

HCV NS3/4A 3 Protease Inhibitors: Simeprevir, Process Patents and Evaluation

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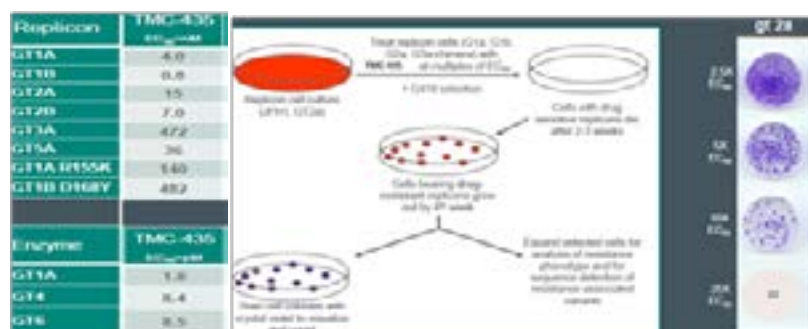
Abstract

In polymerase and protease drug targets and drug development, which has resulted in a strong infectious disease R&D portfolio,” and has given the company its key pipeline asset, simeprevir (TMC435), a novel protease inhibitor in Phase III clinical development for hepatitis C that is being developed in collaboration with Janssen Pharmaceuticals. Currently, hepatitis C virus (HCV) infection is considered a serious health-care problem all over the world. A good number of direct-acting antivirals (DAAs) against HCV infection are in clinical progress including NS3-4A protease inhibitors, RdRp, RNA-dependent RNA polymerase inhibitors, and NS5A inhibitors as well as host targeted inhibitors. Two NS3-4A protease inhibitors (telaprevir and boceprevir) have been recently approved for the treatment of hepatitis C in combination with standard of care (pegylated interferon plus ribavirin). The new therapy has significantly improved sustained virologic response (SVR); however, the adverse effects associated with this therapy are still the main concern. In addition to the emergence of viral resistance, other targets must be continually developed. One such underdeveloped target is the helicase portion of the HCV NS3 protein. This review article summarizes our current understanding of HCV treatment, particularly with those of NS3 inhibitors and patent status and approvals of Simeprevir was clearly explained.

Keywords: Hepatitis C virus; NS3/4A protease; Macrocyclic inhibitors; Protease inhibitors

Introduction

Simeprevir (formerly TMC435) is a second-generation HCV NS3 / 4A serine protease inhibitor marketed under the trade names Olysio, Galexos (in Canada) and Sovriad (in Japan). Hepatitis C virus (HCV) is the major etiological agent of 90 % of all cases of non-A, non- B hepatitis. The incidence of HCV infection is becoming an increasingly severe public health concern with 2-15 % individuals infected worldwide. While primary infection with HCV is often asymptomatic, most HCV infections progress to a chronic state that can persist for decades. Of those with chronic HCV[1-10] infections, it is believed that about 20- 50 % will eventually develop chronic liver disease (e.g. cirrhosis) and 20-30 % of these cases will lead to liver failure or liver cancer. As the current HCV-infected population ages, the morbidity and mortality associated with HCV are expected to triple. The use of protease inhibitors, particularly those selectively targeting HCV serine protease, has great potential to be useful in treating HCV infections in patients by inhibiting HCV replication.



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Tmc-435 colony formation-Assay

In the mid-1970s, it was noticed that supply of blood was contaminated with an unidentified agent causing posttransfusion non-A, non-B hepatitis. This unknown infectious agent struck intravenous drug users and blood transfusion recipients[11]. The offender agent identified in 1989 was hepatitis C virus (HCV) and the first sequences of HCV were reported HCV is one of the leading agents that cause liver failure and hepatocellular carcinoma and is the most relevant reason for liver transplantation. HCV infects about 3 % of the world population; 130–200 million people are estimated to be chronically infected globally. Alarming news is that 350,000 people worldwide die from HCV-related disease every year. For more than 20 years, HCV has been taking the attention of the health professionals, and now, well recognized that HCV is actually a major global health problem. Recently, health professionals determined the worldwide prevalence of HCV 3in comparison with HIV[12,13]. The global prevalence of HCV estimates is 400,000 chronically infected subjects in Australia and Oceania, 14 million in the United States of America, 16 million in the Middle East, 17.5 million in Europe, 28 million in Africa, and 83 million in Asia. Therefore, novel and effective inventions with fewer adverse effects are required for the prevention and control of HCV. The main goal of this review article is to be updated with the current treatments of HCV, putting an emphasis on the HCV NS3 protease and NS3 helicase inhibitors.

Patent approvals

Simeprevir was approved in 2013 for use in the United States, Japan and Canada as a combination treatment for chronic genotype 1 HCV infection. The Committee for Medicinal Products for Human Use of the European Medicines Agency has authorized use of simeprevir in the European Union in a combination treatment for chronic HCV.

On November 5, 2014, the U.S. FDA approved the use of simeprevir in combination with sofosbuvir4 as an all-oral, interferon- and ribavirin-free treatment option for patients with genotype 1 chronic hepatitis C. The recommended treatment duration of simeprevir with sofosbuvir is 12 weeks for patients without cirrhosis or 24 weeks for patients with cirrhosis..

Simeprevir reaches high sustained virologic10response when given in combination with pegylated interferon and ribavirin in patients with HCV genotype 1 infection. Simeprevir is considered a “second-generation compound” as it is a peptidomimetic compound, a small protein-like chain designed to mimic a peptide. It was developed by Medivir and Johnson & Johnson’s pharmaceutical division, Janssen Pharmaceuticals Inc. (hereby referred to as the ‘Sponsor’).

