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HCV Infection and NS-3 Serine Protease Inhibitors

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Abstract

Infection with Hepatitis C Virus (HCV) is a major health problem affecting 270 million people worldwide and 10 million people in Pakistan. Currently, there is no vaccine available for prevention of HCV infection due to the high degree of strain variation. The current standard of care is a combination of Pegylated interferon α (PEG-INF α) with ribavirin and Boceprevir/Telaprevir. Hence, there is a need to develop new antiviral agents from different targets. HCV NS3, a nonstructural protein has serine/protease and helicase domain, and these domains are considered as important antiviral drug targets to combat HCV. In the past 15 years, major scientific advances have enabled the development of many novel serine/protease and helicase inhibitors by using *in vivo* as well as *in vitro* model systems. Most of NS3 protease inhibitors have entered in different clinical trials phases but still there is a need to improve the functionality of these inhibitory compounds. This review summarizes potent antiviral compounds against HCV NS3 protease and their activity in combination with standard therapy. In conclusion, it will be very exciting to see HCV NS3-4A serine protease/helicase inhibitors progress through clinical developments and, hopefully, provide hepatitis C patients with much needed, more effective therapies.

Keywords: HCV; NS3; Inhibitors; Protease; Antiviral; Drugs; Therapies

Introduction

HCV represents a global health concern as it is responsible for a significant number of hepatitis cases worldwide [1]. Approximately, 270 million people are affected worldwide from which 3.2 million people belong to the United States, and 10 million people are from Pakistan [2]. HCV is a plus-stranded RNA virus with genome of 9.5 kilobases. The HCV genome with a large open reading frame encodes a poly protein precursor of about 3010 amino acid residues having an internal ribosome entry site at 5` untranslated region (UTR), vital for the translation. This poly protein precursor is cleaved to generate at least 10 proteins in the order of NH(2)-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS 5B-COOH (Figure 1). These viral proteins are responsible for viral replication and various cellular functions [3-5]. The HCV genome shows a remarkable sequence variation, especially in the hypervariable region 1 of the E2 protein-encoding region [4]. Currently no vaccine is available for prevention of HCV infection due to the high degree of strain variation. The approved treatment for HCV infection is a weekly injection of pegylated alpha interferon (IFN-α) alone or in combination with ribavirin. This leads to clearance of HCV in 50% and 80% of the cases of HCV genotype 1 and 2 infection, respectively but this treatment has certain side effects and slow response rate, especially in patients infected with HCV genotype 1a and 1b [6-8]. Recently, two NS3 protease inhibitors Bocepreir and Telaprevir have been approved as a standard of care for HCV genotype 1 patients and can be used with triple therapy (PEG-IFN- α, ribavirin and Boceprevir or Telaprevir) [9]. This combination treatment can be used to decline HCV infection cases and people who do not respond to monotherapy. Due to increase in HCV infection cases and lack of effective therapies, there is a need to develop specific compounds that can target important factors of the HCV life cycle [10].

HCV NS3 Protein

The NS3 is a 67 kDa protein with multifunctional activity. The NS3 N - terminal has serine protease activity and C terminal has NTPase/helicase activity. NS3 protein is also involved in RNA binding activity [11]. The mature NS3 protein comprises 5 domains: the N-terminal 2 domains form the serine protease along with the NS4A cofactor, and the C-terminal 3 domains form the helicase (Figure 2). The helicase portion of NS3 can be separated from the protease portion by cleaving a linker [12]. Last 185 amino acids at the N-terminus end of NS3 is involved in the cleavage between NS3-4A, 4A-4B,4B-5A and 5A-5B [13]. NS3 catalytic activity is due to three amino acid residues, His-57, Asp-81 and Ser-139 [14]. Most work has focused on the protease, rather than the helicase, activities of the enzyme [15]. Therefore, NS3 can serve as an important drug target in the effort to combat HCV [15].

