell

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/052398

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE LA FUENTE M A ET AL: "Molecular characterization and expression of a novel human leukocyte cell-surface marker homologous to mouse Ly-9.", BLOOD 1 JUN 2001 LNKD- PUBMED:11369645, vol. 97, no. 11, 1 June 2001 (2001-06-01), pages 3513-3520, XP002648670, ISSN: 0006-4971 page 3519, right-hand column, line 4 - line 27 page 3514, right-hand column, last paragraph -----	1-15
A	DEL VALLE JUANA M ET AL: "The cell surface expression of SAP-binding receptor CD229 is regulated via its interaction with clathrin-associated adaptor complex 2 (AP-2).", THE JOURNAL OF BIOLOGICAL CHEMISTRY 9 MAY 2003 LNKD- PUBMED:12621057, vol. 278, no. 19, 9 May 2003 (2003-05-09), pages 17430-17437, XP002648671, ISSN: 0021-9258 abstract -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2012/052398

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐

 on paper
 - ☒

 in electronic form
 - b. (time)

☒

 in the international application as filed
 - ☐

 together with the international application in electronic form
 - ☐

 subsequently to this Authority for the purpose of search
2.

☐

 In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/052398

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010035292	A1	01-04-2010	NONE

WO 2010039154	A1	08-04-2010	AU 2008362482 A1 08-04-2010
			CA 2738644 A1 08-05-2010
			CN 102197050 A 21-09-2011
			EA 201170353 A1 30-12-2011
			EP 2331573 A1 15-06-2011
			JP 2012504602 A 23-02-2012
			US 2012039846 A1 16-02-2012
			WO 2010039154 A1 08-04-2010