Patent application WO2007014926A1 relates to the base compound of simeprevir. The application claims a general structural formula of macrocyclic compounds[14,15] which act as inhibitors of HCV infections. The application also claims a process for preparation of simeprevir and its method of use. It includes a pharmaceutical combination of simeprevir with ribavirin. This patent, if granted, serves as a blocking patent preventing any other competitor from making the product. The claims are very broad, covering a Markush structure of antiviral agents along with its process of preparation and method of use.

There are three patents granted in the United States: US8148399B2, US8153800B2 and US8349869B2. US8148399B2 relates to the base compound of simeprevir. US8153800B2 is a divisional of US8148399B2 and relates to the macrocyclic compounds as well as processes for preparing these compounds and compositions. US8349869B2 is a divisional of US8148399B2 and relates to a macrocyclic compound, it’s N-oxide, pharmaceutically acceptable salt or stereoisomer. It claims a combination of the compound with interferon- α , pegylated interferon- α , and / or ribavirin.

Patent application WO2010072742A1 is a process patent. The application covers a process for the preparation of antiviral agents as well as intermediates for the preparation of bicyclic lactone amides, which are then converted into the desired products used for treating HCV infections, particularly simeprevir. The process claims are moderately narrow, claiming the process and various intermediates for preparation of antiviral compounds.

Patent application WO2011113859A1 covers a process for the preparation of intermediates useful in the preparation of macrocyclic compounds which are used for treating HCV infections, preferably simeprevir. The application also claims various intermediate compounds.

The patent application WO2008092955A1 covers processes for preparing and further processing quinoline compounds to obtain the desired product, preferably simeprevir.

Patent application WO2013041655A1 is a process patent, covering processes for the preparation of salts of intermediate compounds used in the synthesis of simeprevir[16]. The claimed process is a multi-step synthesis involving a number of reactants.

Patent application WO2013061285A1 is a process patent, claiming an improved process for the preparation of intermediate compounds used in the synthesis of HCV inhibitor compounds, particularly simeprevir. The process is claimed to be a straightforward, quick and economic procedure to formulate intermediates for the production of simeprevir. The application also claims various new intermediate compounds.

This patent is listed in the US Orange Book with patent numbers US7671032. Patent application WO2005073195A2 is a product patent, claiming simeprevir derivatives, their salts and prodrugs along with the compositions comprising them, as well as the use of the derivatives for the treatment or prevention of flavivirus infections including HCV infection. These compounds are stated to be useful as NS3 serine protease inhibitors. The application discloses a Markush structure of the general formula along with various substituents.

Patent application WO2008092954A2 is a formulation patent, originally filed by Tibotec Pharmaceuticals, now part of Janssen Pharmaceuticals. The application claims a crystalline form of a substituted macrocyclic compound, preferably simeprevir, for use in HCV treatment. The application also claims a combination of the compound with a pharmaceutically acceptable excipient. The patent is not relevant to the current version of simeprevir sold by the Sponsor since the European Medicines Evaluation Report states that the crystalline form of the drug is poorly soluble. Therefore, an amorphous form of simeprevir was developed. Patent application WO2010031829A1 is a formulation patent, claiming a combination of two compounds,

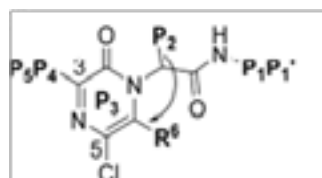
simeprevir and a nucleoside, as well as a combination of these compounds with ribavirin or pegylated interferon. The combination is claimed to produce a synergetic effect to treat HCV infections. Patent application WO2010097229A2 is a product patent, claiming a sodium salt of simeprevir in solid amorphous form, useful for the treatment of HCV infections. Patent application WO2011128378A1 is a formulation patent, claiming a combination of a macrocyclic HCV protease inhibitor, a macrocyclic [17,18] non-nucleoside HCV polymerase inhibitor, and a nucleoside HCV polymerase inhibitor. It is preferably a combination of TMC-647055 and simeprevir. The claims are limited to a combination of specific compounds. TMC647055 is a potent non-nucleoside inhibitor of the HCV NS5B polymerase currently developed by Janssen.

Patent application WO2014033668A2 claims a combination compound comprised of simeprevir, ritonavir and TMC-647055 for treating HCV infection. As per the WIPO ISR, the application is novel and not obvious in comparison to the closest prior art retrieved during the search.

This patent collection comprises 12 different patents (patent families). The majority of these patent applications are still pending in the respective national and regional patent offices.

HCV NS3 protease inhibitor simeprevir

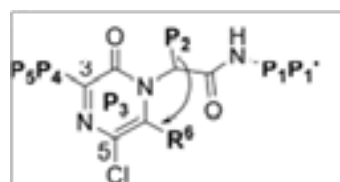
During the development of the now approved HCV NS3 protease inhibitor simeprevir, which contains a quinoline P2 substituent other P2 heterocycles were also evaluated (e.g. pyrimidines). The ether linkage found in simeprevir, which connects the P2 core and the P2 heterocyclic



