Pharm Res (2010) 27:1184–1202
DOI 10.1007/s11095-010-0112-z

EXPERT REVIEW

Drug Delivery Systems and Liver Targeting for Improved Pharmacotherapy of the Hepatitis B Virus (HBV) Infection

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Received: 15 December 2009 / Accepted: 1 March 2010 / Published online: 24 March 2010

ABSTRACT In spite of the progress made in vaccine and antiviral therapy development, hepatitis B virus (HBV) infection is still the most common cause of liver cirrhosis and hepatocellular carcinoma, with more than 400 million people chronically infected worldwide. Antiviral therapy with nucleos(t)ide analogues and/or immunomodulating peptides is the only option to control and prevent the progression of the disease in chronic hepatitis B (CHB)-infected patients. So far, the current antiviral monotherapy remains unsatisfactory because of the low efficacy and the development of drug resistance mutants. Moreover, viral rebound is frequently observed following therapy cessation, since covalent closed circular DNA (cccDNA) is not removed from hepatocytes by antiviral therapy. First, this review describes the current pharmacotherapy for the management of CHB and the new drug candidates being investigated. Then, the challenges in the development of drug delivery systems for the targeting of antiviral drugs to the liver parenchyma are discussed. Finally, perspectives in the design of a more efficient pharmacotherapy to eradicate the virus from the host are addressed.

KEY WORDS antiviral pharmacotherapy · drug delivery systems · hepatitis B virus (HBV) · liver targeting · nanotechnology

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INTRODUCTION

Hepatitis B virus (HBV) is a major cause for acute and chronic hepatitis in humans (1). The prevalence of chronic HBV (CHB) infection has declined recently in endemic regions, mostly as a result of successful mass immunization programs (2). However, CHB still affects approximately 400 million people worldwide (3), and 20–30% of them will die due to exacerbations of chronic liver disease, such as cirrhosis and hepatocellular carcinoma (HCC) (4). As a consequence of the high morbidity and mortality related to CHB, the World Health Organization (WHO) includes this infectious disease as one of the ten leading causes of death (5). Since in CHB the elapsed time period between the infection and the clinical illness is usually several years, there is a great chance for implementing anti-HBV therapy (5).

There are currently seven drugs approved for the management of CHB in Argentina and other developing countries and six by the US FDA. These drugs can be classified into two main families: (i) immunomodulating peptides (IMPs), such as interferon-α (6,7), and (ii) nucleos(t)ide analogues (NAs), such as lamivudine (8–10).

IMPs are effective in about 30% of the infected patients (6). They are sensitive to the acid gastric environment and

are administered by subcutaneous (s.c.) injection. Considering that this is a chronic treatment, this represents a main drawback affecting patient compliance and adherence. Also, IMPs are associated with serious adverse effects, and they are very expensive (7).

NAs target the HBV polymerase, inhibiting viral replication. They are administered by oral route and do not present serious adverse effects. They have clinical benefits, such as reduction in hepatic necroinflammatory activity and, thus, improvement of liver histology, normalization of serum alanine aminotransferase (ALT) levels, and enhanced rates of hepatitis B e antigen (HBeAg) loss and seroconversion to HBeAg antibodies. However, long-term treatment may induce viral resistance (8–10). Factors associated with resistance include dynamics of viral production and clearance, fidelity and efficiency of the viral polymerase, patient compliance and adherence, genetic factors that relate to drug metabolism and bioavailability, and features of the antiviral agent itself, including structure and cross-resistance profile.

A common adverse effect that is frequently observed following therapy interruption with all these anti-HBV agents is viral rebound. This phenomenon stems from the presence of the episomal covalently closed circular form of the HBV genome (cccDNA); cccDNA is not removed by the current antiviral therapy during the course of chronic infection (11). So far, the current monotherapy remains unsatisfactory. Thus, there is an urgent need for better treatment strategies that can (i) actively eradicate cccDNA, which is the primary source of HBV perpetuation (12), and (ii) improve the activity of the existing anti-HBV agents by targeting them to HBV-infected cells (13).

First, the present review describes the pathogenesis and the current pharmacotherapy for the management of CHB and the new drug candidates being investigated. Then, the challenges in the development of drug delivery systems (DDS) for the targeting of antiviral drugs to the liver parenchyma are thoroughly discussed. Finally, perspectives in the design of a more efficient pharmacotherapy to eradicate the virus from the host are addressed.

