Small Molecule and Biologic Inhibitors of Hepatitis C Virus: A Symbiotic Approach

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Abstract: Chronic infection with hepatitis C virus (HCV) remains a global health concern. Using both *in vitro* and cell-based assays, a series of small molecule agents specific for the viral RNA-dependent RNA polymerase have been shown to interfere with viral RNA replication. Although no agents targeting this viral enzyme have demonstrated sustained efficacy in infected patients as measured by reduction in viral load at 72 weeks post-treatment, proof-of-concept has been achieved in the clinic. A comprehensive account of the structure-activity relationship for nucleoside and non-nucleoside inhibitors of HCV polymerase, as well as consideration of early discovery biologic approaches targeting NS5B are reviewed.

Key Words: Nonstructural protein 5B (NS5B), RNA-dependent RNA polymerase (RdRp), hepatitis C virus (HCV), non-nucleoside inhibitors (NNI), nucleoside inhibitors, acyl pyrrolidine, benzofuran.

INTRODUCTION

Hepatitis C virus (HCV), the causative agent of blood-borne non-A, non-B hepatitis, is estimated to infect over 170 million people worldwide. Liver inflammation, scarring, fibrosis, cirrhosis and hepatocellular carcinoma represent the sequela associated with chronic HCV infections. Although a significant reduction in the levels of viral RNA has been demonstrated with pegylated interferon-alpha and ribavirin combination therapy, major unmet medical need still exists. Limitations associated with this standard of care are poor efficacy in patients with genotype 1 virus and adverse events including flu-like symptoms and neuropsychological complications that often require dosage adjustment or discontinuation of therapy [1-3]. Thus, the identification of improved anti-HCV agents having broad-spectrum clinical efficacy across all HCV genotypes with enhanced tolerability are needed.

The viral genome encodes several nonstructural proteins with enzymatic activities potentially amenable to intervention with inhibitory agents. These proteins include the viral protease (NS3/NS4a), helicase (NS3), and an RNA-dependent RNA polymerase (NS5B; RdRp) [4]. Herein we detail a comparison of published information available on two recent nonnucleoside inhibitor (NNI) discovery efforts with several leading nucleoside inhibitors of the viral RNA polymerase, an essential enzyme for HCV replication. Comprehensive coverage of all HCV polymerase inhibitors has been reported elsewhere [5,6].

NS5B RdRp

This essential enzyme, the viral RdRp, replicates HCV RNA and has no host equivalent, suggesting the potential for exquisite selectivity in antiviral agents [7]. Replication is initiated by synthesis of a complementary minus-strand RNA from the positive-strand genome, which subsequently serves as the template to produce additional positive-strand genomic RNA. In the host cell, several parameters can modulate the catalytic activity of NS5B and RNA synthesis, a process comprised of three distinct steps: initiation, elongation and termination [7, 8]. The viral polymerase is localized on the cytoplasmic side of the endoplasmic reticulum membrane and attached *via* a C-terminal hydrophobic tail of 21 residues [9]. Crystal structures of the viral apoenzyme from two genotype 1b strains confirm similarities in architecture to other DNA and RNA polymerases, resembling a 'right hand' having 'palm', 'fingers' and 'thumb' subdomains as originally described for the *Escherichia coli* DNA polymerase [10-13]. However, unique features distinguishing NS5B from other polymerases are apparent in the extensive thumb-finger interactions encircling the active site as well as a β-hairpin loop which protrudes into the active site to modulate RNA binding [14]. High-order structural information of NS5B in the presence of substrate and RdRp inhibitor has furthered our understanding of structure-activity relationships (SAR) controlling enzymatic activity.

Nucleoside Inhibitors

Ribavirin, a nucleoside analog used for treating HCV in combination with pegylated interferon-alpha, represents a non-selective agent that confers a high frequency of mutations during RNA replication to potentially reduce viral fitness (Table **1**) [15]. Viramidine, a pro-drug of ribavirin with reduced accumulation in red blood cells, is proposed to be less prone to develop ribavirin treatment-associated hemolytic anemia (Table **1**) [16]. These agents represent competitive inhibitors of guanosine triphosphate, and thus interfere with elongative RNA synthesis. Recent advances in pharmaceutical discovery led to the identification of several novel nucleoside analogs with improved selectivity and affinity for NS5B compared to ribavirin [reviewed in 5].