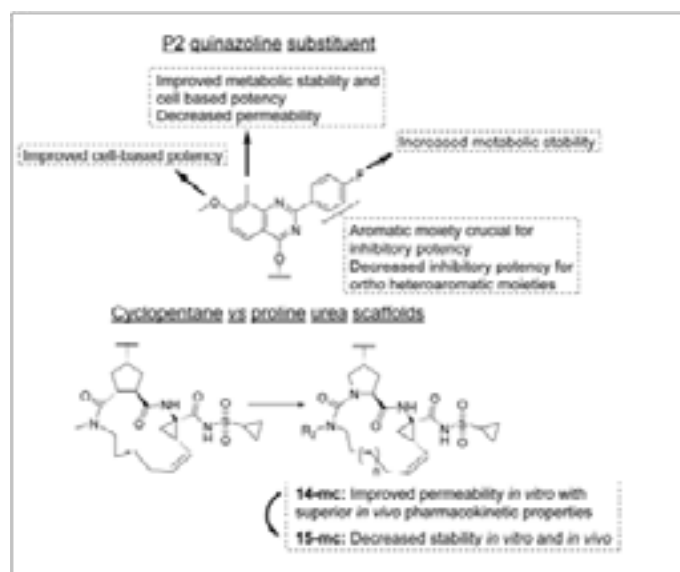
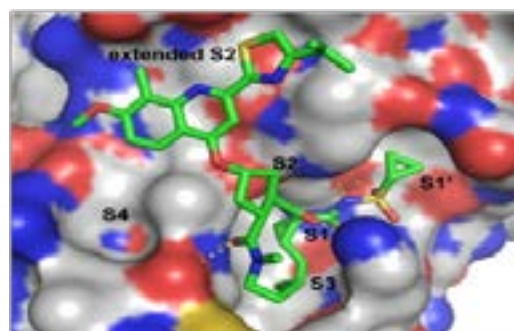
A urea moiety in the C3 position improved both stability and inhibitory potency compared with the carbamate analog. Inhibitors containing P4P5-ureas were prepared and evaluated and indicated allowance for substituents in this area. Relocation of the P2 group to the R6 position was well accepted and resulted in achiral inhibitors with improved inhibitory potencies for elongated R6 moieties. Moreover, the R6 substituents influenced the PK, with favorable properties for a pyridyl moiety.

The resistance profile for this class of inhibitors showed retained inhibitory potencies against known drug-resistant variants of the virus, i.e. R155K, A156T and D168V. Initial evaluation against genotype 3a displayed promising inhibitory potencies for a set of inhibitors with K_i values 0.6-3.4 μM .

• Based on evaluation of several P1P1' building blocks, preliminary results suggested that the acyl sulfonamide did not improve the inhibitory potency. The P1' aryl did not appear to have any specific interactions with the S1' pocket, as supported by comparable inhibitory potencies for truncated derivatives. It was found that the P1 aryl in combination with the P3 pyrazinone and a C3 urea were important for sub-micromolar K_i values, suggesting that this could be the new lead structure.



An efficient Pd-catalyzed C-N urea arylation to the C3 position of the pyrazinone was developed and successively applied to inhibitors with elongated P4P5 urea substituents. In line with our interest in identifying carboxylic acid bioisosteres, a novel Pd-catalyzed carbonylation protocol for sulfonamides yielding acyl sulfonamides was developed. Paper was based on two drug discovery projects within the HCV area, both aiming to inhibit the drug target, NS3 protease. The criteria differed with respect to the various stages of discovery they represented. In the P2 quinazoline macrocyclic series, the lead structure was optimized for improved PK properties along with sub-nano molar K_i and Nano molar EC_{50} values. The pyrazinone series, on the other hand, represents an early stage of drug discovery aiming for new lead compounds, which could be further optimized into coming generation of HCV NS3 protease inhibitors. The main findings are summarized below



Crystal structure of HCV NS3 / NS4A protease in complex with TMC-435 (PDB ID: 3KEE)[19]. Binding subsites of S1' -S4 in the active site are indicated on the surface representation and labelled in black. The bound inhibitor, TMC-435, is shown as a ball-and-stick model and is colored by atom type. For clarity, hydrogen atoms are omitted.

Susceptibility of Clonal HCV Replicons with Variants Identified to TMC-435*

Replicon	TMC-435 EC ₅₀ (nM)
Contra-1	10
Contra-2	10
Contra-3	1000
Contra-4	100
Contra-5	10
Contra-6	10
Contra-7	10
Contra-8	10
Contra-9	1000
Contra-10	100
Contra-11	1000
Contra-12	100
Contra-13	100
Contra-14	100
Contra-15	100
Contra-16	100
Contra-17	100
Contra-18	100
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Contra-92	100
Contra-93	100
Contra-94	100
Contra-95	100
Contra-96	100
Contra-97	100
Contra-98	100
Contra-99	100
Contra-100	100

The Functions of HCV NS3 Proteins

NS3 is a multifunctional protein with serine protease activity at the N-terminal and a nucleoside-triphosphatase- (NTPase)-dependent RNA helicase activity (NS3 NTPase/helicase) at the C-terminal (aa 181–631). Both enzyme activities have been well defined and high-resolution structures have been solved. The C-terminus of NS3 encodes a DExH / D-box RNA helicase. NS3 helicase hydrolysed NTP as an energy source to unwind double-stranded RNA in a 3' to 5' direction during replication of viral genomic RNA. Structural analysis of NS3 revealed the unidirectional translocation and proposed a new function of NS3 as translocase, considering feasible strategies for developing specific inhibitors to block the action of NS3 helicase. The activity of NS3 helicase can be regulated by interactions between the serine protease and helicase domains of NS3, indicating that these two enzyme activities may be somehow coordinated during replication. The function of the HCV helicase is unknown; it has been shown that without functional helicase domains, HCV cannot replicate in cells. It may be involved in the initiation of RNA synthesis on the HCV genome RNA, which contains stable 3'-terminal secondary structure in dissociation of nascent RNA strands from their template during RNA synthesis or in displacement of proteins or other trans-acting factors from the RNA genome. It has been now well recognized that both activities of NS3 protein are required for the replication of virus; they are considered as attractive target sites for the development of direct-acting antivirals (DAAs) therapies. NS5B is the viral RNA-dependent RNA polymerase, another promising anti-HCV target site. NS5A is a phosphoprotein specifically capable of interacting with the 3'-NTR of the HCV genome, other non-structural proteins, and numerous cellular proteins. NS5A also functions in virus assembly. NS4B is an integral membrane protein that is required for the assembly of the "membranous web," the organelle used for RNA replication. NS4A is a cofactor for NS3 that directs the localization of NS3 and modulates its enzymatic activities.