PATHOGENESIS OF HBV INFECTION

HBV is the prototype member of the *Hepadnaviridae* family (hepatotropic DNA virus). Three modes of HBV transmission (perinatal, sexual and parenteral/percutaneous) have been recognized among humans, its only known natural host; blood is the most important vehicle for transmission, but semen and saliva have also been implicated (14,15). Approximately 1–5% of the patients infected as adults and more than 90% of those infected as neonates fail to mount an effective immune response to clear the virus and develop a lifelong chronic infection (16). The quality, quantity, and kinetics of the host innate and adaptative immune responses shape the clinical outcomes of HBV infection (17,18). The resolution of acute infection is related to a vigorous, polyclonal and multi-specific immune response to viral antigens, while an inappropriate quali-quantitative immune response may lead to chronicity (19).

Viral clearance requires the synergistic action of several components of the immune system: (i) a potent B cell response to neutralize circulating viruses, (ii) an efficient cytotoxic T-cell response (CD8+) against infected hepatocytes and (iii) a T-cell secretion of antiviral cytokines (e.g., interferon gamma and tumor necrosis factor alpha, under the regulation exerted by helper T-cells (CD4+) (20). Activation and recruitment of non-HBV-specific innate immune effector leukocytes into the infected liver also contributes to the immunopathogenesis of liver injury (21).

CHB is the result of a continuing attack of infected cells by the host immune system that is not vigorous enough to eradicate all the infected hepatocytes (20,22). It was recently reported that platelets also contribute to the pathogenesis of liver disease by promoting the recruitment of virus-specific cytotoxic T-cells into the liver (23). Taking into account the pathogenesis of HBV infection, two main strategies may be pursued to eradicate the viral infection: (i) enhancement of the host immune response or/and (ii) inhibition of viral replication.

HBV GENOME AND REPLICATION

The infectious virion circulates as a 42-nm Dane particle that comprises a nucleocapsid (core) surrounded by a lipid bilayer studded with complexes of viral glycoproteins (Fig. 1) (16); the nucleocapsid contains the viral genome and the viral polymerase (5,24) (Fig. 2). Cellular proteins, including chaperones and protein kinases, are packaged into nucleocapsids as well (25). The HBV genome is a partially double-stranded relaxed circular DNA of 3,200 nucleotides that consists of a full-length negative strand and a shorter positive one. The 5′ end of the negative strand is covalently linked to the viral polymerase, whereas the 5′ end of the positive strand bears an oligoribonucleotide. The HBV genome contains four major overlapping open reading frames (ORFs) encoding for the envelope proteins (pre-S1, pre-S2 and S), the nucleocapsid proteins (precore and core), the polymerase (P), and the promiscuos transcriptional transactivator X protein (26).

HBV replicates its DNA genome via a reverse transcription step by using its own polymerase. The template for reverse transcription is pgRNA, an RNA intermediate synthesized by the cellular RNA polymerase II. HBV is considered a pararetrovirus, since it exhibits a DNA

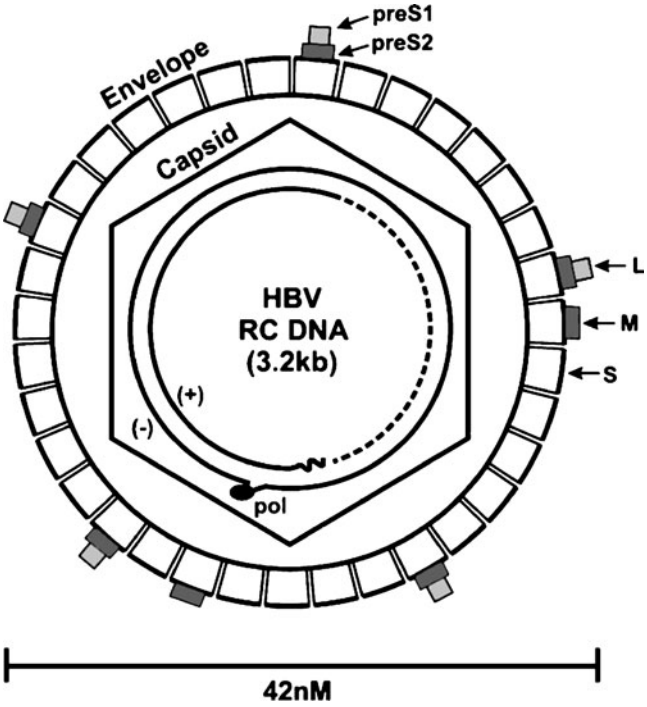

Fig. 1 Structure of the HBV virion (reproduced from ref 16 with permission from Elsevier).

genome together with a reverse transcriptase. It is also the major etiologic agent of HCC, even though it does not contain known oncogenes (16).

