Approaches for the Development of Antiviral Compounds: The Case of Hepatitis C Virus

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Abstract Traditional methods for general drug discovery typically include evaluating random compound libraries for activity in relevant cell-free or cell-based assays. Success in antiviral development has emerged from the discovery of more focused libraries that provide clues about structure activity relationships. Combining these with more recent approaches including structural biology and computational modeling can work efficiently to hasten discovery of active molecules, but that

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is not enough. There are issues related to biology, toxicology, pharmacology, and metabolism that have to be addressed before a hit compound becomes nominated for clinical development. The objective of gaining early preclinical knowledge is to reduce the risk of failure in Phases 1, 2, and 3, leading to the goal of approved drugs that benefit the infected individual. This review uses hepatitis C virus (HCV), for which we still do not have an ideal therapeutic modality, as an example of the multidisciplinary efforts needed to discover new antiviral drugs for the benefit of humanity.

Abbreviations

AAG	Alpha-1 acid glycoprotein
ADK	Aryl α-γ-diketo acids
CYP450	Cytochrome P-450 isoenzymes
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
NNI	Nonnucleoside inhibitors
NTP	Nucleoside triphosphate
P-gp	P-glycoprotein
RdRp	RNA dependent RNA polymerases
SAR	Structure activity relationship

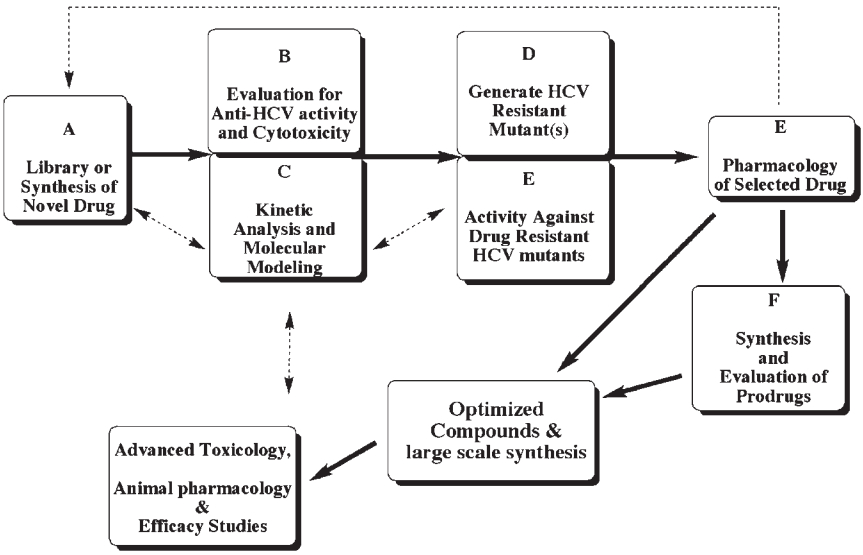
1 Introduction

The history of antiviral drug development has taken numerous circuitous routes from the discovery and development of the first US Food and Drug Administration (FDA) approved antiviral agent, 5-iodo-2′-deoxyuridine (Idoxuridine) by William H. Prusoff (Prusoff 1959; Prusoff et al. 1979), to the development of simplified but powerful triple combinations for HIV such as Atripla and Trizivir (De Clercq 2007; Gallant et al. 2006; Schinazi 1991). Many challenges are encountered during the development of antiviral agents, including adverse events and the development of drug resistant viruses, which necessitate chemists, biologists, and pharmacologists to develop improved, more potent, and less toxic medicines with "high genetic barrier." Although there are major differences among viruses, specific virological and pharmacological approaches used to develop novel antiviral agents are similar across many viral diseases. In this review, we use hepatitis C virus (HCV) as a prominent example for different strategies employed in drug discovery. Sophisticated technologies such as liquid chromatography (LC)-mass spectrometry, real-time PCR, pharmacokinetic and pharmacodynamic modeling, cryo-electron microscopy, crystallographic structure determination and modeling have advanced our capacity to develop antiviral agents in recent years, but the available tools still need to be further optimized.

HCV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It affects an estimate of 170–200 million people worldwide (Shepard et al. 2005) and there is still no vaccine available. HCV will conduce as a model in this chapter to describe the approaches to develop antiviral compounds. Although impressive progress has been made in treating HCV infection, the standard therapy of (pegylated) interferon alpha and ribavirin has low efficacy against some genotypes and is associated with important side effects. Many persons do not tolerate or respond to interferon-based therapy with or without ribavirin (Falck-Ytter et al. 2002). For example, half of the genotype 1-infected subjects are nonresponders to standard pegylated interferon–ribavirin treatment. Therefore, new therapeutic strategies are urgently needed (Manns et al. 2006), including small orally bioavailable molecules as well as combined modalities to prevent or delay the development of drug-resistant HCV.

1.1 Approaches to Discovery

Improved treatment for HCV requires the development of novel agents with unique biological profiles that are effective against a broad range of viral genotypes. While drug or target discovery represents the beginning of the process, multiple studies are required before an antiviral agent becomes a useful, and in particular, safe drug for humans. Using a modern systematic drug discovery and development process, the risk of failure in the clinic can be markedly reduced (Scheme 1).



Scheme 1 The path of least resistance in HCV drug discovery and development

The process of antiviral drug development generally consists of several steps that must be performed prior to filing an Investigational New Drug (IND). The first step is the evaluation of existing libraries of compounds against the target and/or the design and synthesis of new molecules based on knowledge of potential target site structures (Scheme 1). Then the relative potency of potential antiviral agents can be determined rapidly and reproducibly using enzymatic and cellular systems coupled with quantitative real-time polymerase chain reaction (PCR). Efficacy models include cell culture and whole animal systems, which support HCV replication and can assay antiviral molecules for their capacity to inhibit it. This can be performed in normal liver cells (including primary hepatocytes), adapted liver cells (e.g., Huh-6 or 7), and human bone marrow cells. It is critical to determine the mitochondrial toxicity of the compound, after at least 2 weeks in culture, and its effect on various human enzymes such as cellular elastases [human neutrophil elastase (HNE) and human leukocyte elastase (HLE)] and polymerases (alpha, beta, and gamma). The stability of the chemical entity and its protein binding capacity must be evaluated under various conditions (pH, temperature, whole blood, human liver homogenate, etc.). The extent of protein binding and antiviral effect in the presence of alpha-1 acid glycoprotein (AAG) is essential especially for protease inhibitors. Protease inhibitors and certain small molecules can be substrates that may inhibit or induce cytochrome P-450 isoenzymes (CYP450) (Lim et al. 2004; Yeh et al. 2006). The mechanisms of action of the compound must be explored with appropriate enzymatic tools. In vitro selection and characterization of viral variants resistant to the drug candidate is absolutely critical. These experiments may provide clues on what could happen in humans and also confirm the primary antiviral target of the agent at the genetic level. Cross-resistance studies with known site-directed mutagenized viruses having specific clinically relevant mutations are also essential. Ideally, resistance testing should include both cell culture (using viruses with defined mutations) and enzyme-based assays.

