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(54) Title: MODULATORS OF HCV REPLICATION

(57) Abstract: The present invention is directed to the use of a 2,4,5-trisubstituted imidazole derivative in modulating the replication of Hepatitis C virus RNA and/or virus production in cells.

Modulators of HCV replication

The present invention is directed to the use of a certain 2,4,5-trisubstituted imidazole derivative in modulating the replication of Hepatitis C virus RNA and/or virus production in cells.

It is estimated that about 3% of the world's population are infected with the Hepatitis C virus (HCV) (Wasley, *et al.*, 2000, *Semin. Liver Dis. 20*, 1-16). Exposure to HCV results in an overt acute disease in a small percentage of cases, while in most instances the virus establishes a chronic infection causing liver inflammation and slowly progresses into liver failure and cirrhosis (Iwarson, 1994, *FEMS Microbiol. Rev. 14*, 201-204). In addition, epidemiological surveys indicate an important role of HCV in the pathogenesis of hepatocellular carcinoma (Kew, 1994, *FEMS Microbiol. Rev. 14*, 211-220, Alter, 1995. *Blood 85*, 1681-1695).

Investigating the effects of HCV and antiviral compounds is complicated by the absence of a way to reproduce infection in laboratory small animal models as well as in cultivated cells. HCV infects human and chimpanzees, but does not infect small animals such as mice and rats. Similarly, HCV does not efficiently propagate in any cultivated cells or tissues.

Lohmann *et al., Science 285*, 110-113, 1999 disclose a HCV cell culture system where the viral RNA self-replicates in the transfected cells efficiently, and illustrate the ability of a biscistronic HCV subgenomic replicon to replicate in a hepatoma cell line. An HCV replicon is an RNA molecule able to autonomously replicate in a cultured cell and produce detectable levels of one or more HCV proteins.

HCV replicons can thus be used to produce a cell culture providing detectable levels of HCV RNA and HCV protein. In order to replicate efficiently, however, these replicons require the presence of adaptive mutations (see for example, Lohmann *et al., J Virol 77*, 3007-3019, 2003).

Adaptive mutations are mutations in HCV RNA that enhance the ability of an HCV replicon to be maintained and expressed in a host cells. Examples of adaptive mutations can be found in US6630343 B1; WO2002059321 A2; WO0189364 A2; Bartenschlager *et al.*, *Antiviral Res. 60*, 91-102, 2003, and references therein.

Many adaptive mutations map in the viral protein NS5A, in some cases affecting its phosphorylation status. HCV NS5A is a 446-amino acid phosphoprotein, which is phosphorylated on serine/threonine residues and that exists in two distinct species, termed p56 (phosphorylated) and p58 (hyperphosphorylated). Adaptive mutations can result in a significant reduction of the formation of p58, i.e. the hyperphosphorylated form of NS5A.

It has now surprisingly been found that pharmacological agents can prevent NS5A hyperphosphorylation, and therefore can be used to support replication of HCV RNA in cell culture without the need to introduce adaptive mutations. Such cell culture system is a better mimic of *in vivo* replication and is useful in supporting replication of naturally occurring HCV sequences and assisting the establishment of HCV viral infection assays in cultured cells and test animals.

It has been found that pharmacological agents that prevent NS5A hyperphosphorylation can modulate the replication of HCV RNA also to the extent that HCV RNA replication is inhibited. Inhibitors of NS5A hyperphosphorylation may thus have therapeutic applications to treat individuals infected with HCV.

Thus, in one aspect, the present invention provides the use of compound (**A**) to inhibit the formation of hyperphosphorylated NS5A in a cell, a tissue or an organism, wherein compound (**A**) is:
4-[4-(3,4-dichlorophenyl)-2-piperidinyl-3-yl-1*H*-imidazol-5-yl]pyrimid-2-amine (**A**),
or a suitable salt thereof.

In a further aspect, the present invention provides the use of compound (**A**), or a suitable salt thereof, to modulate the replication of HCV RNA and/or viral production of HCV in a cell, a tissue or an organism.

In a further aspect, the present invention provides a method for modulating the replication of HCV RNA and/or viral production of HCV in a cell, a tissue or an organism comprising administering to the cell, the tissue or the organism compound (**A**), or a suitable salt thereof, to inhibit the formation of hyperphosphorylated NS5A.

The skilled addressee will appreciate that references herein to "modulation" and the like of replication of HCV RNA or viral production of HCV is intended to include the inhibition and enhancement of HCV RNA replication or HCV production.

Thus, in one embodiment, there is provided the use of compound (**A**), or a suitable salt thereof, to enhance HCV RNA replication and/or viral production of HCV in a cell.

In a further embodiment, there is provided a method of enhancing HCV RNA replication and/or viral production of HCV in a cultured cell by treating the cell with compound (**A**) or a suitable salt thereof.

In a further aspect, the present invention provides a cell culture obtainable by treatment with compound (**A**) or a suitable salt thereof.

The skilled addressee will appreciate that references herein to HCV RNA are intended to include sub-genomic replicons and full length HCV RNAs. Full length HCV RNA can be introduced into a cell by transfection of HCV RNA or by inoculating the cell with HCV virus obtained from infected individuals or produced in cell culture.

Enhancing HCV RNA replication in a cell with the compounds of the present invention brings about at least one of the following: an increase in maintenance of HCV RNA replication, an increase in the rate of HCV RNA replication, an increase in HCV RNA expression, an increase in HCV protein expression, and an increase in virus production.

Enhancing replication and expression of HCV RNA in a cell culture system using the compounds of the present invention has a variety of different uses, including being used to study HCV replication and expression, to study HCV and host cell interactions, to produce HCV RNA, to produce

HCV proteins, to assist in establishing HCV viral infection in cell culture and to provide a system for measuring the ability of a compound to modulate one or more HCV activities.

In a further aspect, the present invention provides a method of screening a compound for its effect on HCV replication which comprises administration of the compound to a HCV cell culture that has been treated with compound (**A**) or a suitable salt thereof.

The compound described in this invention can be used to produce a cell culture providing detectable levels of HCV RNA and HCV protein in the absence of adaptive mutations that are specific for given cell culture conditions, cell lines or HCV viral isolates. Moreover, the compounds described in the present invention can be exploited to enable replication, in cultivated cells, of HCV RNA with naturally occurring sequences representing different isolates and genotypes.

Thus, in a further aspect, the present invention provides the use of compound (**A**) or a suitable salt thereof in the production of a cell culture which has detectable levels of HCV RNA and HCV protein in the absence of adaptive mutations in the HCV RNA.

In a further aspect, the present invention provides a method of producing a cell culture which has detectable levels of HCV RNA in the absence of adaptive mutations in the HCV RNA by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) or a suitable salt thereof,

c) evaluating the treated cell for HCV RNA replication.

In a further aspect, the present invention provides a method for producing a cell culture which has detectable levels of HCV protein in the absence of adaptive mutations in the HCV RNA by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) or a suitable salt thereof,

c) evaluating the treated cell for HCV protein expression.

In a further aspect, the present invention provides a method of producing a cell culture which has detectable levels of virus production in the absence of adaptive mutations by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) or a suitable salt thereof,

c) evaluating the amount of viral particles secreted in the cell medium.

The compound described in this invention can also be used in combination with selected adaptive mutations present in HCV variants in order to assist the establishment of detectable HCV RNA replication and HCV protein expression in cultivated cells.

