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Review

Molecular virology of chronic hepatitis B and C: Parallels, contrasts and impact on drug development and treatment outcome ☆

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ABSTRACT

Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are highly prevalent worldwide, causing significant liver disease and thus representing high unmet medical needs. Accordingly, substantial pharmaceutical and clinical research efforts have been made to develop and improve treatments for these viruses. While HBV and HCV are both hepatotropic viruses that can cause similar disease in chronically infected patients, they belong to different viral families. There are substantial differences in the molecular virology of HBV and HCV that have profound implications for therapeutic strategy. In particular, HBV has a long-lived nuclear form of its genome (covalently closed circular DNA) that is able to persist in the face of potent inhibition of viral replication. In contrast, HCV does not have a long-lived genome form and depends on active replication to maintain infection; HCV is therefore much more susceptible to eradication by potent antiviral agents. Additional differences between HBV and HCV with therapeutic implications include the size, structure and heterogeneity of their respective viral genomes. These factors influence the number of targets available for therapeutic intervention, response to therapy among viral genotypes and the emergence of viral resistance. Substantial progress has been made in treating each infection, but unique challenges remain. In this review, key differences in the molecular virology of hepatitis B and C will be presented, highlighting their impact on antiviral therapy (particularly with respect to direct-acting antivirals) and the challenges they present to the cure of each disease.

Contents

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1. Introduction

Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) represent an enormous unmet medical need. Worldwide, there are an estimated 350 million and 160 million people chronically infected with HBV and HCV, respectively (Lavanchy, 2004; Lavanchy, 2011). Both viruses can establish infections in the liver which last for decades and lead to persistent liver injury. Over time, serious liver complications can occur with both infections; these include fibrosis, cirrhosis and hepatocellular carcinoma. Infections with these viruses are the most common indication for liver transplantation worldwide (Crespo et al., 2012).

HBV and HCV are both bloodborne pathogens. However, the host response to these infections and, consequently, the natural history of HBV and HCV infection differ markedly. The outcome of HBV infection is strikingly dependent on the age of the host. HBV is spread primarily by transmission from mother to infant during birth. Exposure of neonates to HBV results in the establishment of chronic infection in the vast majority of cases (>90%) (Fattovich, 2003). In contrast, immunocompetent adults exposed to HBV have the opposite outcome with >90% resolving acute infection and establishing lifelong protective immunity. Once established, chronic HBV infection has a complex natural history comprised of several distinct stages through which the infected individual can progress over a period of decades, including an "immunotolerant" phase, $HBeAg^+$ and $HBeAg^-$ phases of chronic active hepatitis, an "inactive carrier" phase, and finally clearance of infection (Chen and Yang, 2011). Although clearance can occur naturally, it is rare (1–2% of patients/year). Furthermore, HBV infection can reactivate during periods of immune suppression, suggesting that HBV is controlled immunologically, rather than by sterilizing immunity. In contrast to HBV, infection with HCV progresses from acute to chronic infection in 50–90% of adults (Maasoumy and Wedemeyer, 2012). Once established, chronic HCV infection rarely resolves spontaneously.

The immune response to acute infection with HBV and HCV also differs substantially, as observed during seminal chimpanzee studies by Wieland and Chisari (Wieland and Chisari, 2005). Remarkably, infection with HBV was noted to be silent with respect to host gene induction early in infection (Wieland et al., 2004). This suggests HBV is highly effective in avoiding detection by the innate immune system, allowing it to replicate and spread prior to the onset of adaptive immunity. Failure of HBV to be detected efficiently by the innate immune system may be due to the fact that HBV does not present effective targets to the innate immune system or, as reviewed by Chang et al. (2012), that viral proteins may actively counter host responses. Resolution of acute HBV infection or progression to chronic infection is dictated by the adaptive immune response. It has been well documented that resolution of infection is characterized by vigorous, multi-specific T-cell responses as well as the appearance of neutralizing antibodies. In contrast, only weak and narrowly focused T cell responses are observed in chronic hepatitis B patients, and neutralizing antibodies fail to emerge (Rehermann and Nascimbeni, 2005). The upregulation of inhibitory T-cell markers (e.g. PD-1) is likely to play a significant role in the failure of the adaptive immune system to mount an appropriate response to HBV (Grimm et al., 2013).

HCV, unlike HBV, robustly triggers activation of innate immunity as a substantial number of IFN-stimulated genes are upregulated in the liver (Lemon, 2010). Nevertheless, HCV can overcome the stimulation of innate immunity by using its own gene products to specifically counter host immune components (Lemon, 2010). The evasion of adaptive immunity by HCV during chronic infection appears to be due to the tremendous genetic diversity of HCV, which allows it to escape recognition, as well as upregulation of T-cell inhibitory markers (Burke and Cox, 2010; Thimme et al., 2012). The differences in virology and immune response to HBV and HCV are also underscored by the success and failure of prophylactic vaccines for these viruses, respectively.

Historically, hepatitis B and C have both been treated with IFN-α-based therapies, and pegylated IFNs are currently licensed for the treatment of both chronic infections. Unfortunately, for both indications, IFN-based regimens are only partially effective and carry a substantial side-effect burden. The pharmaceutical and medical communities have therefore been highly focused on identifying improved therapies. Substantial progress has been made in recent years. Safe, highly effective, and durable chronic suppressive nucleotide and nucleoside therapies became available for the treatment of HBV with the approvals of entecavir and tenofovir disoproxil fumarate in 2005 and 2008, respectively. The treatment of HCV is in a state of rapid evolution in which the addition of direct-acting antivirals (DAAs) to pegylated IFN-based regimens has demonstrated substantial improvements in efficacy. However, combinations of multiple DAAs appear likely to rapidly supplant IFN-based therapies entirely. The purpose of this review is to provide a high-level comparison of hepatitis B and C from a virologic perspective, and to highlight how differences in the viruses impact therapeutic strategies and outcome, particularly with respect to DAAs.

2. Similarities and differences in HBV and HCV from a virologic perspective

Although both HBV and HCV are hepatotropic viruses that establish chronic infection in man, they belong to different viral families. HBV is a DNA virus which replicates by reverse transcription and belongs to the family *Hepadnaviridae*, while HCV is a positive-strand RNA virus belonging to the family *Flaviviridae*. Below is a brief overview of virus ultrastructure, genome organization, replication strategy and genetic diversity. More detailed information on the molecular virology of HBV and HCV can be found in several excellent reviews (Bartenschlager et al., 2011; Lindenbach and Rice, 2005; Lindenbach et al., 2007; Seeger and Mason, 2000; Seeger et al., 2007).

2.1. Virus structure

HBV and HCV share several structural features at a gross level. Both viruses are enveloped, contain capsids comprised of multimers of single core proteins and are roughly the same size (Fig. 1). The infectious HBV particle, also known as the Dane particle, is 42 nM in diameter and its envelope contains three related surface antigens (hepatitis B surface antigen, or HBsAg). These are referred to as the small (S), middle (M) and large (L) surface antigens and are derived from a set of overlapping reading frames

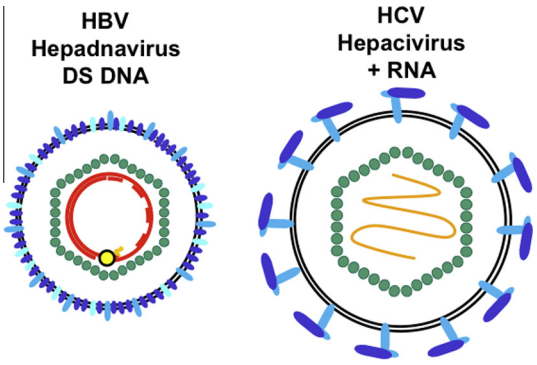

Fig. 1. Structures of hepatitis B and C virions. HBV (left) and HCV (right) belong to different viral families but have some similarity in basic ultrastructure. They are similar in size, with diameters of 42 and 50–60 nM, respectively. Both viruses are enveloped, with multiple viral surface proteins in the envelope, and both contain a capsid which is a polymer made up of a single viral core protein. However, the HBV genome resides within the capsid as a partially double-stranded DNA, whereas the HCV genome is present as a single strand of positive-sense RNA.