Toxicology studies must be performed in at least two animal species. If the toxicity profile of the compound is acceptable, then it joins the "hit or lead list" of compounds to proceed. The metabolism of the compound must be understood and pharmacokinetic studies must be performed in small and large animals. Efficacy studies must be performed in relevant animal models, especially in chimpanzees when more than one candidate is identified and a choice has to be made before proceeding to studies in humans. The ultimate preclinical steps include various studies testing drug combinations in vitro and in vivo, selection of resistant viruses, viral fitness, pyrophosphorolysis, and others.

2 Specific HCV Enzyme Inhibitors

HCV drugs with a direct antiviral mode of action are needed for the treatment of chronic HCV infection. The NS3 protease, NS3 helicase (which localizes to the carboxy-terminal domain of NS3 and catalyzes the unwinding of the double stranded RNA in a 3′ to 5′ direction (Tai et al. 1996)), and NS5B RNA polymerase

are all nonstructural (NS) proteins of HCV. These enzymes are essential for viral replication and are currently the key targets for the design of specific inhibitors, with the major focus on the NS3 protease and NS5B polymerase (Table 1).

The NS3 protease localizes to the amino-terminal domain of NS3 and requires the small NS4A cofactor for its function. This enzyme is a distinct serine protease that is responsible for the downstream cleavage events of the NS polypeptide at four junctions (Failla et al. 1994). It is a heterodimeric protease and a member of the chymotrypsin serine protease family. However, the substrate specificity of the NS3 protease is very distinct from that of the related host enzymes (Urbani et al. 1997). Even so, it has been a challenge to design potent and selective inhibitors against the NS3 protease, as the substrate-binding cleft of the protease is shallow and lacks cavities (Yan et al. 1998). Despite this, several groups have been successful in designing highly selective inhibitors of this enzyme. However, the relatively high sequence difference between genotypes in the protease region limited the design. For instance, many protease inhibitors in the pipeline are effective only against HCV genotype 1, and may show up to 100-fold less activity against genotypes 2 and 3 relative to genotype 1 (Reiser et al. 2005). Genotype 1 is the most common genotype in the Western world, and individuals infected with genotype 1 are less likely to respond to pegylated interferon, making this selectivity a problem. However, there is a need for more and broader acting protease inhibitors to cover all six genotypes of HCV. Specific inhibitors of NS3 protease are discussed in chapter by R. Swanstrom et al., this volume.

The NS5B RNA polymerase is highly conserved and contains a Gly-Asp-Asp motif, which is characteristic for RNA-dependent RNA polymerases (Lohmann et al. 1997). NS5B is the key enzyme responsible for the synthesis of negative strand RNA, using the genome as template and for the subsequent synthesis of genomic positive strand RNA from this template (Yamashita et al. 1998). It is an attractive target to identify selective inhibitors (e.g., nucleoside analogs), since no RNA-dependent RNA polymerase activity is present in mammalian cells. Reminiscent of HIV-1 reverse transcriptase (RT) inhibitors, two classes of HCV polymerase inhibitors, namely, nucleoside analogs and nonnucleoside inhibitors (NNI) are under development. Nucleoside analogues in their metabolically activated 5′-triphosphate form inhibit NS5B by competing with the natural 2′-nucleosides-5′-triphosphate (NTP) and/or by chain termination (see also chapter by Neyts et al., this volume). This action prevents further elongation of the nascent RNA. Intracellular phosphorylation of the nucleoside analogs to the corresponding mono-, di-, and triphosphates, respectively, is mediated by cellular kinases. For example, the phosphorylation of the C-analog PSI-6130 was demonstrated with dCK, UMP-CMP kinase, and nucleoside diphosphate kinase (Murakami et al. 2007). The highly conserved region of the polymerase catalytic site enables broad genotype specificity for nucleoside analogs in contrast to the protease inhibitors and presumably the NNIs.

It is important to note that the intracellular levels of NTP are approximately 100-fold higher than the dNTP levels. Therefore, much higher median effective

Table 1

Class of drug/compound	Mutation	Fold-resistance	Cross-resistance	Comments	Reference
Protease inhibitors	(in vitro)	(in vitro)			
BL 2021	R155Q				(Courcambeck et al. 2006)
	A156V	> 100	VX-950		(Lin et al. 2004)
	A156T	>> 100			
	D168A	400		Indirect contact with inhibitor	
	D168V	1,000		Indirect contact with inhibitor	
VX-950	A156S	10		Dominant mutation	(Lin et al. 2004)
	A156V	> 60	BL 2021		
	A156I	> 60	BL 2021		
SCH 503034	T54A	< 20			(Tong et al. 2006)
	A156S	< 20			
	A156T	> 100	BL 2021		
	V170A	< 20			
ITMN B					
Polymerase inhibitors					
NA					
NM 283	S282T	21	2′C-Methyl-Adenosine	Reduces binding with inhibitor	(Dutartre et al. 2006)
PSI 6130				Non-obligate chain-terminator	(Murakami et al. 2007)
R-1479	S96T				(Le Pogam et al. 2006a)
	N142T				
MK 0608					
A-782759 (Abbott)	H95Q	44			(Mo et al. 2005)
	N411S	28			
	M414L	70	Thiophene-2-carboxylic acid		
	M414T	> 200	Benzo-1,2,4,-thiadiazine		
	Y448H	35			
Benzo-1,2,4,-thiadiazine (GSK)	M414T	> 100	A-782759		(Nguyen et al. 2003)
NNI					
JTK-003					
Thiophene-2-carboxylic acid	M414L	10	A-782759		(Le Pogam et al. 2006b)
	L419M	20			
	M423T	10			
	I482L	20			

concentrations (EC_{50}) were expected for nucleosidic NS5B inhibitors than those observed for HIV RT inhibitors due to competition by the high concentration of natural nucleosides. However, current investigational nucleoside analogs against HCV have demonstrated reasonable potency with EC_{50} values of approximately 1 μM in replicon assays.

Resistance to nucleoside analogs has been observed using replicon assays. Biochemical studies with HIV-1 RT resistant against nucleoside analogues demonstrated two different resistance mechanisms (see also chapter by Nijhuis et al., this volume, for resistance discussion). Besides substrate discrimination, resistance could also be achieved by ATP dependent excision of nucleoside analog-monophosphates after their incorporation (Meyer et al. 1999). The same mechanism of excision (but pyrophosphate dependent) has been described for a related polymerase – bovine viral diarrhea virus RNA-dependent RNA polymerase (D'Abramo et al. 2004). Pyrimidine-based chain-terminating analogs, that is, C and U analogs, were more easily selected for excision, implying that purines (A and G analogs) are the preferred choice for further drug development. However, these compounds would then have to compete with ATP and GTP, which exhibit a much higher intracellular concentrations than at least CTP (Deval et al. 2006).

