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Review

Current drug discovery strategies for treatment of hepatitis C virus infection

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Abstract

Objectives Hepatitis C virus (HCV) infection represents a major worldwide-health problem. The current standard of care is combination therapy with pegylated interferon and ribavirin, which achieves a successful response in only approximately 40% of genotype I patients.

Key findings The biology of HCV infection has been under intensive research and important progress has been made in understanding the replication cycle of the virus. Several therapeutic targets have been under investigation, such as NS3 protease, NS4A replicase and NS5B polymerase. New potential targets, such as NS2 protease, as well as CD-81 and claudin-1 entry co-receptors, have also been identified.

Summary Clinical evaluations of drug candidates targeting NS3 protease, NS4A cofactor, and NS5B polymerase have demonstrated the potential of developing small molecules that interfere with the replication of the virus. Additional issues, including genotype coverage, resistant mutations, and combination therapy represent major challenges for future drug discovery efforts.

Keywords antiviral therapy; HCV molecular targets; hepatitis C virus (HCV); lead optimization; new drug discovery

Introduction

Hepatitis C virus (HCV), the etiologic agent of non-A, non-B hepatitis, represents a worldwide-health problem, with approximately 170 million people accounting for approximately 3% of the total worldwide population chronically infected with the virus.[1,2] In Europe and the USA, HCV is the most common infectious cause of chronic liver disease. In the United States alone, the infection causes 8000–10 000 deaths each year due to liver failure or hepatocellular carcinoma.[3] The virus is transmitted in a number of ways, however the most common sources of infection are through injected drug use and contaminated blood products. Infection with HCV often leads to a chronic form of hepatitis. Without therapeutic intervention chronic infection frequently leads to cirrhosis, hepatic failure or hepatocellular carcinoma. The rate of disease progression is variable but most often slow with many infected subjects remaining asymptomatic over 20–50 years, therefore it is difficult to accurately estimate the prevalence rates of the disease. Approximately 20–30% of patients initially infected develop cirrhosis within 20 years and a small percentage of these are at high risk of hepatocellular carcinoma.

There are six genotypes of the HCV virus that are less than ~70% identical at the nucleotide level.[4,5] Within each genotype, subtypes are defined by their differences in the nucleotide sequences by 20–25%. Genotype I is more difficult to treat than genotypes II and III. The current therapy for chronic HCV infection is subcutaneous injection of pegylated (PEG) interferon (IFN) α-2b or PEG-IFN α-2a in combination with oral ribavirin.[6] Although differences exist between the commonly used PEG-IFNs with respect to the structural modification and doses (weight-adjusted vs fixed) there is generally only small differences in clinical outcomes.[7–9] Combination therapy with PEG-IFN and ribavirin can result in a sustained virologic response (SVR), which is defined as undetectable HCV RNA levels 24 weeks after the end of therapy. Although this combination therapy is reasonably successful with the majority of genotypes, its efficacy against genotype I, the major genotype affecting North America, Europe and Japan, is modest.[8,9] Only approximately 40% of genotype I patients achieve sustained virological response after the standard combination of

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PEG-IFN and ribavirin for 48 weeks.[7,8] The lack of complete response, relapse following standard therapy, and premature termination of therapy due to adverse events complicates the treatment among genotype I-infected patients. Therefore, there is a critical need for new therapies for HCV infection that are more tolerable and effective. Several new therapeutic approaches are being assessed to address this unmet medical need. In this review, we present a comprehensive review on the advancement of understanding the biology of HCV, identification of molecular targets, drug discovery efforts in medicinal chemistry and lead optimization, and critical issues in clinic trials.

Biology of the Hepatitis C Virus

HCV belongs to the family of *Flaviviridae*, which includes other human pathogens, such as Yellow Fever and West Nile Virus. The HCV genome consists of a single-stranded, enveloped positive stranded, uncapped RNA virus of approximately 9600 nucleotides in length. The HCV genome contains a large open-reading-frame flanked by structure 5′ and 3′-nontranslated regions. Upon entering a suitable host cell, the HCV genome serves as a template for cap-independent translation through its 5′ internal ribosome entry site.[10,11] The open-reading-frame translates to produce a single ~3000 amino acid polypeptide through an internal ribosome entry site. This large polypeptide is further cleaved by cellular and viral proteases into at least 10 mature viral proteins which are organized in the following order: H_2N-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. The first three polypeptides are structural proteins, C, E1 and E2, which are directed to the endoplasmic reticulum-Golgi complex and processed by resident enzymes to generate the components for assembly of viral progeny.[12] The small hydrophobic p7 (63 amino acid) protein has been shown to be an ion channel with unknown function in the replication of the viral DNA or formation of the viral particle.[13] The amino- and carboxyl-termini of p7 are released from the polyprotein by host signal peptidase. In chimpanzees, p7 was found to be essential for HCV infectivity.[14] Moreover, in an in-vitro system, disruption of either uncleaved E2-p7 or p7-NS2 abolished infectivity.[15] The nonstructural (NS) proteins are processed by two distinct viral protease activities.[16] The integral membrane NS2 protein, together with the amino-terminal region of the NS3 protein, constitutes the NS2/3 cysteine protease that catalyses the cleavage of NS2 and NS3 proteins. Afterward, the NS2/3 protease is not known to serve any additional function. The NS3 protein, in conjunction with the NS4A cofactor, serves as a serine protease for the cleavage of the remaining NS proteins.[17] The NS3 protease forms a heterodimer with the NS4A protein, which is a cofactor for enhanced protease activity and a facilitator for the heterodimeric complex to anchor into the endoplasmic reticulum.[18] Following auto proteolysis of the NS3 protease, the enzyme continues to act on the NS4A-4B, NS4B-5A, and NS5A-5B junctions to release the downstream individual proteins. Once these NS proteins are cleaved, they assemble into the membrane associated HCV replicase complex. The complex has been shown to contain all HCV NS proteins as well as actively replicating HCV RNA.[19] Based on a functional HCV replicon system, it identifies NS3, NS4A, NS4B, NS5A and NS5B as essential components of the replicase system. Subsequently, the replisome uses the HCV viral genome as the template to generate the negative strand viral RNA intermediates, which in turn are used to synthesize new positive-strand genomic RNA. These newly synthesized viral RNA are either translated to yield more polyproteins for replisome assembly or, in late stage of the infection cycle, are encapsidated to produce new progeny.