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Table 1. Nucleoside Inhibitors of HCV NS5B

Compound	Company	Structure
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Ribavirin	Roche, Ribapharm, ICN Pharma	
Viramidine	Valeant Pharma, Inc.	
2'-C-methyladenosine	Merck/IRBM/ISIS	
2'-C-methylguanosine	Merck/IRBM/ISIS	
2'-O-methylcytidine	Merck/IRBM/ISIS	
3'-L-valine ester prodrug of 2'-O-methylcytidine	Idenix	

Merck-IRBM/ISIS. Merck IRBM/ISIS compounds focus on 2'-O- and branched 3'- and 2'-C-modified analogs including 2'-C-methyladenosine, 2'-C-methlyguanosine and 2'-O-methylcytidine, all nucleoside triphosphate competitive inhibitors of NS5B. Cell-based potency varies widely, often depending on the efficiency of *in vivo* conversion to the corresponding 5'-triphosphate esters [17, 18]. Although a resistant mutant in the active site of the polymerase (S282T) was readily selected *in vitro* using 2'-C-methyladenosine, susceptibility to 2'-O- methylcytidine was retained suggesting the potential of a sugar moiety to modulate the affinity of nucleosides to the polymerase [19]. Consistent with this observation, the 2'-C-methyl ribose nucleosides contain a 3'-hydroxyl; however, steric hindrance and sugar moiety-induced conformations confer RNA chain-termination properties. Exploration into development of prodrug derivatives of these nucleosides and other structurally related analogs such as modified purine/pyrimidine, 2'-C-fluoromethyl and carbocyclic nucleosides are ongoing [20].

Idenix. Proof of concept for NS5B inhibitors has been obtained recently with a 3'-L-valine ester prodrug of 2'-C-methylcytidine, showing modest (< 1 $\log_{10}$) reductions in serum HCV RNA levels after 100 mgs/day oral administration for 15 days (Table **1**) [21]. Initial demonstration of antiviral activity was also confirmed by treating chronically infected chimpanzees for 7 days with 16.6 mg/kg/day resulting in 1.05 $\log_{10}$ copies/ml reduction of serum HCV RNA.

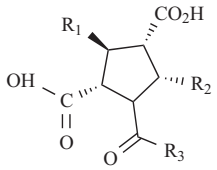
Valeant. A series of adenosine 5'-phosphonate analogues able to mimic naturally occurring adenosine monophosphate were explored for chain termination activity of NS5B. Phosphonates possess enhanced metabolic stability compared with their phosphate counterparts, since the phosphorous-carbon bond is not susceptible to phosphatase hydrolysis. Although poor competitors with ATP, proof of principle has been obtained that nucleoside phosphonates can function as chain terminators [22]. Since the presence of a 5'-phosphonate obviates the need for the first phosphorylation step, the rate-limiting step in the conversion to 5'-triphosphates, development of novel chemistries capable of identifying potent nucleoside phosphonate chain terminators for HCV are needed.

Non-Nucleoside Inhibitors

Mechanistically, NNIs can be classified as active-site inhibitors or non active-site, allosteric binding, inhibitors. Agents including the Merck/IRBM diketo acid series, Japan Tobacco benzimidazole series, and GSK benzothiadiazines have been extensively reviewed elsewhere [5]. This report focuses on the recent introduction of two novel nonnucleoside agents.

N-acyl pyrrolidine. High-throughput screening of the GlaxoSmithKline compound collection for HCV RdRp antagonists was performed using an RNA extension assay with olio-rG primed poly-rC substrate and a C-terminal truncation of genotype 1b enzyme (strain J4). This screening resulted in the identification of two distinct racemic N-benzoyl pyrrolidine libraries varying in the substituent on the benzoyl phenyl ring. Although the mixtures were poor inhibitors, with IC_{50}s between 7 – 12 μM, deconvolution was performed to identify individual components responsible for the inhibition (Table **2**). Synthesis of N-acylpyrrolidines was performed and the structure activity relationship confirmed with substitutions at R^1, with isobutyl or benzyl derived from amino acids leucine or phenylalanine being preferred over smaller or non-branched alkylgroups. The favored derivative with isobutyl at R^1 conferred a 17-fold improvement in inhibition compared with the combinatorial mixture (IC_{50} = 0.7 μM) [23].

Table 2. NS5B Inhibitory Activity of Acylpyrrolidines



Position	Substituent	IC_{50} (μM)
R1	Isobutyl-	0.7
R2	2-thienyl-	0.3
R3	4-CF_3-phenyl-	0.3

Since a range of substitutions were tolerated at the pyrrolidine 5-position (R^2), extensive contacts with the RdRp probably occur elsewhere on the compound. Substitution of R^2 with 2-Thienyl or 2-Thiazoyl conferred additional enhancements to inhibition in the biochemical assay, with IC_{50}s between 0.3 – 0.4 uM. Lastly, a lipophilic, electron withdrawing group such as trifluoro-methyl was identified as the preferred substituent at the meta- and para- positions in the N^1-acyl group, while placement in the ortho- position impaired inhibition (IC_{50} > 20 μM) [23]. Amongst the most active N^1 substituents was the 4-(tri-fluoromethyl)benzoyl group, and resolution into the corresponding pure enantiomers showed exclusive activity with the (+) enantiomer (IC_{50} = 190 nM; Table **2**).