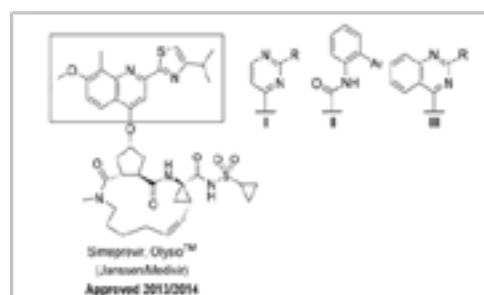
Pharmacokinetic Aspects in Drug Discovery

A revealing paper published in 1988 presented the reasons for the failure of drugs in development. Alarmingly, 39% of drugs failed due to poor PK properties and bioavailability. Years of invested money and time were lost, and the introduction of new drugs on the market was delayed. This ultimately affected the patients in need of new pharmaceuticals. Contemporary drug discovery and development has a different approach. At its best, a drug discovery program is a highly iterative process, where

properties such as solubility, permeability and metabolic stability are evaluated in parallel with optimizations in terms of binding to the target. A less active compound could have advantageous PK properties which enable a better in vivo therapeutic response and, eventually, might offer more convenient dosage regimens for the patient. A successful research program needs to consider and attempt to anticipate how the various properties of a drug cooperate at its final destination inside the human body. Lipinski's well known "rule-of-five" has, since it was presented in 1997, guided the choice of compounds that will proceed in the discovery process. While favourable PK properties and solubility can be predicted from the molecular qualities, the emerging area of demanding and novel targets as well as poor outcomes from big pharma have challenged researchers to think "outside the box", and this can be rewarding. In the HCV research field, for example, the approved HCV NS3 protease anti-HCV drugs violate at least one of the rules, since they have a molecular weight of > 700. One could consider the drug-like properties as guidelines but should also bear in mind that the success of a drug depends on how well various properties are balanced with each other. Moreover, oral drug space is likely to expand with improved formulation techniques.

Harmonizing Antiviral Potency with PK Properties in the Development of HCV NS3

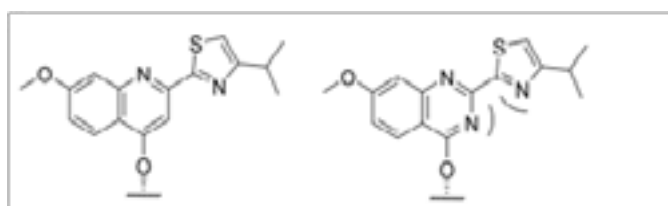
Protease Inhibitors: During the development of the now approved HCV NS3 protease inhibitors simeprevir, which contains a quinoline P2 substituent other P2 heterocycles were also evaluated (e.g. pyrimidines). The ether linkage found in simeprevir, which connects the P2 core and the P2 heterocyclic group, was replaced with a carbamate moiety (II, Figure 14) in another series. 98 However, neither the pyrimidine- nor the carbamate-linked P2 aromatic substituents yielded optimal properties for the inhibitors. During these explorations, a novel P2 quinazoline substituent was identified (III, Figure 14); this was combined with a cyclopentane core (as in simeprevir) and a proline urea core in further optimizations. The quinazoline substituent was modified with the goal of balancing antiviral potency with the PK properties.



Initial modifications: The initial lead in this series which contained a 2-phenylquinazoline on the cyclopentyl scaffold, showed excellent permeation through Caco-2 cells, moderate stability in human liver microsomes, high potency in the enzyme assay and moderate potency in the cell-based assay. Introduction of a methoxy moiety in position 7 of the quinazoline improved the cell-based potency. The metabolic stability, on the other hand, decreased. This property was adjusted by a fluoro-moiety on the para position of the phenyl substituent in the quinazoline.



Initial modifications on the quinazoline substituent.Ki (NS3f-11a): inhibition constant. EC50 (NS31b): cell-based activity. The cut-off values for stability in human liver microsomes (HLM), intrinsic clearance (μL / min / mg): Clint < 30: **norisk**; 30 < Clint < 92: **moderate risk**; Clint > 92: **high risk**. The cut-off values for Caco-2 permeability (cm/s): Papp < 2×10⁻⁶: **low**; 2×10⁻⁶< Papp < 20×10⁻⁶: **moderate**; Papp > 20 × 10⁻⁶: **high**. Interestingly, the introduction of a thiazolyl substituent reduced the enzyme- and the cell-based activity drastically (4 and 5), in contrast to the outcomes found in the quinoline series, where such a moiety improved the potency. A likely reason for the lower potency is that repulsion between the hetero atoms in the thiazolyl moiety and the nitrogens in the quinazoline impeded the bioactive co-planar conformation of the thiazolyl substituent, leading to reduced interactions with the enzyme



A similar reason could possibly explain the drastic decrease in both enzyme and cell-based assays for compounds 6 and 7, i.e. that the non-aromatic rings did not adopt a bioactive conformation, leading to reduced potencies. The main improvements for the initial optimizations were the addition of a methoxy group in position 7 of the quinazoline, which improved the cell-based potency (2), and the introduction of a fluoromoiety on the phenyl group, which increased the metabolic stability