HCV polymerase, like other *flaviviridae* NS5B enzymes, is able to initiate RNA synthesis without an RNA primer, which is unique to viral RNA polymerases (Zhong et al. 2000). When designing an enzymatic NS5B assay, consideration must be given to the choice of initiation nucleotide (GTP), metal ions (Mg^{2+} and Mn^{2+}), and template design with unique hairpin loops and sequence in the 3′ end (Ranjith-Kumar et al. 2002; Kim and Kao 2001). Currently, a number of nucleosides are in clinical development, including PSI-6130 (β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine; Pharmasset/Roche) and its prodrug R7128 as well as MK-0608 (Merck). In addition, a number of NNI of NS5B polymerase are also in development, including JTK-003 (Japan Tobacco). Some of these nucleoside analogs and NNI will be discussed further. For more information, refer to Chaps. 3 and 6.

The high rate of viral turnover in HCV infection, coupled with the absence of proofreading by the NS5B polymerase, results in a rapid accumulation of mutations (Martell et al. 1992). Therefore, one can anticipate that the use of specific enzyme inhibitors as a monotherapy will likely result in viral resistance. This suggests that, like HIV treatment, combination therapy will be necessary for HCV as well (see chapter by Hofmann, this volume). Initial regimens would be combined with pegylated interferon, as that agent is the backbone of the present drug regimens for HCV. In vitro assays have allowed investigators to identify a variety of resistance mutations against current investigational compounds. These mutations are located in the catalytic sites of HCV enzymes and result in enhanced binding discrimination over natural substrates. Therefore, as for HIV, the residue changes in the resistant viruses usually result in reduced viral fitness. For more information on resistance, refer to Chaps. 4 and 11 in this volume.

3 Drug Discovery Tools for HCV

Several excellent reviews have appeared recently (Condon et al. 2005; Huang et al. 2006; Koch and Narjes 2006; Meanwell et al. 2005; Neyts 2006; Shim et al. 2006; Wu et al. 2005), which summarize the development of agents to treat HCV infection. In this report we highlight the application of modern drug discovery tools and techniques to inhibit HCV replication by describing selected examples that have appeared recently in the scientific literature.

As illustrated in Scheme 1, antiviral discovery begins with drugs that may be used as probes of biological function. The structure-based analysis and modeling described in the next two subsections can provide a context for understanding the molecular patterns responsible for a drug's action at its receptor. To effectively evaluate structure activity relationships (SAR, focused libraries of chemically related compounds need to be developed and such methods are discussed in Sect. 4. Anti-HCV activity assays, kinetic and pharmacodynamic modeling are introduced in Sect. 5. Approaches for understanding advanced pharmacological and physiological factors that influence drug delivery are key to efficacy in humans and are described in Sect. 6. Systems for sharing and combining these different data types are the discovery tools of the future.

3.1 X-Ray Crystallographic Analysis and Structure-Based Drug Design

In a structure-based drug design program, the three-dimensional structure of a drug target interacting with small molecules is used to assist the drug discovery process. Structure-based drug design allows one to investigate the interaction of a small molecule with its target protein and to explore the possibilities to chemically modify the molecule to obtain the desired properties. The focus of this section will be on specific discovery programs where the structural information was obtained by X-ray crystallography and applied to the drug discovery process.

Crystal structures of the NS5B polymerase alone and in complexes with nucleotide substrates have been solved and applied to discovery programs (Ago et al. 1999; Bressanelli et al. 2002; Bressanelli et al. 1999; Lesburg et al. 1999; O'Farrell et al. 2003). From these studies, HCV polymerase reveals a three-dimensional structure that resembles a right hand with characteristic fingers, palm, and thumb domain, similar to the architectures of the RNA polymerases of other viruses. However, none of these experimental structures contained the ternary initiation complex with nucleotide/primer/template, as obtained with HIV RT. Accordingly, HCV initiation models have been built using data from other viral systems in efforts to explain SAR (Kozlov et al. 2006; Yan et al. 2007).

Recently, many NNI of HCV NS5B RNA polymerase have been discovered and these were recently reviewed (Condon et al. 2005). One allosteric binding site has

been identified via X-ray crystallographic analysis of NS5B complexed with several different noncovalently bound inhibitors. This predominantly hydrophobic depression is located approximately 35 Å from the polymerase active site in the thumb domain (Biswal et al. 2005; Di Marco et al. 2005; Love et al. 2003; Wang et al. 2003). A more polar area of this pocket contains Ser^{476} and Tyr^{477} residues that appear to provide critical hydrogen bonds to the carboxylate of these inhibitors. Although mechanistic details of inhibition through this allosteric site are unclear, it has been proposed that the enzyme must be forced into an inactive conformation involving the prevention of interaction between the finger-tip region and the thumb domain (Di Marco et al. 2005). A more recent crystallographic analysis of this hydrophobic allosteric binding site revealed some disruption to the integrity of the GTP binding site, thus contributing to a RNA polymerase state incapable of carrying out a polymerization cycle (Biswal et al. 2006).

Like the corresponding HIV enzyme, the HCV NS3 protease is amenable to structure-based design. Starting from the lead hexapeptide DDIVPC, the research group at Boehringer–Ingelheim rationally designed the BILN 2061 family of compounds (LaPlante and Llinas-Brunet 2005) (Fig. 1). BILN-2061 has an IC_{50} of 3 nM for the HCV NS3 protease with its shallow and relatively featureless binding pocket. When administered to HCV-infected patients for 2 days, BILN-2061 produced an unprecedented and rapid decrease in viral load, thus demonstrating the first proof-of-concept for a new class of HCV antiviral agents.

Initially, medicinal chemists undertook a systematic strategy of single amino acid changes of the DDIVPC-type hexapeptide, with the goal of improved potency and reduction in the peptidic nature of the series. Important substituents that directly contact the protease pocket were identified by a combination of differential line-broadening NMR experiments and docking of hexapeptides into the active site of an X-ray structure of the *apo* NS3 protease. The resulting model was the first liganded NS3 protease complex described (Tsantrizos et al. 2003). The most critical observation from these efforts was that only substituents of the P1–P4 regions experienced binding interactions with the protease.

