Hepatitis C, a chronic disease affecting the global population significantly is caused majorly by Hepatitis C virus [HCV]. Among the several druggable targets explored for Hepatitis C, the viral protein, non-structural protein 5B [NS5B] is the target of choice for researchers as it is the key enzyme in the HCV replication and its active site is conserved among all genotypes. In the recent years the landscape of Hepatitis C therapies, have evolved from Peg-Interferon [PEG-INF]/Ribavirin, to directly acting anti-virus along with PEG-INF and finally, INF free regimens with greater than 90% sustained virological response [SVR]. The launch of Sofosbuvir, a nucleotide inhibitor of NS5B marks the major paradigm in hepatitis C research. Sofosbuvir exhibits, pan-genotypic activity, low barrier to resistance, highly effective and safe. However, the high prices of these medications limit their universal access. This review will focus on progress towards the discovery and development of NS5B inhibitors targeting allosteric sites and active site, covering the chemical class and structure-activity relationships.

**Keywords:** Hepatitis C virus, NS5B, Inhibitors, Structure-Activity relationships, Genotypic variance, Allosteric site, Active site

**INTRODUCTION**

Hepatitis means inflammation (a painful reddish swelling) of the liver and Hepatitis C virus (HCV) is one of the major causes. HCV can spread through many ways ranging from a blood transfusion, poorly sterilized medical equipment, from the mother (HCV carrier) to the baby, being tattooed with unsterilized tools, having unprotected sex with an infected person and sharing of personal care items [1]. About 80% of those infected with HCV develop chronic infection [2]. Patients can relapse and develop infection due to the presence of variants of the virus [quasispecies] arising from error-prone replication [3].

The World Health Organization [WHO] has estimated that almost 3.3% of world’s population has been suffering from HCV infection. About 2-3 million people in the U. S. A, 4-10 million people in Europe and close to 12 million people in India suffer from HCV infection [4, 5]. A combination of Peglated Interferon [weekly injections] and Ribavirin [daily oral], given for a period of 24 or 48 w, depending on the HCV genotype was the potential treatment option available till 2011 [6]. Improved outcomes are seen in 50-60% of patients infected with genotype 1 and 4, but were effective in 70-90% of genotype 2 and 3 infected population. However, adverse effects like flu like symptoms are more common. Later in 2011 first generation direct acting anti-virals [DAAs] NS3/4A protease inhibitors namely Telaprevir [Incivek], Boceprevir [Vtcvrels] were launched, but their use is restricted to patients infected with HCV genotype 1 [6, 7]. Over a decade efforts were focused towards development of specific inhibitors of the viral RNA-dependent RNA polymerase NS5B as it is the key enzyme for viral replication, conserved among the HCV genotypes 1, 2, 3, 4, 5 and 6, has an active site and many allosteric binding pockets [8]. A major breakthrough in Hepatitis C research came with the launch of Sofosbuvir [Sovaldi] in December 2013, an NS5B inhibitor developed by Gilead. Sofosbuvir is safe and potent first in class nucleoside inhibitor of NS5B potency with pan-genotypic activity against HCV genotypes 1, 2, 3 and 4 [9]. Simeprevir [Olysio] an NS3/4A protease inhibitor was also launched in 2013. Since then many combination therapies involving NS3 protease inhibitors, NS5A inhibitors, and NS5B inhibitors were approved by U. S. FDA. Hepatitis C therapies launched from 2014 to till date were furnished in Table 1 [10].

### Table 1: FDA approved drugs for hepatitis C launched since 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Brand name/Drug name</th>
<th>Manufacturer</th>
<th>Genotype [SV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>Sovaldi/Olysio/RBV</td>
<td>Janssen</td>
<td>Genotype 1 [Up to 92%]</td>
</tr>
<tr>
<td>2014</td>
<td>Harvoni</td>
<td>Gilead</td>
<td>Genotype 1,4,5,6 [Up to 100%]</td>
</tr>
<tr>
<td>2014</td>
<td>VIEKIRA PAK</td>
<td>AbbVie</td>
<td>Genotype 1 [Up to 100%]</td>
</tr>
<tr>
<td>2015</td>
<td>Daklinza</td>
<td>Bristol-Myers Sqibb</td>
<td>Genotype 3 [Up to 98%]</td>
</tr>
<tr>
<td>2015</td>
<td>Technivie</td>
<td>AbbVie</td>
<td>Genotype 4 [Up to 100%]</td>
</tr>
<tr>
<td>2016</td>
<td>ZEPATIER</td>
<td>Merck</td>
<td>Genotype 1,4 [Up to 100%]</td>
</tr>
<tr>
<td>2016</td>
<td>Epclusa</td>
<td>Gilead</td>
<td>Genotype 1-6 [95 %]</td>
</tr>
</tbody>
</table>

But the high cost of these medications limits their use, and there still exist a need for cost-effective therapies, therapeutic agents for Hepatitis C patients with liver cirrhosis or carcinoma, and most important anti-HCV agents with potent activity against genotype 3 which is the second most predominant HCV variants. The purpose of this review is to discuss the reported active site and the allosteric site directed NS5B inhibitors identified till date.

### Hepatitis C virus

Hepatitis C virus is small, spherical, enveloped, the hepatotropic RNA virus that causes acute and chronic hepatitis in humans. HCV belongs to Flaviviridae family. HCV is classified into seven different genotypes (1, 2, 3, 4, 5, 6 and 7) which differ in nucleotide sequentially by 30%; these genotypes are further classified into...
Mechanism of RNA synthesis by NS5B

Different subtypes [a, b, c, d... etc.] that differs in nucleotide sequence by 20-25%. Different HCV genotypes also differ in worldwide distribution, transmission and disease progression [11]. It has a 5'-triphosphate (rNTP) structure that cannot be cleaved by host proteases. HCV induces the membranous web that can be used as replication site [17, 18]. NS5B synthesizes complementary Ve-stranded RNA from Ve-stranded RNA template. RNA synthesis with NS5B can occur either by de novo initiation or primer extension [Self primer] [8]. NS5B uses divalent metal ions as co-factors such as magnesium or manganese for RNA synthesis.