Thus, in a further aspect, the present invention provides the use of compound (A) or a suitable salt thereof in the production of a cell culture which has detectable levels of HCV RNA and HCV protein in the presence of selected adaptive mutations in those cells.

In a further aspect, the present invention provides a method of producing a cell culture which has detectable levels of HCV RNA in the presence of selected adaptive mutations in those cells by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying selected adaptive mutations,

b) treating the cell with compound (A) or a suitable salt thereof,

c) evaluating the treated cell for HCV RNA replication.

In a further aspect, the present invention provides a method of producing a cell culture which has detectable levels of HCV protein in the presence of selected adaptive mutations in those cells by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying adaptive mutations,

b) treating the cell with compound (A) or a suitable salt thereof,

c) evaluating the treated cell for HCV protein expression.

In a further aspect, the present invention provides a method of producing a cell culture which has detectable levels of virus production in the presence of adaptive mutations by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying adaptive mutations,

b) treating the cell with compound (A) or a suitable salt thereof,

c) evaluating the amount of viral particles secreted in the cell medium.

Cell systems suitable for use in the present invention include, but are not restricted to, primary human cells, for example hepatocytes, T-cells, B-cells and foreskin fibroblasts, as well as continuous human cell lines, for example HuH7, HepG2, HUT78, HPB-MA, MT-2, MT-2C, and other HTLV-1 and HTLVII infected T-cell lines, Namalawa, Daudi, EBV-transformed LCLs. In addition, cell lines of other species, especially those that are permissive for replication of flaviviruses or pestiviruses, for example SW-13, Vero, BHK-21, COS, PK-15, MBCK, etc., can be used.

Preferred cell systems are hepatoma cell lines such as Huh -7, Hep3B and HepG2.

The skilled person will appreciate that the uses and methods described herein to modulate HCV RNA replication and/or HCV virus production in cell cultures can be adapted to modulate HCV RNA replication, HCV virus infection and/or HCV virus production in test animals.

Test animals suitable for use in the present invention include mammals such as rodents. Preferred test animals are rodents such as rats and mice.

The presence of replicating HCV RNA can be evaluated by conventional methods such as, for example, RT-PCR, quantitative RT-PCR, Northern blotting, or by measuring the activity of an HCV protein or protein encoded by reporter gene engineered into the HCV RNA.

HCV protein expression can be evaluated by conventional methods such as, for example, ELISA assays, Western Immunoblots, or radioactive protein labeling followed by immunoprecipitation assays.

The presence of HCV viral particles secreted in the cell medium can be evaluated by conventional methods, such as, for example, real-time reverse transcription PCR amplification (TaqMan), b-DNA, or by utilizing the cell medium to infect naïve cells or laboratory animals.

The compounds described in this invention can also be used in order to identify the cellular kinase(s) responsible for the hyperphosphorylation of HCV NS5A.

Thus, in a further aspect, the present invention provides the use of compound (**A**) or a suitable salt thereof in the identification of cellular kinase(s) responsible for the hyperphosphorylation of HCV NS5A.

In a further aspect, the present invention provides a method of identifying cellular kinase(s) responsible for the hyperphosphorylation of HCV NS5A by:

a) covalently binding compound (**A**) or a suitable salt thereof to a chromatography matrix,

b) using the chromatography matrix to purify kinase(s) from cellular protein extracts,

c) eluting the kinase(s) from the affinity matrix,

d) identifying the eluted kinase(s).

The level of NS5A hyperphosphorylation needs to be tightly regulated during the viral replication. It has been found that varying the concentration of compound (**A**) can modulate the replication of HCV RNA to the extent that HCV RNA replication is inhibited. Inhibitors of NS5A hyperphosphorylation may thus have therapeutic applications to treat HCV patients.

Thus, in a further aspect, the present invention provides the use of compound (**A**) or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of HCV infection.

In another aspect of the invention, there is provided a method of inhibiting replication of HCV RNA and/or of treating or preventing an illness due to hepatitis C virus, the method involving administering to a human or animal (preferably mammalian) subject suffering from the condition a therapeutically or prophylactically effective amount of the pharmaceutical composition described above or of compound (**A**) as defined above, or a pharmaceutically acceptable salt thereof. "Effective amount" means an amount sufficient to cause a benefit to the subject or at least to cause a change in the subject's condition.

In a further embodiment of the present invention, there is provided the use of compound (**A**), or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment or prevention of infection by hepatitis C virus, in combination with one or more other agents for the treatment of viral infections such as an antiviral agent, and/or an immunomodulatory agent such as α-, β- or γ-interferon, particularly α-interferon. Suitable antiviral agents include ribavirin and inhibitors of

hepatitis C virus (HCV) replicative enzymes, such as inhibitors of metalloprotease (NS2-3), serine protease (NS3), helicase (NS3) and RNA-dependent RNA polymerase (NS5B).

Suitable pharmaceutically acceptable salts of the compounds of this invention include acid addition salts which may, for example, be formed by mixing a solution of the compound according to the invention with a solution of a pharmaceutically acceptable acid such as hydrochloric acid, fumaric acid, p-toluenesulfonic acid, maleic acid, succinic acid, acetic acid, citric acid, tartaric acid, carbonic acid, phosphoric acid or sulfuric acid. Salts of amine groups may also comprise quaternary ammonium salts in which the amino nitrogen atom carries a suitable organic group such as an alkyl, alkenyl, alkynyl or aralkyl moiety.