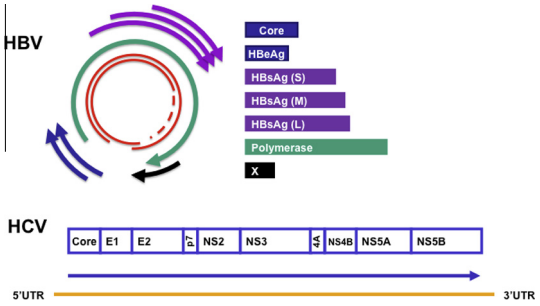

Fig. 2. Genomic structures of HBV and HCV. HBV (top) and HCV (bottom) have substantially different genomic organization. The partially double-stranded DNA genome of HBV encodes four major overlapping reading frames (ORFs) in a compact 3.2 kilobase genome. The reading frames include the surface antigen ORF (purple), which gives rise to three related surface antigens (large, middle and small); the core and precore reading frames (blue), which give rise to HBcAg and HBeAg, respectively; the polymerase reading frame (green), which gives rise to the polymerase; and the X reading frame (black), which gives rise to the X protein. In contrast, the 9.6 kilobase single-stranded, plus-sense HCV RNA genome is directly translated into a single polyprotein, which is subsequently processed into 10 distinct viral proteins, including the structural proteins core, envelope 1 (E1) and envelope 2 (E2) and the nonstructural (NS) proteins, p7, NS2 protease, NS3/4 protease, NS4B, NS5A and the NS5B polymerase.

(described in Section 2.2). The HBV nucleocapsid is approximately 28 nM in diameter. The capsid is comprised of hepatitis B core antigen (HBcAg) which initially forms dimers and then polymerizes to form icosahedral capsids. The HBV genome resides within the capsid in the form of a partially double-stranded, relaxed circular 3.2 kb DNA, which has the unusual property of being covalently linked to the 70 kD viral polymerase. HBV infection is also marked by the secretion of HBsAg particles without nucleocapsids (i.e. subviral particles) from infected hepatocytes in large excess (several orders of magnitude) over infectious particles.

The infectious HCV particle is spherical and approximately 50–60 nM in diameter. Its envelope contains two proteins, E1 and E2, which are heavily glycosylated and associate to form heterodimers which mediate attachment and entry into hepatocytes. The capsid of HCV is made up of the HCV core protein which, like HBcAg, forms dimers and then further polymerizes to encapsidate the HCV genome. The HCV genome resides in the virion as a single 9.6 kilobase positive strand of RNA.

2.2. Genome organization and viral proteins

HBV and HCV differ markedly in their genome organization (Fig. 2). As indicated above, HBV has a partially double-stranded DNA genome arranged in a relaxed circular conformation. During viral infection, the genome is repaired to form a covalently closed circular DNA (cccDNA), which serves as the transcriptional template. The circular nature of the genome allows HBV to encode four open reading frames and a total of seven proteins from a compact 3.2 kb DNA. The largest open reading frame encodes the viral polymerase, a multifunctional protein involved in priming and reverse transcription of the genome. The three HBsAg envelope proteins S, M, and L are derived from a second open reading frame. A third open reading frame encodes the HBV core (capsid, HBcAg) protein as well as a second protein known as hepatitis B e antigen (HBeAg). Despite sharing sequence with HBcAg, HBeAg protein is functionally distinct, being processed differentially, secreted from hepatocytes and playing a role in immune evasion. The final open reading frame encodes the X protein, which is essential for productive infection and transcription *in vivo* and in cell culture models based on authentic infection (Chen et al., 1993; Zoulim et al., 1994; Lucifora et al., 2011). The role(s) of the X protein during viral replication include transcriptional regulation and several other reported functions (Benhenda et al., 2009).

In contrast to HBV, the single-stranded, positive-sense RNA genome of HCV is organized in a linear fashion, and can be directly translated in the host cell cytoplasm. The genome is flanked by 5′ and 3′ untranslated regions (UTRs). These sequences have extensive secondary structure, with the 5′ UTR encoding an internal ribosome entry site (IRES) and both the 5′ and 3′ UTRs being important for replication of the genome. The sequence between the UTRs encodes a single continuous polyprotein which, after translation, is cleaved by host- and virus-encoded proteases into 10 distinct proteins. The first three are structural: the core protein as well as the envelope proteins E1 and E2. The remaining seven proteins are nonstructural. The p7 protein is membrane-bound and putatively functions as an ion channel. NS2 is an autoprotease which also plays a role in viral assembly. NS3 encodes a bi-functional protein with serine protease and helicase activities. The NS3 protease cleaves and separates the nonstructural proteins with NS4A being a necessary co-factor. The NS3 helicase is required for viral RNA replication. The NS4B and NS5A proteins are also critical for viral replication, though their exact roles have not been completely defined. NS4B is an extremely hydrophobic membrane protein and appears to be crucial in the formation of the membranous complexes in which HCV genome replication takes place. NS5A is a dimeric phosphoprotein with RNA binding capability and is also an essential component of the viral replication complex. The final protein is the NS5B RNA-dependant RNA polymerase, which catalyzes the replication of negative- and positive-sense genome copies.

2.3. Viral replication cycles

Although both HBV and HCV target hepatocytes, they have dramatically different replication strategies (summarized in Fig. 3). HBV infection initiates with attachment of the virion to the hepatocyte, mediated by the surface antigens, with the large HBsAg (L) playing a crucial role. The host receptor has long remained elusive although a recent report suggests a sodium taurocholate-transporting protein as a candidate receptor (Yan et al., 2012). Following binding, the virus is internalized and uncoated, and the DNA genome is delivered to the nucleus. The processes of internalization, uncoating and nucleocapsid disassembly are poorly understood due to the historical lack of robust *in vitro* infection systems. After delivery to the nucleus, the partially double-stranded DNA is converted into cccDNA. A critical feature of the HBV lifecycle is that cccDNA persists in the hepatocyte nucleus as supercoiled, chromatinized episomes. cccDNA is transcribed into four co-terminal

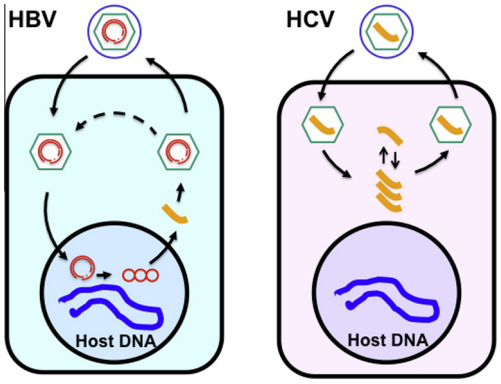

Fig. 3. Replication cycles of HBV and HCV (simplified view). HBV (left) and HCV (right) have substantially different replication strategies. HBV enters hepatocytes in a receptor-mediated process, is uncoated and delivers its DNA to the nucleus. The incomplete double-stranded genome is then completed and repaired to form cccDNA, which is maintained without active replication in the nucleus as a chromatinized episome. Transcription of cccDNA by host RNA polymerases gives rise to viral mRNA molecules which are exported to the cytoplasm and translated; the resulting proteins and pregenomic RNA assemble to form new nucleocapsids, which can either cycle back to the nucleus to amplify the copy number of cccDNA or acquire an envelope and be secreted as progeny virions. While HCV also enters the cell by a receptor-mediated process, its genome is delivered to the cytoplasm, where it is translated and viral proteins assemble to form replication complexes for genome replication. Within the replication complexes, negative-sense RNA strands are copied from the infecting genomes, and from these additional positive-stranded genomes are replicated. Progeny positive RNA strands can be encapsidated, acquire an envelope and leave the cell as progeny virions. Note that no nuclear or DNA form of the HCV genome is generated during infection.

mRNAs which are capped, polyadenylated and exported to the cytoplasm. Each viral mRNA has a promoter driving transcription. Two enhancer elements embedded in the viral genome also regulate transcription. A variety of cellular transcription factors, including ubiquitous and hepatocyte-specific transcription factors as well as nuclear receptors, regulate viral transcription (Quasdorff and Protzer, 2010). The viral X protein is also regulates viral transcription at an epigenetic level by modulating the acetylation status of histones bound to cccDNA (Belloni et al., 2009).

Viral mRNAs are then translated into the various protein products in the cytoplasm. The core protein, polymerase and largest HBV transcript (the "pregenomic" RNA) then associate to form an immature nucleocapsid. Within the nucleocapsid, the polymerase catalyzes a complex set of reactions including priming, reverse transcription of the pregenomic RNA into a first (minus) strand of DNA, degradation of the template RNA, and DNA-dependent synthesis of a partial second (plus) strand of DNA. At this point the mature nucleocapsid is functionally equivalent to the capsid which entered the cell during primary infection, and has two potential fates. Newly formed nucleocapsids can deliver their genomes back to the nucleus, resulting in amplification in the pool of cccDNA. Alternatively, nucleocapsids can bud into the endoplasmic reticulum, acquire an HBsAg-containing envelope and be exported from the cells as progeny virions.