Unlike DNA-dependent DNA polymerases, HBV reverse transcriptase (RT) does not hold proofreading activity, leading to a high rate of mutations (1.4 to 3.2×10^{-5} nucleotide substitutions/site/year) (27), which represents a 10^4-fold increase with respect to other DNA viruses and more closely resembling retroviruses (e.g. Human Immunodeficiency Virus, HIV). This fact, together with the large amount of viral particles produced (about 10^{12}/day) may result in the selection of HBV quasispecies containing several mutations within its genome (28). Some of these mutations might be detrimental to the virus, while others might improve its survival in specific environments (29). Furthermore, some of them may alter the overlapping gene and its translational product as well. For example, since the envelope gene is completely overlapped by the polymerase gene, nucleotide substitutions within the S gene selected as a consequence of immunotherapy may or may not be translated into an amino acidic substitution within the viral polymerase (Fig. 1). These amino acidic changes may influence viral replication capability and produce resistance to antiviral drugs. Similarly, nucleotide changes selected within the polymerase gene as a consequence of therapy with NAs may or may not result in amino acidic subtitutions within the S protein. As a consequence, reduction in the antigenicity of this envelope glycoprotein and emergence of immune escape mutants may occur (29–32).

HBV replicates predominantly in host hepatocytes, while replication in other cell types is still a matter of discussion (33,34). A simple outline of the HBV replication cycle is described in Fig. 3. After virions enter hepatocytes through a still unknown receptor, the virus uncoats, and the relaxed circular genome is directed to the nucleus. The cellular machinery collaborates in repairing the partially relaxed circular genome to cccDNA (35). The degree of capsid proteins phosphorylation may be critical for this step. In sharp contrast to retroviruses, integration of viral HBV-DNA to the host genome is not essential for viral replication (16). However, it is considered an important factor associated with liver carcinogenesis, since almost all HBV-related HCCs contain randomly integrated viral DNA into the cell's genome (7). Experimental evidence indicates that viral integration during CHB may lead to chromosomal deletions, translocations, transpositions or stimulation of cellular oncogenes expression (7). Fortunately, viral DNA integration to the host genome occurs at low frequency.

The cccDNA is present as a viral minichromosome in low amounts (from 10–50 genomes per infected hepatocyte). Moreover, stable cccDNA is not lost during mitosis and usually persists during effective antiviral therapy. However, entecavir (ETV) was reported to reduce cccDNA levels in duck hepatitis B virus (DHBV)-infected hepatocytes (36). The durability and stability of cccDNA is a key factor in the long-term maintenance of CHB infection and in the viral rebound after therapy cessation, as it serves as the template for the transcription of all the viral mRNAs: (i) pgRNA that serves as the template for reverse transcription and the synthesis of the core and the polymerase and (ii) 3 subgenomic mRNAs, necessary for the translation of the

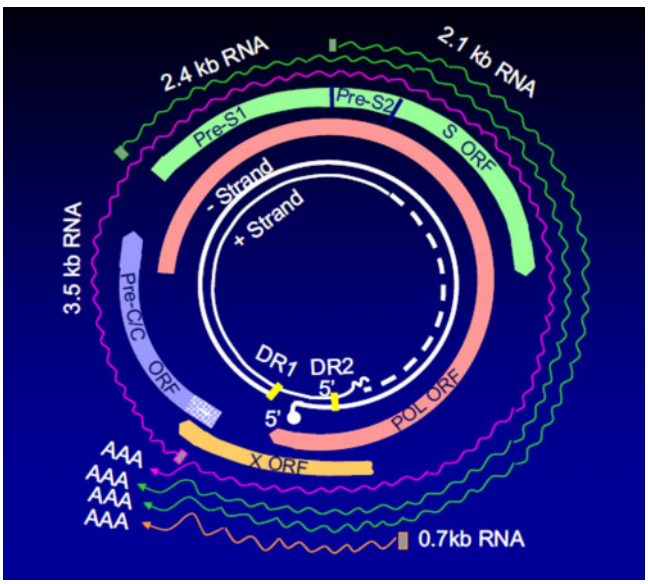

Fig. 2 HBV genome (reproduced from Ref 5 with permission from Elsevier).