NS3 Inhibitors

After the success of protease inhibitors in the treatment of human-immunodeficiency virus-1 (HIV) infection, it is thought that development of a specific inhibitors of NS3 protease activity would be an attractive target for new anti-HCV drugs [10,16]. The inhibition of NS3/4A protease will interfere with the viral life cycle and restore the pathways of innate immunity [17]. There are several HCV NS3 protease inhibitors in development, such as telaprevir (VX-950), boceprevir (SCH- 503034), ITMN-191, TMC435350, MK-7009 and ACH-806 (GS-9132) [17]. Teleprevir and Boceprevir have recently completed clinical trials and have been approved as a standard of care for the treatment of HCV in combination with pegylated interferon α (PEG-INF α) and ribavirin. Teleprevir and boceprevir both agents are CYP 3A4 substrates and inhibitors (as well as P-glycoprotein substrates), so they may have significant pharmacokinetic interactions with ARVs [18].

SCH 503034 (Boceprevir) is a novel linear peptidomimetic ketoamide and potent oral hepatitis C virus (HCV) protease inhibitor, which showed potent (overall inhibition constant, 14 nM) time-dependent inhibition of the NS3 protease in cell-free enzyme assays as well as robust *in vitro* activity in the HCV Replicon system, as monitored by immunofluorescence and real-time PCR analysis. SCH 503034 in combination with IFN was more effective in suppressing

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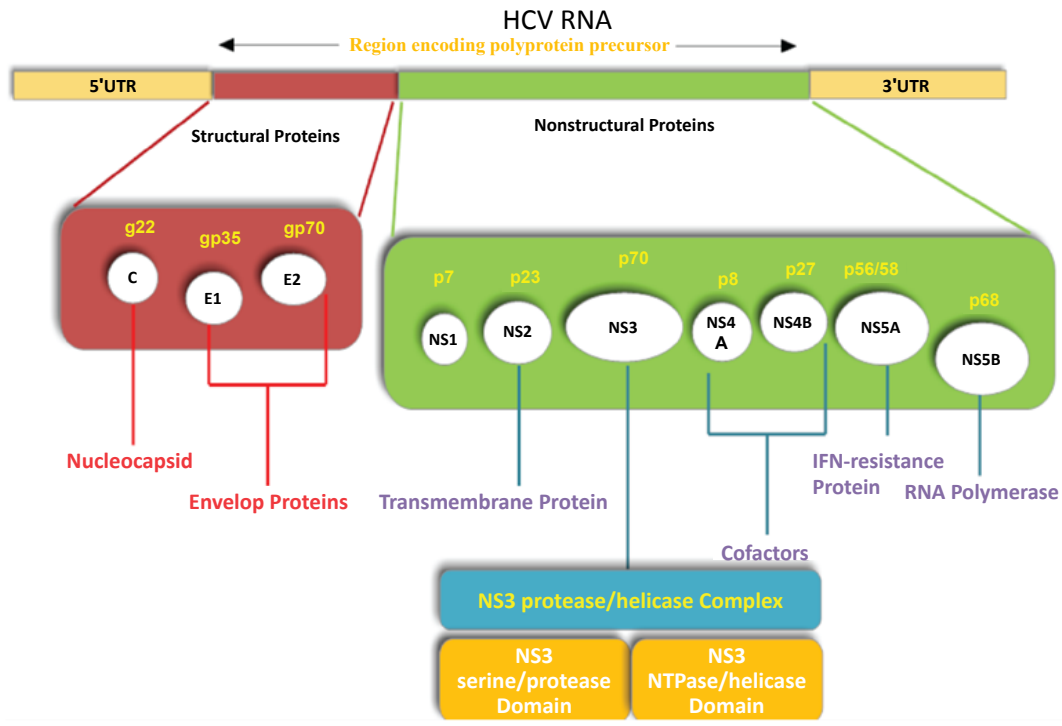

Figure 1: HCV proteins. The HCV genome carries a long open reading frame (ORF) encoding a polyprotein precursor of 3010 amino acid. This polyprotein precursor is cleaved to generate at least 10 proteins in the order of NH(2)-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS 5B-COOH (25, 51).

replicon synthesis than either compound alone. SCH 503034 plus PEG-IFN-alpha-2b was well tolerated in patients with HCV genotype 1 nonresponders to PEG-IFN-alpha-2b+/-ribavirin. These results of antiviral activity of the combination suggested a new antiviral drug to target HCV infected patients [19]. The rate of sustained virological response was significantly increased in previously untreated adults of genotype 1 infection, when boceprevir was added to standard therapy of peg interferon and ribavirin. The rates were similar with 24 weeks and 44 weeks of boceprevir [20].