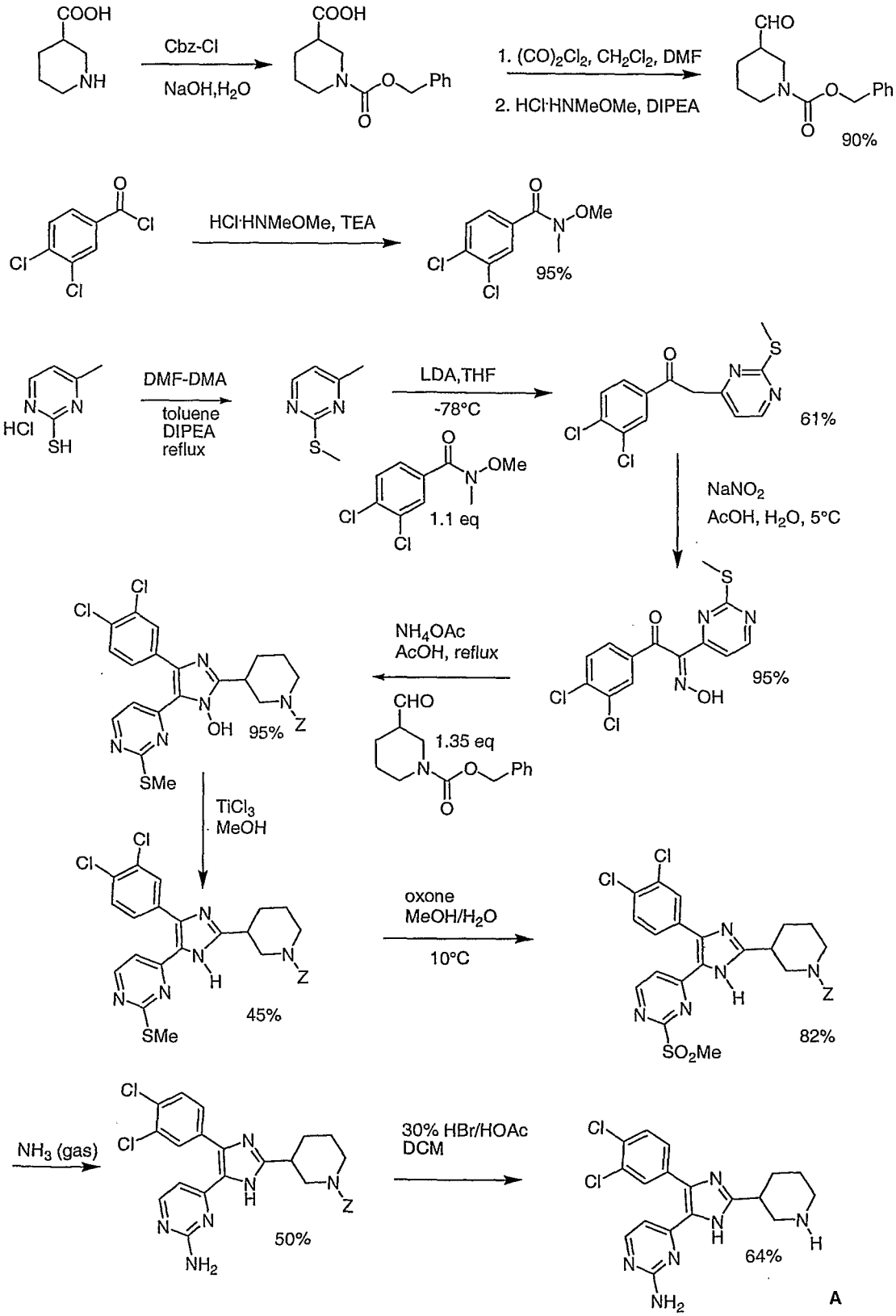
Suitable salts of the compounds of the present invention include, not only the pharmaceutically acceptable salts thereof as hereinbefore described, but also any common salts or quaternary ammonium salts formed, e.g., from inorganic and organic acids. Suitable salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like: and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, malic, tartaric, citric, ascorbic, mapoic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methane-sulfonic, ethane disulfonic, oxalic, isethionic, trifluoroacetic and the like. The salts are generally prepared by reacting the free base or acid with stoichiometric amounts or with an excess of the desired salt-forming inorganic or organic acid or base in a suitable solvent or solvent combination.

The present invention includes within its scope prodrugs of compound (**A**) above. In general, such prodrugs will be functional derivatives of compound (**A**) which are readily convertible *in vivo* into the required compound (**A**). Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in "Design of Prodrugs", ed. H. Bundgaard, Elsevier, 1985.

A prodrug may be a pharmacologically inactive derivative of a biologically active substance (the "parent drug" or "parent molecule") that requires transformation within the body in order to release the active drug, and that has improved delivery properties over the parent drug molecule. The transformation *in vivo* may be, for example, as the result of some metabolic process, such as chemical or enzymatic hydrolysis of a carboxylic, phosphoric or sulfate ester, or reduction or oxidation of a susceptible functionality.

Compound (**A**) is disclosed in published International patent application WO 97/47618 (Merck & Co., Inc.). The synthesis of compound (**A**) is shown in the following scheme:

Scheme 1: Compound (A)



The abbreviations used in this scheme are as follows:

Ac = acetyl; Cbz or Z = benzyloxycarbonyl; DCM = dichloromethane; DIPEA = N,N-diisopropylethylamine; DMF = N,N-dimethylformamide; DMF-DMA = N,N-dimethylformamide dimethylacetal; LDA = lithium diisopropylamide; TEA = triethylamine

The invention is illustrated by the accompanying Figures.

Figure 1 - *Inhibition of NS5A hyperphosphorylation in cell culture*

The presence of hyperphosphorylated NS5A (p58) was evaluated in Huh7-HB68 cells. Proteins were labeled either with ^{35}S-methionine (lanes 2-4) or with ^{32}P-orthophosphate (lanes 6-8) in the presence of DMSO (lanes 2 and 6) or with 5 μM of Compound (A) (lanes 3 and 7) or **SB203580** (lanes 4 and 8). After the radiolabelling, protein extract was prepared, NS5A was immunoprecipitated and proteins were loaded on a 7.5 % SDS-PAGE and autoradiographed. The sizes of molecular weight marker proteins are indicated in lanes 1 and 5.

Figure 2 - *Detection of HCV-RNA and HCV-specific proteins after treatment of the cells with compounds described in the invention*

RNA was transcribed from the plasmids wt, wt-GAA, m17, SA, m17/SA and m17-GAA and electroporated into 10A-IFN cells. Cells were incubated for four days without or with 8 μM of Compound (A) or **SB203580**.

(A) HCV-specific RNA analysis using quantitative RT-PCR. Total cellular RNA was extracted and HCV-specific RNA was quantified as described in Materials and Methods. On the Y-axis is shown the fold-induction with respect to the DMSO control (black bar), Compound (A) (grey bar) or **SB203580** (dotted bar). Replicon RNA is indicated at the bottom of the figure.

(B) Western Blot of total protein extract. Cell extract was prepared and 50 μg of protein were loaded onto SDS-PAGE for each lane. Specific anti-NS5A antibody was used as primary antibody and a Peroxidase-conjugated antibody (Pierce) was used as secondary antibody. The Western Blot was developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

NS5A Hyperphosphorylation assay

Inhibition of NS5A hyperphosphorylation in intact cells was measured by determining the amount of hyperphosphorylated NS5A (p58) in cells expressing HCV NS5A in the context of a polyprotein comprising at least NS3, NS4A, NS4B, and NS5A (Neddermann et *al., J Virol 73*, 9984-9991, 1999). Suitable cells are, for example, cells stably expressing an HCV replicon with adaptive mutations that do not affect NS5A hyperphosphorylation, such as HBI10 or HB68. HBI10 and HB68 are Huh-7 derived-cell lines described in WO2002/059321.

Hyperphosphorylated NS5A was detected as a protein that i) migrates with an apparent molecular weight of about 58 kDa in SDS PAGE, and ii) is immunoreactive with anti-NS5A antibodies.

Thus, the amount of hyperphosphorylated NS5A was detected by immunoprecipitation of radioactively labeled proteins.

MATERIALS

Cell culture

HBI10 or HB68 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) in the presence of 0.8 mg/ml of G418 (Geneticin; Gibco/BRL). For starvation prior to radioactive protein labeling, minimal essential medium without methionine (Gibco/BRL) was used. For protein labeling, 100 μCi/ml of ^{35}S-labelled methionine (Promix, Amersham, Cat. No. SJQ0079, 1000 Ci/mmole) was added to the cells.

Cell Lysis Buffer

25 mM sodium phosphate pH 7.5, 20% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF).

Immunoprecipitation buffer

20 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100

NDET buffer

10 mM Tris-HCl pH7.50, 4% sodium deoxycholate, 0.5% Triton X-100, 10 mM EDTA

Protein A-Sepharose resin

Protein A-Sepharose resin was obtained from Amersham Biosciences

Antibodies

NS5A-specific antisera were obtained as described in Tomei *et al., J. Virol. 67*, 4017-4026, 1993.