HCV viral entry into hepatocytes requires sequential interaction between viral proteins and hepatocellular components. HCV viral particles may consist of a nucleocapsid surrounded by a lipid bilayer filled with two envelope glycoproteins, E1 and E2. Antibodies generated against the envelope glycoprotein could elicit cross-reactive neutralizing antibodies, suggesting that these envelope proteins are involved in cell entry.[20] In addition to cell entry via receptor-mediated endocytosis, HCV has recently been shown to spread by direct cell-to-cell transfer. Receptor-mediated endocytosis may involve specific interaction between the cellular receptors, such as CD81 and co-receptor, and the HCV envelope glycoproteins E1 and E2.[21] A recent study suggested that claudin-1 was an HCV co-receptor required for a late step in cell entry, after virus binding and interaction with the HCV co-receptor CD81.[22]Since multiple factors are involved in viral entry and replication there exits numerous opportunities for future antiviral drug development. A thorough understanding of how these factors interact during HCV infection and replication will help guide rational drug discovery efforts.

Molecular Targets in Drug Discovery

A schematic diagram depicting the molecular targets of the HCV is shown in Figure 1. These targets include the following nonstructural (NS) proteins: NS2 and NS3 proteases, NS4A, NS4B, NS5A and NS5B.

NS2/3 protease

The NS2/3 protease is also known as the autoprotease. It is dispensable for RNA replication *in vitro* but is essential for the complete replication cycle both *in vitro* and *in vivo*.[23,24] The catalytic activity of the NS2/3 protease resides in the carboxyl terminal portion of NS2 and the amino-terminal portion of NS3. NS2 protein is anchored into the intracellular membrane through at least one *trans*-membrane domain. A crystal struc-

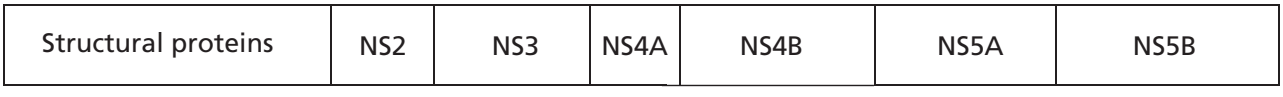


Figure 1 Schematic diagram of the hepatitis C virus gene product. The structure of the hepatitis C virus genome (from 5′ to 3′ direction) and its encoded proteins are shown.

ture of the NS2 protease catalytic domain (amino acid 94–217) has recently been solved.[25] The catalytic domain of NS2 appears to form a dimmer with two composite active sites. Cys184 and two additional residues, His143 and Glu163, appear to be essential for catalytic activity of this cysteine-protease. Based on the requirement of dimmer formation, it was postulated that the NS2-3 cleavage and formation of active replication complex may be a NS2-concentration dependent phenomenon. In light of the essential role of NS2 for the production of infectious virus, it is likely that NS2 may play additional functions that will be delineated in the future. So far, no drug discovery report has been published targeting NS2 protease.

NS3 protease/helicase

NS3 is a multifunctional protein, with a serine-protease located in the amino-terminal one-third and a RNA helicase located in the carboxyl terminal two-thirds of the protein. Both enzyme activities have been well studied and X-ray crystal structures have been solved.[26] The NS4A polypeptide also functions as a co-factor for the NS3 serine protease. The mid-section of NS4A is incorporated as an integral component of the NS3 serine protease and its amino-terminal domain is serving as a membrane anchor for the NS3-4A complex. The NS3 serine protease is known to have a very shallow substrate binding site which is formed by a catalytic triad-His57, Asp81 and Ser139.