VX-950 (Telaprevir) is a highly selective and potent linear peptidomimetic inhibitor of the HCV NS3-4A protease having α-ketoamide group serine trap warhead forming a covalent but reversible complex with a steady-state inhibition constant (Ki) of 7 nM against the enzyme. VX-950 and alpha interferon combination reduced HCV RNA in replicon cells with no significant increase in cytotoxicity [21]. It caused up to 4.4 $\log_{10}$ IU/ml median viral titer reduction in HCV genotype 1 patients at doses of 750 mg every 8 hours in a 14-day study period [22]. Mutations associated with resistance to telaprevir occurred in the NS3 catalytic domain either as single mutation (V36A/M, T54A, R155K/T, A156S/T/V) or as double mutation (at positions 36+155 or 36+156 [23]. The combination of interferon and telaprevir can reduce HCV RNA. Therefore, the combination of telaprevir and peginterferon alfa can result in greater antiviral activity than telaprevir or peginterferon alfa alone and can decrease the incidence of viral breakthrough [24].

Teleprevir and boceprevir have been approved in May 2011 by US Food and Drug Administration and then by European Medicines Agency for the treatment of chronically infected HCV genotype 1 patients. Activity of Teleprevir and boceprevir varies among different genotypes as it was designed to target genotype 1 HCV. It was effective against genotype 2 in small clinical trials. It was revealed through *in vitro* studies that teleprevir and boceprevir were efficient for genotype 2,5 and 6 but not against 3 [25].

C8-bromo derivatives studies led to the discovery BI 201335, a very potent and linear tripeptide inhibitor of HCV genotype-1 NS3 protease [26]. Protease inhibitor BI201335, polymerase inhibitor BI 207127 and ribavirin when combined showed rapid and strong activity against HCV genotype-1 in phase I trial and did not show any serious side effects [6]. In Caco-2 cell assays, BI 201335 showed excellent permeability and high metabolic stability after incubation with human, moneky, dog and rat liver microsomas. It showed good absorption, distribution, metabolism, and excretion (ADME) profile *in vitro*, as well as in rat, monkey, and dog and showed good pharmacokinetics in humans. BI 201335 is in phase 3 of development [27].

SCH 900518 (Narlaprevir) is a novel linear ketoamide inhibitor that was originally developed as an HIV protease inhibitor. It is currently undergoing phase III clinical trials. It showed improved potency (~10-fold), pharmacokinetic profile and physicochemical characteristics [27]. SCH 900518 (± ritonavir) alone or in combination with PegIntron resulted in trough plasma concentrations of SCH 900518 above the EC90. Majority of patients had plasma HCV RNA below the LLQ (<25 IU/mL) during combination therapy with PegIntron (± ritonavir) on Day 15. Therefore, SCH 900518 alone or in combination with PegIntron is safe and well tolerated. Currently, it is in phase II of clinical trials [28].

Introduction of various modified prolines at P_2 and optimization of the P_1 side chain led into the discovery of SCH6 (SCH446211) [27], which is a ketoamide peptidomimetic inhibitor that showed potent inhibition of HCV NS3/4A protease *in vitro* with an extended interaction in Huh-7 hepatoma cells stably transfected with a subgenomic HCV RNA replicon. SCH6 inhibited the HCV replication and protein expression in replicon cells. In particular, based on quantitative real-time RT–

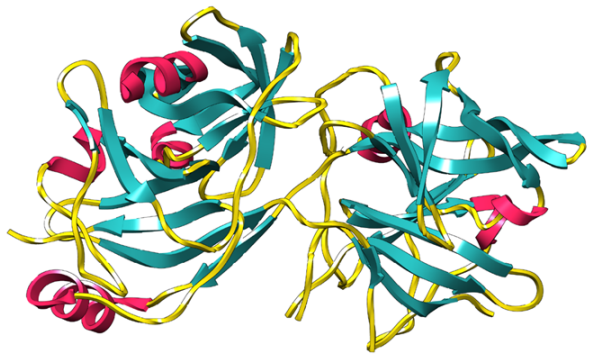

Figure 2: NS3-4A protein structure. Helix are shown in pink, Strands are in blue and Coils are shown in yellow color.