METHOD

1. HBI10 or HB68 cells were grown to 80 % confluency in 6-well plates.
2. Compound to be tested, dissolved in DMSO at 100x concentration, was added to each well.
3. One hour later, the medium was removed and replaced with Minimal Essential Medium without methionine. This step is omitted in the case of 32P-orthophosphate labeling.
4. Compound to be tested, dissolved in DMSO at 100x concentration, was freshly added to each well.

5. One hour later 100 μCi of ^{35}S-labelled methionine per ml of Minimal Essential Medium without methionine was added to each well for 35S-metabolic labeling together with the compound to be tested, dissolved in DMSO at 100x concentration. In the case of 32P-labelling, cells were washed once with Dulbecco's modified Eagle's medium without phosphate (ICN) and labeled for 4 hours in the same medium containing 500μCi/ml of [32P]-orthophosphate (285.5 Ci/mg, NEN) together with the compound to be tested, dissolved in DMSO at 100x concentration.
6. After 4 hours, cells were harvested from each well and individual cell extracts were prepared in 100 μl of cell lysis buffer.
7. 50 μl of each extract were then heated at 95°C for 4 min after the addition of 2% sodium dodecyl sulfate (SDS) and 10 mM DTT.
8. Aliquots of antibody coated protein A-Sepharose for immunoprecipitation of the extracts were prepared by mixing 5 μl of HCV NS5A-specific antisera and 50 μl of protein A-Sepharose 50% suspension in 300 μl of immunoprecipitation buffer and incubating under gentle stirring for 1 h at 4°C.
9. The antibody-coated protein A-Sepharose thus obtained was then washed twice with 300 μl of immunoprecipitation buffer and resuspended in 500 μl of the same buffer.
10. The radiolabelled protein extracts obtained at step 7 were added to the suspension and the mixtures incubated under gentle stirring for 1 h at 4°C
11. The immunoprecipitate was collected and resuspended and the mixture layered on 0.7. ml of 0.5× NDET buffer containing 30% sucrose and pelleted by centrifugation for 5 min at 5,000 × *g*.
12. The immunoprecipitate was then washed once with 500 μl of NDET and once with 500 μl of PBS and
13. Protein was detached from the PAS-resin by boiling in SDS sample buffer and loaded on a 7.5 % SDS-PAGE for electrophoresis. When the dye front reached the bottom of the gel, the gel was fixed, soaked in Amplify (Amersham Bioscience) for 30 minutes, dried, and autoradiographed on an X-ray film or a Phosphoimager (Storm 820, Amersham Pharmacia Biotech) in order to evaluate the amount of hyperphosphorylated NS5A. The intensities of the bands corresponding to hyperphosphorylated NS5A and non-hyperhosphorylated NS5A were compared to determine the percent inhibition of NS5A hyperphosphorylation.

Agents that inhibit the formation of hyperphosphorylated NS5A were tested for inhibitory activity in the assay described above and the compounds were generally be found to have IC_{50} values in the range from about 0.001 μM to about 50 μM.

Methods to detect HCV replication

To determine the biological consequences of inhibition of NS5A hyperphosphorylation, the effect of the compounds of the present invention were tested on HCV RNA replication in cell culture.

Methods for detecting HCV RNA replication include those measuring the production or activity of HCV RNA, production or activity of viral proteins or production of viral particles. Measuring includes qualitative and quantitative analysis.

Techniques suitable for measuring RNA production include those detecting the presence or activity of RNA. The presence of RNA can be detected using, for example, complementary hybridization probes or quantitative RT-PCR or Northern blotting. Techniques for measuring hybridization between complementary nucleic acid and quantitative PCR are well known in the art (see for example, Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-1998, Sambrook, *et al., Molecular Cloning, A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, and U.S. Patent No. 5,731,148).

Techniques for measuring protein production include those detecting the presence or activity of a produced protein. The presence of a particular protein can be determined by, for example, immunological techniques such as ELISA assays, Western Immunoblots, or radioactive protein labeling followed by immunoprecipitation assays. Protein activity can be measured based on the activity of an HCV protein or a reporter protein sequence.

Techniques for measuring HCV protein activity vary depending upon the protein that is measured. Techniques for measuring the activity of different non-structural proteins such as NS2/3, NS3, and NS5B, are well known in the art (see, for example, references hereinbefore provided).

Assays measuring HCV RNA replication also include those detecting virion production from a replicon that produces a virion. The presence of HCV viral particles secreted in the cell medium can be evaluated by conventional methods, such as, for example, real-time reverse transcription PCR amplification (TaqMan), b-DNA, or by utilizing the cell medium to infect naïve cells or laboratory animals. Assays measuring HCV RNA replication also include those detecting a cytopathic effect from a replicon producing proteins exerting such an effect. Cytopathic effects can be detected by assays suitable to measure cell viability.

A reporter sequence can be used to detect HCV RNA replication or protein expression. Preferred reporter proteins are enzymatic proteins whose presence can be detected by measuring product produced by the protein. Examples of reporter proteins include luciferase, beta-lactamase, secretory alkaline phosphatase, beta-glucuronidase, green fluorescent protein and its derivatives. In addition, a reporter nucleic acid sequence can be used to provide a reference sequence that can be targeted by a complementary nucleic acid. Hybridization of the complementary nucleic acid to its target can be determined using standard techniques.

Assays measuring HCV RNA replication can be used to evaluate the ability of a compound to modulate HCV RNA replication. Such assays can be carried out by providing one or more test compounds to a cell expressing an HCV RNA and measuring the effect of the compound on RNA replication.

EXAMPLES

Examples are provided below to further illustrate different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Materials and Techniques

This example illustrates the techniques employed for evaluating the biological effects of the compounds of the present invention

Cells and cell culture

HBI10A, HB68 and 10AIFN were derived from Huh-7 cells as described in WO2002059321 A2; Mottola *et al., Virology 293,* 31-43, 2002; and Trozzi *et al., J Virol 77,* 3669-3679, 2003.

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and in the case of HBI10A and HB68 in the presence of 0.8 mg/ml of G418 (Geneticin; Gibco/BRL). For routine work, cells were passed 1 to 5 twice a week using 1x trypsin/EDTA (Gibco, BRL).

Nucleic Acids and Construction of Recombinant Plasmids

Manipulation of nucleic acids was done according to standard protocols (Sambrook, *et al.,* 1989. *Molecular Cloning: A Laboratory Manual,* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) Plasmid DNA was prepared from ON culture in LB broth using Qiagen 500 columns according to manufacturer instructions.