The NS3 serine protease structure provided necessary details to permit rational structure-assisted inhibitor design.[27,28] This endeavor targeting the enzyme-substrate binding site resulted in the discovery of boceprevir, a structurally novel ketoamide protease inhibitor.[29] Recent proof-of-concept clinical studies with boceprevir and other HCV protease inhibitors BILN-2061 and telaprevir demonstrated the feasibility of targeting the protease.[30,31] Boceprevir and telaprevir are in phase III clinical trials.

The NS3 helicase is a member of the superfamily 2 DExH/D-box helicases. It unwinds double-stranded RNA or single-strand RNA with significant secondary structure in conjunction with the hydrolysis of ATP.[32] This activity is essential in separating the complementary RNA during the replication of viral progeny. It is unknown why the serine protease domain and the RNA helicase domain are linked together in a single polypeptide. A recent finding seems to shed some light on the need of the protease domain for unwinding efficiently RNA duplex by the helicase. Most of the drug discovery activity has been focusing on NS3 serine protease. Recent success in the late clinical evaluation of NS3 protease inhibitor may shift attention to the helicase target.

NS4A

NS4A is a small (54 amino acid) polypeptide that anchors the NS3 protein to the cellular membrane. The NS4A polypeptide also functions as a co-factor for NS3 serine protease. Its central domain is part of the integral component of the enzyme, and its amino-terminal is embedded in the membrane for the anchor of the NS3-4A complex. Complete folding and serine protease activity of NS3 requires the interaction of a β-strand present in NS4A.[33,34]

NS4B

NS4B contains three structural domains. The amino-terminal domain interacts in-plane with the membrane, the central transmembrane domains containing several transmembrane loops are embedded into the membrane, and the carboxyl-terminal cytosolic domain is exposed to the cytosolic surface of endoplasmic reticulum. In cell culture, a specific membrane alteration, known as the membranous web, was identified as the predominant site of RNA replication that harbours subgenomic HCV replicons.[35] The membranous web may be induced by NS4B alone. This structure closely resembles the 'sponge-like inclusions' that were found by electron microscopy in the livers of HCV-infected chimpanzees.[36] There are several potential roles for the membranous web in HCV replication.[37] For example, the membranous web was found to contain the nonstructural NS3 and NS5A HCV proteins as well as HCV RNA. Therefore, it may serve as a replication complex. In addition, it may serve as a site to conceal the viral RNA and help the virus to evade double-stranded RNA-triggered host antiviral responses. It is possible that drugs targeting NS4B may be able to disrupt formation of the membranous web, and hence, prevent the HCV RNA replication.

NS5A

NS5A is a phosphoprotein that exists in either a basally phosphorylated state or in a hyper-phosphorylated state. Phosphorylation of NS5A is a conserved feature among hepaciviruses and pestiviruses, as well as flaviviruses such as HCV. Although the relevance of this conserved phosphorylation is not well understood, it is considered to play an important role in the HCV life cycle.[38] In the HCV in-vitro replicon model, NS5A mutations that blocked interaction with human vesicle-associated membrane protein-associated protein A (hVAP-A) strongly reduced HCV RNA replication.[39] In addition, adaptive mutations may suppress NS5A hyper-phosphorylation, and consequently promoted hVAP-A interaction. A subset of adaptive mutations suppressed NS5A hyper-phosphorylation and promoted hVAP-A binding.

NS5A is anchored to the membrane by an amino-terminal amphipathic α-helix domain, which has a hydrophobic, tryptophan-rich region embedded in the cytosolic membrane interface.[40] Sequence homology analyses and limited proteolysis of recombinant NS5A suggested that it contained three structural domains. The crystal structure of the conserved domain I showed the property of a zinc-binding domain that functioned as RNA-binding for replication purposes.[41]

NS5B RNA-dependent RNA polymerase

NS5B is a RNA-dependent RNA polymerase responsible for the synthesis of a complementary negative-stranded RNA using the HCV genome as the template and the subsequent synthesis of genomic positive-stranded RNA from this negative-stranded RNA template.[42] The HCV5B protein contains motifs that are shared by all RNA-dependent RNA polymerases (RdRps), which include the hallmark GDD sequence within the motif C and similarity of the enzyme structure with the shape of a right hand, the classical fingers, palm and thumb domains. An extensive interaction between the fingers and thumb domains resulted in a completely enclosed active

site. The NS5B has become a major target for drug discovery research. Both the active site comprising the finger and thumb domains and the palm domain are targets for specific antagonistic interaction.[43]

Viral entry co-receptors

HCV only infects higher primates, such as humans and chimpanzees. Only some specific cell types could be targets, which include hepatocytes, B cells and dendritic cells. A few recent studies have improved our understanding of the mechanism for HCV to enter the host cells.[44] The low-density lipoprotein receptor, together with glycosaminoglycans, may serve as the initial and primary collectors of the hepatitis C viral particles. Further association between HCV E2 and CD81 and/or scavenger receptor class B member I (SR-BI) may be necessary, but not sufficient for directing the entry of the HCV particles. Blocking antibodies against CD81 or SR-BI may reduce infectivity, while expression of CD81 and SR-BI in nonhepatocyte-derived cell lines did not confer susceptibility to HCV infection.[11] In a recent study using an iterative expression cloning approach, the tight junction component claudin-1 was identified as an HCV co-receptor.[22] Claudin-1 appears to be essential for HCV entry into hepatocytes and renders nonhepatocytes permissive to HCV infection. However, there are cell types that are still nonpermissive despite expression of CD-81, SR-BI and claudin-1, suggesting the presence of additional factor(s) for HCV entry.