PCR measurements, the IC_{50} and IC_{90} of SCH446211 were estimated to be 40 ± 20 and 100 ± 20 nM (*n*=17), respectively. Long-term culture of replicon cells with SCH446211 reduced replicon RNA to <0.1 copy per cell. SCH6 did not show cellular toxicity at concentrations up to 50 μM. SH6 is potentially a useful inhibitor against HCV NS3 protease. Currently, its therapeutic use is on hold [29].

BILN 2061 (Ciluprevir) is a potent and non-covalent specific macrocyclic inhibitor of HCV serine protease genotype 1 *in vitro* [30]. BILN 2061 was the first NS3 protease inhibitor ever tested on human to inhibit the HCV infection. BILN 2061, when administered to patients infected with HCV genotype 1 for two days resulted in an impressive reduction (2-3 $\log_{10}$ copies/mL) of HCV RNA plasma levels, and established proof-of-concept in humans for an HCV NS3 protease inhibitor [30,31]. In rhesus monkeys a cardiac histologically toxicity was identified on receiving high doses of BILN-2061 for four weeks duration. BILN 2061 was helping to understand these toxicities and thus contributing in combating HCV disease but due to its cardiac toxicity, its trial has been stopped [32].

Another highly specific and strong macrocyclic inhibitor of NS3/4A protease is TMC435 (Medivir/Tibotec). HCV replication was inhibited by TMC435 in a cellular assay (subgenomic 1b replicon) with a half-maximal effective concentration (EC_{50}) of 8 nM and a selectivity index of 5,875. When HCV-1 infected patients were administered with TCM-435, alone or in combination with pegylated interferon alpha and ribavirin, they produced significant reductions in HCV RNA and suppressed the emergence of drug resistant replicon colonies. TMC435 has recently entered clinical trials and is in phase 3 of development [33-35]. TMC-435 is effective for genotype 1, 2, 5, and 6 and has adverse effects as an asymptomatic increase in level of bilirubin [25].

ITMN-191 (Danoprevir), a macrocyclic inhibitor of the NS3 protease active site achieves high liver concentrations following oral administration [36]. ITMN-191 inhibited genotype 1 NS3/4A protein in a time-dependent fashion. Peg interferon alfa-2a showed a significant degree of antiviral synergy with ITMN-191 and reduced the concentration of ITMN-191 required for HCV replicon elimination [37]. Maximal decreases in HCV RNA using ITMN-191 were: −3.9 $\log_{10}$ IU/ml and −3.2 $\log_{10}$ IU/ml in TN receiving 200 mg q8 h and 200 mg q12 h, respectively. The overall incidence of viral rebound was low (10/37) and was associated with the R155K substitution in NS3 regardless of the HCV genotype [38]. Danoprevir is currently in phase II of clinical trials [39].

MK-7009 (Vaniprevir) is a macrocyclic inhibitor of genotype 1a and 1b proteases at subnanomolar concentrations and also has tremendous selectivity against a range of human proteases [40]. MK-7009 also had excellent selectivity against a broad range of pharmacologically relevant ion channels, receptors, and enzymes [41]. Administration of vaniprevir for four weeks with Peg-IFN/Ribavirin achieved Rapid viral response rate of 69-82% [42]. Due to its favorable profile, it is currently in phase II clinical trial and being tested with both healthy volunteers and HCV-infected patients [41].

GS-9256 is a macrocyclic NS3 serine protease inhibitor that showed antiviral activity against HCV genotype 1. HCV genotype 1 patient was given GS-9256 plus RBV 1000-1200 mg daily. It showed a rapid virologic response (RVR), HCV RNA <25 IU/mL at day 28 and after 28 days, all patients received pegylated interferon/ribavirn. This resulted in HCV RNA <25 IU/mL at Week 24 in 67% (10/15), 100% (13/13) and 94% (13/14) of patients in the 3 treatment groups [6]. In the series of phosphinic acid derived NS3 protease inhibitors, GS-9256 has best combination of potency (Ki 0.089 nM). GS-9256 did not inhibit CYP-450 enzymes and did not block the hERG channel at concentration up to 25 lM. GS-9256 is currently in phase 2b for the treatment of HCV infection [43].