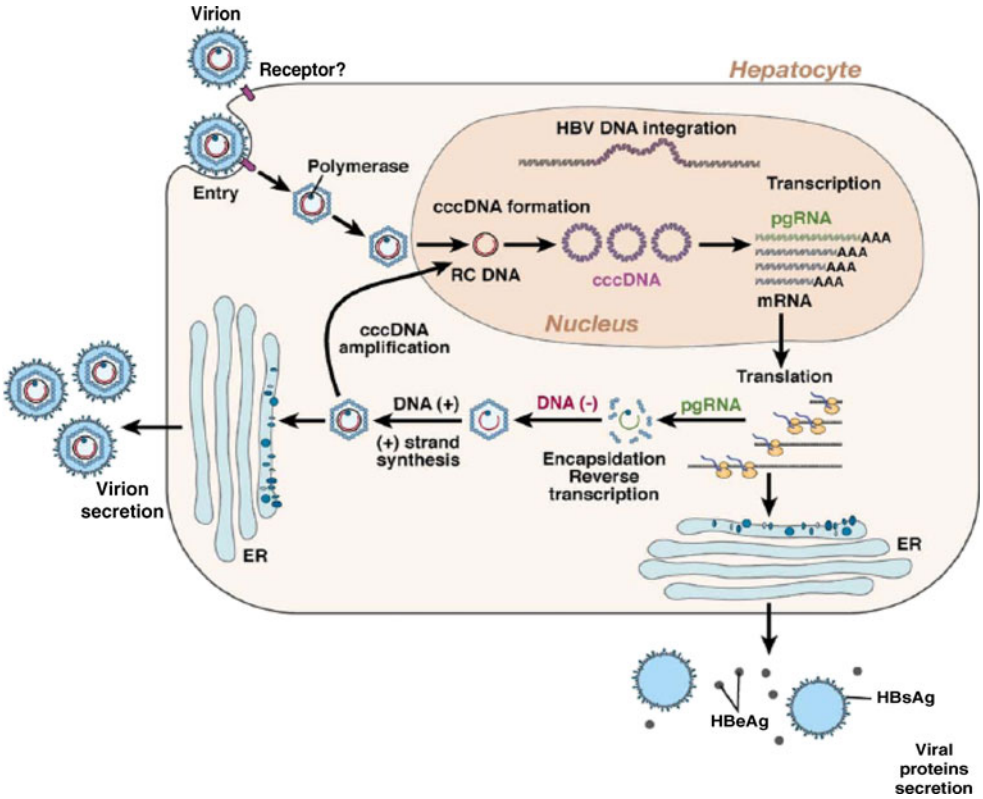

Fig. 3 HBV replication cycle (reproduced from Ref 35 with permission of Elsevier).

envelope proteins and the synthesis of X protein. This latter protein is capable of transactivating a wide range of viral and cellular genes, and it may play a role in liver oncogenesis and in viral replication as well.

Viral mRNAs are then transported to the cytoplasm where translation of the correspondent HBV proteins, nucleocapsid assembly, and viral replication occurs. Nucleocapsid formation requires the binding of the viral polymerase to a stem-loop structure located at the 5′ end of the pgRNA called *epsilon*. The polymerase bound to *epsilon* serves as a protein primer for viral DNA synthesis. After completing the synthesis of negative-strand DNA, the RNA is degraded by the activity of the viral RNase-H associated to the Pol protein, followed by positive-strand synthesis and circularization of the viral genome. Then, the capsid containing partially double-stranded circular genome may be involved in one of the two following pathways: (i) formation of mature virions after envelope assembly in the endoplasmic reticulum of the host cells and secretion into serum or (ii) recycling to the nucleus to maintain or amplify the number of cccDNA copies in the infected cell.

The potential targets for antiviral therapy are then deduced from the viral life cycle.

Since the HBV polymerase is an essential and multifunctional protein for viral replication and has been the main target of anti-HBV drug development, the following section describes the functions and structure of this highly versatile viral enzyme.

HBV POLYMERASE

The replication of the viral genome is a multi-step process catalyzed by the virus-encoded polymerase, which is responsible for (i) encapsidation of the viral pgRNA, (ii) priming of DNA synthesis, (iii) reverse transcription of the pgRNA to the double-stranded DNA, and (iv) RNase-H activity for the degradation of the pgRNA (22).