HCV NS3 NTPase / Helicase Inhibitors: The structure of the NS3 helicase is also available and well characterized. However, the developments of NS3 helicase inhibitors have been slow. This target is traditionally difficult as evidenced by the fact that no helicase inhibitors have been approved for clinical use. The main issue might be toxicity because the motor domains of HCV helicase are conserved to that of cellular proteins. As a result, more attention should be given to find inhibitors that bind sites rather than the conserved regions of cellular enzymes without affecting cellular ATPases or GTPases. Recently, a good number of high-throughput screening systems (HTS) have been developed to screen potential inhibitors that specifically inhibit essential activities of NS3. Many world renowned laboratories are engaged to study the helicase portion of NS3 as a possible HCV drug target over the last 17 years. Several studies have revealed that NS3 is essential for viral replication, both in whole animal

and replicon model. Mutations in HCV RNA are unable to replicate in sub genomic replicons, which further validates the necessities of NS3 helicase in viral life cycle. NS3 helicase has unique property that plays a more complex role in viral replication. NS3 helicase unwinds both double-stranded DNA and duplex RNA, but typically most helicases do not unwind both. It is known that there is no DNA stage in HCV replication and replication occurs outside the nucleus; the biological importance of the NS3 helicase's ability to unwind DNA remains elucidate.

The ATP[20-23] and RNA binding sites are the most promising targets on HCV. To the best of our knowledge, very limited numbers of small molecules have been reported in the literature over the past years and fewer structure-activity relationships data are available. Because NS3 helicase seems to be a key cellular motor proteins, monitoring ATP hydrolysis is the early screening assays to screen potential inhibitors that yielded few specific hits. However, recent screens of small chemical libraries through HTS have identified some valuable compounds that inhibit HCV catalyzed DNA unwinding, NTPase-dependent RNA helicase, and RNA binding ability, some of which also prevent HCV replicon in cells. Major NS3 helicase inhibitors with their helicase inhibitory activity employing both DNA or RNA substrate and ATPase activities are discussed in Table 1

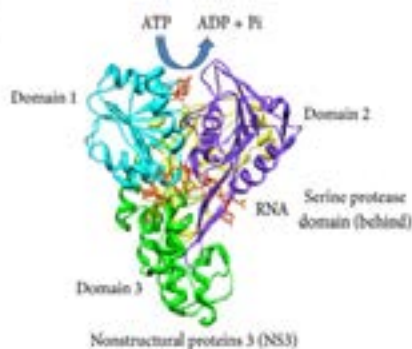
Table 1: Inhibitory effects of some NS3 helicase inhibitors.

NS3 helicase inhibitor	IC50 (μM)		
	Helicase		ATPase
	DNA	RNA	
DRBT	1.5	500	No inhibition
TBBT	20	60	No inhibition
Soluble blue HT	40	Inhibition	23.8
Ring-expanded (fat) nucleoside analogues	7–11	5.5–12	Activation
AICAR analogue	37	No inhibition	ND
QU663	, 0.75	ND	No inhibition
p14	0.2	ND	No inhibition
DBMTr	17.6	No inhibition	No inhibition
Acridone derivatives	1.5–20	ND	No inhibition
Thiazolpiperazinyl derivative	110	ND	1000
(BIP)2B	5.4	0.7	Inhibition (in the presence of RNA)
Tropolone derivatives	3.4–17.8	ND	ND
Tetrahydroacridine derivative, 3a	, 0.02	ND	ND
Manoalide	ND	15	70
Thioflavin S	10	12	ND
SG1-23-1	ND	11.7 μg/mL	No inhibition
LOPAC compounds	0.6–3.7	0.8–8.9	ND
C-29EA	ND	18.9 μg / mL	No inhibition
Psammaphin A	ND	17	32
Cholesterol sulfate	ND	1.7	No inhibition

ND: not determined.

HCV Translation and Polyprotein Processing

HCV belongs to the founding member Hepacivirus genus of the family Flaviviridae; it is a positive sense single-stranded RNA virus with seven genotypes and more than 90 different subtypes. The viral genome is 9600 nucleotides (nt) in length, which contains a 5'-nontranslated region (NTR) with an internal ribosome entry site (IRES), 3'-NTR and encode a single polyprotein containing 3000 amino acids, and is positioned between 5'-NTR and 3'-NTR. The translation of the polyprotein is initiated by an internal ribosome entry site (IRES) present at the 5'-NTR. Unlike eukaryotic mRNA, HCV genome which lacks a 5' cap translation depends on IRES that directly binds with 40S ribosomal subunits, inducing conformational changes in the 40S subunits. The IRES-40S complex then recruits eukaryotic initiation factor (eIF) 3 and the ternary complex of Met-tRNA-eIF2-GTP[24] to form a noncanonical 48S intermediate before a kinetic slow transition to the translationally active 80S complex. Once the formation of initiation complex takes place, the genome of HCV is translated to produce a large polyprotein that undergoes proteolytic cleavages with specific viral and cellular proteases to form 10 individual viral proteins, each of which has specific functions in viral life cycle. The N-terminal one-third of the polyprotein encodes the virion structural proteins; the core protein (C) forms the viral nucleocapsid and envelopes glycoproteins E1 and E2, involved in receptor binding required for viral entry into the hepatocyte. A small integral membrane protein, p7, functions as an ion channel. The remaining portion of the genome encodes 6 important non-structural (NS) proteins: NS2, NS3, NS4A, NS4B, NS5A, and NS5B, which coordinate the intracellular processes of the viral life cycle. Host endoplasmic reticulum (ER) derived signal peptidase cleavages the mature structural proteins among the junctions C / E1, E1 / E2, and E2 / p7. Signal peptide peptidase releases core from E1 signal peptide. The p7 / NS2 junction is also cleaved by signal peptidase within the NS region. Two viral enzymes, the NS2 autoprotease and the NS3-4A serine protease, are involved further in the proteolytic processing of NS proteins. The NS2 autoprotease cleaves at the NS2/3 site, whereas the NS3-4A serine protease, which requires the NS4A protein as cofactor for functioning properly, cleaves at all downstream junctions. Another small protein that encodes HCV genome is called F (frame shift) or ARFP (alternative reading frame protein), but its precise roles in viral life cycle are unknown.