HCV enters the hepatocyte by a clathrin-mediated endocytosis process.[45] This process transits through an endosomal, low pH compartment and endosomal membrane fusion. A common feature for all related flaviviruses appears to involve the structural changes of the envelope proteins to expose an internal fusion peptide (class II fusion proteins) in a low pH environment. Although the detailed mechanism is unknown, the fusion between the envelope proteins and the endosomal membrane is believed to lead to the release of the HCV genome. With the most recent findings in identifying CD-81 and claudin-1 as co-receptors for HCV entry, it may become possible to target these co-receptors for drug therapy.

Medicinal Chemistry in Lead Optimization

Potential structure-assisted drug design targeting NS2/3 protease

The crystal structure of the catalytic domain of the HCV NS2 protease has been solved. This protein is a cysteine protease with His143, Glu163 and Cys184 being the three resides crucial for proteolytic activity. The NS2/3 protein is an autoprotease responsible for the intramolecular cleavage of NS2 from NS3 between amino acid 1026 and 1027 (at the sequence of WRLL – APIT). It has a uniquely formed catalytic site (Figure 2); the catalytic triad is contributed to by two monomers, the His143, Glu163 from one monomer and the Cys184 from the other. Hence, NS2 requires dimerization for proteolytic activity and each NS2 dimer contains two active sites.

NS2/3 cleavage has been shown to be absolutely required for genome replication and viral infectivity. The classical cysteine protease inhibitor iodoacetamide and N-ethylmaleimide showed strong inhibition of NS2/3 activity. Furthermore, NS4A derived peptides are potent inhibitors of NS2/3 activity.[46] Therefore, NS2/3 has been suggested to be a promising target for future HCV drug development. The available structure shows the post-catalytic state of the protease with the C-terminal residues of the NS2 protein remaining coordinated in the active site. Figure 2b shows the surface model of the binding site of the three NS2 C-terminal residues. The S1 (val) and S2 (val) pockets are hydrophobic in nature with well defined shapes. The prime sites are unoccupied in the crystal structure and they also have well defined shapes. These well defined binding pockets make the NS2 active site an attractive target for structure-based drug design.

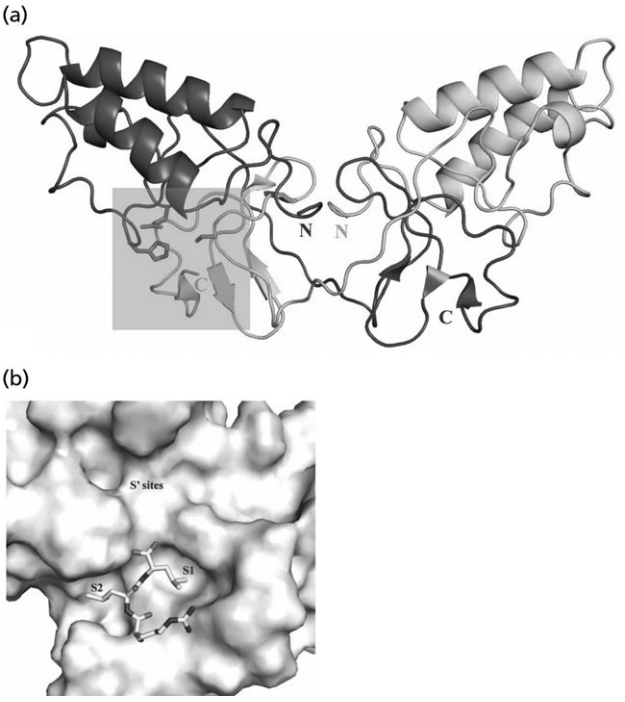
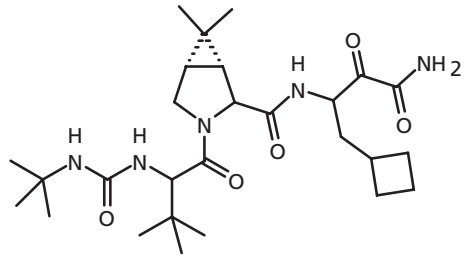


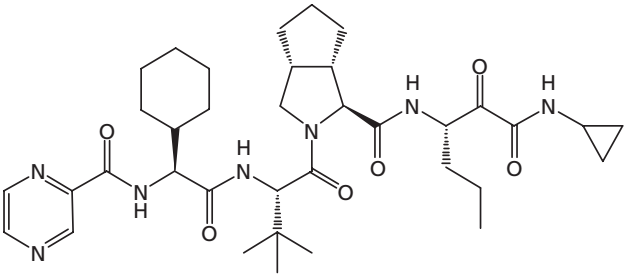
Figure 2 The structure of NS2 protease catalytic domain (PDB code: 2hd0). (a) The NS2 catalytic domain functions as a homodimer (monomer A in dark grey, monomer B in light grey), with two residues His143, Glu163 from one monomer and Cys184 from the other monomer forming the catalytic triad. (b) Surface representation of the NS3 active site. The N-terminal three residues in the NS2 protein was shown as stick model to illustrate the binding sites. Carbon atoms are shown in light grey, oxygen in medium grey and nitrogen in dark grey. The figures are generated with Pymol.