The structure of the HBV polymerase consists of four conserved domains that have been determined by comparison with the HIV RT sequence and confirmed by genetic and functional studies: (i) the terminal protein involved in the priming for reverse transcription, (ii) the spacer region, (iii) the RT/DNA polymerase domain, and (iv) the RNAse-H region (Fig. 4). The terminal protein harbors the primer for reverse transcription; the synthesis of the minus strand-DNA is initiated in this domain. Point mutations in this region are reflected as the inability of the viral polymerase to encapsidate the viral pgRNA, precluding HBV replication (37). The spacer region is dispensable for enzymatic activity and tolerates mutations, but its own function is still unknown. The RT is a functional domain where reverse transcription and synthesis of the second DNA strand take place. It can be divided into seven subdomains labelled A, B, C, D, E, F and G. Its catalytic site is placed within the subdomain C, around the YMDD (tyrosine-methionine-aspartate-aspartate), a highly conserved motif among hepadnaviruses and retroviruses that seems to be the

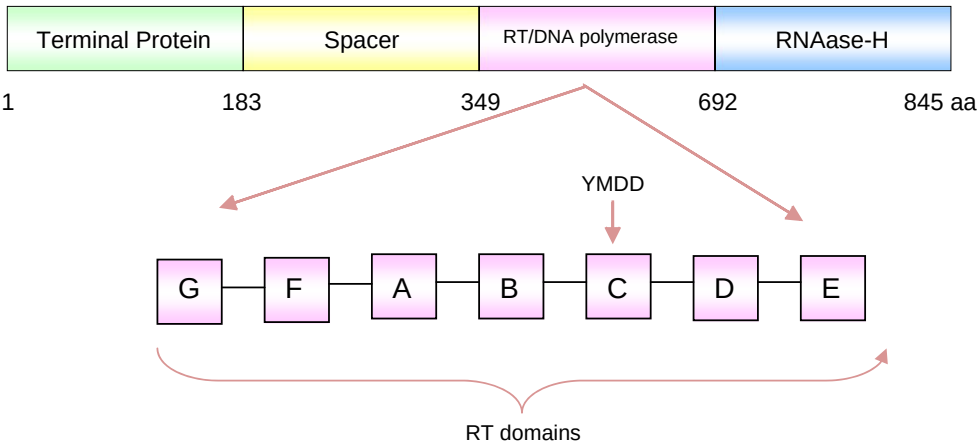

Fig. 4 Schematic representation of the HBV polymerase. The four conserved domains are shown (Terminal Protein, Spacer, RT/DNA polymerase, RNAse-H) as well as the seven RT/DNA polymerase domains (A, B, C, D, E, F and G). The catalytic site of the HBV RT is indicated with an arrow pointing to the YMDD motif of the RT/DNA polymerase domain C; aa = aminoacid.

nucleotide recognizing site. Drug-resistant mutants harbor point mutations in C and A or B subdomains. Moreover, point mutations in the YMDD motif are the most common and clinically relevant; many of them may be selected during antiviral therapy with NAs (e.g., lamivudine), contribuiting to therapy failure. Finally, the RNAse-H region of the viral polymerase is another functional domain where degradation of the pgRNA takes place. Some amino acid sequences located within the RT and the RNAse-H domains of this enzyme are highly conserved and harbor CD8+ and CD4+ T-cells epitopes that might contribute to both the viral clearance and the development of subclinical forms of hepatitis B disease (38). In addition, the HBV DNA polymerase is significantly different from both nuclear and mitochondrial human DNA polymerases regarding the inhibitory specificity of NAs (22), providing an excellent target for antiviral drug design.

CURRENT PHARMACOTHERAPY

Many strategies have been envisioned to eradicate persistent viral replication and prevent progression to active liver disease, liver failure and death (39). Since many stages of the viral life cycle have not been elucidated yet, few targets are available for the development of novel antivirals. For instance, HBV polymerase has been the main target of anti-HBV drug development due to its active function in viral replication.

On the other hand, considering the significant role that the host immune system plays in eradicating the pathogen, the immune-based therapy has become a good option for CHB-infected patients. This kind of strategy is built on the concept of restoring HBV-specific T-cell response in chronic carriers as well as stimulating an HBV-specific immune response followed by an appropriate reduction of the viral load. Since immunomodulators target a host function (e.g. protein kinase activated by double-stranded RNA or PKR, an interferon (IFN)-induced mediator of the cellular antiviral response), the development of antiviral resistance is significantly lower than that of drugs directly targeting a viral function (e.g., NAs). The currently available immunotherapies are interferon alpha, its pegylated derivatives and thymosin alpha-1. Experimental immune-oriented approaches include interleukin 2 (IL-2) and 12 (IL-12) cytokines and new formulations of potential therapeutic vaccines (40).