De novo initiation involves recognition of the 3'end of template RNA by NS5B and synthesis of RNA without the use of a primer.

Primer extension involves elongation of the primer along the length of the template, which corresponds to the elongation step in the de novo initiation. The primer is self-primer [3’ end of the template RNA folds back intra-molecularly]. Since NS5B lacks the proofreading function. As a result, there is a high rate of error-prone replication leading to mutations [8].

**NS5B inhibitors**

There are several classes of NS5B inhibitors that target the active site and the allosteric sites of the enzyme thus inhibiting HCV replication. NS5B inhibitors are broadly categorized into Nucleoside analogues (bind to active site) and Non-nucleoside analogues (bind to allosteric sites)[19].

**Nucleoside/Nucleotide analogues (NI)**

Nucleoside/Nucleotide analogues (NI) target the highly conserved active site of the polymerase and terminate the RNA synthesis by getting incorporated into the RNA chain in lieu of naturally occurring nucleotide triphosphates (NTP's), hence they are also called chain terminating inhibitors [8, 20]. The ionic nature of the phosphate group present in nucleotides prevents them from penetrating into the cell membrane; hence they are delivered as uncharged nucleosides. These nucleosides are phosphorylated to 5'-triphosphate form (active form) by cellular kinases, where the formation of the monophosphate form is the rate limiting step [21, 22]. Many of these compounds have progressed to clinical trials and they act in synergy with interferon and Ribavirin combination.

**Purine nucleoside inhibitors**

Researchers at the Merck Research Laboratory designed 2'-β-methyl analogues by modifying the Ribose moiety of a nucleoside, namely 2'-C-Methyladenosine (1) and 2'-C-methylguanosine (2) and tested their anti-HCV activity [23]. 2'-C-Methyladenosine displayed potent activity against NS5B (IC<sub>50</sub> = 1.9 µM) [24, 25], but its efficacy was hampered by poor oral bioavailability and stability. In order to improve the efficacy Hecker et al. From Merck developed 4-pyridyl pro-drugs which showed high aqueous solubility, bioavailability, and stability.

Several hetero based modified analogues of 2'-C-Methyl-adenosine were also developed with reduce polarity and good cell permeability, but they showed poor stability. Hence the focus was moved towards the development of phosphoramidate analogues to enhance stability. Compound 3 was identified as most potent among the series with replicon EC<sub>50</sub> of 0.26 µM and is a potential candidate for further development [26].

2'-C-methylguanosine (2) was active against NS5B with IC<sub>50</sub> of 0.13 µM and replicon EC<sub>50</sub> of 3.5 µM; it also showed enhanced bioavailability [27]. Structure-activity relationship [SAR] studies revealed that β-orientation of a methyl group at 2'-C position is required for the activity.

Either alpha-orientation of the methyl group or movement of a methyl group to 3' position diminishes the activity [24]. Several heteros based modified analogues of 2'-C-methylguanosine such as 7-deazaguanosines, 2'methyl-6-O-methyl-guanosine were mentioned in the literature but none of them have been progressed to further development.
Pyrimidine nucleoside inhibitors

Idenix discovered 2'-C-Methylcytidine (4) which displayed potent anti-HCV activity with EC\textsubscript{50} of 0.26 µM in the replicon assay \[29, 30\]. But the efficacy of 2'-C-Methylcytidine was hampered by poor oral bioavailability and in order to improve the efficacy Pierra et al. developed 3'-O-L-valinyl ester analogue valopicitabine (NH283) (5) \[31\]. NH283 reduced viral load when given alone or in combination with pegylated interferon, but its development was discontinued due to gastrointestinal side-effects \[32\]. A series of 3'-deoxyribonucleosides have also been described as chain terminators, among them 3'-deoxycytidine displayed submicromolar potency in viral replicon assay \[29,33\]. However, these 2'-C-methyl ribonucleosides are not effective against the S282T mutant, so 2'-O-methyl ribonucleotides were developed and among them 2'-O-methylcytidine (6) showed no difference in activity against the mutant and the wild-type \[25\].

Klumpp et al. At Roche identified R1479 (4'-azidocytidine) as an NS5B inhibitor through targeted screening of nucleotide library. R1479 displayed potent activity in sub-genomic replicon assay \[IC\textsubscript{50} = 1.28 µM\] but showed no effect in cytotoxicity assay \[34\] and also showed limited cell permeability and poor bioavailability. Further, R1626 was developed as a prodrug for R1479 (4'-azidocytidine) which displayed potent anti-HCV activity with IC\textsubscript{50} of 1.28 µM in HCV subgenomic replicon system and had no effect on cell viability or proliferation of Huh-7 cells \[35, 29\].

4'-azidouridine (7) the corresponding analogue of R1479 [4'-azidocytidine] was also found to be inactive in inhibiting HCV replication. Later, Perrone et al. developed permeable membrane phenyl ProTide form of 4'-azidouridine (8) which showed good antiviral activity \[EC\textsubscript{50} = 0.61 µM\]. Naphthyl ProTide form (9) was more active than the phenyl counterpart in inhibiting HCV replication \[34\]. Meanwhile, other pyrimidine analogues such as beta-D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI 6130) \[33\], N4-hydroxycytidine \[36\] and 2'-fluororibonucleosides \[37\] also appeared in literature as anti-HCV agents. PSI-6130 is a highly potent anti-NS5B agent and is effective against the S282T mutant. Currently, RG7128 the prodrug of PSI-6130 is in Phase 3 and is the first agent to show a broad genotypic coverage \[33\].

Sofosbuvir (a prodrug of 2'-F-2'-C-methyluridine monophosphate), developed by Gilead Sciences, has been shown to be effective across HCV genotypes 1b, 2a, 3a and 4a \[IC\textsubscript{50} = 0.7-2.6 µM\] and also provided a genetic barrier to resistance \[38, 39\]. In a randomized, open-label study, Sofosbuvir showed sustained virologic response in patients infected with HCV genotype 1, 2 and 3 when given in combination with Daclatasvir, an NS5A inhibitor \[40\]. Jonckers et al. identified a series of phosphoramidate prodrugs of 2'-deoxy-2'-spirooxetane ribonucleosides. These prodrugs showed antiviral activity in HCV replicon assay (Huh-7 cells) with an EC\textsubscript{50} value ranging from 0.2 to 98 µM, but failed to show potency in cytotoxicity assay \[41\].