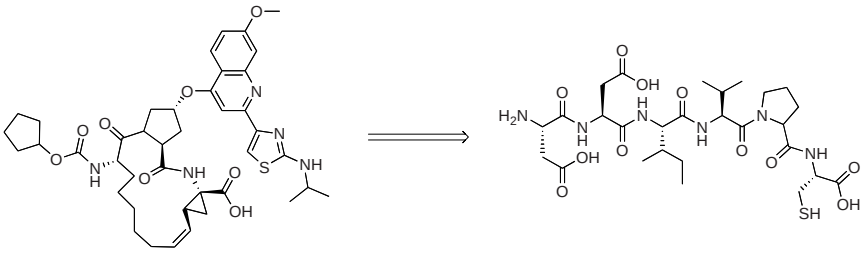


Fig. 1 Structure of BILN 2061 and initial lead peptide DDIVPC. Activities were determined in a radiometric assay against the NS3 protease domain

With the goal of reducing the entropic costs of binding, the macrocyclic nature of the BILN-2061 series was designed to rigidify the free-state conformation and to resemble the bound state observed in the peptide NS3 protease complex (see chapter by Anderson et al., this volume). The Boehringer–Ingelheim researchers were able to solve a crystal structure of NS3 protease complexed to an 11 nM macrocyclic inhibitor. The binding properties of each of the regions of the macrocyclic inhibitors could be investigated relative to the known SAR in the macrocyclic series. The *t*-butyl carbamate capping group sits in a shallow, wide, solvent exposed, and partially hydrophobic groove. The replacement of the *t*-butyl group with a variety of hydrophobic groups resulted in only minor changes in potency, which is consistent with the lack of a deep defined pocket in that region. It was also clear that the proline ring is mostly solvent-exposed and has very little direct contact with the protease; however, its role is critical in providing proper positioning of P1, P3, and the macrocycle, which are the three key binding groups (Goudreau et al. 2004).

Researchers at Schering–Plough reported an X-ray crystallographic analysis of two potent macrocyclic inhibitors bound to the NS3 protease (Chen et al. 2006). The macrocycles (Fig. 2) were prepared based on earlier modeling and X-ray crystallographic analysis, which indicated that an enhanced binding to the protease might result from a ring size of approximately 15–17 atoms. The ligand–protease complexed crystals were obtained by soaking the compound into preformed enzyme crystals. Subsequent analysis showed good binding of the macrocyclic ring with the Ala^{156} methyl group, which formed a nice doughnut-shaped crown by encircling the methyl group and strong interaction into the S4 pocket. Surprisingly, this study also revealed the formation of a covalent bond between the inhibitor P1 carboxylamide carbonyl and the Ser^{139} hydroxyl of the protease rather than by the hemiketal oxygen atom. Instead, the hemiketal oxygen was interacting with and stabilized by a catalytic histidine residue (His^{57}).

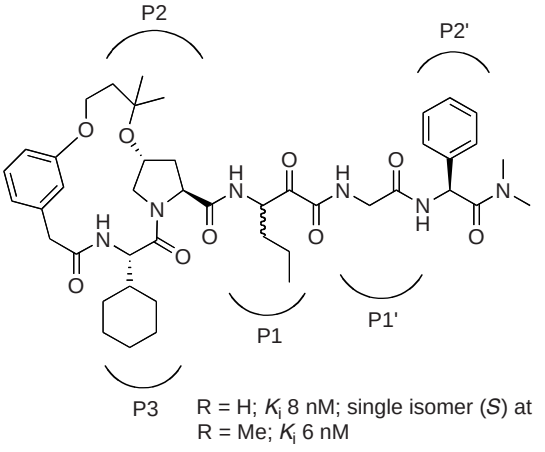


Fig. 2 Structure two Schering–Plough compounds with binding affinities for HCV

3.2 Molecular Modeling, Small Molecule Docking, and Computational Analysis

A recent molecular modeling study involving the HCV NS5B RNA-dependent RNA polymerase investigated its strong substrate specificity for RNA (Kim and Chong 2006b). Given this specificity, it may not be so surprising that HCV NS5B is not inhibited by most known inhibitors of DNA dependent DNA polymerases or reverse transcriptases. The authors state that "HCV polymerase has not been successfully inhibited by nucleoside analogs due to its strong substrate specificity for RNA." However, this conclusion fails to take into account the higher intracellular concentrations of rNTP relative to dNTP (Traut 1994) as discussed earlier.

The goal of this modeling study was to determine the specific interaction of the 2′-OH group of the RNA substrate and the active site residues to better understand the RNA-specificity. The study started from the three dimensional crystal structures of HIV-1 RT and HCV NS5B polymerase. Comparing the active site sequence alignment of HIV-1 RT and HCV NS5B showed that the "steric gate" of HIV-1 RT (Tyr^{115}) corresponds to Asp^{225} in HCV NS5B, but the small side chain of Asp^{225} provides an open space at the active site big enough to accommodate the ribose sugar moiety of a RNA substrate. In HCV NS5B, the side chain carboxylate of Asp^{225} was found to be in close proximity to the 3′-OH of a rNTP. A similar hydrogen bonding interaction occurs between the amide backbone of Tyr^{115} and the 3′-OH of NTP in HIV-1 RT. No specific interaction with an active site residue could be found close to the 2′-OH group of a rNTP. The researchers proposed an interaction with the enzyme by bridging water molecules inside the active site. The water molecules could act either as a hydrogen bond donor or acceptor to the 2′-OH group of the RNA substrate. If proven correct, the previously unknown 2′-OH binding pocket at the active site of RdRp could provide invaluable information for the development of novel anti-HCV nucleoside analogs.

Scientists at Merck constructed a binding model, based on structures of the NS5B protein, to understand the mechanism of inhibition within a series of dihydroxypyrimidines that act as pyrophosphate mimics in the NS5B active site (Fig. 3) (Koch et al. 2006). The binding of a triphosphate of a cocrystallized nucleotide with NS5B has been shown crystallographically to be mediated in the active site by two Mg ions. In other polymerases, during elongation, one Mg ion chelates the primer ribose hydroxy group and the alpha-phosphate of the incoming nucleotide. The second Mg ion is bound between the beta- and gamma-phosphate of the incoming nucleotide triphosphate and is cleaved off with the pyrophosphate after addition of this nucleotide to the primer.

The pyrimidine core of Merck's pyrophosphate mimic series is proposed to interact with the Mg ion chelating the beta- and gamma-phosphate groups of the triphosphate (Fig. 3). A binding model of the pyrimidine bound to the Mg ion in the crystal structure of NS5B was generated from the Mg–oxygen interaction geometry from crystallographic information. The model suggests the 2-thiophene of the pyrimidine to be stacked against Arg^{158} with mainly lipophilic interactions. In the crystal