An important feature of hepadnaviral replication is the secretion of high levels of HBeAg and HBsAg into circulation. These viral antigens play a significant role in the evasion of the host immune response. HBeAg is a toleragen that plays a role in establishing chronic infection during vertical transmission (Chen et al., 2004). HBsAg particles, which can be present in the blood at levels 10–1000× higher than infectious virus, may have multiple roles in immune evasion, including blocking the host antibody response, blunting innate immune responses (Xu et al., 2009) and driving HBV-specific T cell exhaustion in the liver (Fisicaro et al., 2010).

Like HBV, the HCV lifecycle initiates with attachment of the virus particle to the hepatocyte. Unlike HBV, considerably more is known about this process, owing to the establishment of robust cell culture models for HCV. The E1/E2 envelope proteins have direct interactions with cellular receptors, including CD-81 and the scavenger receptor B-1 (SRB-1). Claudin-1 and occludin are also critical cellular co-receptors for HCV infection. Entry is pH-dependent, suggesting that it occurs through endocytosis. The nucleocapsid is then delivered to the cytoplasm and disassembles to release the genome. The positive-sense RNA genome is directly translated into a single polyprotein of approximately 3000 amino acids, which is subsequently cleaved by proteases (including the host signal peptide peptidase and the viral NS2 and NS3/4A proteases) to yield the individual proteins described above.

HCV genome replication requires the NS3/4A, NS4B, NS5A and NS5B proteins and takes place in membranous compartments induced by viral proteins, particularly NS4B. Within these replication complexes, the viral positive-strand RNA is first replicated into a negative strand. The negative strand can then be copied to produce new positive strands of RNA. Within infected cells, positive RNA strands are found in excess of negative strands, typically in a ratio of 10:1. Newly synthesized positive strands can then either be used for additional translation and genome replication, or for the assembly of new virions. RNA genome encapsidation may be facilitated by the NS5A protein and the core protein which associates with lipid droplets within the cell. Nucleocapsids can then acquire an E1/E2-containing envelope and be secreted from the cell, completing the replication cycle. Recently, HCV subviral particles composed predominantly of host lipid and viral envelope proteins were found to be common in the circulation of HCV patients. As with HBV infection, circulating subviral HCV particles may represent a mechanism for evading the host immune response (Agaugue et al., 2007; Perrin-Cocon et al., 2008). Finally, it is important to note that HCV replication does not involve a DNA intermediate, takes place entirely in the cytoplasm, and does not establish a long-lived form of the viral genome.

2.4. Genetic diversity

HBV and HCV have both evolved into multiple genotypes as they have distributed across the human population. HBV is separated into eight different genotypes (A–H) which differ by a minimum of 8% at the nucleotide level (Araujo et al., 2011; Jazayeri et al., 2010; Kahila Bar-Gal et al., 2012). HCV is classified into six major genotypes (1–6) and each genotype can be further classified

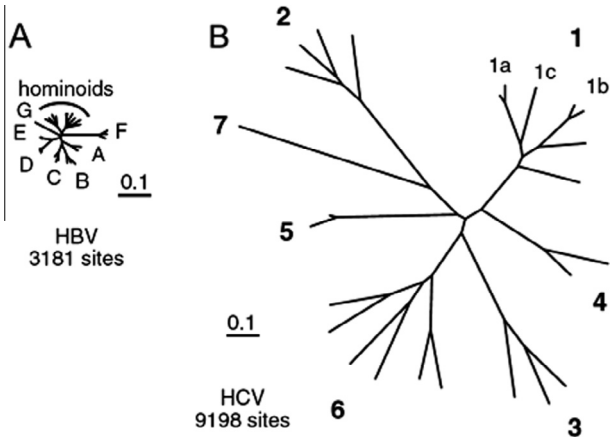

Fig. 4. Genetic distance between genotypes for HBV and HCV. HBV and HCV are each divided into multiple genotypes. A comparison of genetic distance between HBV and HCV genotypes is indicated, with nucleotide genetic distance drawn on the same scale. The analysis is based on full-length genome sequences using sequences available in GenBank. Alignmants were performed using ClustaX and Maximum likelihood trees were inferered using PAUP v 4b10. Reproduced from (Ray and Howard, 2010), with permission.

into multiple subtypes (e.g. genotypes 1a, 1b, 1c, 2a, 2b, etc.). HCV genotypes and subtypes diverge by an average of 30% and 20% at a nucleotide level, respectively (Kuiken and Simmonds, 2009). HCV is therefore much more genetically diverse than HBV (Fig. 4).

A major mechanism underlying the generation of diversity for both viruses is the error rate of their polymerases during genome replication. Interestingly, enzymatic studies with HBV and HCV indicate that their error rates are similar overall. For HBV, nucleotide misincorporation rates range from 6.6×10^{-4} up to 1.1×10^{-3} (Park et al., 2003). For HCV, NS5B error rates range from 2.3×10^{-6} (for transversions) up to 3.2×10^{-3} (for transitions) (Powdrill et al., 2011). However, a critical difference between the two viruses is that HBV has a circular genome with multiple overlapping reading frames which are translated in different frames, whereas the genome of HCV is organized in a linear manner. The overlapping reading frames of HBV likely restricts the number of viable mutations, since many mutations would have to be tolerated by two different proteins. For example, the effect of various HBV polymerase mutations on the HBsAg open reading frame has been reviewed in depth by Locarnini and Yuen (2010).

3. Consequences of virologic differences for antiviral therapy

The molecular differences between HBV and HCV highlighted above have substantial implications for therapeutic strategy and treatment outcomes. These implications range from the number of viral targets present for drug discovery, to the fundamental effect drugs have toward resolving infection, and the ability of each virus to evade antiviral therapy, both through natural genetic diversity and the emergence of resistance.

3.1. Impact of genome content on antiviral discovery

Examination of the genomic structure and protein content of HBV and HCV immediately reveals that HBV presents fewer viral targets for antiviral intervention than HCV. HBV has only 4 open reading frames, and of its 7 encoded proteins (some of which are highly related), only the polymerase has a classic enzymatic function. In contrast, HCV has 10 distinct proteins, with at least 4 well described enzymatic functions (the NS5B polymerase, NS3 helicase, and NS2 and NS3/4 proteases). Below is a brief overview of targets for each virus, with an emphasis on those that have been clinically validated.

3.1.1. Viral targets for the treatment of hepatitis B

Of the potential targets in the HBV genome, the polymerase stood out initially as a prime candidate for antiviral intervention. Not only had polymerases already been successfully targeted for other chronic viral infections, but nucleoside analogs originally developed for herpesvirus and human immunodeficiency virus infections demonstrated activity against HBV *in vitro,* in animal models and in early clinical studies (Mason et al., 1998; Nevens et al., 1997; Shaw and Locarnini, 1995; Shaw and Locarnini, 1999; Trepo et al., 2000; Tsiquaye et al., 1994). During the 10 year period 1998–2008, 5 nucleoside or nucleotide analogs were approved for the treatment of chronic hepatitis B (Table 1). They can be divided into 3 groups, based on structure and resistance profile:

- nucleoside phosphonate (nucleotide analog) prodrugs, which include tenofovir disoproxil fumarate (tenofovir DF) and adefovir dipivoxil;
- pyrimidine L-nucleosides, including lamivudine and telbivudine (several other L-nucleosides have also demonstrated potent clinical activity against HBV, including emtricitabine, clevudine and torcitabine, but they are not licensed); and
- the guanosine analog entecavir (Fig. 4).

Each of these nucleoside/tide analogs was approved based on its ability to safely suppress serum viremia and improve patient disease state (e.g. normalize serum ALT, improve liver histology) (Papatheodoridis et al., 2012). However, the more recently approved agents are clearly superior in achieving durable virologic suppression and clinical benefits. Most notably, tenofovir DF and entecavir have the highest virologic response rates in both $HBeAg^+$ and $HBeAg^-$ patients (Table 1), and they also have the highest barrier to resistance (see below). Accordingly, tenofovir DF and entecavir are the most widely prescribed agents in the United States and the European Union, and their use results in durable suppression of serum viremia to below the limit of detection in most patients. As discussed below, nucleoside/tide analog therapy is rarely curative. This class of drugs requires chronic dosing to maintain suppression of viremia, and is also largely ineffective at reducing HBs antigenemia.

Besides the polymerase, no other direct viral targets have been clinically validated for the treatment of hepatitis B. The most advanced efforts have been made at targeting HBcAg and viral RNAs. Several classes of compounds that interfere with the normal polymerization of the viral capsid have been identified, some of which have antiviral activity *in vitro*, and at least one has demonstrated efficacy in animal models (Delaney et al., 2002; Deres et al., 2003; Wang et al., 2012c; Weber et al., 2002). To date, no clinical results have been reported for any capsid-targeted compound. Small interfering (si) RNA has shown promise as a potential therapeutic by potently knocking down one or more viral transcripts (and consequently, antigens), for prolonged periods of time in cell culture and animal models (Klein et al., 2003; McCaffrey et al., 2003; Morrissey et al., 2005). While challenges remain in delivery, the most advanced nucleic acid-based therapeutics may enter the clinic in the near future.