Resistant mutations to NI include S282T, S96T, and N142T; of these S282T confer resistance to Sofosbuvir, 2'-C-methylcytidine and 2'-C-methyladenosine while S96T and N142T confer resistance to 4'-azidothymidine (R1479). The S282 residue is in close proximity to the residues binding at NTPi (initiation nucleotide) \[42\]. Hence it is clear that NTP binding site is altered during de novo initiation of RNA synthesis and elongation.

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Polamreddy et al.

Miscellaneous nucleoside analogues

These are the nucleoside mimics who do not possess either a Purine or a Pyrimidine nitrogenous base. Ribavirin is the best example of this class. In an attempt to develop a more potent analogue of Ribavirin, Witkowski et al. discovered Taribavirin [viramidine] [43]. Taribavirin targets the liver and has a better safety profile [less likely to cause anemia] than Ribavirin, therefore Taribavirin is considered as a potential candidate for further development and is currently in Phase 3 clinical trials [44]. However, in the VISERI study taribavirin given at 600 mg BID, was able to improve survival rates in only 44% of patients and is inferior to Ribavirin which improved survival rates in 55% of patients [45]. Other miscellaneous nucleoside analogues include acyclic triazole analogues synthesised by Zhu R et al [46], pyrrolopyrimidines designed at Valeant [47], alpha-gamma-diketo acids [48] but none of them had entered clinical trials.

M. Emilia Di Francesco et al. designed a series of nucleoside inhibitors bearing a 7-heterocyclic substituted 7-deaza-adenine nucleobase. The most potent of this series is the 1, 2, 4-oxadiazole analogue which showed potent anti-viral activity in vivo and in vitro [49]. Manfroni et al. reported that one of the 6-aminquinolone derivatives 6-amino-7-[4-[2-pyridinyl]-1-piperazine]quinolone showed good anti-HCV activity in an enzymatic assay (IC50 = 0.069 µM) and replicon assay (EC50 = 3.03 µM) with low cytotoxicity [50]. The nucleoside inhibitors currently in different phases of development are given in Table 2.

Table 2: Nucleoside inhibitors in the market and under investigation

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Clinical phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-7977 [Sofosbuvir]</td>
<td>Launched</td>
<td>Gilead</td>
</tr>
<tr>
<td>VX-153</td>
<td>Phase 2</td>
<td>Vertex Pharmaceuticals</td>
</tr>
<tr>
<td>ACH-3422</td>
<td>Phase 1</td>
<td>Achillion</td>
</tr>
<tr>
<td>PSI-938</td>
<td>Discontinued</td>
<td>Gilead</td>
</tr>
<tr>
<td>RG7128 [Mericitabine]</td>
<td>Discontinued</td>
<td>Roche</td>
</tr>
<tr>
<td>IDX21437 [MK 3682]</td>
<td>Discontinued</td>
<td>Idenix/Merck</td>
</tr>
<tr>
<td>IDX84</td>
<td>Discontinued</td>
<td>Idenix</td>
</tr>
<tr>
<td>PSI-7851 [Isomer of Sofosbuvir]</td>
<td>Discontinued</td>
<td>Pharmasset</td>
</tr>
<tr>
<td>TMC649128</td>
<td>Discontinued</td>
<td>Medivir and Tibotec</td>
</tr>
<tr>
<td>GS-6620</td>
<td>Phase 1</td>
<td>Gilead</td>
</tr>
<tr>
<td>BCX5191</td>
<td>Discontinued</td>
<td>Biocyst</td>
</tr>
<tr>
<td>Valopicitabine</td>
<td>Discontinued</td>
<td>Idenix/Novarties</td>
</tr>
</tbody>
</table>

GS-6620 demonstrated potent activity in phase 1 clinical study but showed high pharmacokinetic and pharmacodynamic variability [51, 52]. VX-153 in combination with Daclatasvir was well tolerated in HCV genotype 1 infected patients but showed a moderate SVR in a phase-2 study [NCT01042541]. ACH-3422 showed pan-genotypic activity in vitro and was well tolerated in phase-1 randomized double-blind study [53, 54]. TMC649128 developed by Tibotec pharmaceuticals entered phase 1b trials where safety, tolerability, pharmacokinetics and antiviral activity of the compound are evaluated [55]. IDX21437 a uridine nucleotide analog generated high triphosphate levels and showed potent pan-genotypic activity and is currently being evaluated in phase II trials [NCT0232707] [56].

Non-nucleoside analogues (NNI)

Non-nucleoside analogues are the alternative means for targeting viral polymerases. These analogues target allosteric sites of NS5B and cause change in the conformation of the active site, thereby inhibiting the initiation phase of the RNA synthesis. The allosteric sites on the HCV polymerase namely Site I is located in the interface of the finger loop (Δ1 loop) and thumb domain, Site II is located in the thumb region beneath the site I, site III is in the template channel region in the palm domain and includes ‘primer-grip site’ and Beta-loop, Site IV located near the active site in the palm region. There is also another allosteric site located in the finger loop i.e. Site V.

Site I (Thumb I)

Site I is at the upper section of the thumb domain which is 30 Å away from the active site and is at the junction of the thumb and the finger domain [11]. The site I also overlap with the allosteric GTP binding site. When the inhibitor binds to site I, it displaces the Δ1 loop and brings a change in the conformation of the enzyme thereby disrupting the inter-domain communication. This change in conformation prevents the formation of the NS5B-RNA complex, thereby inhibiting RNA synthesis in an NTP non-competitive manner. But the compounds targeting this site do not inhibit elongation phase of RNA synthesis and they also do not have any effect on the performed NS5B-RNA complex [57, 58]. Amino acid substitutions at P495, P496 and V499 with Ala, Leu, and Ser respectively render resistance to this class of inhibitors. Substitution at P495 position confers a high level of resistance to inhibitors and substitution at P496 and V499 residues renders low-level of resistance [58].