Lead hopping targeting NS3 protease

The structures of two phase III clinical candidates from Merck and Vertex are shown in Figure 3. Due to the shallow binding site of the NS3 protease, the current leads are fairly peptidic by maintaining the basic peptide backbone. Boceprevir is a tripeptide, while telaprevir is a tetrapeptide. Most of the chemistry efforts during the drug discovery stage focused on the modifications of the side chains, as shown in Figure 4, to improve antiviral potency and pharmacokinetic behaviour of the molecule. Lead hopping strategy for future lead optimization may emphasize in the following areas. In the P1'



Boceprevir



Telaprevir

Figure 3 Chemical structures of boceprevir and telaprevir.

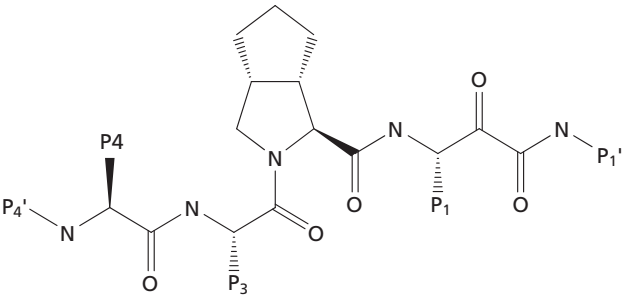
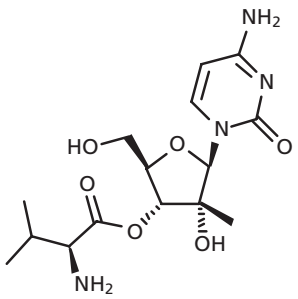


Figure 4 Lead hopping strategy for identifying new NS3 protease inhibitors.

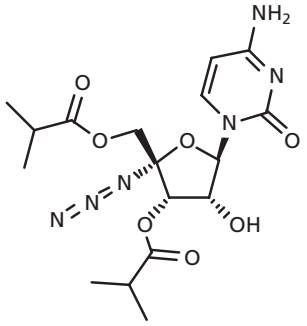
capping position, the substitutions could be selected from the group consisting of: aryl, alkyl, alkylaryl, heteroalkyl, cycloalkyl, alkylamino, arylamino, and cycloalkylamino. In the P1 side chain, substitutions may be limited to short to medium length alkyl and cycloalkyl groups. In the P3 position, the substitutions may be consisting of: alkoxyl, alkyl, alkenyl, aryl, alkylamino, and arylamino. In the P4 side chain and P4′ capping group, the substitutions may be consisting of alkyl, cycloalkyl, aryl, alkoxy, aryloxy, alkylsulfonyl, arylsulfonyl, akylsulfonamido, heteroalkylcarbonyl, and heteroarylcarbonyl.

Optimization of pro-drugs targeting NS5B polymerase and NS5A replicase

For NS5B polymerase inhibitors, two nucleoside analogues in early clinical evaluation are prodrugs (Figure 5). The rationale for this approach is using the prodrug for enhancing the absorption and delivery to the liver target site. This approach



NM 283
Valopicitabine



R1626
Prodrug of R1479

Figure 5 Chemical structures of valopicitabine and R1626.

may take advantage of the presence of two major types of carboxyesterase activity in various tissues in humans.[47] Carboxyesterase-1 (CES-1) is expressed in most tissues including liver, with the exception of low levels of expression in intestine. On the other hand, carboxyesterase-2 (CES-2) is expressed in intestine, liver and kidney. By designing a prodrug that is sensitive to CES-1, but resistant to the CES-2, may allow the prodrug to pass the intestinal barrier and to hydrolyse in the target liver tissue. In humans, the substrate specificities for CES-1 and CES-2 are significantly different. CES-1 prefers substrates with a small alcohol group and a large acyl group. In contrast, CES-2 recognizes a substrate with a large alcohol group with a small acyl group. In addition, CES-1 shows high *trans*-esterification activity, especially with hydrophobic alcohol. For CES-2, the *trans*-esterification activity is very negligible.

Hence, one may tailor the prodrugs in the following manner. As shown in Figure 5, the R1 group may consist of analogues of adenine, quinine, cytidine and thymine. In the R2 acyl side chain, the functional group may consist of alkyl, aryl, alkylaryl, alkylsulfonyl, arylsulfonyl, heteroalkyl and heteroaryl.