Finally, Myrcludex B, a novel peptide therapeutic that blocks viral entry *in vitro* and in animal models, has advanced to the clinical validation stage (Petersen et al., 2008). Although Myrcludex B likely targets a host, not a viral protein, it is a highly potent inhibitor of HBV entry, and has already completed a Phase 1 study in

Table 1
Nucleoside and nucleotide analog antivirals approved for the treatment of chronic hepatitis B. Data summarized from Current EASL clinical practice guidelines (Papatheodoridis et al., 2012).

Drug	Approval year	% Patients suppressed (<400 copies HBV DNA/mL) after one year of therapy		% Patients with HBsAg seroconversion after one year of therapy	
		$HBeAg^+$ patients	HBeAg patients	$HBeAg^+$ patients	HBeAg patients
Tenofovir disoproxil	2008	76	93	3	0
Telbivudine	2006	60	88	0.5	0
Entecavir	2005	67	90	2	0
Adefovir dipivoxil	2002	21	63	0	0
Lamivudine	1998	44	73	1	0

healthy volunteers (Haefeli et al., 2012). Intravenous doses of 0.1 μg to 5 mg and a subcutaneous dose of 0.8 mg were well tolerated. At the 5 mg intravenous dose, peak plasma concentrations reached >100 ng/mL. Future development of Myrcludex B including therapeutic studies in chronic hepatitis B patients are eagerly anticipated.

3.1.2. Viral targets for the treatment of hepatitis C

HCV encodes 10 distinct proteins, including several with enzymatic functions that provide attractive targets for antiviral therapy. Indeed, every HCV protein has been investigated, and *in vitro* data suggest that all are potential points for intervention. However, the most intensive efforts for drug development have focused on the NS3 protease, the NS5A protein and the NS5B polymerase (Fig. 6); each has been validated as a target by potent efficacy in clinical studies with specific inhibitors. It appears likely that some combination of antivirals targeting these three proteins will evolve into the future standard of care. Additional targets for antiviral intervention include viral entry (by targeting either viral proteins or host receptors such as SRB1), the viral NS4B protein and the host protein cyclophilin A (which interacts with NS5A). As these are less explored in both drug design and clinical studies, they will not be covered in this review.

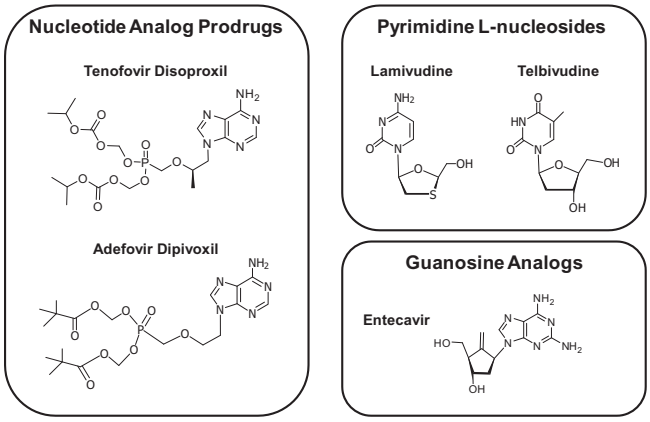

Fig. 5. Approved anti-HBV nucleotide and nucleoside analog antivirals. The nucleosides and nucleotides approved for the treatment of chronic hepatitis B infection fall in three structural classes (1) the acyclic phosphonate (nucleotide analog) prodrugs, which include tenofovir disoproxil and adefovir dipivoxil; (2) the pyrimidine L-nucleosides, which include lamivudine and telbivudine; and (3) the guanosine analog entecavir.

3.1.2.1. NS3 protease inhibitors. The HCV NS3 protease was the first clinically-validated viral target using small molecules. Based on the observation that NS3 was unusually sensitive to inhibition by its own cleavage products, Lamarre and colleagues were able to design and optimize highly potent macrocyclic NS3 inhibitors that act through reversible, non-covalent binding to the protease active site (Pause et al., 2003). These efforts culminated in the discovery of BILN-2061 which was demonstrated in Phase 1 studies to elicit multi-log reductions of HCV RNA during short-term monotherapy (Lamarre et al., 2003). BILN-2061 was unfortunately discontinued due to non-clinical toxicity findings, but was soon followed by many other macrocyclic and acyclic product-like protease inhibitors that demonstrated potent activity in early clinical studies. The most advanced include the Phase 3 inhibitors simeprevir (TMC-435), faldaprevir (BI-201335) and asunaprevir (BMS-650032), as well as several compounds in Phase 2 studies (Fig. 5).

A second class of NS3 protease inhibitors that act by covalently binding to the NS3 protease active site was also discovered. These efforts eventually gave rise to telaprevir (VX-950) and boceprevir (SCH-503034), two compounds which recently became the first approved DAAs to treat HCV in combination with pegylated-IFN and ribavirin. Although less potent than BILN-2061 *in vitro*, telaprevir demonstrated >4log viral load reductions in HCV patients during short-term monotherapy studies when dosed 3× daily at 750 mg (Perni et al., 2006; Reesink et al., 2006). Boceprevir, which has potency similar to telaprevir *in vitro*, had less pronounced activity when tested as a monotherapy in patients at a dose of 400 mg 3× daily (−1.6log reductions in HCV RNA) (Malcolm et al., 2006; Sarrazin et al., 2007b). Boceprevir was subsequently developed and approved at a dose of 800 mg three times daily in combination with pegylated IFN and ribavirin. Interestingly, few if any novel covalent NS3 protease inhibitors are currently being explored clinically, perhaps because of the challenges in optimizing them into low-dose, once-daily therapeutics.

Mechanistically, NS3 protease inhibitors act by preventing the NS3/4 protease from processing the HCV polyprotein during replication. After translation from the plus strand of HCV RNA, the HCV

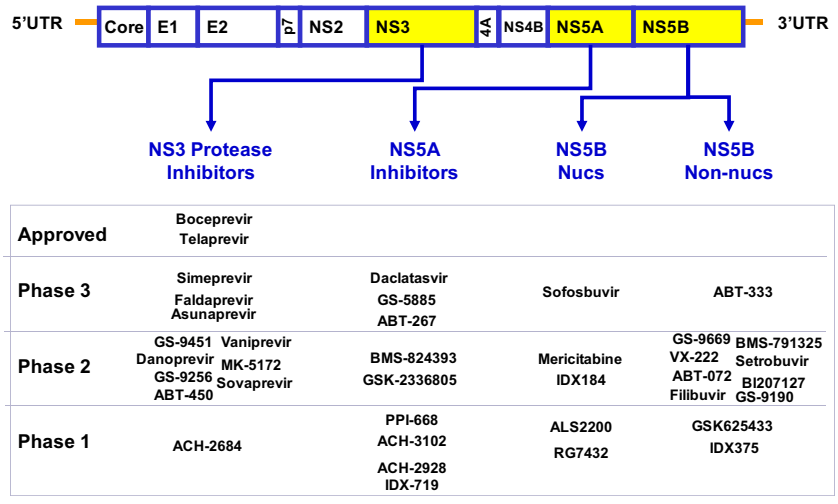

Fig. 6. HCV drug targets and compounds in clinical development. While the HCV genome encodes 10 distinct proteins, 3 have been the most successfully targeted, in terms of the development of clinical drug candidates. These include the NS3 protease, the NS5A replication complex protein and the NS5B polymerase, which has been targeted by both nucleoside and non-nucleoside inhibitors which bind at distinct sites. Compounds approved or in clinical development for each class are indicated, along with the stage of development.

polyprotein is first cleaved by host proteases to separate the structural proteins, and then by the NS2 protease, which liberates itself. The remaining cleavages at the junctions between NS3, NS4A, NS4B, NS5A, and NS5B are catalyzed by the NS3 protease, with NS4A as a co-factor. All of them are potentially blocked by NS3 protease inhibitors. Failure of the polyprotein to be separated into individual nonstructural proteins prevents assembly into replication complexes and replication of the RNA genome.