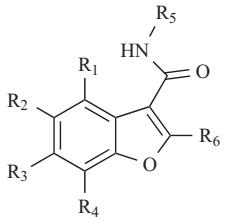
Mechanistic studies performed with the most active compound at steady-state conditions confirmed a reversible, non-competitive inhibition profile with respect to GTP. Further, although specificity for the viral polymerase and not the RNA substrate was confirmed, broad inhibition activity across HCV genotypes other than type 1b or to other viral and mammalian polymerases was not reported [23]. The mechanistic studies suggest that the acyl pyrrolidine class of inhibitors do not bind to the nucleotide-binding pocket of the viral RdRp although whether they are active site inhibitors remains to be determined. Moreover, exploration into the mechanism of action, inhibition of the initiation or elongative phase of RNA synthesis, will be essential to guide the development of combination therapy, pairing of nucleoside and non-nucleosides, for maximal efficacy and minimization of drug resistance. Lastly, optimization of the acylpyrrolidines to identify analogues capable of inhibiting NS5B in a cell-based HCV replicon assay is awaited.

Benzofuran derivatives. Recently, a novel class of HCV RdRp inhibitors, benzofuran derivatives, were detailed by Viropharma, Inc./Wyeth. [24]. Inhibition of viral RdRp

activity was measured using a standard biochemical assay with NS5B derived from the consensus sequence of a patient infected with HCV genotype 1b virus (strain BB7) [25]. Cell-based antiviral activity was evaluated in the Huh-7 HCV replicon system using ELISA to measure intracellular NS5A protein levels using an 8-point dose response curve. Preferred compounds were those having IC_{50}s around 500 nM or less in either assay.

SAR on the two-membered benzofuran ring derivatives conferred improvements in antiviral activity with varying substituents on R1 and R6 of the benzofuran core (Table **3**) with IC_{50}s reported as less than 1 uM for cell-based inhibition. Specifically, hydrophobic groups appear well tolerated in the R1 position whereas bulky nonpolar substituents such as phenyl or di-fluorophenyl groups on R6 correlated with enhanced activity. Lastly, the presence of a carboxylic acid methylamide on R5 contributed to optimal activity. The paucity of information published in the patent regarding specific compound IC_{50}s, selectivity for other polymerases, binding affinity to RNA, mechanism of action, and inhibition profile for other HCV genotypes does not readily facilitate determination of the preferred compounds for advancement into the clinic, although one member of this compound class, HCV-796, is currently in phase 1b trials.

Table 3. NS5B Inhibitory Activity of Viropharma, Inc./Wyeth Benzofurans



Position	Substituent	IC_{50} (μM)
R2	-O-CH_3	<1
R5	-CH_3	<1
R6	-phenyl	<1

The orally bio-available compound, HCV-796, has demonstrated potent inhibition of HCV RNA replication in cell-based assay systems with a reported IC_{50} of 5nM against genotype 1a and 9 nM against genotype 1b. This compound reportedly also showed significant antiviral activity in a chimeric human-mouse model that supports HCV infection [26], and appears to be additive in combination with alpha interferon. The initial phase 1b study showed that this agent was well tolerated and has a favorable pharmacokinetic (PK) profile. A further randomized, double blind, multiple ascending dose, placebo controlled phase 1b study involving dosing of 96 treatment-naïve chronic HCV patients over fourteen days to assess safety, antiviral activity, and PK profile is underway [27].

The N-acyl pyrrolidine and benzofuran derivatives were found to be highly selective for the HCV RdRp, with IC_{50}s > 50 uM for a panel of polymerases (BVDV, Dengue, HIV RT, Human DNA polymerase α, β and γ and RNA polymerase II). Although the binding site within the RdRp for these compounds has yet to be disclosed, previous compounds highlighted herein bind either in the NTP binding site (competitive inhibitors including Merck/IRBM and Idenix nucleosides) or binding in the allosteric site (nonnucleoside inhibitors including Merck/IRBM diketo acid series, Japan Tobacco benzimidazole series, and GSK benzothiadiazines) as shown by the arrow in the ribbon model of the HCV RdRp adapted from [28] (Fig. **1**).

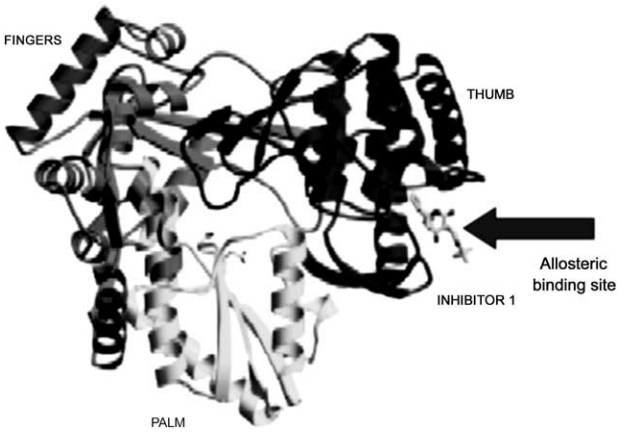


Fig. (1). A ribbon diagram of HCV NS5B adapted from JBC (2003) 278: 9489.