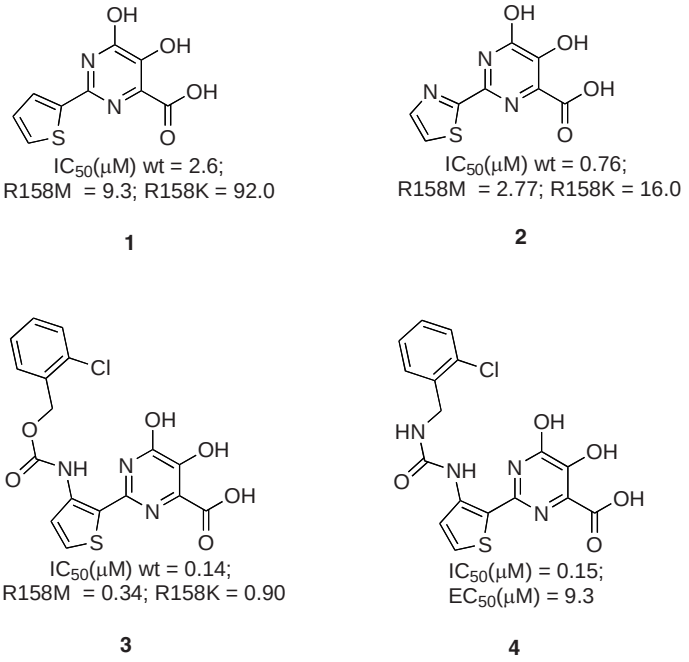


Fig. 3 Activity of **1, 2**, and **3** wild type HCV NS5B and site-directed mutations. Binding and replicon activity of **4**

structure with GTP, Arg^{158} seems to be interacting lipophilically with the base of the incoming nucleoside triphosphate and forms hydrogen bonds to the alpha-phosphate, and most likely plays a critical role in activating the alpha-phosphate for nucleophilic attack by the 2′-OH of the primer. Two mutations of Arg^{158} were prepared to test the importance of these interactions. It was suggested that mutation into Met, a more lipophilic amino acid without a charge but with similar size, should maintain the affinity of the pyrimidine. Mutation into the more polar Lys would maintain a positive charge while decreasing the capability to interact lipophilically with the thiophene. A decrease of inhibition for pyrimidines **1, 2**, and **3** for both mutations was observed upon testing (Fig. 3). The data suggested that the lipophilic component of the interaction between Arg^{158} and 2-thiophene is particularly important for thiophene **1**, as the decrease in potency is significantly larger for the Lys mutation (35-fold) than for the Met (threefold). The same kind of behavior is observed for thiazoles **2** and **3** each with a potency enhancing side-chain. Here the loss in potency on the Arg^{158}Lys mutant is less severe (sixfold), but still larger than on the Arg^{158}Met mutant (twofold).

The K_m for the substrate UTP has been measured and does not show significant differences between wt and mutant enzymes. The model shows that the space available to substituents in positions 4 and 5 of the thiophene is limited, in agreement with SAR studies. Interaction with a number of basic and lipophilic residues bound

in a cavity by some lipophilic substituents in the 3-position is responsible for the increase in potency. Urea **4** emerged from these studies as one of the most potent compounds, which also inhibited viral RNA generation in a cell-based HCV replicon assay. The dihydroxypyrimidine class of inhibitors appears to be a promising starting point for future development of drugs that could be utilized to combat HCV.

A broader docking study of the pyrophosphate mimic binding site was undertaken utilizing 88 different aryl $\alpha-\gamma$-diketo acids (ADK) (Kim and Chong 2006a). Pharmacophore-guided docking (FlexX-SYBYL 7.2) study of ADK molecules revealed two binding sites: a hydrophobic pocket and a hydrophobic groove that has excellent three-dimensional arrangements to accommodate substituted aromatic rings. The pocket is located around Leu^{159} while the hydrophobic groove is located at the opposite side of the pocket and becomes hollow at the end of a channel that provides binding sites for bulky atoms such as chlorine or bromine. Additionally, the Mg binding site in the polypeptide chain of HCV RdRp discussed earlier was also found to be critical. A nice correlation was observed in that the ADK, with potent antiviral activity and highly substituted aromatic rings, mapped well onto the hydrophobic binding sites to reinforce the hydrophobic interaction. Conversely, the lack of hydrophobic binding with ADK that were devoid of critical aromatic substitution coincided with compounds less potent toward HCV RdRp.

4 Medicinal Chemistry Approaches and the Role of Quantitative Structure Activity Relationships

4.1 One at a Time Traditional Synthesis

Because of the complexities associated with the synthesis of nucleoside analogs as active site inhibitors of NS5B polymerase, parallel and combinatorial techniques have made few inroads into these drug discovery programs. Traditional one at a time synthesis techniques are still the mainstay of nucleoside analog antiviral research. In this section, we provide one recent example of a discovery program relying on traditional synthetic methods to prepare nucleoside analog inhibitors of HCV replication. The most important nucleoside analogs currently in late development or clinical trials for treatment of HCV are shown in Fig. 4. Studies have demonstrated that the 2′-Me compounds function as chain terminators due to steric clashes with incoming nucleosides.

The Merck compound MK-0608 is a 2′-C-Me-7-deaza-adenosine analog, which has recently been reported to show a 5.7 log drop in viral load in HCV-infected chimpanzees after dosing QD at 2 mg/kg (Olsen 2006). An efficient and practical process for preparing kilogram quantities has been described (Bio et al. 2004). The 12-step synthesis provides an impressive 35% overall yield and starts from the inexpensive diacetone-D-glucose. The synthesis features a novel acyl migration in route to prepare the key crystalline furanose diol intermediate (Fig. 5). The conditions

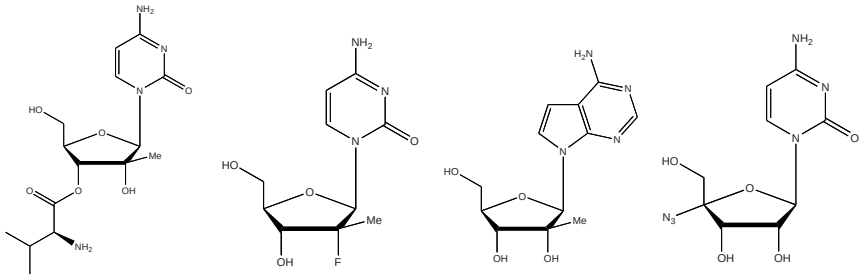


Fig. 4 Important nucleoside analogs that have undergone clinical trials for treatment of HCV. Both PSI-6130 (R7128) and R1479 were administered to humans as prodrugs

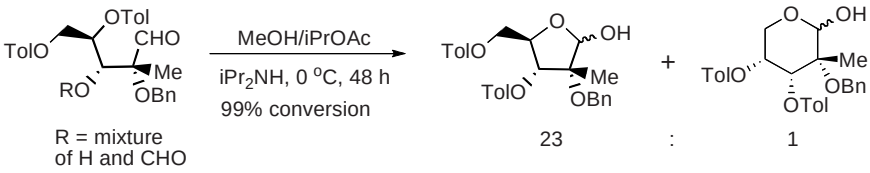


Fig. 5 Selective acyl migration and furanose formation

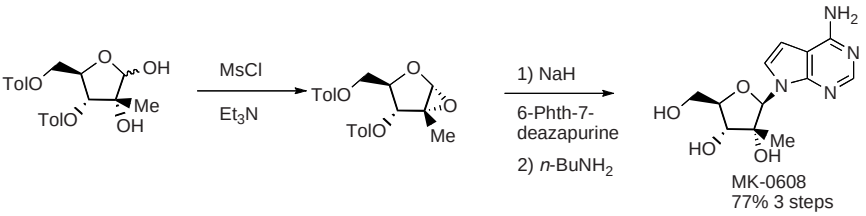


Fig. 6 Highly β-selective glycosidation to couple the C-2 branched furanose epoxide with the 7-deazapurine

required promoting acyl migration and formation of the furanose rather than the corresponding pyranose. Much experimentation resulted in the use of diisopropyl amine in methanol/isopropyl acetate to give a 23:1 ratio of the desired furanose relative to the pyranose with 99% conversion.