Halogenated benzimidazoles and benzotriazoles such as dichloro(ribofuranosyl) benzotriazole (DRBT) and tetrabromobenzotriazole (TBBT) both inhibit HCV helicase catalyzed DNA unwinding with IC₅₀ of 1.5 and 20 μM, respectively. When employing RNA substrate, only TBBT inhibits RNA unwinding with IC₅₀ of 60 μM.

In another report, the efficacies of TBBT and DRBT were tested in four different HCV genotype 1b replicon systems. Depending on the cell line, TBBT inhibits HCV replicons with IC₅₀ ranging from 40 to 65 μM and DRBT inhibits HCV replicons with IC₅₀ ranging from 10 to 53 μM].

Soluble blue HT inhibits NS3 catalyzed DNA unwinding with an IC₅₀ of 40 μM. After several rounds of structural refinement, discovered one of the soluble blue HT derivatives, compound 12, which is a good anti-HCV agent with an IC₅₀ of 10.1 μM and EC₅₀ value of 2.72 μM against HCV NS3 catalyzed DNA unwinding and replicon Ava.5 / Huh-7 cells, respectively.

Ring-expanded “fat” nucleosides (RENs) inhibit HCV and related Flavivirus helicase, including the West Nile virus (WNV) and Japanese encephalitis virus (JEV). They catalyzed HCV DNA unwinding with IC₅₀ in the 7–11 μM range and HCV helicase catalyzed RNA unwinding with IC₅₀ of 5.5–12 μM. In this paper, RENs demonstrated different selectivity profiles between the viral enzymes[25].

Another nucleoside, the compound 4 (4-carbamoyl-5-[4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl]imidazole-1-β-D-ribofuranoside), inhibits helicase catalyzed DNA unwinding against WNV and HCV with IC₅₀ of 23 and 37 μM, respectively, but it had no effect on helicase catalyzed RNA unwinding. It was a surprise that no activity was observed against the NTPase / helicase of either DENV or JEV irrespective of whether RNA or a DNA substrate was employed.

QU663 inhibits HCV helicase catalyzed DNA unwinding with a 750 nM, competing with the nucleic acid substrate without affecting ATPase function, even at high concentrations. Docking studies showed that by interacting with the putative binding site QU663 induced a similar conformational shift.

Small peptide inhibitor, 14 amino acid-long peptide (p14), revealed a basic amino acid stretch corresponding to motif VI of HCV, WNV, and JEV of NTPase / helicase. This peptide inhibited the HCV unwinding activity of the enzyme with an IC₅₀ of 0.2 μM employing DNA substrate. The order of inhibitory effects was HCV > WNV > JEV. The binding of the peptides does not interfere with the[26] NTPase activity of the enzymes.

Tropolone derivatives have been screened as inhibitors of HCV helicase catalyzed DNA unwinding. The derivative of tropolone, called 3,7-dibromo-5 morpholinomethyltropolone (DBMTr), acts with an IC₅₀ of 17.6 μM. It has no effect on HCV helicase catalyzed ATP hydrolysis nor HCV helicase catalyzed RNA unwinding. The authors also mentioned that DBMTr might be developed as potent inhibitor of the HCV helicase due to its low toxicity to yeast cells.

Acridone derivatives have also been screened as inhibitors of HCV helicase catalyzed DNA unwinding with IC₅₀ between 1.5 and 20 μM. These compounds also inhibit replication of HCV (EC₅₀ 1–10 μM) and are not particularly toxic to cells. Thethiazolpiperazinyl derivative compound 23 inhibits the helicase activity with an IC₅₀ of 110 μM, using DNA substrate. None of the compounds were able to inhibit the NS3 NTPase activity. Testing in the subgenomic HCV replication, it exhibited EC₅₀ of 3 μg / μL and CC₅₀ > 50 μg / μL. 1-N,4-N-bis[4-(1H-Benzimidazol-2-yl)phenyl]benzene-1,4-dicarboxamide, designed as (BIP)2B, is a potent and selective inhibitor of HCV NS3 helicase, which inhibits unwinding reaction regardless of DNA or RNA substrate, but not ATP hydrolysis without RNA or at satu-

rated level of RNA. (BIP)2 B inhibited NS3 helicase from HCV genotypes 1a, 1b, 2a, and 3a. Evidence presented here shows that it directly and specifically binds to NS3 protein.

Other new tropolone derivatives, compounds 2, 6, and 7, inhibit HCV catalyzed DNA unwinding ($IC_{50} = 3.4\text{--}17.8\ \mu\text{M}$). They are also effective in RNA replication ($EC_{50} = 32.0\text{--}46.9\ \mu\text{M}$) and exhibit the lowest cytotoxicity. The derivatives 2 and 7 have been shown to be resistant mutants. The effects of the compound 2 plus IFN- γ and compound 2 plus ribavirin combinations were evaluated in cell culture, indicating that both combinations result in an additive effect with a very slight tendency to synergy. The tetrahydroacridinyl derivative 3a is the most potent inhibitor reported to date (nM). It did not show inhibition towards the ATPase activity of NS3 up to $100\ \mu\text{M}$. Manoalide[27] was originally identified as an inhibitor of phospholipase A2, but later it was reported that it inhibits HCV NS3 helicase activity with RNA substrate ($IC_{50} = 15\ \mu\text{M}$). In addition, it inhibits the NS3 ATPase and RNA binding to NS3. A direct interaction between manoalide and NS3 was presented to explain the inhibition of NS3 activities through the structural change upon its binding.