Lead Optimization in Drug Discovery

Recent advancements in chemistry, such as combinatory synthesis, result in a multifold increase in the number of compounds that are available for evaluation in drug discovery. This advancement, coupled with other improvements by structure chemists using a variety of tools, such as X-crystallography, structure modelling and ligand/substrate docking logarithm, and by molecular biologists developing high-throughput binding targets and cell-base activity assays,

provides an unprecedented level of structure-based rational design to further guide the synthesis of new chemotypes as potential drug leads. In concert with the advancement of chemistry and biology, new automated tools became commercially available which carried out complex, programmable and adaptable robotic operations to test hundreds of thousands of compounds in a speedy and precise manner. As a result, these new forces have worked synergistically to increase our ability to discover new compound entities (NCEs) that exhibit pharmacological activity.

To understand the needs of lead optimization, it is important to define the basic characteristics of the drug-like leads.[48,49] There are five general properties of the compounds to be drug-like: potency, availability, duration, safety and reasonable pharmaceutical properties. For a successful clinical drug, each of the five properties need to be present to a certain extent. A major deficit in one of the properties may prevent the compound from moving from drug discovery to the market. During the discovery phase of lead optimization, an optimal (acceptable) range of each of the five properties needs to be met. Among the five essential properties, three belong to the domain of drug metabolism and pharmacokinetics (DMPK): bioavailability, duration, safety issues. Hence, the lead optimization in discovering DMPK could be divided into three categories. First, for oral administration, the primary goal would be improving the oral bioavailability. This could be achieved by improving either the oral absorption or reducing the first-pass metabolism, or both. Secondly, improving the duration of the drug in the body could reduce the frequency of the dosing regimen. The duration of the drug in the body as measured by the half-life could be directly affected by the systemic clearance of the compound. Improving the systemic clearance may extend the half-life in a reciprocal manner. Lastly, reducing the DMPK related toxicity could be approached by several fronts, such as reducing the drug–drug interaction due to cytochrome P-450 (CYP) inhibition or induction, minimize the generation of reactive metabolites that causes covalent binding. CYPs are the major enzymes involved in the metabolism of drugs in the liver. Many well-established assays/screens are available for lead optimization in DMPK. In general, these screens could be divided into two major categories: *in vivo* and *in vitro*. A more detail discussion will be focusing on using these tools in lead optimization.

Improving antiviral potency

The development of a replicon system for screening the antiviral potency of a compound represents a significant step in drug discovery for HCV therapy.[50] The replicon system is the first in-vitro system for understanding the HCV RNA elements that are necessary for viral replication. The prototype subgenomic replicon system was a bicistronic RNA in which a portion of the structural and the nonstructural proteins was replaced by a neomycin phosphotransferase gene marker. A second heterologous internal ribosome entry site was used for the initiation of the translation of NS3-5B proteins. The replicon can propagate in the Huh-7 human cell line and maintain for many passages. Since the original functional genotype 1b replicon, replicons for genotypes 1a and 2a have also been constructed. In addition, full-length replicons have been developed and the ranges of the permissive host cell have been expanded to nonhepatic as well as other hepatic-base cell lines. High-throughput replicon screening has become the gold-standard in the pharmaceutical industry for the evaluation of antiviral activity of new chemical entities. Secondary screenings targeting the subtype 1a and 2b, as well as the emergence of resistant clones have become common practice when the antiviral drug discovery extends to more difficult issues. One of the observations was that the replicon system with full-length HCV genomes and adaptive mutations were still unable to produce infectious virus.

Recently an isolate of HCV genotype 2a (JFH-1) was identified to replicate in Huh-7 cells and other cell lines without the requirement for adaptive mutations.[51] It was subsequently found that cloned JFH-1 genomes transfected into Huh-7 cells produced viruses that were infectious for naïve Huh-7 cells. This observation allowed the in vitro studies of the complete life cycle of HCV. A more efficient isolate called J6 was produced by chimeric cloning in tissue culture for infecting chimpanzees and in immunodeficient mice with partial human liver.[52] These advancements in in-vitro systems greatly improved the capability of assessing the antiviral activity of NCEs during lead optimization in drug discovery.

Improving bioavailability

Bioavailability after oral dosing usually is governed by the absorption in the gastrointestinal tract and the extent of the first pass in the gastrointestinal tract and the liver. A combination of in-vitro and in-vivo screens may be employed to assess the absorption through the gastrointestinal tract and used to predict human absorption.

The in-vivo approach in the discovery phase is to rely on animal pharmacokinetics. For instance, if the absorption of the lead compound is within the acceptable ranges in rodent and nonrodent species, such as dogs and monkeys, it may provide certain assurance that human absorption may be within the acceptable range as well. To support this hypothesis, several publications have suggested that there is a correlation between animal and human absorption, especially considering that some distinct differences exist in the gastrointestinal physiology between species, such as the transit time.[53] The in-vitro approach mostly relies on using the Caco-2 system for screening the permeability of the lead compound. Numerous reports have documented the correlation between the Caco-2 permeability and absorption in humans.[54] The Caco-2 system appears to be most predictable for compounds that are absorbed by the transcellular mechanism. Due to the small pore size of the tight junction, the Caco-2 system is less permeable to compounds that are absorbed by the paracellular mechanism. However, treatment of Caco-2 cells with calcium-chelating reagents, such as EDTA, could increase the pore size of the tight junction. This approach has been used to understand the potential paracellular permeability of lead compounds.[55]