MK-5172 is a novel P2-P4 quinoxaline macrocyclic NS3 inhibitor [44] active in genotype 1–3 NS3/4a and has good plasma exposure and excellent liver exposure in several species [45]. The rapid viral load reduction was observed for seven days of monotherapy with 800 mg multiple oral doses of MK-5172. MK-5172 inhibits viral replication (IC_{50}=2 nM) in the genotype 1b Replicon assay. Genotype 2a replicon is also inhibited by MK-5172 (EC_{50}=5 nM). MK-5172, when administered to chimpanzees having genotype 1a and 1b, showed viral load reduction between 4-5 logs at a dose of 1 mg/kg of body weight twice daily for 7 days. MK5172 because of its preclinical profile is being anticipated to be effective for multiple HCV genotypes and has entered in clinical trial phase II [46].

ABT-450, a recently developed compound by Abbott is a macrocyclic acrylsulfonamide inhibitor of NS3 protease. ABT-450 is administered in combination with ritonavir to allow once daily dosing. ABT-450/or in combination with the polymerase inhibitor ABT-333 and ribavirin was administered to patients. After 12 weeks of dosing in treatment naïve patients, ABT-450/ritonavir (ABT-450/r) was well tolerated and showed potent antiviral activity when combined with Peg-IFN/Ribavirin. About 91% genotype 1 infected patients achieved SVR at 24 weeks. ABT-450 did not show any adverse events during its administration and is currently in clinical trial phase II [47].

IDX320, a potent non-covalent macrocyclic inhibitor has entered clinical trial phase II. IDX320 is actually a liver-targeted nucleotide analog pro-drug preventing the HCV polymerase from copying viral genetic material [48]. This has shown effective results against genotype 1a, 1b, 2a, and 4a (0.8 to 1.9 nM IC50) and also with activity against genotype 3a (23 nMIC50) [49].

BMS-650032 (Asunaprevir) is potent and selective NS3 protease inhibitor tested in combination with NS5A replication complex inhibitor BMS-790052. Patients infected with HCV genotype 1b were given BMS-790052 (60 mg once a day) and BMS-650032 (initially 600 mg twice a day, then reduced to 200 mg twice day). For genotype 1b, a higher level of asunaprevir-associated resistance was observed at the same selection pressures; ranging from 170- to 400-fold relative to wild-type control [50]. All patients achieved SVR12 and SVR24. There

was no viral breakthrough. BMS-650032 is currently in phase II of clinical trials [51].

Achillion and Gilead in collaboration developed ACH-806 (GS-9132) [52], which is a potent and specific inhibitor of HCV. ACH-806 inhibits HCV replication by a novel mechanism. When the entire coding regions of ACH-806-resistant Replicon variants were sequenced, several consensus mutations were yielded. Furthermore, reverse genetics identified two single mutations in NS3, a cysteine-to-serine mutation at amino acid 16 and an alanine-to-valine mutation at amino acid 39, that are responsible for the resistance of the replicon variants to ACH-806. Both mutations are located near the N terminus of NS3 where extensive interactions with the central hydrophobic region of NS4A exist [52]. Reversed-phase High-performance Liquid Chromatography (HPLC) assay system was used to test the inhibitory efficiency using a NS3-NS4A protein as the HCV protease. Nine derivatives of thiazolidine showed more than 50% inhibition at 50 µg/ml concentration RD4-6205 was the most selective derivative with 50% inhibition at 6.4 µg/ml concentration. Therefore, RD4-6205 derivative is an important structure for inhibitory activity on the HCV protease NS3-NS4A [53]. ACH-1625 is a linear, non-covalent inhibitor of NS3 protease with similar potency for all genotypes except GT-3 [54]. At high drug doses, it showed high potency and excellent safety profile. It is equipotent against HCV genotypes 1a and 1b at IC50 ~1 nM. At the doses of 200 mg, 400 mg and 800 mg of ACH-1625 in combination with SOC achieved 75-81% RVR and ACH-1625 did not show any serious adverse effects. It is in the clinical trial II phase [55].

Recently, PHX1766 has successfully entered the clinical trial phase 1 and has shown robust potency and high selectivity in replicon studies (50% maximal effective concentration 8 nM). By using dose-adaptive overlapping clinical trial design, the safety, tolerability, antiviral activity and pharmacokinetics of PHX1766 has been successfully checked in healthy volunteers and chronic hepatitis C patients. There was a mean maximal observed HCV RNA decline of 0.6 $\log_{10}$ IU/ml in the first 24 hours and 1.5 $\log_{10}$ IU/ml after 6 days of PHX1766 dosing [56].