Plasmids containing desired mutations were constructed by restriction digestion using restriction sites flanking the mutations or by PCR amplification of the area of interest, using synthetic oligonucleotides with the appropriate sequence. Site directed mutagenesis was carried out by inserting the mutations in the PCR primers. PCR amplification was performed using high fidelity thermostable polymerases or mixtures of polymerases containing a proofreading enzyme (Barnes, *et al.,* 1994. *Proc. Natl. Acad. Sci. 91,* 2216-2220.) All plasmids were verified by restriction mapping and sequencing.

pHCVneo17.wt is described in Trozzi *et al., J Virol 77,* 3669-3679, 2003. It contains the cDNA for an HCV bicistronic replicon identical to replicon I_{377}neo/NS3-3'/wt described by Bartenschlager (SEQ. ID. NO. 3) (Lohmann *et al.,* 1999. *Science 285,*110-113, EMBL-genbank No. AJ242652). The plasmid comprises the following elements: 5' untranslated region of HCV comprising the HCV-IRES and part of the core (nt1-377); neomycin phosphotransferase coding sequence; and EMCV IRES; HCV coding sequences from NS3 to NS5B; 3' UTR of HCV. pHCVNeo17.C is a variant of pHCVneo17.wt as described in Trozzi *et al., supra.* The other plasmids are identical to pHCVNeo17.wt but contain the following mutations: (i) SA, S2204A in NS5A; (ii) SI, S2204I in NS5A; (iii) AT, A2199T in NS5A; (iv) m17/SA, S2204A in NS5A and E1202G in NS3; (v) m17-GAA, E1202G

in NS3 and D2737A / D2738A in NS5B; (vi) wt-GAA, D2737A / D2738A in NS5B. For the Bla-reporter HCV replication assay the neomycin phosphotransferase (*neo*) gene of plasmid pHCVneo17.wt and pHCVneo17.SA was replaced by the β-lactamase (*bla*) gene as described in WO2003089672 A1 and in Murray *et al.*, *J Virol 77*, 2928-2935, 2003, resulting in plasmid wt-BLA and SA-BLA, respectively.

RNA Transfection

Plasmids were digested with the ScaI endonuclease (New England Biolabs) and transcribed *in vitro* with the T7 Megascript kit (Ambion). Transcription mixtures were treated with DNase I (0.1 U/ml) for 30 minutes at 37°C to completely remove template DNA, extracted according to the procedure of Chomczynski (Chomczynski *et al.*, 1987. *Anal. Biochem. 162*, 156-159), and resuspended with RNase-free phosphate buffered saline (rfPBS, Sambrook *et al.*, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

RNA transfection was performed as described by Liljestrom *et al.*, 1991. *J. Virol. 6*, 4107-4113, with minor modifications. Subconfluent, actively growing cells were detached from the tissue culture container using trypsin/EDTA. Trypsin was neutralised by addition of 3 to 10 volumes of DMEM/10%FCS and cells were centrifuged for 5 minutes at 1200 rpm in a Haereus table top centrifuge at 4°C. Cells were resuspended with ice cold rfPBS by gentle pipetting, counted with a haemocitometer, and centrifuged as above. rfPBS wash was repeated once and cells were resuspended at a concentration of 1-2 x 10^7 cell/ml in rfPBS. Aliquots of cell suspension were mixed with RNA in sterile eppendorf tubes. The RNA/cell mixture was immediately transferred into the electroporation cuvette (precooled on ice) and pulsed twice with a gene pulser apparatus equipped with pulse controller (Biorad). Depending on the experiment, 0.1, 0.2 or 0.4 cm electrode gap cuvettes were used, and settings adjusted (see Table below).

Table

Cuvette gap (cm)	**Volume (µl)**	**Voltage (Volts)**	**Capacitance (µFa)**	**Resistance (ohm)**	**RRNA (µg)**
0.1	70	200	25	infinite	1-10
0.2	200	400	25	infinite	5-20
0.4	800	800	25	infinite	15-100

After the electric shock, cells were left at room temperature for 1-10 minutes (essentially the time required to electroporate all samples) and subsequently diluted with at least 20 volumes of DMEM/10%FCS and plated as required for the experiment. Survival and transfection efficiency were monitored by measuring the neutral red uptake of cell cultured for various days in the absence or in the

presence of neomycin sulfate (G418). With these parameters, survival of Huh-7 cells was usually 40-60% and transfection efficiency ranged between 40% and 100%.

Real-time reverse transcription PCR amplification (TaqMan)

Replicon RNA was extracted from selected clones either using the Qiagen RNAeasy minikit following manufacturer instructions or as described by Chomczynski *et al.*, 1987. Anal. Biochem. *162*, 156-159. TaqMan analysis was typically performed using 10 ng of RNA in a reaction mix (TaqMan Gold RT-PCR kit, Perkin Elmer Biosystems) either with HCV specific oligos/probe (as disclosed in published International application WO02/059321) or with human beta-actin specific oligos/probe (Pre-Developed TaqMan Assay Reagents, Endogenous Control Human beta-actin, Part Number 4310881E, Applied Biosystems). PCR was performed using a Perkin Elmer ABI PRISM 7700 under the following conditions: 30 minutes at 48°C (the RT step), 10 minutes at 95°C and 40 cycles: 15 seconds at 95°C and 1 minute at 60°C. Quantitative calculations were obtained using the Comparative C_T Method (described in User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystem, Dec 1997) considering the level of GAPDH mRNA constant. All calculations of HCV RNA are expressed as fold difference over a specific control.

Beta-lactamase gene reporter assay (BLA –assay)

The BLA-assay was performed after 4 days of incubation in the presence of the compounds to be tested according to Murray *et al., J Virol 77,* 2928-35, 2003. Briefly, medium was removed, and cells were stained for 90 min with CCF4-AM (Aurora Biosciences Corp.) in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, pH 8.0. For quantitation of the fraction of cells harboring *bla* replicons, cells were photographed by using a digital charge-coupled device color camera and green and blue cells were counted. Another method for measuring beta-lactamase activity is using a fluorescence plate reader that quantitates the amount of green (530 nm) or blue (460 nm) fluorescence emitted by cells stimulated with light of 405 nm.

Cell ELISA assays

The effect of the compounds of invention on viral replication and the replication proficiency of the mutant replicons was estimated by monitoring expression of the NS3 protein by Cell-ELISA with the anti-NS3 mab 10E5/24 as described by Trozzi *et al.* J. Virol. 2003, 77:3669-79). Compounds were dissolved and serially diluted in dimethyl sulfoxide (DMSO) in such a way that the final DMSO concentration was 1%. Transient transfection assays were performed with 10AIFN cells, prepared and transfected by electroporation as described by Trozzi *et al.* J. Virol. 2003, 77:3669-79). Cells were supplemented with the compounds between 1 and 4 hours after transfection

Example 2: Compound synthesis

Compound SB203580 was purchased from Calbiochem (San Diego, CA 92121). Compound (A) was obtained as described above.

Example 3: Inhibition of NS5A phosphorylation in cell culture by compounds of the invention

Compound (A) of the present invention was evaluated in cell culture in order to assess its effect on NS5A phosphorylation in the context of live cells and active HCV replication using HB68 cells, which stably carry an adapted HCV replicon.

In order to follow NS5A hyperphosphorylation, cells were metabolically labeled with ^{35}S-methionine, or with ^{32}P-orthophosphate to investigate phosphorylation efficiency. Compound (A) inhibited the formation of the hyperphosphorylated form of NS5A (p58; Fig. 1, lanes 3 and 7 when used at a concentration of 5 μM. No compound inhibited basal NS5A phosphorylation without affecting NS5A expression. SB203580 was used as a negative control as it had no effect either on NS5A expression or on NS5A phosphorylation in cells at a concentration of 5 μM.

Example 4: Activation of replication of wt Con1 replicon in the presence of compounds of invention – Detection by the *bla*-gene reporter assay

To determine the biological consequences of inhibition of NS5A hyperphosphorylation, the effect of compounds of the present invention on HCV RNA replication effect was assessed in cell culture.