The present section summarizes the drugs currently approved for the management of CHB (Table I).

Nucleos(t)ide Analogues (NAs)

NAs need to be activated via a phosphorylation process to the corresponding nucleoside triphosphate or nucleotide diphosphate; they are first phosphorylated by cellular kinases to nucleoside monophosphate and then further phosphorylated by cellular enzymes to the diphosphate and the triphosphate. The initial phosphorylation step is usually the rate-limiting step in the activation process and may account for some of the differences in potency among the various antivirals. Three categories of NAs are currently available in clinics: (i) L-nucleosides (lamivudine, LMV and telbivudine, LdT), (ii) acyclic phosphonate nucleotides (adefovir dipivoxil, ADV and tenofovir, TDF), and (iii) cyclopentane deoxyguanosine analogues (entecavir, ETV).

Advantages of this group of compounds are oral administration, excellent safety profile, rapid antiviral effect, histologic improvement and relatively low cost. The main drawback is that long-term treatment may induce resistance. Despite the potent inhibition of viral replication, prolonged therapy with this group of compounds rarely cures HBV infection, and viral rebound is common after discontinuation of therapy (16).

Lamivudine (LMV)

β-L-(-)-2′,3′-dideoxy-3′-thiacytidine (LMV, 3TC) was the first FDA-approved NA for the treatment of CHB, although it was first developed for the treatment of HIV infection.

Table I FDA-Approved Drugs for the Pharmacotherapy of HBV Infection

Drug family	Drug	Commercial name (Company)	FDA approval[a]	Dosage forms	Adult dose
Immunomodulators (IMPs)	IFN-α2B	Intron-A® (Schering Corporation)	1992	Powder for injection (10 MIU/ml; 18 MIU/mL; 50 MIU/mL), solution for injection in vials (10 MIU single dose, 18 and 25 MIU multidose), solution in multidose pens (3 MIU)	5 to 10 MIU thrice a week, for 16–24 weeks
	PEG IFN-α2A	Pegasys® (Roche)	2005	Solution for injection in vials (180 μg/mL), solution for injection in prefilled syringes (180 μg/0.5 mL)	180 μg s.c. once weekly for 48 weeks
Nucleos(t)ide analogues (NAs)	Lamivudine	Epivir-HBV® (GlaxoSmithKline)	1998	Tablets (100 mg)	100 mg once daily
	Adefovir Dipivoxil	Hepsera® (Gilead Sciences)	2002	Tablets (10 mg)	10 mg once daily
	Entecavir	Baraclude® (Bristol-Myers Squibb)	2005	Tablets (0.5 mg and 1 mg); Oral Solution (0.05 mg/mL)	0.5–1 mg once daily
	Telbivudine	Tyzeka® (Novartis)	2006	Tablets (600 mg)	600 mg once daily
	Tenofovir Disoproxil Fumarate	Viread® (Gilead Sciences)	2008	Tablets (300 mg)	300 mg once daily

s.c.: subcutaneous

[a] approval year for HBV treatment

LMV is a dideoxy analogue of cytidine and displays a non-natural configuration. Contrary to its dextrorotary counterpart, LMV exhibits a more potent activity against HIV and HBV reverse transcriptases as well as a lower toxicity (22). LMV has a solubility of approximately 70 mg/mL in water at 20°C; it is rapidily absorbed after oral administration in patients with CHB, and its bioavailability is approximately 86% with low binding to plasmatic proteins (<36%). The drug is intracellularly phosphorylated to its active metabolite, LMV triphosphate (LMV-TP).