3.1.2.2. NS5A inhibitors. NS5A burst onto the scene as a critical new HCV target in 2008 when Nettles and colleagues reported efficacy from a proof-of-concept study in patients (Nettles et al., 2008). A single dose of an optimized NS5A inhibitor, daclatasvir (BMS-790052), elicited 3-log reductions in viral RNA in genotype 1 patients at modest doses (10–100 mg); this was later followed by a 14-day monotherapy study, which confirmed the finding (Nettles et al., 2011). This series of inhibitors was originally identified by high-throughput screening using a GT1b HCV replicon cell line; resistance mapping of the initial hit revealed mutations in NS5A (Gao et al., 2010). A great leap forward in potency (down to picomolar levels) was realized when the initial inhibitor scaffold was dimerized. Interestingly, crystal structures of NS5A indicate that the protein also exists as a dimer (Tellinghuisen et al., 2005). The mechanism of action of NS5A compounds is not entirely clear. However, treatment with NS5A inhibitors appears to relocalize the protein within the cell, presumably disruping replication complexes and terminating viral replication (Lee et al., 2011).

Like the protease inhibitors, daclatasvir has demonstrated the ability to significantly improve SVR rates in combination with pegylated-IFN plus ribavirin and has progressed to Phase 3 studies (Pol et al., 2012). Daclatasvir was also used in crucial combination studies (with either the protease inhibitor asunaprevir or the nucleoside NS5B inhibitor sofosbuvir) which demonstrated that IFN was not required to cure HCV patients (Lok et al., 2012). In addition, many other NS5A inhibitors, (e.g. GS-5885, ABT-267) have progressed into Phase 2 development and have shown great promise in enhancing SVR rates in both IFN-containing and IFN-free regimens (Fig. 6) (Lawitz et al., 2012b).

3.1.2.3. NS5B polymerase inhibitors. As with HBV, the HCV NS5B polymerase was immediately viewed as a prime target for antiviral intervention. Intense drug discovery efforts have given rise to two classes of inhibitors: nucleoside analogs and allosteric, non-nucleoside inhibitors.

3.1.2.3.1. Nucleoside NS5B inhibitors. Nucleoside analog inhibitors of the HCV polymerase were first validated clinically by valopicitabine (NM-283), a prodrug of 2′-C-methylcytidine, which demonstrated a modest $1.2\log_{10}$ decrease in viral load at high doses (800 mg once daily) during two-week monotherapy studies (Toniutto et al., 2007). More potent suppression was later observed for R1626, a prodrug of 4′-azidocytidine (3.7log mean reduction in viral load at a dose of 4500 mg twice daily) (Roberts et al., 2008).

Mechanistically, nucleoside analog triphosphates act as substrates for the NS5B polymerase, causing chain termination following incorporation into replicating HCV RNA genomes. In general, nucleoside analogs are not phosphorylated as well as parent ribonucleosides, and have therefore been developed as prodrugs that deliver nucleoside monophosphates to hepatocytes. Addition of the second and third phosphates occurs effectively within cells, generating antiviral concentrations of the triphosphate inhibitors. As an inhibitor class, nucleoside analogs must be carefully assessed for selectivity (inhibition of viral polymerases vs. host polymerases). The development of NM-283, R1626 and other nucleoside analog prodrugs has been halted, based on clinical toxicities that may be related to a lack of selectivity over host polymerases.

Two nucleoside analog prodrugs have progressed into advanced clinical studies: sofosbuvir (GS-7977, in Phase 3) and mericitabine (R1728, in Phase 2) (Fig. 5). Sofosbuvir, in particular, has shown great promise. As a monotherapy, sofosbuvir demonstrated highly potent antiviral activity, with the ability to reduce viral load >4logs within 7 days (Lawitz et al., 2011). In combination with pegylated IFN and ribavirin, sofosbuvir has demonstrated the ability to elicit high rates of SVR, with abbreviated treatment durations (12–24 weeks) in genotype 1, 4 and 6 patients (Hassanein et al., 2012). Recent pilot studies have also resulted in 100% SVR rates in treatment-naïve genotype 2/3 patients in combination with ribavirin alone (Gane et al., 2011). These promising results can be attributed both to the potency of sofosbuvir and the high genetic barrier to resistance for the nucleoside class.

3.1.2.3.2. Non-nucleoside NS5B inhibitors. NS5B has also proven to be highly susceptible to inhibition by allosteric polymerase inhibitors. Indeed, at least four distinct inhibitor binding sites (designated sites I–IV) have been discovered. Distinct inhibitors have been optimized against all four NS5B sites, and each site has been validated in the clinic (demonstration of multi-log HCV suppression) and has representatives currently in clinical development (Fig. 5). Recent reviews cover non-nucleoside NS5B inhibitor in depth (Mayhoub, 2012; Membreno and Lawitz, 2011). Briefly, site I is located in the "thumb" region of NS5B (based on the analogy of the polymerase structure as a "right hand") and can be bound by a class of benzimidazole inhibitors. BI-207127 is the most advanced compound targeting NS5B site I, and is currently in Phase 2 development, in combination with the protease inhibitor BI-201335 and ribavirin. Site II is nearby, also in the thumb region, and is the target of several clinical stage inhibitors, including the thiophene inhibitors VX-222 and GS-9669. Site III is in the "palm" region, and is targeted by several clinical stage inhibitors, including the benzothiadiazine inhibitor setrobuvir (ANA-598), as well as ABT-333 and ABT-072. Site IV is nearby in the palm and include the prototype benzofuran inhibitor HCV-796 (no longer in development) and the current clinical stage inhibitor IDX375. Finally, tegobuvir (GS-9190) represents a unique inhibitor that spans the Site III and Site IV pockets, forming a covalent interaction with the polymerase (Hebner et al., 2012).

3.2. Consequences of differences in replication cycles

As described above, HBV and HCV have fundamentally different replication strategies. With respect to therapy, the quintessential difference is that HBV establishes a long-lived nuclear form of its genome (cccDNA) that persists without active replication, whereas HCV has no long-term archive, so that maintenance of infection relies on continuous viral replication. Potent antiviral inhibition therefore results in different outcomes for the two infections: hepatitis C is curable by a pure block of viral replication within a practical timeframe, whereas hepatitis B is not.

3.2.1. Outcome of therapy with potent replication inhibitors: HBV

For HBV, potent antiviral therapy with nucleoside/tide analogs drives serum HBV DNA to below detectable limits, because the incorporation of nucleoside analog triphosphates into actively replicating viral genomes by the HBV polymerase results in chain termination of viral genomes. As a result, cytoplasmic nucleocapsid particles cannot synthesize new DNA genomes and secrete mature viral particles into circulation. *In vitro* and *in vivo* studies have confirmed this, by demonstrating profound inhibition of both the cytoplasmic replicating forms of the virus and extracellular virions. However, despite highly potent inhibition of active replication, cccDNA reservoirs within infected cells will continually transcribe viral mRNA for export to the cytoplasm. These viral messenger RNAs are sufficient to produce new virus; as soon as active therapy

is stopped, viral production resumes and serum HBV DNA levels rebound. In addition, cccDNA continuously gives rise to other viral antigens, including high titers of serum HBsAg, despite potent nucleoside analog inhibition of new genome replication.

Blocking active HBV replication through nucleoside/tide analog treatment conceptually prevents the cycling of new genomes back to the nucleus to amplify, or replenish, cccDNA levels. However, existing pools of cccDNA have a sufficiently long half-life to maintain chronic infection despite years of potent antiviral suppression. This property of cccDNA was demonstrated *in vitro* and in animal models and, later, in the clinic (Dandri et al., 2000; Delaney et al., 1999; Moraleda et al., 1997; Zhu et al., 2001). Notably, analysis of cccDNA in biopsies from patients undergoing therapy with adefovir dipivoxil demonstrated that, despite potent suppression of serum viremia (4–5$\log_{10}$ copies/ml reductions in viremia) for 48 weeks, cccDNA levels were reduced by only 0.8$\log_{10}$ (Werle-Lapostolle et al., 2004). Similar results were later observed in patients treated with entecavir or lamivudine (Cheng et al., 2011; Wong et al., 2006).

3.2.2. Outcome of therapy with potent replication inhibitors: HCV

Because HCV is an RNA virus that replicates solely in the cytoplasm, a complete block in replication for a time period sufficient to allow decay of all existing RNA genomes is conceptually sufficient to cure infected cells. Indeed, it has been known for two decades that a proportion of patients can achieve lasting remission after a finite course of IFN-α (an antiviral and immunomodulatory therapy) (Shindo et al., 1992). However, it has only recently been demonstrated that the use of direct-acting HCV replication inhibitors alone is sufficient to cure infection without the need for immunomodulatory therapy (IFN and/or ribavirin). For example, the combination of an NS5A inhibitor (daclatasvir) plus an NS3 protease inhibitor (asunaprevir) was able to cure 4/11 patients in a pilot study (Lok et al., 2012). More recently 24 weeks of treatment with the combination of the nucleoside inhibitor sofosbuvir plus daclatasvir demonstrated a 100% cure rate in genotype 1 patients ($n = 29/29$) and 93% in genotype 2/3 patients ($n = 28/30$) after 24 weeks of therapy (Sulkowski et al., 2012). Therefore, it is clear that the goal of HCV treatment is to induce a sterilizing cure, *not* chronic suppression of the virus. It is also worth noting that the specific point at which HCV replication is interrupted (i.e. the specific viral target) is not critical. However, a main point of differentiation between inhibitor classes is the genetic diversity of the target, which can greatly influence the likelihood that resistance will emerge. Indeed, the primary threat to the efficacy of direct-acting antivirals is the development of resistance, as will be discussed in the next section.