A subgenomic replicon was used in which the original neomycin phosphotransferase (*neo*) gene was replaced by the β-lactamase (*bla*) gene (WO2003089672 A1 and Murray *et al.*, *J Virol 77*, 2928-2935, 2003). Cells actively replicating HCV express β-lactamase and show a fluorescent blue staining after incubation with a diffusible β-lactamase substrate (BLA-assay). Replicon RNA was electroporated in 10A-IFN cells and compounds were added at a concentration of 8 μM two hours after electroporation. The BLA-assay was performed after 4 days of incubation in the presence of the compounds to be tested according to Murray et al., J Virol 77, 2928-35, 2003. Briefly, medium was removed, and cells were stained for 90 min with CCF4-AM (Aurora Biosciences Corp.) in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, pH 8.0. For quantitation of the fraction of cells harboring *bla* replicons, cells were photographed by using a digital charge-coupled device color camera and green and blue fluorescent cells were counted. Alternatively, fluorescence was measured by using a CytoFluor 4000 fluorescence plate reader.

Electroporation of the wild type Con1 replicon did not generate any fluorescent blue cells, whereas the addition of compound (A) used at a final concentration of 8 μM resulted in the production of fluorescent blue cells as a consequence of HCV replication. The control compound SB203580 had no effect on HCV replication. In order to demonstrate that the blue staining is a result of HCV replication and not a result of a longer half life of the electroporated HCV RNA or β-lactamase enzyme, the cells

were incubated, in addition to the compounds, with an inhibitor of the HCV RNA-dependent RNA polymerase (Tomei *et al., J. Virol. 78*, 938-946, 2004). In the presence of a cell-permeable inhibitor of the HCV RNA-dependent RNA polymerase the number of observable fluorescent blue cells is significantly reduced.

Example 5: Activation of replication of wt Con1 replicon in the presence of compounds of invention – Detection of HCV-RNA and HCV-specific proteins

It was investigated whether the compounds of the present invention activated HCV replication to an extent sufficiently efficient to allow the detection of viral proteins or viral RNA in the total cell population. RNA of wt Con1 replicon was electroporated into 10A-IFN cells and compounds were added two hours after electroporation. After 4 days of incubation, cells were collected and cellular extracts were assayed for the presence of NS5A by immunoblot (Fig. 2B) or for HCV RNA by quantitative PCR (Fig. 2A). As expected, no NS5A was visible in untreated cells or in cells incubated with the control inhibitor SB203580 at a concentration of 8 μM (Fig. 2B, lanes 11 and 13). NS5A could be detected only in the presence of compound (**A**) (8 μM). During the characterization of the replicon, several mutations were identified that were synergistic to adaptive mutations, thus increasing replication efficiency (Krieger et al., *J. Virol* 75, 4614-24, 2001; Trozzi *et al., J Virol.* 77, 3669-79, 2003). One of these mutations was E1202G, which maps in NS3 (herein described as m17). By itself this mutation had little if any effect in promoting replication of the Con1 replicon (Fig 2B, lanes 2 and 5). However, it had a strong synergistic effect on replication when combined with the S2204A mutation (Fig. 2B, compare lanes 3 and 4). It was thus investigated whether the mutation E1202G in NS3 acted synergistically with the kinase inhibitor in a similar way to that observed with the adaptive mutation (lanes 5-7). Compound (**A**), used at a final concentration of 8 μM, activated replication of the replicon m17 more efficiently than the replication of the wt-replicon. The presence of NS5A was due to active HCV replication and not due to protein stabilization, because replicons containing the RdRP-inactivating mutation GAA (Lohmann *et al., J. Virol.* 71, 8416-28, 1997) did not show any detectable NS5A protein (lanes 8-10).

A similar experiment was carried out in order to detect HCV-specific RNA using real-time reverse transcription PCR amplification (Fig. 2A). Expression of β-actin was used as internal control in order to standardize for total amount of RNA. Shown is the fold-induction of HCV-RNA with respect to the DMSO control. Induction of replication of the wt Con1 replicon could not be detected by quantitative PCR by compound (**A**). However, as observed in Fig. 2B for protein expression, cells supporting subgenomic replicons with the synergistic mutation in NS3 (m17) contained significantly more HCV RNA upon incubation with the kinase inhibitor than those expressing the wt Con1 replicon. Replication was induced 30-fold for compound (**A**). The presence of HCV RNA was due to active replication as the HCV RNA polymerase-minus mutants (wt-GAA and m17-GAA) did not show any induced amount of RNA.

Claims

1. Use of compound (**A**), or a suitable salt thereof, to inhibit the formation of hyperphosphorylated NS5A in a cell, a tissue or an organism, wherein compound (**A**) is 4-[4-(3,4-dichlorophenyl)-2-piperidinyl-3-yl-1*H*-imidazol-5-yl]pyrimid-2-amine (**A**).

2. Use of compound (**A**) as defined in Claim 1, or a suitable salt thereof, to modulate the replication of HCV RNA and/or viral production of HCV in a cell, a tissue or an organism.

3. A method for modulating the replication of HCV RNA and/or viral production of HCV in a cell, a tissue or an organism comprising administering to the cell, the tissue or the organism compound (**A**) as defined in Claim 1, or a suitable salt thereof, to inhibit the formation of hyperphosphorylated NS5A.

4. Use of compound (**A**) as defined in Claim 1, or a suitable salt thereof, to enhance HCV RNA replication and/or viral production of HCV in a cell.

5. A method of enhancing HCV RNA replication and/or viral production of HCV in a cultured cell by treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof.

6. A cell culture obtainable by treatment with compound (**A**) as defined in Claim 1 or a suitable salt thereof.

7. A method of screening a compound for its effect on HCV replication which comprises administration of the compound to a HCV cell culture that has been treated with compound (**A**) as defined in Claim 1 or a suitable salt thereof.

8. Use of compound (**A**) as defined in Claim 1 or a suitable salt thereof in the production of a cell culture which has detectable levels of HCV RNA and HCV protein in the absence of adaptive mutations in the HCV RNA.

9. A method of producing a cell culture which has detectable levels of HCV RNA in the absence of adaptive mutations in the HCV RNA by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the treated cell for HCV RNA replication.

10. A method for producing a cell culture which has detectable levels of HCV protein in the absence of adaptive mutations in the HCV RNA by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the treated cell for HCV protein expression.

11. A method of producing a cell culture which has detectable levels of virus production in the absence of adaptive mutations by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus not carrying adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the amount of viral particles secreted in the cell medium.

12. Use of compound (**A**) as defined in Claim 1 or a suitable salt thereof in the production of a cell culture which has detectable levels of HCV RNA and HCV protein in the presence of selected adaptive mutations in those cells.

13. A method of producing a cell culture which has detectable levels of HCV RNA in the presence of selected adaptive mutations in those cells by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying selected adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the treated cell for HCV RNA replication.

14. A method of producing a cell culture which has detectable levels of HCV protein in the presence of selected adaptive mutations in those cells by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the treated cell for HCV protein expression.

15. A method of producing a cell culture which has detectable levels of virus production in the presence of adaptive mutations by:

a) contacting a cell in tissue culture with HCV RNA or HCV virus carrying adaptive mutations,

b) treating the cell with compound (**A**) as defined in Claim 1 or a suitable salt thereof,

c) evaluating the amount of viral particles secreted in the cell medium.