LMV is highly effective in inhibiting HBV replication, and it is well tolerated after oral administration. Severe side effects, such as lactic acidosis, hepatomegaly with steatosis and pancreatitis, have been reported at very low frequency. This profile contributes to high patient compliance and adherence to the regimens. Clinical trials have provided evidence that during therapy with this antiviral drug, improvements of liver histology and inflammation are achieved and that the rates of HBeAg disappearance and seroconversion to anti-HBe are similar to those observed with IFN-α therapy (see below). Viral DNA levels in plasma were intimately associated with histologic disease progression in livers of HBV-infected patients (8). Despite the primary clincial benefits, the majority of the patients relapse, and viral rebound accompanied by severe exacerbation of the liver disease appears upon treatment interruption. Furthermore, long-term monotherapy with LMV results in emergence of resistant viruses in 24% and 70% of the patients after 1 and 4 years of therapy, respectively (41). This phenomenon is associated with loss of clinical effectiveness and acute exacerbation of liver disease that can result in hepatic decompensation and even death. Two types of mutation account for LMV resistance. Mutation at codon M204 of the YMDD motif results in the substitution of isoleucine for methionine (M204I). An alternate mutation at this site is methionine for valine (M204V). Both mutations are sufficient to confer resistance not only to LMV but also to other structurally related antivirals such as telbivudine. The M204V mutation almost invariably occurs in combination with a second mutation at codon 180 located in the B domain: substitution of leucine by methionine (L180M). M204I may occur alone or occasionally, in combination with L180M. Double mutants L180M+M204V and L180M+M204I replicate better than single mutants M204I and M204V *in vitro*. In addition, the L180M mutation renders them more resistant to LMV. A fourth well-characterized mutation, V173L, was only observed in combination with the double mutant L180M + M204V, suggesting that it only emerges in HBV strains with this pattern of resistance to LMV (38,42,43). Summarizing, the four major patterns of HBV-resistant mutants selected during treatment with LMV are in order of frequency: (i) L180M+M204V, (ii) V173L+L180M+M204V, (iii) M204I, and (iv) L180M+M204I.

Adefovir Dipivoxil (ADV)

(9-[2-[bis[(pivaloyloxy)methoxy]phosphenyl],ethoxy]-ethyl adenine), an oral prodrug of adefovir (an acyclic nucleotide analogue of adenosine monophosphate) was the first of its kind approved by the FDA for the treatment of CHB. ADV

displays an aqueous solubility of 19 mg/mL at pH 2.0 and 0.4 mg/mL at pH 7.2. After absorption, the bis-(pivaloyloxymethyl) moiety is removed, resulting in the active drug adefovir, which is twice phosphorylated intracellularly to the active metabolite adefovir triphosphate. This metabolite acts as (i) an inhibitor of HBV polymerase by blocking the priming reaction and (ii) a chain terminator when incorporated into the viral DNA (40). Like LMV, treatment with ADV also leads to an improved liver histology, reduced serum HBV DNA levels, and normalized ALT values. ADV is active against wild-type, pre-core mutant (HBeAg-negative patients) as well as LMV-resistant HBV strains. It would also be active against mutants with resistance to ETV. ADV pharmacotherapy shows a biphasic clearance of the virus with a first decline corresponding to the clearance of HBV particles from serum followed by a second slower phase corresponding to the loss of infected cells (40). ADV is well tolerated, reduces serum HBV DNA levels by 4 logs, and leads to seroconversion in 20–27% of patients treated over 12 weeks at a daily dose of >30 mg (44). The structural similarity of ADV to its natural substrate (dATP) and its small flexible linker allow a greater accessibility to the polymerase and may account for the lower resistance rate compared to LMV; i.e., the resistance profile is 3%, 9%, 18% and 28% after 2, 3, 4, or 5 years of treatment, respectively (9). *A priori*, these clinical outcomes consolidate ADV as more advantageous than LMV. On the other hand, dose-related side effects, such as nephrotoxicity, lactic acidosis and severe hepatomegaly with steatosis, are more frequent than with LMV. In addition, severe hepatitis within 12 weeks after therapy cessation has been reported in approximately 25% of patients (40). The best documented resistant mutants selected during treatment with ADV are the substitution of asparagine at codon 236 for threonine (N236T) and of alanine at codon 181 for valine (A181V), the latter conferring cross-resistance to LMV.

Entecavir (ETV)

1S-(1a,3a,4b)-2-amino-1,9-dihydro-9-[4-hydroxy-3-(hydroxyl-methyl)-3-methylenecyclopentyl]-6H-purin-6-one) (entecavir, ETV) is a carbocyclic analogue of 2-deoxyguanosine in which the oxygen of the furanose ring is replaced by a vinyl group. Due to its potent and selective activity against HBV, FDA approved its use in 2005 for the treatment of CHB. ETV is slightly soluble in water (2.4 mg/mL). After oral administration, ETV is phosphorylated thrice intracellularly to ETV triphosphate (ETV-TP). This active metabolite inhibits the activity of the RT and the DNA polymerase as well as the priming step. It also acts as a DNA chain terminator (45). As opposed to LMV, ETV shows a slow rebound of viremia upon therapy interruption, probably due to its effect on the pool of nuclear cccDNA (40). In coincidence with ADV, ETV leads to lower resistance rates than LMV, and its incidence increases with the duration of therapy. Resistance rates are 10% and 25% after 2 and 3 years, respectively, in patients with primary LMV-resistance, and 0.8% in naïve patients over 3 years (9). Thus, the presence of L180M and/or M204V/I mutants selected during LMV monotherapy seems to be a prerequisite for the development of ETV resistance. In this context, the best known resistant mutants selected during therapy with ETV are I169T, S184G, S202I, and M250V (46,47). ETV proved to be effective in inhibiting replication of wild-type, and LMV- and ADV-resistant mutants. Lactic acidosis and severe hepatomegaly with steatosis have been reported after cessation (40). Although ETV (like tenofovir) is the most effective of all the anti-HBV agents, it is not widely used due to its remarkably higher price. Also, long-term efficacy data are not available yet because of its relatively recent implementation.