3.3. Consequences of differences in genetic variability

The impact of the genetic diversity of HBV and HCV on treatment can be manifested in two main ways: the primary response to treatment for different viral genotypes/subtypes and the emergence of viral resistance during treatment.

3.3.1. Impact of viral genotype on treatment

3.3.1.1. Impact of HBV genotype on DAA therapy. The impact of HBV genetic diversity on the outcome of treatment with DAAs can only be assessed for nucleoside analogs, because this is the only approved class. Although it is not a well studied area, HBV genotype does not appear to have a significant impact on the *in vitro* efficacy of nucleoside analogs. For example, examination of the *in vitro* efficacy of adefovir in patient clinical isolates spanning multiple genotypes did not identify significant differences in EC_{50} (Yang et al., 2003). Clinical efficacy data for adefovir dipivoxil also indicated that the antiviral response (defined as reduction in serum HBV DNA) did not differ significantly between genotypes (Westland et al., 2003). For other nucleoside analogs, examination of primary antiviral responses by genotype either has not identified differences, or they have not been studied explicitly (Liu and Kao, 2008; Raimondi et al., 2010). The homogenous response of different HBV genotypes to nucleoside analogs would be expected *a priori*, because the polymerase active site is typically very highly conserved. However, this response should not be extrapolated to other viral targets. Potential therapies targeting other viral proteins or nucleic acids may encounter differences in response between genotypes, depending on the degree of conservation of the target.

3.3.1.2. Impact of HCV genotype on DAA therapy. Unlike HBV, multiple classes of DAAs for HCV infection have been studied in the clinic, and it became clear very early that the patient's genotype would have a strong impact on the response to therapy. Indeed, this was anticipated, given the differential response of HCV genotypes to pegylated-IFN plus ribavirin (Fried et al., 2002; Manns et al., 2001). The substantial impact of genotype on antiviral response to DAAs was observed when the protease inhibitor BILN-2061 was tested in genotype 2 and 3 patients. Treatment of genotype 1 patients with 500 mg BILN-2061 twice daily for two days led to a homogenous, multilog viral load reduction (Hinrichsen et al., 2004). In contrast, treatment of genotype 2/3 patients with the same regimen resulted in highly variable responses, ranging from no significant viral load reductions to potent responses approximating those observed in genotype 1 patients. (Reiser et al., 2005). These differences can be attributed at the molecular level to polymorphisms in the active site of the protease, which reduce inhibitor binding affinity and thus clinical potency (Thibeault et al., 2004).

The BILN-2061 clinical data were not unique, as other first- and second-generation noncovalent, product-like NS3 protease inhibitors have reduced potency against both genotypes 2 and 3. For example, simeprevir was shown to have reduced biochemical potency against genotype 3, and this translated into a lack of efficacy in genotype 3 patients during short-term monotherapy (Moreno et al., 2012). This phenomenon also extends to the covalent protease inhibitor class, as telaprevir was shown to lack efficacy against genotype 3 HCV in pilot clinical studies (Foster et al., 2011). Recently, a third-generation, noncovalent product-like protease inhibitor discovered by Merck (MK-5172) was shown to have greatly improved *in vitro* potency against genotype 3 NS3 protease (Summa et al., 2012). This translated into potent suppression of HCV RNA in genotype 3 patients during early clinical studies, suggesting that the genotype barrier can be overcome for protease inhibitors (Brainard et al., 2010).

The differential antiviral response of HCV genotypes extends beyond the protease class. First-generation NS5A inhibitors were also noted to have differential *in vitro* activity across HCV genotypes, with genotypes 2 and 3 being less sensitive than genotypes 1 and 4 (Fridell et al., 2011; Huang et al., 2011; Link et al., 2010; Wang et al., 2012a, 2012b). Interestingly, despite *in vitro* potency differences between genotypes, daclatasvir has proven to be highly efficacious in genotype 2 and 3 patients when combined with sofosbuvir (Sulkowski et al., 2012). Although the antiviral activity of daclatasvir has not been delineated in monotherapy studies in genotype 2 and 3 patients, combination studies suggest it has sufficient activity at a 60 mg once-daily dose to be effective in combination with 400 mg of sofosbuvir. Similar to protease inhibitors, optimized second- and third-generation NS5A inhibitors appear to be able to overcome the genotype barrier *in vitro* (Yang et al., 2012). Clinical validation of the pan-genotype activity of next-generation NS5A inhibitors is eagerly awaited.

With regard to potency against different HCV genotypes, *in vitro* experience with non-nucleoside NS5B inhibitors has generally

been similar to that with NS3 protease and NS5A inhibitors. Specifically, substantial decreases in potency (in some cases, >1000-fold compared to genotype 1) are commonly observed when site I–III polymerase inhibitors are tested against non-genotype 1 HCV strains (Herlihy et al., 2008; Paulson et al., 2009). As with NS3 protease inhibitors, differences in drug susceptibility can be explained at the molecular level by amino acid polymorphisms in each non-nucleoside inhibitor binding site on NS5B. As a result, clinical studies with most non-nucleoside NS5B inhibitors have been restricted to genotype 1 patients. Site IV benzofuran inhibitors appear to be unique among the allosteric NS5B inhibitors, in that they retain activity across HCV genotypes (Herlihy et al., 2008); indeed, HCV-796 was observed to have activity in non-genotype 1 patients during Phase 1 studies (Kneteman et al., 2009).

In contrast to NS3, NS5A, and non-nucleoside NS5B inhibitors, nucleoside NS5B inhibitors were recognized early onto be active across all HCV genotypes. In fact, due to the highly conserved nature of the NS5B nucleotide binding pocket, all nucleosides with selective HCV activity have demonstrated consistent activity across genotypes during *in vitro* and animal model studies (Carroll et al., 2009; Herlihy et al., 2008; Lam et al., 2012; Lam et al., 2010; Paulson et al., 2009). These findings have been translated into the clinic. As an example, sofosbuvir demonstrated similar antiviral activity during 7-day monotherapy studies, regardless of whether patients were infected with genotype 1, 2, or 3 (Gane et al., 2011; Lawitz et al., 2011).

3.3.2. Emergence of drug resistance during therapy

3.3.2.1. Emergence of HBV resistance during nucleoside therapy. Based on experience of treating human immunodeficiency virus (HIV) infection with nucleoside analogs, resistance was expected to emerge during nucleoside therapy of hepatitis B. This proved to be the case with lamivudine, first in transplant patients (Bartholomew et al., 1997; Ling et al., 1996) and later in immunocompetent patients (Chayama et al., 1998; Honkoop et al., 1997). Resistance mapped to the nucleotide binding pocket of the HBV polymerase, with multiple different mutational patterns observed clinically. In general, lamivudine resistance is characterized by a mutation in the YMDD motif, with the methionine being mutated to either isoleucine or valine (rtM204I/V, Fig. 6). Mutations at rt204 are typically accompanied by mutations at nearby positions 180 (rtL180 M) and 173 (rtV173L). Although the latter are not strictly required, they augment resistance, and may also enhance the replicative fitness of YMDD mutant viruses (Delaney et al., 2003). These mutations are well documented to confer phenotypic resistance to lamivudine in cell-based drug susceptibility assays.

Lamivudine resistance mutations were also later demonstrated to confer broad cross-resistance to other, structurally related L-nucleoside analogs, including telbivudine, emtricitabine and clevudine (Yang et al., 2005). In large studies, lamivudine resistance emerged after several months of therapy and was observed in 20–25% of patients after one year of treatment; the number increased with treatment duration, with resistance emerging in approximately 80% of patients after five years. Unfortunately, resistance is associated with rebounds in serum viremia and the loss of clinical benefits, including increases in serum transaminase levels and progression of liver fibrosis. Consistent with its *in vitro* profile, telbivudine appears to have a similar resistance profile to lamivudine clinically, although longer term data (i.e. beyond two years) have not been reported (Liaw et al., 2009).