The commercially available dye thioflavine S is identified as the most potent inhibitor of NS3 catalyzed DNA and RNA unwinding. After separating into their active components, P4 inhibits unwinding, sub genomic replication with IC_{50} of 2 and $10\ \mu\text{M}$, respectively, and was not toxic.

SG1-23-1, isolated from ethyl acetate extract from marine feather star, *Alloecomatella polycladia*, exhibits the strongest inhibition of NS3 helicase activity using RNA substrate ($IC_{50} = 11.7\ \mu\text{g} / \text{mL}$). Interestingly, the extract inhibits interaction between NS3 and RNA but not ATPase of NS3. Moreover, it also inhibits the RNA replication with EC_{50} of 23 to $44\ \mu\text{g} / \text{mL}$. Four LOPACs Sigma's library of pharmacologically active compounds[28] (ATA, AG 538, NF 023, and Suramin) were identified. All but AG 538 have the ability to unwind DNA ($IC_{50} = 0.6\text{--}3.7\ \mu\text{M}$) and RNA ($IC_{50} = 0.8\text{--}8.9\ \mu\text{M}$). All but NF 023 inhibited replication of sub genomic HCV replicons ($EC_{50} = 18\text{--}98\ \mu\text{M}$). Unfortunately, none of these inhibitors were specific to NS3 helicase.

Recently, it has been reported that an ethyl acetate extract from marine sponge *Amphimedon* sp., called C-29EA, inhibits both protease ($IC_{50} = 10.9\ \mu\text{g} / \text{mL}$) and helicase ($IC_{50} = 18.9\ \mu\text{g} / \text{mL}$) activities of HCV[28], but not ATPase activity. Importantly, it has been shown that the highest inhibition on viral replication is derived from genotypes 1b and 2a with EC_{50} values of 1.5 and $24.9\ \mu\text{g} / \text{mL}$, respectively [Psammaplin A (PsA) has antibacterial and antitumor activity and also inhibits a wide range of enzymes reported to date. PsA has the ability to inhibit HCV helicase catalyzed RNA unwinding ($IC_{50} = 17\ \mu\text{M}$) in addition to ATPase and RNA binding activity. PsA inhibited the subgenomic viral replication derived from genotype 1b and genotype 2a, with EC_{50} 6.1 and $6.3\ \mu\text{M}$, respectively.

Cholesterol sulfate might be a potential inhibitor of HCV NS3 helicase, with IC_{50} of $1.7\ \mu\text{M}$ using RNA substrate. However, it exerted no ATPase and serine protease activity. A structure-activity study revealed that anion binding and hydrophobic region in NS3 may be targets of cholesterol sulfate.

Despite the great efforts, no potent and selective NS3 helicase inhibitors have been entered for clinical use. However, some good candidates, for example, soluble blue HT deriva-

tive, compound 12, QU663], and acridone derivatives have been identified to be suitable for further development as NS3 helicase inhibitors. It is not a surprise to imagine that NS3 helicase inhibitors will dominate HCV research in the near future.

Current Treatment for HCV Infection

A combination of pegylated interferon and ribavirin is still the only choice for the treatment of hepatitis C. Depending on the genotypes, this standard of care (SOC) increased the sustained virologic response (SVR) and defined the HCV RNA levels undetectable in the blood 24 weeks posttreatment, from $\sim 5\%$ to $\sim 40\text{--}80\%$. In HCV genotype 1 infected patient, those with high viral loads, mostly null responders or relapsers, the SOC treatment with pegylated interferon plus ribavirin for 48 weeks achieves 50% SVR. On the other hand, the SOC treatment with pegylated interferon plus ribavirin for 24 weeks up to 80% achieves SVR in the HCV genotype 2 infected patients. However, current SOC is associated with severe side effects including rash, nausea, anaemia, and depression.

The preventive measures against HCV include the development of HCV vaccine which may be one good idea. This is a challenging job because HCV has a great ability to change its amino acid and evade the immune response, which is 10 fold higher than HIV. The development of HCV vaccine[29] is now in progress. In 2011, the US Food and Drug Administration approved two new antivirals, boceprevir and telaprevir, which was a milestone in HCV research. They inhibit an important viral protein, the NS3-4A protease. The drugs are designed in such a way that specifically attack HCV genotype 1, which is considered one of the most prevalent genotypes, accounting for about 60% of global infections, and the least responsive to current treatment. This new standard of care, a combination of boceprevir or telaprevir with peg-IFN plus ribavirin[30], has been approved for elimination of HCV infection in the USA, Europe, and Japan.

Anti-HCV DAAs can be classified into several categories:

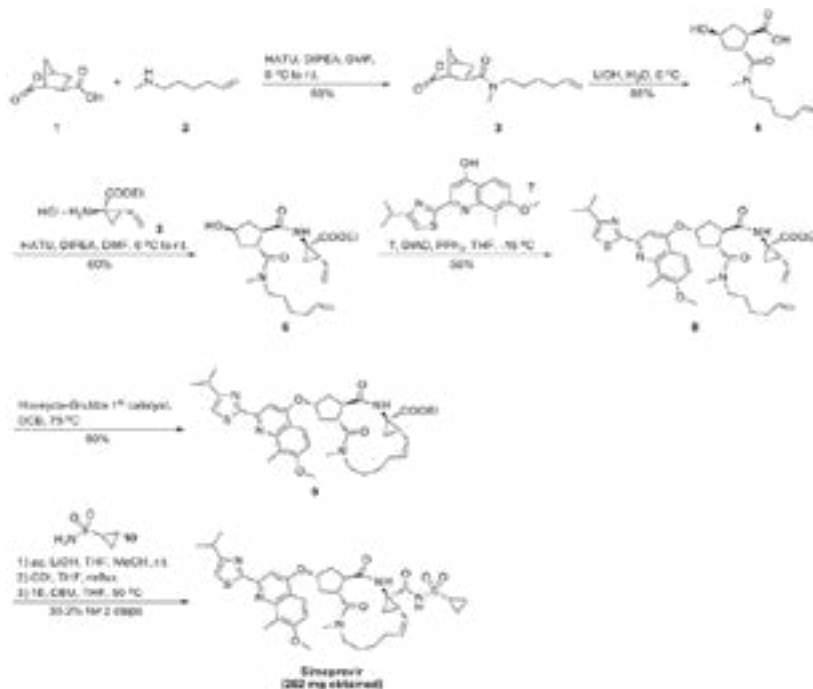
- HCV NS3-4A serine protease inhibitors,
- HCV NS3 NTPase/helicase inhibitors,
- HCV NS5B polymerase inhibitors,
- HCV NS5A inhibitors, and others