The second element involved in the oral bioavailability is the first-pass effect. For a compound to enter the systemic circulation from the gastrointestinal tract it needs to pass through two barriers, the intestinal wall and the liver. Both the intestinal mucosa and the liver are enriched in drug metabolism enzymes. It has been well accepted that, due to species difference, animal metabolism may not be suitable in predict-

ing first pass in humans. Hence, the most prevailing screening for estimating the first pass is using the microsomal preparations from human intestine or liver. The extraction ratio based on how quickly the parent compound disappears in the microsomal incubation is often used to estimate the extent of the liver first pass. A interesting approach to estimate the oral bioavailability was recently presented by using a Caco-2/hepatocyte hybrid system.[56] This novel system combines the Caco-2 permeability assay and the liver first-pass assay into one system. Further validation of the system may prove the general usefulness of this system.

Extending half-life

The half-life of a lead compound is determined by the clearance and the volume of distribution. The higher the clearance the lower is the half-life. Conversely, the higher the volume of distribution the higher is the half-life. For orally administered compounds, it may be possible on some occasions to develop slow release formulations to extend the apparent half-life. However, the primary goal in drug discovery is to improve the clearance of the compound. Since there are several ways to predict human clearance, the screening assays are designed according to these approaches. For example, allometry using animal clearance data has been often used to estimate human clearance. This approach requires clearance in at least three animal species. Therefore, using allometry to predict human clearance is hardly a high-throughput process. The most frequently used high-throughput assays aim to predict the hepatic clearance in the lead optimization process. These assays use either hepatic microsomes or primary hepatocytes derived from humans or animals. The general screening procedure monitors the disappearance of the parent compound in an incubation which contains the compound and certain amount of the microsomes or hepatocytes. Assuming the disappearance of the parent compound follows first-order kinetics, the rate of the process which is also called the intrinsic clearance rate is calculated based on the time course of the incubation. A number of recent publications suggested that using pooled hepatocytes from human donors represented a reasonable correlation between the intrinsic clearance rates and the in-vivo clearance rates of a number of marketed compounds.[57]

Future improvement of new compound entities

A summary of recent clinical trials is shown in Table 1.[58–62] Boceprevir is one of the lead candidates in late stage of clinical trials. The pharmacokinetic properties of this molecule have been reported.[49] The oral bioavailability in humans was estimated to be approximately 20%, resulting in the requirement of a fairly large oral dose of 400–800 mg. Improvement of the oral bioavailability by an increase in the absorption and decrease in the clearance will have a major impact on the dosage of NCEs. The effective half-life of boceprevir in humans is approximately 2–3 h. This short half-life would require a three-times a day dosing regimen for providing sufficient exposure for viral load reduction. Improvement of the half-life of NCEs in humans would reduce the dosing frequency to once a day or twice a day.

Reducing drug metabolism and pharmacokinetic related safety issues

Several potential safety issues related to DMPK have been well-understood. For example, the drug–drug interaction due to CYP isozyme inhibition and metabolism related toxicity are two of the common reasons for a lead compound to fail in development. Compounds are usually screened for their ability to inhibit major human CYP isozymes using either pooled human microsomes or supersomes, which contain individual isozymes.[63] Measurement of the CYP activity could be accomplished by either LC/MS-MS or a fluorimetric method. An important aspect is to differentiate whether the inhibition is direct or metabolism/mechanism-based. The distinction between these two possibilities is that direct inhibition is reversible and noncovalent binding that results in the alteration of the Michaelis–Menten kinetic parameters, whereas mechanism-based inhibition is irreversible, due to covalent binding to the enzyme by a chemically reactive intermediate.

Potential mechanism-based related safety issue

Many of the identified HCV targets belong to two classes of enzymes: protease and polymerase/replicase. One potential mechanism-based safety issue for protease inhibitor is cross-activity with human proteases, such as cathepsins. Cathepsins are protease localized in lysosomes and in responding to certain physiological signals may be released to the cytoplasm. Inhibition of cathepsins may lead to unknown interruption of physiological functions. Cross-activity of polymerase inhibitor with human enzymes may interfere with the transcription/translation mechanism. To reduce the potential safety issue a cytotoxicity assay should be routinely used to evaluate NCEs.

Clinical Evaluations

NS3 protease inhibitors

Since the first isolation of a cDNA clone derived from a blood-borne non-A, non-B hepatitis genome in 1989, a great deal of progress has been made on the biology of HCV and the small molecule drug discovery efforts.[2] The first proof-of-

Table 1 Summary of some recent drug candidates in clinical trials

Drug candidate	Target	Clinical trial	Company
Boceprevir[29]	NS3 protease	Phase III	Schering-Plough
Telaprevir[31]	NS3 protease	Phase III	Vertex
BILN 2061[30,58]	NS3 protease	Phase I/II	Boehringer-Ingelheim
Valopicitabine[59,60]	NS5B polymerase	Phase IIb	Idenix
R1626[61]	NS5B polymerase	Phase I	Roche
ACH 806[62]	NS4A replicase	Phase I	Achillian

concept clinical study was reported regarding the NS3 protease inhibitor.[30] In subsequent reports, BILN2061, a specific NS3 protease inhibitor from Boeringer-Ingelham, was shown to be effective in patients with genotype I, II, and III HCV infections.[58] Although further clinical study on BILN2061 was halted due to toxicity, two additional protease inhibitors were entering the clinical evaluations.