Biologic Inhibitors of HCV Replication

Biopharmaceutical agents that interfere with HCV RNA metabolism have recently begun exploration into early clinical trials. Antisense oligonucleotides which represent one biologic platform inhibit viral RNA translation or RNA replication *via* formation of an RNA-RNA antisense or RNA-DNA antisense hybrid. ISIS 14803 when administered intravenously 3 times weekly conferred greater than 1 log_{10} decrease in HCV RNA levels [29]. Although proof of principle was obtained in approximately one-third of patients, frequent elevations in hepatic transaminase levels, a marker of liver inflammation, will likely preclude further development of this compound.

Ribozymes, a second class of biologic agents representing catalytic enzymes composed of RNA, have been demonstrated to bind to and catalyze the cleavage of specific RNA sequences. This metabolic cleavage directly interferes with viral protein synthesis that is essential for viral RNA replication. At present, toxicity findings have hindered pre-clinical development for this platform [29].

A third class of biopharmaceutical agents capable of disrupting HCV replication is RNA interference, small interfering RNAs (siRNA) that bind to complementary regions of RNA and effectively silence expression [30]. The application of RNA interference using short interfering RNAs directed against the NS5B coding region has recently been reported. Double-stranded siRNAs can block gene expression and RNA synthesis from HCV replicons propagated in human liver cells, albeit incompletely. After repeated exposure to siRNA, drug-resistant replicon RNAs were amplified and mutations within the siRNA target sequence confirmed. Similar to using combination therapy to minimize the development of escape mutations, combination

of two siRNAs were confirmed to severely limit this mutational evolution in cell-based assays [31].

NS5B Sequence Variability

The extremely rapid replication cycle and lack of a proof-reading activity in NS5B contributes to the high genetic variability present amongst the HCV genotypes. A comparison across the six major genotypes shows greater than 30% divergence based on RNA sequence [6]. This poses significant limitations for optimization of siRNAs or chemically modified antisense oligonucleotides to confer broad inhibition across genotypes or even inhibition across the multiple quasi-species within a single infection. For small molecule chemistry development, lead optimization chemistries need to account for incorporating broad-spectrum activity against NS5B RdRp from the major genotypes.

Pair-wise comparison of NS5B amino acid sequence shows about 75% identity amongst the six major genotypes and up to 85% identity within subtypes of the same genotype [6]. Although two motifs, A and F, are most highly conserved within NS5B, sequence variation is indeed present even within these conserved motifs. Compound modifications to alter particular side chain substituents at contact points with the RdRp represent critical factors affecting binding affinity and the evolution of drug resistance. The recent development of systems for propagating HCV in culture will facilitate studies of the development of resistance [32,33].

Although the acyl pyrrolidine and benzofuran classes were identified by conventional screening techniques, with the advancement of NS5B crystal structures, exploration of the conserved active site and protein core structure could be exploited for rationale compound design. Targeting regions with high degree of homology amongst HCV genotypes with a computational structural approach, rather than solvent-exposed residues found on the surface of the RdRp remains a viable approach to identify next-generation RdRp inhibitors for treatment of chronic HCV.

CONCLUSION

Similar to the inhibitors described for HIV reverse transcriptase (RT), both nucleoside/nucleotide and NNIs have been identified for the HCV RdRp. As with HIV RT, multiple NNIs from distinct chemical classes interacting at an allosteric site have been identified, highlighting the evolutionary and structural conservation among this group of enzymes. In recent years, small molecule chemistry has achieved many early successes in identifying inhibitors of the NS5B RNA polymerase, with early clinical proof of principle attained for several of these compounds. As the potential to reduce viral RNA in chronically HCV-infected patients is realized, new challenges will begin to emerge regarding drug resistance. At present, as with HIV, the anticipated optimal therapeutic approach remains combination therapy with an armamentarium of agents directed at different aspects of the viral life cycle. Co-administration of agents that inhibit various viral enzyme targets (protease, polymerase, helicase) or viral processes (translation, replicase complex formation or viral assembly), or use of multiple agents targeting the same viral enzyme having additive or synergistic benefit (nucleoside and nonnucleoside polymerase inhibitors) are expected to represent future therapies. Regardless of the mechanism of action, small molecule compounds are likely to be used preferrably in combination biologic agents to attain maximal clinical efficacy.

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