All NS3 protease inhibitor compounds (Table 1) are safe and well tolerated in monotherapy as well in combination and can be further investigated to improve HCV antiviral therapies.

Resistance to Ns3 Protease Inhibitors

The emergence of drug-resistant viruses is a main hurdle in the development of antiviral therapy [57]. Telaprevir, boceprevir, and ITMN-191 are the most promising protease inhibitors but resistance against them has emerged in clinical trials [58]. Using HCV replicon system four mutations in the HCV protease (R155Q, A156T, D168A and D168V) have been identified that shows resistance to BILN-2061. However, the molecular mechanism of drug resistance is still unclear [57]. In particular, R155 and A156, which mutate to give severe resistance against ITMN-191, TMC435, and boceprevir, interact closely with the P2 drug molecules. Molecular changes at these residues confer resistance by weakening the inhibitor binding [58]. The major BILN 2061-resistant mutations at Asp^{168} are fully susceptible to VX-950, and the dominant resistant mutation against VX-950 at Ala^{156} remains sensitive to BILN 2061. Modeling analysis suggests that there are different mechanisms of resistance to VX-950 and BILN 2061 [59]. Using G418 selection in the presence of SCH6, resistant replicon RNAs were generated in a dose-dependent fashion. Sequencing showed remarkable consistency in mutations conferring SCH6 resistance in genotype 1b replicons. A156T conferred high level resistance to SCH6 and a related ketoamide, SCH503034, as well as BILN 2061 and VX-950 [60]. Two mechanisms were evidenced for viral resistance to BILN-2061. A 'direct' resistance mechanism is based on contacts between the mutated R155Q and A156T protease residues and its inhibitor [57]. Recently, one group reported that Solanum nigrum and Accacia nilotica extracts showed antiviral activity against NS3 protease [61,62].

In future, HCV antiviral drugs will be developed such that they will not only be a combination of multiple drugs but will also suppress the emergence of resistance [63].

Conclusion

To date, it appears that HCV protease inhibitors will be developed as the next generation of anti-HCV drugs. The combination of specific HCV NS3/4A inhibitors with Peg-IFN or Peg-IFN+RBV treatment regimes may be synergistic, opening the door to future combination therapies. Nevertheless, it will be very exciting to see HCV NS3-4A serine protease/helicase inhibitors progress through clinical developments and, hopefully, provide hepatitis C patients with much needed, more effective therapies.

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NS3 Inhibitor	Development Phase	Genotype	Company	Nature
SCH 503034 (Boceprevir)	FDA Approved	1 ,2,5,6	Merck	Linear peptidomimetic
VX-950 (Telaprevir)	FDA Approved	1 ,2,5,6	Vertex Pharmaceuticals	Linear peptidomimetic
BI 201335	Phase III	1	Boehringer Ingelheim	Linear tripeptide
SCH 900518 (Narlaprevir)	Phase II	1	Merck & Co	Linear ketoamide
SCH6 (SCH446211)	On hold	2	Merck & Co	Ketoamide peptidomimetic
BILN 2061 (Ciluprevir)	Trial stopped	1	Boehringer Ingelheim	Macrocyclic
TMC435 (Simeprevir)	Phase III	1 ,2,5,6	(Medivir / Tibotec)	Macrocyclic
ITMN-191/RG7227 (Danoprevir)	Phase II	1	InterMune/Roche	Macrocyclic
MK-7009 (Vaniprevir)	Phase II	1	Merck & Co	Macrocyclic
GS-9256	Phase II	1	Gilead	Macrocyclic
ACH-1625	Phase II	1	Achillion	Macrocyclic
MK-5172	Phase II	1,2	Merck & Co	Macrocyclic
ABT-450	Phase II	1	Abbott/Novartis	Macrocyclic
IDX320	Phase II	1, 1b, 2a, 3a and 4a	Idenix	Macrocyclic
BMS-650032 (Asunaprevir)	Phase II	1,4	Bristol-Myers Squibb	Macrocyclic
ACH-806 (GS-9132)	Phase II	1	Achillion and Gilead	N/A
PHX1766	Phase I	1	Phenomix	N/A

Table 1: NS3 Serine Protease Inhibitors and their current development phase.

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