In a phase Ib study, the NS3 protease inhibitor VX-950, also known as telaprevir, rapidly reduced the viral load as monotherapy in patients with HCV genotype I infection.[31] In parallel, the NS3 protease inhibitor SCH 503034 (boceprevir) was being explored in a phase Ib study and showed robust antiviral activity. In this study, boceprevir as monotherapy or in combination with PEG-IFN α-2b was well tolerated in patients with HCV genotype I who were prior nonresponders to PEG-IFN plus ribavirin.

NS5B polymerase inhibitors

There are several polymerase inhibitors in phase I and phase II clinical trials. Three of them, two nucleoside analogues (valopicitabine and R1626) and a nonnucleoside analogue HCV796, were advanced into phase II trials. Valopicitabine is a nucleoside prodrug of NM107 that competitively inhibits NS5B polymerase activity.[59] At high oral doses, valopicitabine reduced the viral load by more than one log. When patients were treated with valopicitabine combining with PEG-IFN, the viral load decreased approximately three logs.[60]

R1626 is a prodrug of a nucleoside analogue, R1479, which selectively inhibits the NS5B polymerase. In a phase I study, approximately three log viral load drops were found in patients after oral administration of high doses of R1626.[64] HCV-796 is a nonnucleoside NS5B polymerase inhibitor. In a phase I study, approximately 1–2 log drops of viral loads were observed.[61] In combination with PEG-IFN, more than three log drops of the HCV viral loads were observed compared with only about one log drop for PEG-IFN alone. Further clinical study was discontinued due to elevated liver enzyme activity in approximately 8% of the patients after eight weeks or more of combination therapy with HCV-796.

NS4A replicase inhibitor

ACH-806 is an acyl thiourea compound with binding selectivity to NS4A, resulting in altered protein composition and inactivation of replication complex.[65] A short-term antiviral activity, safety and pharmacokinetics evaluation in patients after oral administration for five days showed an average one log drop of the viral loads. This is the first study showing that NS4A could also be a target for anti-HCV therapy.

In the replicon system, ACH-806 was shown to have a synergistic effect when combined with both HCV NS3 proteases, such as telaprevir and BILN-2061, and NS5B polymerase, such as 2-C′-methyladenosine.[66] If these in-vitro antiviral results could be reproduced in HCV-infected patients, there is potential for an all oral regimen of small molecules.

Resistant Mutants

It has been well documented that about half of HCV patients infected with genotype I do not respond to combination PEG-IFN and ribavirin therapy. With the addition of specific anti-HCV compounds to the standard of care, it also became clear in early clinical trials, before treatment initiation and the application of selective pressure, they had demonstrated the existence of naturally occurring resistance mutations to NS3 protease and NS5B polymerase inhibitors. A recent study analysing HCV genome sequences from 507 treatment-naïve patients infected with genotype I HCV, showed that the prevalence rates of mutations conferred resistance to be 8.6% for genotype Ia and 1.4% for genotype Ib.[67] In a separate study, naturally resistant mutants were detected against the nonnucleoside, but not to the nucleoside, NS5B polymerase inhibitors.[62,68] Thoe studies revealed the existence of naturally occurring mutants in the general population. It is possible that with a combinatory approach the prevalence of dual resistance mutants may be dramatically reduced.

Summary

HCV infections in the USA alone cause 8000–10 000 deaths each year due to liver failure or hepatocellular carcinoma. Recent advancements in our understanding of the molecular mechanism of HCV replication provide numerous opportunities for future antiviral drug development. Several drug candidates, such as HCV NS3 protease inhibitors, NS5B polymerase inhibitors and NS4A replicase inhibitors, are in various stages of clinical evaluation. Despite the promising efficacies demonstrated in the clinical trials, most of the clinical candidates in phase III require fairly large and frequent dosages. A critical issue in any future clinical trial is to understand the efficacy of the drug candidates administrated at different stages of the disease and the treatment/retreatment of patients resistant to previous standard care.

Although as of yet there is no evidence that NCEs are having an impact on other hepatitis viral diseases, there is certainly potential to use the NCEs to target multiple diseases in the future.

Future issues during lead optimization include identifying drug candidates with better potency and more favourable pharmacokinetic properties. More clinically relevant issues include identifying the best combination of therapies and duration of treatment to maximize the rate of sustained viral responses and to minimize the emergence of resistant mutants. The similarities between HCV and human immunodeficiency virus (HIV) in terms of viral biology, molecular targets, resistance mutations and transmission, suggest that lessons learned from development of anti-HIV drugs may be useful for developing combination treatment against HCV. In addition, the knowledge we learn from the drug discovery efforts against HCV infection may be applicable to other viral infections, such as hepatitis B virus.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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