16. Use of compound (**A**) as defined in Claim 1 or a suitable salt thereof in the identification of cellular kinase(s) responsible for the hyperphosphorylation of HCV NS5A.

17. A method of identifying cellular kinase(s) responsible for the hyperphosphorylation of HCV NS5A by:

a) covalently binding compound (**A**) as defined in Claim 1 or a suitable salt thereof to a chromatography matrix,

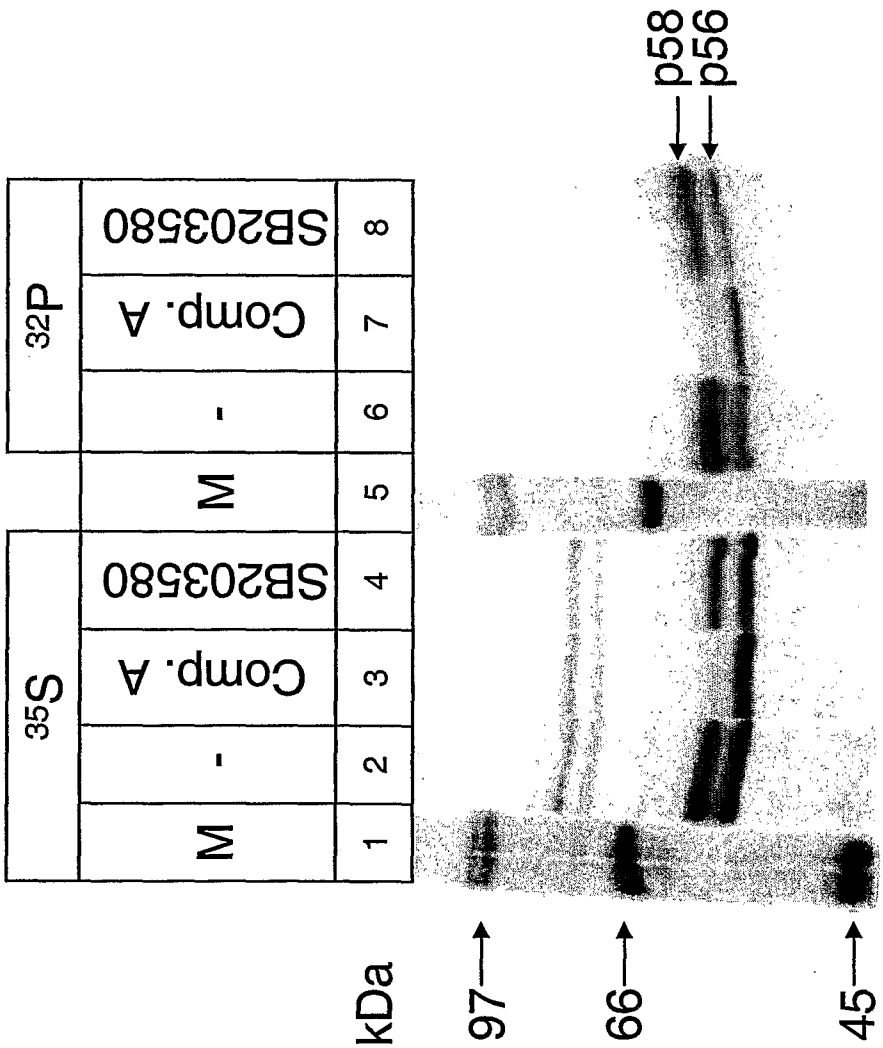
b) using the chromatography matrix to purify kinase(s) from cellular protein extracts,

c) eluting the kinase(s) from the affinity matrix,

d) identifying the eluted kinase(s).

18. Use of compound (**A**) as defined in Claim 1 or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of HCV infection.

19. A method of inhibiting replication of HCV RNA and/or of treating or preventing an illness due to hepatitis C virus, the method involving administering to a human or animal subject suffering from the condition a therapeutically or prophylactically effective amount of compound (**A**) as defined in Claim 1 as defined above, or a pharmaceutically acceptable salt thereof.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p56
kDa
97
66
45
35S
32P
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Comp. A
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Comp. A
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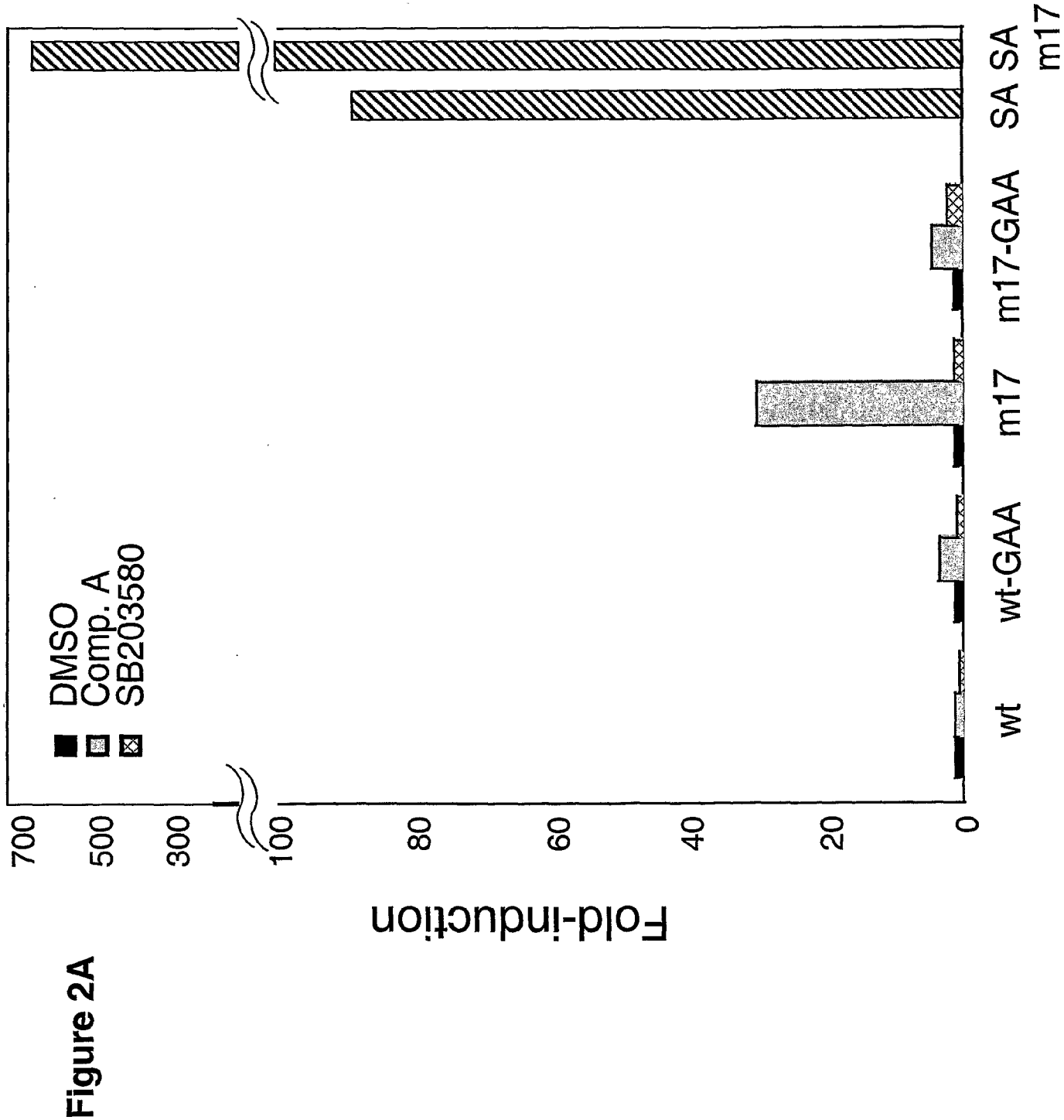
2
3
4
5
6
7
8