A second key development in the synthesis of MK-0608 was the highly β-selective glycosylation to couple the C-2 branched furanose epoxide with the 7-deazapurine. Vorbrüggen glycosylation conditions did not provide any coupled product with the 7-deazapurine presumably due to the inability of silyl migration to the 7-C. The optimized coupling conditions involved the use of sodium hydride to generate the anion of 6-phthalimido-7-deazapurine that provides a β-selective opening of an 2-β-*C*-methyl-1,2-α-anhydroribose to provide, after global deprotection, MK-0608 in 77% yield from the diol furanose (Fig. 6).

4.2 Parallel Synthesis

The use of parallel synthesis techniques in both solution and solid phase has become ubiquitous in the drug discovery process. One recent example that applies these techniques to HCV targets will be discussed. Random evaluation of compound libraries at Valient Pharmaceuticals produced a hit compound with an IC_{50} of 27 μM against HCV NS5B polymerase (Ding et al. 2006). Using the parallel synthesis strategy outlined in Fig. 7, a series of derivatives was synthesized.

A cyclo-condensation approach was used to prepare the oxopyrimidine core from substituted aldehydes, thiourea, and ethyl cyanoacetate. A subsequent sulfur alkylation, mostly with benzyl halides, provided the final compounds. After evaluating these compounds for their anti-HCV activity, it was found that all compounds containing substitutions on the oxopyrimidine ring nitrogen had $IC_{50} > 100\,\mu M$. None of the compounds possessed a significant increase in activity toward NS5B, with the best compound active at 3.8 μM. Better success was realized with a pteridine series of compounds (Fig. 8) (Ding et al. 2005). The initial pteridine hit, again identified from screening compound libraries, had an IC_{50} of 15 μM in an NS5B polymerase assay. SAR studies focused on different substituents at the 6- and 7-positions (R groups in Fig. 4) and substitutions at the 4-position that were conducted in parallel fashion by displacement of the 4-chloropteridine. It was found that NH or OH at the 4-position is critical for the inhibitory activity. The most active compound identified (Fig. 4, $R = 4-F-\mathrm{Ph}$) had an IC_{50} of 0.5 μM in the NS5B polymerase assay. However, this most promising compound, when tested in a replicon assay, had a disappointing EC_{50} of 90 μM – most likely due to poor cell penetration.

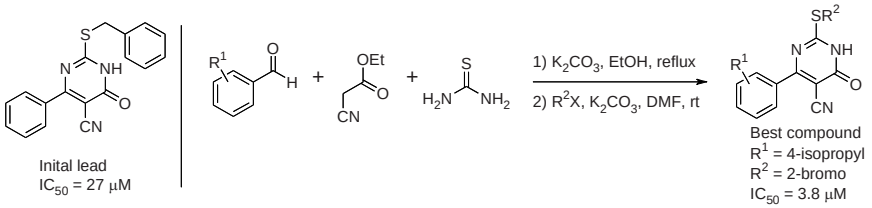
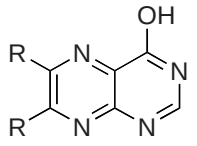


Fig. 7 An efficient cyclocondensation approach to prepare oxopyrimidines



Lead compound: R = Ph; IC_{50} = 15 μM
Best compound: R = 4-F-Ph; IC_{50} = 0.5 μM

Fig. 8 Pteridine series of NS5B RdRp active compounds

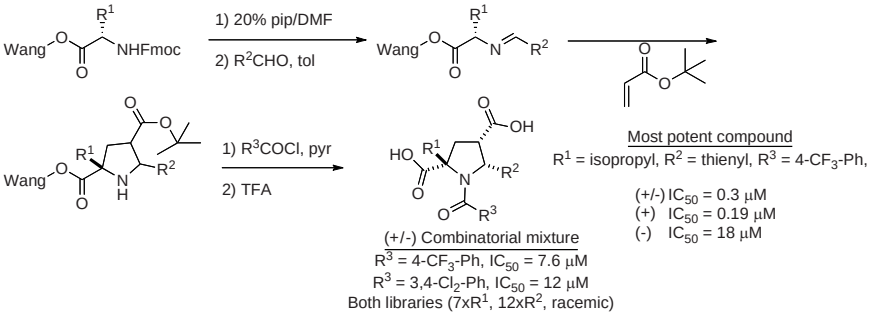


Fig. 9 A combinatorial library mixture of *N*-acylpyrrolidines with modest HCV NS5B activity

4.3 Combinatorial Chemistry

Over the last 10 years, the practice of synthesizing and screening mixtures of compounds has lost interest as a means to identify new leads in drug discovery, mainly due to problems associated with deconvolution, variable yields of components, purity, and inability to identify the source of activity.

The recent example of the identification of a small molecule inhibitor for HCV NS5B from a combinatorial mixture is an indication that these techniques still have value to the drug discovery process (Burton et al. 2005). High-throughput screening of the GlaxoSmith–Kline compound collection using a NS5B polymerase assay identified two racemic *N*-benzoyl pyrrolidine libraries. Both libraries were present as a mixture of 168 components ($7 \times R^1,\ 12 \times R^2$, and racemic) and originated from a combinatorial $[3+2]$ cycloaddition reaction of *t*-butyl acrylate, a series of aromatic aldehydes, and resin bound amino acid esters followed by *N*-acylation (Fig. 9). Enumeration of the library using automated solid phase synthesis and purification techniques led to the synthesis of a 0.3 μM NS5B inhibitor. In addition, only one enantiomer of this racemate was found to possess significant NS5B activity (Fig. 9).