Benzimidazoles

Benzimidazoles identified by Boehringer Ingelheim inhibited NS5B at low micromolar IC50 values [8, 59-61]. Using high-throughput parallel synthetic techniques, 1, 2-disubstituted benzimidazole 5-carboxylic acid scaffold was identified as the minimum core for biological activity [62]. Ishida et al. identified compound 10 of the benzimidazole class with IC50 value of 4.3 µM in an enzyme inhibition assay [63]. Removal of the N-cyclohexyl in benzimidazole scaffold led to a complete loss in the activity. Hence, a six-membered ring at the N1 position is a significant fragment and optimally fits to the hydrophobic pocket [64]. Replacement of 2-pyridyl part of 10 with 2-furyl led to a more potent analogue compound 11 (IC50 = 1.6 µM).
Beaulieu et al. later reported that modifications to the right-hand side of the molecule (at C2 carbon) lead to 800-fold increase in potency. The most potent of them is compound 12 with IC_{50} of 0.008±0.002 µM[65]. Elongation of the alkyl chain from benzyl did not influence the potency. Hirashima et al. reported a series of benzimidazole derivatives with biphenyl substituent at C2 position[66]. Intense modification led to identification of a potent analogue 13 which was effective against genotype 1a (IC_{50} = 62 nM), genotype 1b (IC_{50} = 17 nM) and genotype 3a (IC_{50} = 61 nM). Promising pharmacokinetic (PK) parameters and good safety profiles of compound 13 (JTK-109) led it to its clinical development as an anti-HCV agent. Vaishali Patil et al. designed a series of benzimidazole derivatives by introducing alkyl group at N3 and benzoxy phenyl group at C2 positions of benzimidazole nucleus, of which potent compounds inhibited NS5B activity by 24.2 % at 100 µM concentrations [67].

Weidlich et al. identified Candesartan cilexetil as specific NS5B inhibitor through machine learning approach. Candesartan is an angiotensin II receptor blocker and is currently used to treat hypertension. Candesartan cilexetil, when tested in vitro for anti-NS5B, showed potency in the micromolar range. Preliminary docking results indicated that replacement of benzyl ring in benzimidazole with thiazine-1,1-dioxide can improve the activity of Candesartan cilexetil[68].

**Indoles**

In order to improve the potency of benzimidazoles, Beaulieu et al. replaced the benzimidazole core of inhibitors with a more lipophilic indole group. The indoles showed more than 30 fold improvement in cell-based replicon assay [69]. Harper et al. reported a series of indole-N-acetamide derivatives and determined their anti-HCV potency in Huh-7 cells [70]. Among them morpholine acetamide indole-N-acetamide derivatives and determined their anti-HCV potency in Huh-7 cells [70]. Among them morpholine acetamide indole-N-acetamide analogue (14) exhibited good potency in replicon assay (IC_{50} of 11+/−2 nM) and cell based assay as well [EC_{50} = 0.3+/−0.1 µM] with less cytoxicity (CC_{50} = 50 µM) [70].

A series of indole derivatives were developed by replacing C-6 carboxylic acid group [71]. All the compounds in the series possess activity in nanomolar range against NS5B and micromolar to submicromolar activity in replicon assays, the most potent being Compound 15 with IC_{50} = 4 nM and EC_{50} = 0.12 µM.

Ikegashira et al. developed a series of 6,7-dihydro-5H-benzo[5,6][1,4]diazepino[7,1-a]indoles as NS5B inhibitors [72]. The 6, 5-bicyclic core and the C-2 ring were bridged to get a potent compound. Among them compound 16 was found to be a potent analog with an IC_{50} value of 0.0090 μM [72]. Venkatraman et al. described a series of analogues based on tricyclic indole scaffold and tested them for anti-viral activity. It was found that some of these compounds showed potent activity against NS5B genotype 1b in the biochemical and cellular assay, but showed modest PK in rats. Later ester prodrugs of these compounds were designed to improve the PK [73].

In an attempt to improve the aqueous solubility of indoles, David McGowan et al. designed a series of 1,6 and 2,6 macrocyclic bridging indoles by connecting tether to either the indole nitrogen or phenyl ring in the C2 position of the central indole core [74]. Further optimizations of these macrocyclic indoles generated potent allosteric inhibitors targeting the finger loop domain and eventually lead to the discovery of TMC647055. TMC647055 showed nanomolar potency in the cellular assay, limited toxicity and promising PK properties in rats and dogs and is currently being evaluated in clinical [75,76]. Velázquez et al reported the synthesis of 3, 4-dihydrofuranoindoles which showed inhibitory activity against NS5B with IC_{50} values in the range of 6-15 nM [77].

Gentles et al. through analogue based drug design optimized cyclopropyl-fused indole benzazines and in the process identified BMS-791325 which showed potent anti-NS5B activity, good safety and PK properties. BMS-791325 also showed promising results clinically when given in combination with Daclatasvir (NS5A inhibitor) and Asunaprevir (NS3 inhibitor) [78].

**Quinoxalines**

Rong et al. identified compound 17 via high throughput screening of compound library. Optimization of 17 resulted in more potent analogue (compound 18) with an IC_{50} value of 0.69 µM in NS5B inhibition assay [79].

**Thieno [3,2-b]pyrroles**

Ontoria et al. through replacement of 6, the 5-bicyclic core of benzimidazole developed trinstitutedazaindoles and were tested in enzyme inhibition and replicon assay. The regioisomeric thieno [3, 2-b] pyrrole 20a is nearly as potent as 19 in enzyme inhibition.
assay. However, compound 20a was less active in the replicon assay ($IC_{50} = 1.12 \mu M$) as compared to 19 and introduction of an acetamide group led to a 4-fold improvement in replicon activity [20b, $IC_{50} = 2.9 \mu M$] [80].