Adefovir dipivoxil was the second nucleoside/tide analog to be approved for the treatment of chronic hepatitis B, and has a better resistance profile than lamivudine. Nevertheless, although resistance was not observed in the first year of Phase 3 clinical studies, resistance did emerge in approximately 3% of patients after two years (Angus et al., 2003). Adefovir resistance mapped to different loci in the HBV polymerase than lamivudine resistance, which was not unexpected, since adefovir retained *in vitro* and clinical activity against lamivudine-resistant viruses (Westland et al., 2005; Yang et al., 2005). Specifically, adefovir resistance emerged at positions 236 (rtN236T) and at position 181 (rtA181V/T) in the polymerase. These mutations were confirmed to confer low levels of resistance to adefovir *in vitro*, which are sufficient to cause a rebound in viremia (Qi et al., 2007). Long-term resistance surveillance during clinical studies indicated that the frequency of resistance to adefovir dipivoxil continued to increase with time, with approximately 30% of patients showing evidence of resistance after five years.

Entecavir and tenofovir DF, the current agents of choice for hepatitis B, have significantly higher barriers to resistance than lamivudine, adefovir or telbivudine. In treatment-naïve patients, emergence to entecavir is low (<1% of patients acquire resistance after one year of therapy, and only 1.2% of patients have resistance after 3–5 years). However, in patients previously treated with lamivudine, entecavir resistance emerges at high rates, and up to 50% of patients have resistance after five years (Tenney et al., 2009). These findings are consistent with the mutational patterns identified in entecavir-resistant HBV. Resistance to entecavir requires lamivudine resistance mutations (e.g. rtL180M plus rtM204I/V), plus one or more entecavir-specific polymerase mutations, which may include rtI196L, rtS2024I/G, rtL250I/V or several mutations at position rtT184. The requirement for multiple mutations explains the high barrier to resistance in treatment-naïve patients, while the overlap with lamivudine resistance explains the high failure rate in lamivudine-experienced patients.

In contrast to lamivudine and adefovir dipivoxil, resistance has not been seen to date for tenofovir DF in either treatment-naive (no resistance after five years) or treatment-experienced patients (Marcellin et al., 2013). *In vitro* studies have demonstrated low-level cross resistance of tenofovir to adefovir-resistant HBV (Qi et al., 2007). However, the 300 mg once-daily dose of tenofovir DF appears to provide sufficient drug levels in liver to overcome these small susceptibility changes, as adefovir dipivoxil failure/non-responder patients respond clinically to tenofovir DF (Berg et al., 2010; Patterson et al., 2011). Overall, the availability of entecavir and tenofovir DF has minimized the emergence of resistance in treatment-naïve patients, and tenofovir DF alone or in combination has enabled the management of patients who have failed earlier agents. The clinical management of HBV drug resistance is addressed in further detail in a recent review (Zoulim and Locarnini, 2012).

3.3.2.2. Emergence of HCV resistance during therapy. Given the tremendous natural genetic diversity of HCV, it was anticipated that resistance might emerge rapidly during therapy with specific antiviral agents. This was confirmed clinically during monotherapy studies with multiple classes of DAAs. Initial studies with the covalent NS3 protease inhibitor telaprevir demonstrated that numerous strains of resistant virus emerged within a week of starting therapy (Sarrazin et al., 2007a). Resistance mutations mapped to the active site of the protease, and included single or double mutations at positions V36, T54, R155, and A156. These were confirmed to confer various levels of phenotypic resistance in both biochemical and cell-based assays. Telaprevir mutations confer extensive cross-resistance to boceprevir (also a covalent inhibitor) and a subset of mutations, including mutations at R155 and A156, confer cross-resistance to non-covalent NS3 inhibitors (Lin et al., 2005; Tong et al., 2006). Non-covalent inhibitors such as simeprevir, danoprevir and GS-9451 also select mutations at R155 and A156, as well as multiple mutations at position D168 (Dvory-Sobol et al., 2012; Forestier et al., 2011; Reesink et al., 2010).

Overall, first- and second-generation protease inhibitors have a low genetic barrier to resistance, which is typically manifested as genotypic resistance and viral breakthrough within the first few

days of monotherapy. In fact, the recent application of "deep sequencing" has shed new light on the emergence of resistance. Deep sequencing of clinical samples from monotherapy studies of the non-covalent NS3 inhibitors GS-9256 and GS-9451 indicated that mutations can be detected within 24 h of a single drug dose (Svarovskaia et al., 2012). Multiple resistant strains, such as numerous R155, D168 and A156 variants, were also commonly identified in single serum samples, confirming the quasispecies nature of HCV. Rather than generating *de novo* resistant variants, potent suppression of wild-type HCV reveals pre-existing mutants, which generally circulate at levels below the limit of detection for conventional (population) sequencing techniques. However, the pre-existence of resistance mutations as dominant species (i.e. natural polymorphisms) has also been documented in individual patients, and can cause primary non-response to treatment (Bartels et al., 2008; Kuntzen et al., 2008).

Similar to protease inhibitors, resistance also emerged rapidly to first-generation NS5A inhibitors, including daclatasvir and GS-5885. Mutations emerged in the first domain of the NS5A protein at residues M28, Q30, L31, and Y93 in genotype 1a and at L31 and Y93 in genotype 1b (Lawitz et al., 2012b; Nettles et al., 2011). The story is similar for non-nucleoside NS5B inhibitors, with each of the 4 inhibitor binding sites having a "signature" set of mutations that confer resistance. Site I NS5B inhibitors (e.g. BI 207127) select mutations at position P495, P496 and V499 (Lagace et al., 2010). Site II inhibitors (e.g. filibuvir, VX-222, and GS-9669) select mutations at positions L419, R422, M423, I482, A486, and V494 (Bartels et al., 2010; Lawitz et al., 2012a; Wagner et al., 2011). Site III inhibitors (e.g. setrobuvir) select mutations at positions G554, Y448, and M414 (Lawitz et al., 2010). Finally, the prototype site IV inhibitor HCV-796 selected resistance mutations at position C316 in NS5B during clinical studies, as well as at residues S365 and M414 during *in vitro* studies (Howe et al., 2008). The emergence of resistance to non-nucleoside NS5B inhibitors generally occurs rapidly during monotherapy, indicating a low genetic barrier to resistance for this inhibitor class.

In contrast to NS3 protease and NS5A inhibitors and the non-nucleoside NS5B inhibitors, nucleoside analog NS5B inhibitors do not readily select for resistance. This has been attributed to the virus's inability to mutate in the highly conserved nucleotide-binding pocket without significantly reducing its replicative fitness. *In vitro* resistance studies, which typically require prolonged selection periods, have yielded the NS5B active site mutation S282T for a variety of 2′-substituted ribonucleoside analogs (e.g. sofosbuvir, 2′-C-methyl adenosine, 2′-C-methyl cytosine) (Lam et al., 2012; Le Pogam et al., 2006; Migliaccio et al., 2003). Interestingly, the 2′-substituted guanosine analog GS-938 and the 4′-substituted cytosine analog R1479 select distinct sets of mutations *in vitro*: S15G/C223H/V321I and S96T/N142T, respectively (Lam et al., 2011; Le Pogam et al., 2006). Despite extensive clinical experience with multiple 2′-substituted nucleoside analogs, the S282T mutation has not emerged as a problem. Overall, the high barrier of resistance observed with nucleoside analogs is consistent with the pangenotypic activity observed with this class, suggesting that they are a robust "backbone" for therapy.

3.3.2.3. Differences in drug resistance between HBV & HCV. As summarized above, the kinetics of drug resistance appear to be significantly different for HBV and HCV. On one extreme, the appearance of HCV resistant to some DAAs can be detected within hours. In contrast, resistance to even first-generation HBV therapies such as lamivudine was comparatively slow, taking months to years. However, it is important to note that these differences between HBV and HCV are difficult to compare, because the diversity of drugs used to treat HBV is lower than for HCV. In fact, resistance to nucleotide analogs appears to be infrequent for both HBV (e.g. tenofovir DF) and HCV (e.g. sofosbuvir). Currently, there are no DAAs against HBV proteins other than the nucleotide polymerase inhibitors and, therefore, no data on the kinetics of resistance to other targets, such as the HBV capsid. Nevertheless, differences in genomic organization and replication strategy are likely to influence the emergence of resistance emergence for these two viruses.