Conclusions

The direct-acting antiviral agents (DAAs), particularly NS3 protease inhibitors, telaprevir and boceprevir, which were approved in combination with current SOC (peg-IFN and ribavirin) for the treatment of HCV infection that significantly increased SVR, have opened a new window in HCV therapy. However, the side effects associated with this new therapy are a questionable marker. Anemia is the most frequent adverse effects with either telaprevir or boceprevir. They also exhibit strong inhibitory effect against an important drug metabolism enzyme, cytochrome P4503A4 (CYP3A4) resulting in the development of drug-drug interactions. In addition to drug resistance, the efficacies of these inhibitors differ significantly between HCV genotypes[31]. It is well known that IFN itself has significant side effects. Another important issue arises with their short half-life and frequent dosing. With the advent of different small classes of DAAs, the future aim is to introduce an IFN-free regimen, oral cocktails of

DAA's. The proof-of-concept studies presented some promising data confirming that the achievements of SVR without introducing IFN may be feasible. Thus, the combination of host and viral targeted inhibitors could be an attractive strategy in maximizing anti-viral efficacy.

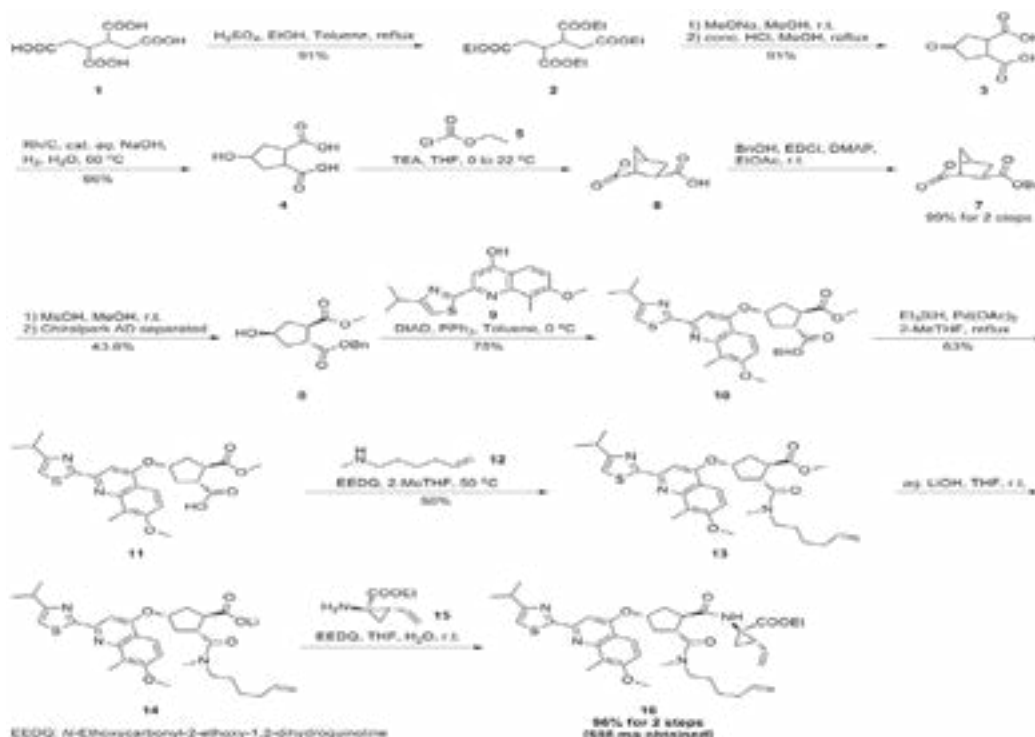
Route 1



Reference:

1. WO2007014926.
2. Bioorg. Med. Chem. Lett. 2008, 18, 4853-4858.
3. WO2008092954 / US2010029715.
4. CN101921269.

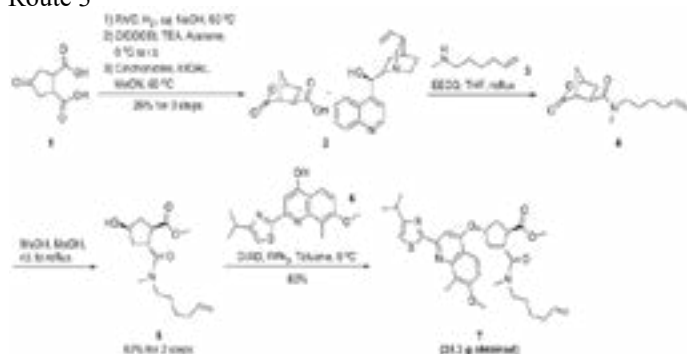
Route 2



Reference:

1. WO2008092955.

Route 3



Reference:

1. WO2010072742.
2. WO2010097229.
3. WO2011113859.
4. WO2013041655.
5. J. Med. Chem. 2014, 57, 1673-1693.

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