![Image](image.png)

**Coumestan derivatives**

Coumestans belong to the flavonoid class of phytoestrogens. Based on the structure-activity relationship investigations on wedelo-lactone and four synthetic coumestan derivatives, it was proposed that these compounds serve as potent anti-HCV agents [81]. Wedelo-lactone and LQB34 ($IC_{50} = 18.5 \mu M$) were active in the NS5B enzyme inhibition assay. The thumb I NNIs that are currently in different phases of development are given in Table 3.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Clinical phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beclabuvir (BMS-791325)</td>
<td>Phase 3</td>
<td>Bristol-Myers Squibb Company</td>
</tr>
<tr>
<td>MK-3281</td>
<td>Discontinued</td>
<td>Merck</td>
</tr>
<tr>
<td>BI 207127 [Deleobuvir]</td>
<td>Discontinued</td>
<td>Boehringer Ingelheim</td>
</tr>
<tr>
<td>TMC67055</td>
<td>Phase 2</td>
<td>Janssen</td>
</tr>
<tr>
<td>BILB 1941</td>
<td>Discontinued</td>
<td>Boehringer Ingelheim</td>
</tr>
</tbody>
</table>

*Clinical phase details of all compounds are captured from AdisInsight*

BMS-791325 inhibited NS5B from HCV genotype 1, 3, 4, and 5 with $IC_{50}$ value of 28 nM and showed a $EC_{50}$ value of 3 nM and 6 nM against genotype 1a and 1b respectively in replicon assay [82]. However, amino acid substitutions at P495 conferred resistance to the compound [83]. BMS-791325 in combination with daclatasvir [DCV], Asunaprevir (ASV) showed a substantial viral response in 94% of treatment-naive HCV patients [84] and is currently in Phase III trials [NCT02098616]. MX-3281 showed potent anti-HCV activity against genotype 1a, 1b and 3b [85]. But in clinical studies, MX-3281 monotherapy (800 mg BID for 7 d) was effective only against genotype 1b [86]. BI207127 developed by Boehringer-Ingelheim was tested in combination with faldaprevir and Ribavirin. Data from an SOUND-C3 study showed that the triple combination gave 95% survival rate in genotype 1b patients [87]. Currently, Phase III trials are being completed for BI207127 in combination with faldaprevir and Ribavirin in chronically infected HCV GT1b treatment-naive patients [NCT01728324]. However, in a phase II study it was found that amino acid substitutions P495/S/T were more common which lead to viral relapse hence the development of BI207127 was halted [88]. TMC647055, an indole derivative showed cross-genotypic activity in in vitro replicon assay, but amino acid residues L392 and P495 were associated with resistance [89]. Findings from Phase 1b suggested that TMC647055 in combination with NS3 inhibitor simprevir was well tolerated and showed potent antiviral activity [76]. Phase II trial evaluating the safety, tolerability, efficacy and pharmacokinetic profiles of TMC647055 in combination with Samatasvir, simprevir and JNJ56914845 (NS5A inhibitor) in HCV genotype 1a and 1b infected patients was completed [NCT01852604]. BILB 1941, when given as monotherapy for 5 d, demonstrated potent antiviral activity, but its development was limited by gastrointestinal tolerance [90].

**Site II [Thumb II]**

The binding pocket of site II inhibitors is a shallow hydrophobic pocket situated at the base of the thumb domain [91]. Inhibitors binding at this site also interfere with interactions between the finger loop and the thumb domain thereby preventing the enzyme from adopting a closed conformation. Mutations at this site, namely L419M, M423T render high-level resistance to Site II inhibitors.

**Thiazolone derivatives**

Yan et al. identified Compound 21 as an allosteric inhibitor of NS5B ($IC_{50} = 3.0 \mu M$) by screening the Valeant in-house compound collection [92]. SAR studies of 21 revealed that replacement of 4-fluorophenyl moiety with other aryl substituents was not tolerated [93]. On examination of the binding mode of thiazolones, it was found that the 4-fluorophenyl group occupies a deep hydrophobic pocket defined by the side chains of Leu419, Met423, Tyr477, and Trp528, the C==O of thiazolone ring forms hydrogen bond with NH of Tyr477, while the lone pair N makes hydrogen bond with NH group of Ser 476. Ethyl-furan moiety of the inhibitors binds to the hydrophobic channel defined by Leu419, Met423, Ile482, Val485, Leu489, and Leu497 [95].
Al-Ansary et al. designed a novel series of thiazolone derivatives by replacing the furan moiety coupled to the thiazolone core with a large spacer and hydrophobic moiety to fill the hydrophobic pocket in the thumb II site and tested this for the anti-HCV activity. The most potent among the tested is compound 22 with EC50 value of 0.75 µM in the HCV genotype 1b replicon assay [94].

**Quinazolín-4-ones**

Beaulieu et al. through structure based drug designing strategy designed 1H-quinazolin-4-one analogue by hybridization of thiazolone and anthranilic acid based inhibitors. These quinazolín-4-ones showed potent anti-HCV activities in replicon assays against genotype 1a and 1b with IC50 values of<200 nM [95].

**Phenylalanines**

Wang et al. had identified N, N-disubstituted phenylalanine analogues as a novel class of NS5B inhibitors. Compound 25 with IC50 value of 5.7 µM was the more potent in the series. This lead compound provided a good starting point for the optimization study [91, 99]. Optimization of compound 25 by introducing nitro and cyano groups at meta position generated more potent analogues 26 and 27 with IC50 values of 0.7 and 0.8 µM respectively, and retained selectivity against human DNA polymerases α, β, and γ (IC50>50μM) [100].

**Pyranoidoles**

Pyranoidoles that putatively bind at Site II inhibit the transition between initiation and elongation stage of RNA synthesis[101]. Researchers from Wyeth have done high throughput screening [HTS] of the various compound libraries. The effort culminated in the identification of pyran oxindole 28 with IC50 value of 3.0 µM in NS5B inhibition assay[102].

**Thiophene-2-carboxylic acid derivatives**

Compounds based on the thiophene scaffold were originally identified by Shire Biochem Inc., as binding to NS5B in the thumb domain and inhibiting its activity [96]. Compounds of this class are hydrophobic, and bind to the hydrophobic binding site [site II] in the enzyme, forming interactions with L419, M423, L474, and W528 residues.

Researchers at Shire Biochem discovered thiophene-2-carboxamide (23) as an allosteric inhibitor of the NS5B polymerase during a screening campaign[97,98]. Optimization of compound 23 generated compound 24 with improved potency.