4.4 High-Throughput Screening for the Identification of New HCV Leads

Scientists at Ibis Therapeutics (A Division of Isis Pharmaceuticals Inc.) recently reported a new class of small molecules that bind the HCV RNA internal ribosome entry site (IRES) IIA subdomain with sub-micromolar affinity. The IRES mediates the initiation of viral-RNA translation and represents a novel drug target for inhibiting HCV replication. However, targeting RNA specifically and selectively with small molecules has met with much difficulty. The benzimidazole hit (Fig. 10) with a K_D of approximately 100 μM to a 29-mer RNA model of domain IIA was identified from a 180,000-member library using mass spectrometry-based screening

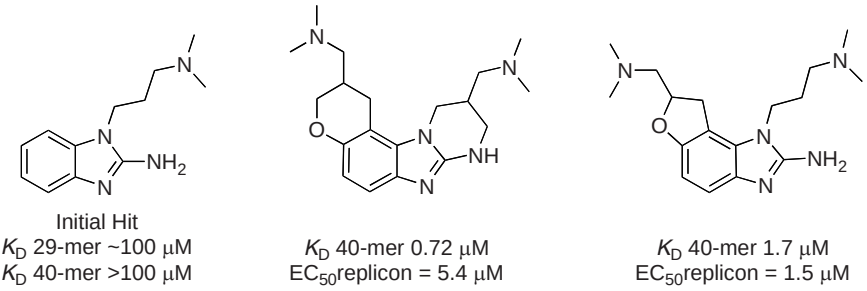


Fig. 10 Screening hit and higher affinity benzimidazoles for HCV RNA internal ribosome entry site (IRES) IIA subdomain

methods. Hits were identified via mass spectrometry as follows: (1) A mixture of the RNA substructure and the potential small-molecule ligands were introduced into the mass spectrometer by electrospray ionization; (2) Ligands that bound to the target were identified by mass shifts; (3) The observed mass equals the mass of the target plus the mass of the ligand. This technique allows one to screen multiple ligands and even multiple targets in a single assay as long as they have different masses. Benzimidazole derivatives with submicromolar binding affinity for the IIA RNA construct were discovered via MS-assisted SAR studies. The optimized benzimidazoles (Fig. 10) reduced viral RNA in a cellular HCV replicon assay and showed minimal toxicity ($CC_{50} > 100\,\mu M$) against Huh-7 cells in an MTT assay.

5 Cell-Based Assays to Predict Toxicity and Resistance Aspects

Before an antiviral agent becomes a drug, advanced toxicity testing, pharmacological combination, and drug-interaction studies are needed. The use of new cell-based assays that can predict mitochondrial toxicity, lactic acidosis, peripheral neuropathy, anemia, hypersensitivity, lipodystrophy, and other potential side effects can alleviate these issues (Stuyver et al. 2002).

Mitochondria-associated toxicities, such as pancreatitis, are frequently demonstrated in HIV/HCV-coinfected individuals, and may significantly influence treatment options (de Mendoza and Soriano 2005). Yet, no cell culture or animal models have been developed to predict nucleoside-induced pancreatitis. Nevertheless, an association of HCV replication and mitochondrial DNA depletion in primary human lymphocytes obtained from HIV/HCV-coinfected individuals under concomitant administration of HCV and HIV medications was demonstrated by de Mendoza and coworkers (de Mendoza et al. 2007). They claimed that the use of HCV medication together with certain antiretroviral agents seemed to enhance mitochondrial damage due to a synergistic deleterious interaction between the anti-HCV and anti-HIV drugs. In contrast, an improvement in mitochondrial content with effective

anti-HCV therapy was also confirmed in this report, and these findings may be valuable for hepatitis B infection as well (de Mendoza et al. 2007).

Testing in cell-based assays has led to the discovery of numerous anti-HCV hits and leads. For example, a base modified ribonucleoside analog called β-D-*N*-hydroxycytidine (NHC) was shown to inhibit HCV RNA replication in culture, but had a low oral bioavailability in non-human primates while metabolizing into two natural nucleosides (Stuyver et al. 2003a, b). More biologically stable nucleosides with improved pharmacological profiles are being evaluated, including a potent and highly selective nucleoside analogs [PSI-6130 (a prodrug R7128, is in clinical trials)] with anti-HCV activity in replicon systems. (Carroll and Olsen 2006; LJ et al. 2006; Murakami et al. 2007; Pierra et al. 2005; Stauber and Stadlbauer 2006, Stuyver et al. 2006a, b)

BILN-2061 (Boehringer Ingelheim) was one of the first compounds identified in the replicon system that was clinically tested (in Phase I studies, it reduced HCV-RNA 2–3 $\log_{10}$ in most patients infected with genotype 1), but trials were put indefinitely on hold due to cardiac toxicity issues. Other examples of NS3 protease inhibitors, including VX-950 (Vertex) and SCH-503034, are in Phase 2 trials (PEG-IFN combination studies) and have demonstrated potent antiviral effects (Lin et al. 2006; Neyts 2006; Reesink et al. 2006). Using the replicon system, the selection of HCV resistant mutants have been reported for BILN 2061 (at 156 and 168), VX-950 (at 156), and SCH 503034 (at 156 and 170). These results suggest that resistance studies in vitro might predict what to expect in future clinical trials (Lin et al. 2004; Tong et al. 2006) (for more details see chapter by Anderson et al., this volume).

6 Pharmacokinetic and Pharmacodynamic Aspects of Drug Development of Agents for the Treatment of HCV Infections

6.1 In Vitro Evaluation

The initial screening and early toxicity studies of novel agents to treat viral hepatitis are usually performed using in vitro assays. Use of in vitro (cell culture) and enzymatic assays to screen for and measure potency (determination of median effective concentration, or 90% effective concentration; EC_{50} and EC_{90}, respectively) at the intended site of action of the compound may include viral polymerase (nucleoside analogs, NA) protease (viral protease inhibitors) or cellular targets (interferons stimulate T and B cells of the immune system). Toxicity studies are conducted against a panel of cell lines or primary cells (e.g., human bone marrow cells) to determine cellular sensitivities (IC_{50}, IC_{90}). Compounds with desirable therapeutic index (high IC_{50}/EC_{50}) ratio are considered candidates for further in vivo studies in animal models.

6.2 Preclinical In Vivo Testing

Preclinical pharmacokinetic studies are conducted in appropriate animal species to model the relationship between plasma and target tissue concentrations vs. time and dosage regimen. Interspecies scaling of pharmacokinetic parameters may be performed to predict pharmacokinetic parameters in humans (Patel et al. 1990). However, estimates for interspecies scaling may not always be reliable, especially for compounds that are activated or metabolized by enzymes that vary significantly between species (Hurwitz et al. 2005). The pharmacokinetic model could also be used to design a dose regimen that produces the desired tissue concentration of the biologically active form of the compound (usually based on the EC_{50} and IC_{50} data derived in vitro) for the desired length of time in an appropriate animal model, for example, woodchucks for hepatitis B (Hurwitz et al. 1998, 2002) or immunocompromised mice bearing liver xenografts for hepatitis C (Feitelson and Larkin 2001). Since hepatitis B and C regimens are prolonged (months for hepatitis C, chronic for B), long-term toxicity studies are warranted to evaluate for toxicities that may occur during extended use.