Figure 1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Comp. A
SB203580
Fold-induction
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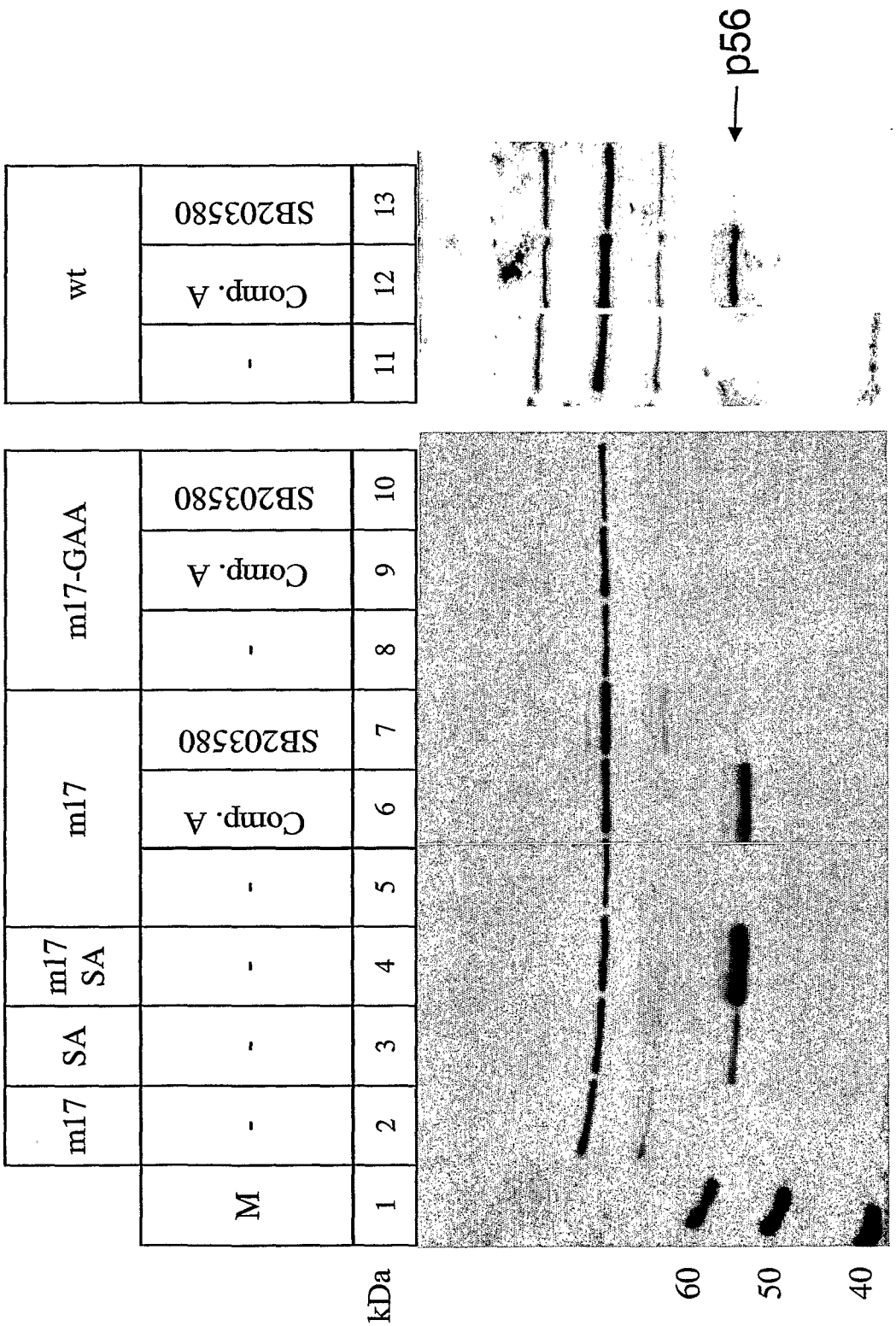
300
100
80
60
40
20
0
wt
wt-GAA
m17
m17-GAA
SA
SA
m17

Figure 2A

Figure 2B


Western
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m17
SA
m17 SA
m17
m17-GAA
wt
Comp. A
SB203580
kDa
60
50
40
p56

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/000584

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/506 A61P31/12

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)
A61K

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 2006/021449 A (ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P ANGELETTI SPA; DE FRANCE) 2 March 2006 (2006-03-02) claims compounds 1-3 ----- -/--	1-19

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
10 April 2006	16/05/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Veronese, A

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEDDERMANN P ET AL: "Reduction of Hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 78, no. 23, December 2004 (2004-12), pages 13306-13314, XP002351158 ISSN: 0022-538X abstract page 13307, column 2 page 13308, column 1 page 13313, columns 1-2	1-19
A	WO 97/47618 A (MERCK & CO., INC; LIVERTON, NIGEL, J; BUTCHER, JOHN, W; CLAREMON, DAVI) 18 December 1997 (1997-12-18) cited in the application page 36, lines 17-19 claims	1-19
A	EVANS M J ET AL: "Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 101, no. 35, 23 August 2004 (2004-08-23), pages 13038-13043, XP002351159 ISSN: 0027-8424 abstract column 13041, line 2 - column 13043, line 2	1-19
A	APPEL NICOLE ET AL: "Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain." JOURNAL OF VIROLOGY. MAR 2005, vol. 79, no. 5, March 2005 (2005-03), pages 3187-3194, XP002376318 ISSN: 0022-538X the whole document	1-19

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FRANCESCO DE R ET AL: "Challenges and successes in developing new therapies for hepatitis C" NATURE, NATURE PUBLISHING GROUP, LONDON, GB, vol. 436, 18 August 2005 (2005-08-18), pages 953-960, XP002351157 ISSN: 0028-0836 the whole document	1-19
A	PAWLOTSKY J M ET AL: "THE NON-STRUCTURAL 5A PROTEIN OF HEPATITIS C VIRUS" JOURNAL OF VIRAL HEPATITIS, BLACKWELL, OXFORD, GB, vol. 6, no. 5, 1999, pages 343-356, XP009056107 ISSN: 1352-0504 the whole document	1-19
A	COITO C ET AL: "High-throughput screening of the yeast kinome: identification of human serine/threonine protein kinases that phosphorylate the hepatitis C virus NS5A protein" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 78, no. 7, April 2004 (2004-04), pages 3502-3513, XP002351160 ISSN: 0022-538X abstract	1-19
A	WO 01/91749 A (MERCK & CO., INC; MERCK FROSST CANADA & CO; MERCK SHARP & DOHME LIMITE) 6 December 2001 (2001-12-06) the whole document	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/000584

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-4, 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/000584

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006021449 A	02-03-2006	NONE	
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