Further optimization of 28 by introducing a fluorine group generated compound 29 with more potent activity. Compound 29 exist as a pair of enantiomers and greater NS5B polymerase inhibitory activity was found with R-isomer[103]. C7 analogues were also designed and the most potent of them is compound 30 with IC50 values of 0.003 µM against both NS5B BK and BB7 strains. This compound showed good antiviral potency in the replicon assay [EC50 = 0.12 µM] [103, 104].
Dihydropyranoles

Hui Li et al. by identified dihydropyranone 31 high throughput screening of in-house compound libraries which inhibited NS5B from HCV genotype 1b (BK strain) with IC_{50} value of 0.93 µM [105]. Truncation of compound 31 by removing phenyl at the right-hand side and combined substitutions at para and meta positions of phenyl ring at left-hand side generated compound 32 with good potency [105].

Thiazolidine-4-one derivatives

Thiazolidine-4-one scaffold has gained prominence in recent years as it possesses antibacterial [106, 107], antifungal [108, 109], anticonvulsant [110, 111], antituberculosis [112, 113] and anticancer activities. The antiviral activity of thiazolidine-4-one scaffold was emphasized in several studies. Ravindra et al. investigated the therapeutic potential of Thiazolidine-4-ones against NS5B and identified a series of 4-thiazolidinones derivatives possessing potent activity against N-terminal Histidine-tagged NS5BCΔ21 (genotype 1b) [Table 4] [114]. Among these, the most potent analogs are compounds 33, 34a and 34b possessing Pyridin-2-yl, Pyridin-3-ylmethyl, and Furan-2-yl methyl at N3 position respectively.

Table 4: Inhibitory activities of 4-thiazolidinone derivatives against NS5BCΔ21 [genotype 1b]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ar</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>%inh</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>Pyridin-2-yl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>98+/−6.9</td>
</tr>
<tr>
<td>34a</td>
<td>Pyridin-3-ylmethyl</td>
<td>Cl</td>
<td>Cl</td>
<td>Cl</td>
<td>CH3</td>
<td>95.6+/−0.8</td>
</tr>
<tr>
<td>34b</td>
<td>Furan-2-ylmethyl</td>
<td>Cl</td>
<td>Cl</td>
<td>Cl</td>
<td>CH3</td>
<td>97.8+/−0.8</td>
</tr>
</tbody>
</table>

Kuçükçügül et al. designed a series of 4-thiazolidinone derivatives, of which the potent compound inhibited NS5B with an IC_{50} value of 5.6 µM. Molecular modeling studies revealed the influence of arylidine moiety on enzyme activity [115].

Anthranilic acid derivatives

Thomas Nittoli et al. identified a lead molecule with IC_{50} value of 1.6 µM (compound 35), by HTS screening of the Wyeth compound library collection. Further optimization of this molecule resulted in analogues showing micromolar potency against the enzyme and in inhibition of viral replication [116]. The most potent among these analogues is compound 36 with an IC_{50} value of 0.010 µM. In an effort to optimize anthranilic acid based inhibitors Timothy A. Stammers et al. discovered compound 37, which showed good activity against NS5B from genotype 1b in the biochemical assay and moderate activity in cell based assay[117].

May MM et al. designed a series of N-phenylbenzene sulphonamides which bind to thumb II allosteric site and showed nanomolar potency against NS5B. Genotype profiling showed the potency of these against HCV genotype 1a and 1b [118]. The thumb II non-nucleoside inhibitors that are currently in different phases of development are given in Table 5.
Filibuvir developed by Pfizer is a potent inhibitor of NS5B from genotype 1. In phase I and II clinical trials, Filibuvir when given as monotherapy or in combination with interferon/Ribavirin for 4 w reduced viral load [119]. But Pfizer halted the development of Filibuvir for business reasons, VCH-759, when given as monotherapy in HCV genotype 1 infected patient, was well tolerated, but had limitations such as gastrointestinal side effects and occurrence of resistant variants [120]. Vertex Pharmaceuticals product VCH-222, when tested in Phase II trials to for anti-viral activity, did not show the promising effect and resistance mutations L419, R422, M423, I482, A486, and V494 were common, hence halted for further development [121]. GS-9669 in combination with Sofosbuvir and Ribavirin, reduced viral load in 92 % of patients in a trial involving a small number of patients [122]. Currently, GS-9669 is in phase II and is being evaluated for efficacy in combination with Sofosbuvir and Ribavirin [NCT01994294].

Site III [Palm I]

Different classes of inhibitors that bind at site III include benzothiadiazides, Acylypyrrolidines, anthranilic acid derivatives, acyclic acid derivatives, indoles and 1, 5-benzodiazepines. These inhibitors bind at a site on the inner thumb/palm domain near the active site and inhibit initiation of RNA synthesis, but do not have any effect on elongation step and already formed enzyme/substrate complex.

Benzothiadiazines

Benzothiadiazines are an important heterocyclic class of compounds showing beneficial effects in many diseases because of their diverse biological properties, and NS5B inhibitory activity is one among them [122]. Derivatives of benzothiadiazides were initially identified from the GlaxoSmithKline proprietary compound library [123, 124]. Benzothiadiazines inhibited de novo initiation with IC50 values of about 5-10-fold less than those for primer extension [125].

Inhibition with these compounds occurs in a non-competitive manner. These compounds are genotype specific; they have strong activity against 1a, 1b and 2a genotypes but weak activity against genotype 3a. Resistant mutations arising in HCV genotype 1b replicons after exposure to benzothiadiazines include M414T, C451R, G558R, and H95R [125]. John K. Pratt et al. identified N-1-alkyl benzothiadiazines by high throughput screening. These were found to inhibit NS5B polymerase at the initiation step of RNA synthesis. SAR data demonstrated that N-1-alkyl benzothiadiazines with quinoline core and 1, 8-naphthyridone core showed equivalent potencies in inhibition of NS5B polymerase [126].

The nature of hydrophobic functionality at N1 position impacts the biochemical potency. The compound 38a with unbranched α-Carbon at N1 position inhibits NS5B with IC50 of 0.1 µM, and branching at alpha-carbon of alkyl group decreases the potency [Compound 38b].