7 Physiological Factors that Influence Drug Delivery for HCV Drugs

The oral bioavailability of drugs may be limited by the compound's stability in the acidic pH of the stomach, the presence of food, enzymes secreted into the lumen of the intestine, its lipophilicity, affinity for uptake proteins embedded within the villi on the surface cells of the small intestine (Patil et al. 1998), and peptide transporters (Balimane and Sinko 1999; Landowski et al. 2003). Furthermore, some compounds are actively extruded (in an ATP dependent manner) from cells lining the intestine into the lumen, by unidirectional transport proteins, such as P-glycoprotein (P-gp), or may undergo intestinal metabolism by enzymes like the cytochrome P450 3A (Cyp3A) enzymes that are located in high concentrations on the intestinal villi (Benet et al. 2004; Frassetto et al. 2003; Wacher et al. 1998). High levels of CYP3A enzymes are also present in the endoplasmic reticulum of hepatocytes (Soars et al. 2006), which are the primary substrates for HCV infection. The microstructure of the healthy liver resembles overlaying plates of hepatocytes surrounding a network of sinusoids (Marieb 2007; Han et al. 2002). The duration of exposure of drugs to the hepatocytes may be limited by susceptibility to drug metabolizing enzymes, since hepatocytes are the major site where drug metabolism occurs. Furthermore, drug exposure in hepatocytes may also be limited by the various unidirectional active transport (ATP dependent) proteins, including P-gp, BCRP\ABCG2, and MRP2 (Castell et al. 2006; Chandra and Brouwer 2004; Elferink and Groen 2002; Gomez-Lechon et al. 2004; Leslie et al. 2005; Pauli-Magnus and Meier 2006; Vermeir et al. 2005).

Although nucleoside analogs are not substrates for P-gp or CYP3A4, most protease inhibitors and NNI are substrates for both the P-gp efflux pump (Aungst 1999;

Storch et al. 2007) and CYP3A 4 metabolism (Sagir et al. 2003; Zhou et al. 2005). The HIV protease inhibitors ritonavir and atazanavir are potent inhibitors of both P-gp and CYP3A4. Therefore pharmacokinetic "boosting" has been used as a strategy in HIV therapy and may be feasible for HCV protease inhibitors for increasing peak plasma concentrations (C_{max}), reducing the rate of elimination (longer $t_{1/2}$), increasing exposure (AUC), and lowering the frequency of dosing needed to maintain sufficient drug levels (Cooper et al. 2003; King et al. 2004; Moyle and Back 2001). However, administration of other drugs with protease "boosting" regimens can result in complex drug interactions, which may require dose modification or drug replacement of either the protease inhibitor or the other drug. Various studies demonstrated the value of developing reliable assays for in vivo pharmacokinetic phenotyping of drug metabolic profiles when using drug combinations (Gerber et al. 2007). Furthermore, the reduction in the activities of drug metabolizing enzymes and active transporters may need to be considered when designing doses for individuals with severe liver disease.

When drugs enter and are distributed into systemic circulation, they partition between various cellular compartments according to their relative affinities for potential binding partners. The initial binding sites of drugs are serum proteins, mainly albumin and alpha-1-acid glycoprotein (AAG) and globulins (Boffito et al. 2003; Herve et al. 1994; Sheppard and Bouska 2005). Drugs that bind protein either covalently or noncovalently with high affinity and a slow rate of dissociation (restrictive binding), usually have small distribution volumes that may be similar to that of the serum compartment. However, some agents may demonstrate nonrestrictive (permissive) binding, characterized by high protein-bound fraction at equilibrium, but accessible to other binding sites as a result of rapid dissociation rates from the protein. Permissive binding often results from several low-affinity interactions with multiple sites on albumin, rather than single high affinity interactions (Herve et al. 1994; Sheppard and Bouska 2005). Experiments conducted in cell culture demonstrated a decrease in the cellular uptake, accumulation, and antiviral potency of anti-HIV protease inhibitors in the presence of physiological concentrations of AAG. This suggests that only the unbound fraction of protease inhibitors in the extracellular fluid is capable of entering the cells by passive diffusion where they exert antiviral effects (Bilello et al. 1996; Jones et al. 2001). Certain drugs bound to certain large molecular weight moieties (e.g., albumin bound interferon) may enter cells through endocytosis. Therefore, binding affinities of drugs to serum proteins and other potential binding partners have to be taken into account when designing new antiviral strategies.

8 Conclusions

Hepatitis C infection has an unpredictable natural history with significant potential for causing severe liver disease and variable response to current therapy based on pretreatment factors. Therefore, HCV is an excellent model to describe the

approaches for the development of antiviral compounds. As we learn more about HCV molecular biology and pathobiology, newer therapies will continue to be developed. Many drugs have already failed in phase 1 or 2 because they lacked potency or showed significant toxicities (gastrointestinal, liver, bone marrow). For example, potency has been an issue with certain nonnucleoside inhibitors of HCV polymerase, as two such compounds have stumbled in the clinic because of efficacy problems. In 2004, Rigel Pharmaceuticals Inc. disclosed that its R803 did not give a significant reduction in viral levels in a Phase 1/2 trial. In June 2007, XTL Biopharmaceuticals Ltd. discontinued development of its XTL-2125 after the compound did not significantly lower viral load vs. placebo in a Phase 1 study. Not much is known about why ViroPharma Inc.'s HCV-796 caused elevations in liver enzymes in some patients who received the nonnucleoside HCV polymerase inhibitor for at least 8 weeks in a Phase 2 trial. Idenix NM-283 was recently discontinued during a phase 2 trial because of gastrointestinal problems associated with this prodrug. Similarly, Roche's R-1626 nucleoside polymerase inhibitor was also discontinued due to bone marrow effects. It is important to understand why drugs fail in the clinic and if there is a scientific or chemical modification that can be made to reduce the adverse untoward effects.

Accordingly, many lessons have been learned and extensive knowledge has been built from these failures. Newer protease and polymerase inhibitors have emerged as potent, specifically targeted therapies against HCV infection, but will need to be used in combination with interferon and probably ribavirin to minimize resistance and increase the sustained virological response. Their introduction into practice will add complexity to the treatment of HCV infection because of the potential development of resistance and drug–drug interactions with other medications. The proper timing for the introduction of these drugs relative to interferon and ribavirin therapy are factors that will need to be individualized according to patient's needs and viral kinetics, requiring specialized infectious disease physicians and gastroenterologists with intimate knowledge of how these compounds act in inhibiting viral replication to maximize their efficacy. In 2007, it became clear that add-on therapy to standard of care is the way to go. Valuable lessons have been garnered from the failures of numerous anti-HCV agents. The development of new agents will require clinical proof that they are safe and produce sustained virological responses in controlled clinical trials, leading eventually to high curative rates. We are closing the gap on this virus and with the knowledge accumulated several potential cures are within sight.

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