While intrinsic polymerase error rates are similar between HBV and HCV, HBV is clearly more constrained genetically, as its compact genome makes extensive use of overlapping reading frames. Most HBV mutations must therefore be tolerated in multiple reading frames to yield viable viruses. In contrast, the HCV genome is less constrained, although RNA secondary structures required for replication may limit some mutations. HBV also has a stable genomic archive (cccDNA), providing a central starting sequence (or set of sequences) from which mutations must arise to generate resistance. The repopulation of the liver with a drug-resistant virus will require establishment of new cccDNA pools encoding the mutation; this is likely be a slow process, as it may require either the decay of "wild-type" cccDNA within infected cells, and/or the generation of new, uninfected target hepatocytes. In contrast, HCV relies on active replication to maintain infection, and it can therefore evolve continuously with each replication cycle, without reverting to a central "archive" sequence. This leads to the constant generation, and therefore the pre-existence, of drug-resistant species. Although most mutant viruses will be less fit, many will be maintained at significant titers, and are readily detectable when drug pressure is applied.

4. Future directions for therapy

4.1. Future directions for hepatitis B

As summarized above, the availability of tenofovir DF and entecavir has enabled serum viral load to be chronically suppressed to the limit of detection using safe, well-tolerated, once-daily medications. Chronic viral suppression translates into significant long-term clinical benefits (normalization of ALT and improvements in liver histology) (Marcellin et al., 2013). However, despite the potent and durable on-therapy effects of these drugs, the reservoir of cccDNA in infected hepatocytes remains largely unaffected. This problem is illustrated by the very low levels of HBsAg seroconversion observed in patients (0–3% after a year of therapy) (Table 1). Five years of continuous potent antiviral suppression in $HBeAg^+$ patients with tenofovir DF yielded HBsAg loss and seroconversion rates of approximately 10% and 8%, respectively (Marcellin et al., 2013). However, HBsAg seroconversion events in $HBeAg^-$ patients are extremely rare, even after many years of therapy. Thus, while the availability of tenofovir DF and entecavir allows the durable management of chronic hepatitis B, they are likely to represent lifelong therapies for most patients.

The current unmet need for chronic hepatitis B is enhancing HBsAg seroconversion. Ideally, new treatment paradigms can be identified that elicit durable suppression of both viremia and antigenemia (HBsAg seroconversion) with finite courses of treatment. This will require a fundamental change in therapeutic approach, and represents a formidable challenge to pharmaceutical and clinical researchers. The occasional spontaneous resolution of infection in untreated patients and the frequency of HBsAg seroconversion observed with nucleoside/tide and IFN-based therapies give hope that it is possible to establish immune control of virus replication. However, the therapeutic strategies needed to enhance HBsAg seroconversion rates are unclear. Several studies are now focusing on the combination of IFN with nucleoside/tide analogs, but new approaches must also be explored. These may include new treatments that target viral or host proteins necessary for establishing

or maintaining infection, or directly target the host immune system to prompt an antiviral immune response.

Because tenofovir and entecavir are highly effective at blocking viral DNA replication, new therapeutics targeting viral or host proteins must do something beyond this. Directly impacting cccDNA copy number would be ideal, but is a difficult problem. Alternatively, it may be possible to "silence" cccDNA through epigenetic modification. Recent studies suggest that epigenetic alteration of cccDNA is an important mechanism of the antiviral activity of IFN-α (Belloni et al., 2012). Modulation of host enzymes that modify or "read" histones bound to cccDNA is therefore a potential therapeutic approach, although selectivity for viral over host histones will present a significant challenge. Because the high antigen loads present in chronic hepatitis B patients are likely to underlie T-cell dysfunction, another possible therapeutic approach is to inhibit viral antigenemia at points downstream of transcription, preventing the production, secretion, or circulation of viral antigens to help restore an antiviral immune response. However, because HBV is highly dependent on host machinery for the production and secretion of its antigens, selectivity will likely be a challenge for this approach as well.

Based on our current understanding, the loss of circulating HBsAg and seroconversion to anti-HBs represent clearance of the vast majority of infected hepatocytes by the host immune system. The restoration of antiviral immunity is therefore likely to be necessary to maintain long-lived remission. This is supported by the observation that a bone marrow transplant from an anti-HBs + donor to a chronically infected recipient led to sustained clearance of infection (Lau et al., 1997). Conceptually, there are many ways to target the immune system to promote an effective antiviral immune response. Modulation of innate immunity could be brought about by targeting innate immune mechanisms, such as toll-like receptors, NOD-like receptors or RIG-I like receptors, through the use of cytokines beyond IFN-α. Adaptive immune responses could be modulated either by overcoming dysfunction of existing HBV-specific T cells, by blocking negative T-cell costimulatory receptors or activating positive costimulatory receptors, or by generating new HBV-specific T cells through therapeutic vaccination.

Some of these areas are already being pursued with some degree of success. IFN-λ which works through a receptor present in hepatocytes, but restricted in many other tissues, may provide antiviral activity with greater selectivity than IFN-α. It is now in active clinical development. The orally available, small-molecule TLR7 agonist GS-9620 has recently demonstrated the ability to induce HBsAg loss and/or full seroconversion in animal models, and is currently in clinical development (Lanford et al., 2011; Menne et al., 2011). However, while immune modulation in chronic hepatitis B is attractive, and may ultimately be necessary, it presents significant safety challenges and must be approached with caution in the clinic.

4.2. Future directions for hepatitis C

The combination of pegylated IFN-α and ribavirin has been the standard of care for many years, and is able to cure approximately 50% of genotype 1 patients and 80% of genotype 2/3 patients. The approval of the NS3 protease inhibitors boceprevir and telaprevir for use in combination with pegylated IFN-α plus ribavirin has further raised cure rates to 70–80% in genotype 1 patients. However, boceprevir and telaprevir have each added to the side-effect burden of IFN/ribavirin, require dosing several times a day, and are not approved to treat genotypes 2–6 HCV. In the near future, it appears likely that a number of new agents, including NS3 protease, NS5A, and NS5B inhibitors, will be approved to treat genotype 1 HCV in combination with pegylated IFN-α and ribavirin. This new wave of compounds is expected to have several benefits over current telaprevir- and boceprevir-based therapies; including better side-effect profiles, once-daily dosing and the potential to shorten the overall course of treatment, and better efficacy, particularly in the case of nucleoside analogs, which have a superior resistance profile compared to the NS5A and NS3 protease inhibitors.

Because recent combination studies with DAAs have made it clear that IFN and ribavirin are not needed to cure hepatitis C, there is now an intense focus on accelerating the clinical development of all-oral, IFN- and ribavirin-free regimens. In the near future, we are likely to see the approval of regimens composed of 1–3 DAAs (with orthogonal resistance profiles) combined with ribavirin to treat genotypes 1, 2 and 3. The number of drugs needed to achieve high cure rates will depend on the potency and genetic barrier to resistance of the agents involved. Numerous studies have demonstrated that it is beneficial to retain ribavirin in IFN-free regimens to achieve the highest SVR rates. As an approved drug, ribavirin is likely to remain an option in this role. Nevertheless, it is a suboptimal drug in terms of convenience (twice-daily, high dose) and side effects (e.g. anemia). It is therefore likely to be supplanted as additional DAAs with superior profiles become available. In this regard, we are likely to see regimens free of both IFN and ribavirin, first for the treatment of genotype 1, for which there are considerably more compounds in development compared to genotypes 2–6). Beyond this, the ultimate goal for HCV therapy will be to achieve cure rates approaching 100% in all patients, regardless of genotype, with safe, well-tolerated and simple treatment regimens. Based on the advances made in identifying "pan-genotypic" NS3 protease, NS5A and nucleoside NS5B inhibitors, it appears that this will be possible in the foreseeable future.

5. Conclusions

Chronic hepatitis B and C continue to represent huge unmet medical needs. Although there are parallels in the diseases, they present different therapeutic challenges, particularly due to their substantially different replication cycles and distinct genomic organization. At the highest level, the fundamental difference in therapy is that hepatitis C is curable with potent DAAs within a reasonable timeframe, based on the lack of a long-lived genome, while hepatitis B is not, since current antiviral agents do not directly impact cccDNA. The availably of potent, safe and well-tolerated nucleoside/tide analogs with high resistance barriers to treat hepatitis B has enabled viral replication to be chronically controlled. However, the next step, enhancing HBsAg seroconversion rates using finite treatment courses (i.e., eliciting a functional cure) represents a formidable challenge for the future. In contrast, the treatment of hepatitis C is advancing at an extraordinarily rapid pace. The development of potent antivirals directed against multiple viral targets with orthogonal resistance profiles will transform therapy. Although new agents are likely to be approved in combination with pegylated IFN-α and ribavirin in the immediate future, the focus is clearly shifting toward eliminating IFN and ribavirin, with their associated side effects. Based on promising early results from combination studies excluding IFN and ribavirin in genotype 1 patients, this appears highly achievable. Looking further ahead, simple, highly effective regimens that can treat patients infected with all genotypes of HCV will be the "ultimate goal".

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