Zhou Y et al. identified a series of 5-hydroxy-3H-pyridazinone derivatives [39] which bind to the NS5B ‘palm’ site [127, 128]. The addition of linear alkyl fragments with 4-5 carbon atoms at R2 position, 2-thiophene at R1 and -NS02Me at R3 position of pyridazinone conferred good anti-NS5B and antiviral activities [compound 40, IC50 < 0.01 µM] [129].

John T. Randolph et al. synthesized a series of Isoamyl-substituted B-ring aminothiadiazine analogues and neoheyxyl-substituted B-ring aminothiadiazine analogues and evaluated them for their ability to inhibit NS5B genotype 1a and 1b [130]. The primary amine of isoamyl analogue 41 is a potent inhibitor in both enzyme and replicon assays, enantiomer [S-enantiomer] of primary amine 41a is more active in biochemical assay [genotype 1a] as compared to the R-form.
In an effort to improve the bioavailability and plasma inhibition assay and are equipotent in replicon assay. The most 

than 43 in enzyme inhibition assay [132].

than 43 in enzyme inhibition assay [132].

with an IC_{50} value of 0.4 µM [134].

Proline sulfonamides

Ariamala Gopalsamy et al. identified proline sulfonamide 49 with an IC_{50} of 3.1 µM as a potent inhibitor of NS5B through high throughput screening. Optimization of compound 49 resulted in many analogues with good potency and selectivity; the most potent of them is compound 50 with an IC_{50} value of 0.08 µM [135].
Acrylic acid derivatives

Pfefferkorn et al. identified a lead compound PNU-248809 (51), a modest inhibitor of NS5B CA21 by high throughput screening. They have drawn the following SAR conclusions by modifying A and B rings in the template (52) [136].

Phenyl ether at A ring (Compound 53) and small hydrophobic substituents at R1 position of B ring are preferred. In order to improve the activity of compound 53, Jeffrey A. Pfefferkorn et al. generated analogues by changing substituents at R1 position of B ring. Substitutions at the meta and para positions of B ring are deleterious. In the case of monosubstituted analogues, halogens were more preferred at R1 position and the activity increased with the size of the halogen. The most potent of this series is compound 54.

Fluoropyridones

Schoenfeld et al. through structure based drug designing discovered a series of non-nucleoside inhibitors and optimization of these analogues generated compound 55, a fluoropyridone derivative which showed nanomolar potency against HCV-NS5B. It inhibited 4 isoforms of Cytochrome P450 [1A4, 2C9, 2C19, 2D6], and exhibited high metabolic stability in both in vitro and in vivo. But the only limitation was poor oral absorption, which was corrected by using a
lipid-based formulation. It showed robust anti-NS5B potency against a panel of 17 different clinical isolates from both genotype 1a and 1b with an EC$_{50}$ value ranging from 0.4-6.8 nM [137].

Indoles

High throughput screening of NS5B using non-trinucleotide primed assay revealed indole derivatives exemplified by compound 56, which inhibited NS5B with an IC50 value of 0.9 µM but showed poor activity in the replicon assay[138,139]. Efforts made by Anilkumar et al. To improve the cell-based activity involved the optimization of substituents at N1 and C3 positions of compound 56. However, none of the designed compounds showed significant activity in the cell-based replicon assay[140].

Further optimization by the addition of cinked heterocycle analogues such as pantothen and pyrimidinedione at C3 position, halogen substituted benzyl group or amino substituted pyridine at N1 position and CF3 at C5 position of compound 56 resulted in compounds 57, 58 and 59 with a good activity profile in enzyme assays and cell-based Replicon assay[140]. These compounds form bidentate hydrogen bond interactions with a backbone of Tyr-448 and Ile-447.

Later the same group of scientists tried to replace the acid group at C2 position to further optimize the indole analogues. Acyl sulphonamide at C2 position improved the binding affinity and replicon activity (Compound 60) [141]. Heterocyclic modification with sulphonamide group also showed an excellent activity profile (compound 61), but acyl groups are more preferred as they act as tethers for the addition of new functional groups.

Further optimization at the C2 position resulted in phenyl acyl sulphonamide series of analogs (compound 62 and 63) with 2log fold improvement in enzyme activity [142].

Cheng et al. developed a series of pyridine carboxamide derivatives which bind to palm I site of NS5B. Some of them showed low nanomolar potency in enzyme inhibition assay [genotype 1b] and submicromolar potency in cell-based replicon assay[142].

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Clinical phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS625433</td>
<td>Phase 1</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>ANA598</td>
<td>Discontinued</td>
<td>AbbVie</td>
</tr>
<tr>
<td>Stribuvir</td>
<td></td>
<td>AbbVie</td>
</tr>
<tr>
<td>ABT-333</td>
<td>Launched</td>
<td></td>
</tr>
<tr>
<td>ABT-072</td>
<td>Discontinued</td>
<td></td>
</tr>
</tbody>
</table>

*Clinical phase details of all compounds are captured from Adisinsight
Currently, Setrobuvir is being studied in combination with peginterferon and Ribavirin in phase 2 trials [NCT01903954]. It is very effective in phase 2 trials but the emergence of M144T, M144L, G534D mutations reduced the efficacy [143]. ABT-333 in combination with ABT-450/r a protease inhibitor, Ombitasvir and ribavirin showed 96% survival rate in HCV genotype 1b infected patients having cirrhosis [144]. In a phase IIa study the triple combination regimen ABT-450/r [combined with ritonavir] and ABT-072 with ribavirin showed survival rate in 9/11 patients infected with HCV genotype 1 at 36 w post-treatment [145].

Site IV [Palm II]

Benzofuran-C3-carboxamide [HCV796] is a class of inhibitors that bind at site IV. The binding site of HCV796 is different from the binding site of benzothiadiazines. HCV796 binds to residues in the primer grip site. S365T, C316Y, and M414T mutations in NS5B rendered resistance to HCV796 both in replicons and in biochemical, enzymatic assays[146]. HCV796 has an excellent inhibitory profile and approved by FDA for phase II trial but was later discontinued due to its hepatotoxic effects.

Another potent inhibitor of this class is a boronic acid derivative GSK5852, which is more potent than HCV796 and was active against C316Y mutants. Voitkunles et al. reported that GSK5852 showed good anti-HCV activity with EC50 values in the nanomolar range against HCV genotypes 1 and 2 and has an excellent resistant profile. This molecule is currently in clinic [147,148]. The Palm II non-nucleoside inhibitors that are currently in different phases of development are given in Table 7.

Table 7: Direct acting non-nucleoside palm II inhibitors

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Clinical phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-9190 [Tegobuvir]</td>
<td>Phase 2</td>
<td>Gilead</td>
</tr>
<tr>
<td>IDX375</td>
<td>Discontinued</td>
<td>Idenix</td>
</tr>
<tr>
<td>HCV-79</td>
<td>Discontinued</td>
<td>Viropharma/Wyeth</td>
</tr>
<tr>
<td>GS5852</td>
<td>Phase 2</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>PPI-383</td>
<td>Phase 1</td>
<td>Presidio Pharmaceuticals, Inc.</td>
</tr>
</tbody>
</table>

*Clinical phase details of all compounds are captured from Adis in site

Tegobuvir has a unique mechanism of action and is distinct from other NNIs of NS5B [149]. Tegobuvir undergoes metabolic activation resulting in glutathione adducts which specifically interacts with NS5B thereby inhibiting viral replication [149]. PPI-383 is a non-nucleoside inhibitor with pan-genotypic activity; it exhibits EC50 value of 8.3nM, 2.2nM against genotypes 1a and 1b respectively in cell-based replicon assays. It is also active against HCV genotypes 2a, 3a and 4a [EC50 in the 4.4-11.7 nM] [150]. Currently, the Phase 1b trials have been completed for this compound [NCT01928147].

Combinations of different classes of drugs are being tried and many of these are in clinical trials. NS5A inhibitors (Ombitasvir, Daclatasvir, Ledipasvir and MK-8742) are tried in combination with protease inhibitors and NS5B inhibitors. Those that have advanced to Phase III are listed in Table 8.

Table 8: Combination therapies targeting HCV in clinical, developmental Phase III

<table>
<thead>
<tr>
<th>Combination</th>
<th>Clinical phase</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asunaprevir+Daclatasvir+Bdelabuvir [BMS-791325]</td>
<td>Phase III</td>
<td>Bristol-Myers Squibb</td>
</tr>
</tbody>
</table>

Effect of NS5B genotypic variation on inhibitor response

HCV exists in 7 different genotypes and within each genotype are several subtypes which differ with respect to viral sequence and geographic distribution [151]. HCV genotype 1 and 3 are more prevalent globally compared to other genotypes [152]. The active site region of NS5B is conserved among all the genotypes and Nucleoside inhibitors target the active site and hence exhibit cross genotypic effect[20]. The Non-nucleoside inhibitors bind to distinct allosteric sites present on thumb I, Thumb II, Palm I and Palm II regions. The sequence at these regions are variable and hence Non-nucleoside inhibitors does not exhibit pan-genotypic activity [20]. In fact, significant differences in response to NNI have been noted within different viral sequences isolated from the same individual [153]. So the differences in viral genotypes can be the major factors for therapeutic failure. Clearly, more genotypic analysis is required to improve the strategies for development of potential anti-HCV agents.

DISCUSSION

Hepatitis C is a chronic inflammatory liver disease which will lead to Liver fibrosis, liver cirrhosis, liver carcinoma and liver failure. Though HCV infection was identified in the 1970’s, the research was stalled due to unavailability of in vitro and in vivo experimental system. Later in 1999 the availability of the HCV replicon system fueled the development of anti-HCV agents, but the HCV drug discovery rate was slow. Till 2011 the standard care of treatment for Hepatitis C was a combination of pegylated interferon and Ribavirin given for a period of 24 to 48 w. But this treatment regimen was effective in only 50-60% of patients and is associated with severe side effects. In 2011 first generation NS3 protease inhibitors namely telaprevir [Incvek], Boceprevir [Vircrivos] were approved by FDA after which a second generation protease inhibitor Simeprevir [Olysio] was launched in 2013. However, these were effective against only HCV genotype I infected patients. Later, many effective NS3/4a inhibitors [Paritaprevir, grazoprevir], NS5B inhibitors [Sofovbuvir and Dasabuvir], and NS5A inhibitors [Ledipasvir, ombitasvir, daclatasvir, elbasvir, velpatasvir] were launched in the recent years. But, unsuccessful response to these therapies was seen in a small percentage of patients and this need to be considered. Treatment failure in this population is due to relapse [viral load is reduced significantly or undetected at the end of the therapy followed by a rebound to pretreatment levels once the treatment is discontinued]. The major cause of relapse is the emergence of resistance-associated variants (RAV).

The RAV can emerge in either the NS3 or the NS5B or the NS5A or sometimes in two regions. The most common RAVs in NS5B region include S282T and C315Y and persistence of these are highly variable. While RAVs in the NS3 region diminish over time, and those in the NS5A region are long lasting. Treatment options in this intolerant patient population depend on the causes of, treatment failure, the composition of the initial treatment regimen and severity of the disease. For example, is a patient is intolerant to PegIFN/RBV the treatment option could be to use DAAs and if it is due to RAVs to NS5A inhibitor then the treatment regimen should be NS5A inhibitor-free or use of a different NS5A inhibitor. However, very less amount of data is available to conclude on which treatment regimens are best fit for treatment failure patients and hence leaving room for drug development opportunities in HCV research.
CONCLUSION
In the recent years, there has been rapid progress in HCV research and effective therapies targeting viral enzymes, namely NS3, NS5A and NS5B were launched in the market. However, the high cost of the medications limits their universal use, hence there exists a need for cost effective therapies. Also, handling of DAA associated RAVs is a major challenge. In future HCV research should aim at the development of therapies for non-responder patient population and treatment regimens with short duration of treatment, even less than 12 w.

ABBREVIATION
HCV-Hepatitis C virus, NS5B-nonstructural protein 5B, NLI-Nucleoside inhibitors, NNI-Non-nucleoside inhibitors, NTP-Nucleotide triphosphate, PK-Pharmacokinetics, GTP-Guanosine-5’-triphosphate, RAV-Resistance-associated Variants, DMA-Direct acting antivirals

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CONFLICTS OF INTERESTS
Declared none

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