Telbivudine (LdT)

β-L-2′-deoxythymidine (telbivudine, LdT) is a nucleoside analogue of thymdine that was approved by the FDA for the treatment of CHB in 2006. Its active metabolite, LdT triphosphate is a potent and selective inhibitor of HBV replication. As opposed to LMV that preferentially inhibits first-strand (RNA-dependent) DNA synthesis, LdT depletes HBV second-strand (DNA-dependent) DNA synthesis. As LMV, LdT is a weak inhibitor of human DNA polymerases (45). The anti-HBV activity is conferred by the common hydroxyl group in the 3′ position of the β-L-2′-deoxyribose sugar of the molecule. It was suggested to be more potent than LMV in reducing HBV DNA levels *in vivo*, though a similar resistance profile is observed (40). The resistance rate reported for LdT was 3% in patients who were HBeAg-positive and 2% in those who were HBeAg-negative after 1 year therapy. After 2 years, these percentages increased to 17.8% to 21.6% in HBeAg-positive patients and 7.3% to 8.6% in HBeAg-negative patients (48). LdT is well tolerated after oral administration, and the most common adverse events are upper respiratory tract infection (14–17%), fatigue and malaise (12–14%), nasopharyngitis (11–15%), headache (11–12%), and abdominal pain (6–12%) (48,49).

Tenofovir Disoproxil Fumarate (TDF)

The FDA approved TDF for the treatment of CHB in 2008. It is the fumaric acid salt of bis-isopropoxycarbonyloxymethyl ester derivative of tenofovir. *In vivo*, TDF is converted to tenofovir, an acyclic (nucleotide) analogue of adenosine 5′-monophosphate. TDF has a solubility of 13.4 mg/mL in distilled water at 25°C, and the

oral bioavailability is approximately 25%. As it is structurally related to ADV, its mode of action as well as its antiviral resistance profile is very similar. However, its greater efficacy and potency in inhibiting viral replication may result in lower resistance rates. According to Marcellin *et al.*, TDF monotherapy is a very good option for the treatment of patients with LMV-resistant HBV (50). TDF is more effective than ADV and ETV in these patients, and it results in much lower renal toxicity than ADV.

Immunomodulating Peptides (IMPs)

The goal of IMPs is to enhance the T-cell-mediated HBV-specific immune response that helps the host immune system mount a defense against hepatitis B (51). Only interferon alpha and its poly(ethylene glycol)-grafted (PEG) counterpart have been approved worldwide.

Interferon Alpha (IFN-α)

IFN-α is a natural water-soluble glycoprotein naturally produced by cells in response to viral infections. One of the commercially available derivatives is recombinant IFN-α2b, the first drug approved by the FDA for the treatment of CHB (Table I). IFN-α has antiviral, immunomodulatory and antiproliferative effects. Its activity relies on two mechanisms: (i) supression of HBV replication in virus-infected hepatocytes by inhibition of synthesis of viral DNA and activation of cellular enzymes with antiviral activity and (ii) enhancement of the cellular immune response against HBV-infected hepatocytes by increasing the phagocytic activity of macrophages and the expression of class I histocompatibility antigens and by stimulating the activity of helper T lymphocytes and natural killer (NK) cells (6). It is worth mentioning that the rate of response to IFN-α is relatively low, being effective in approximately 30–40% of the treated patients; 56% of those who responded relapsed within the first year after discontinuation therapy (52). The most important viral factor that determines the response to IFN-α is the pre-treatment HBV-DNA levels: the lower the level, the better the response. Other viral key factors are the presence of basal core promoter (BCP) and precore mutations (53). In this context, predicting the responsiveness of the patient to IFN-α is crucial in the clinical setting. The level of sensitivity of HBV to IFN-α may be genotype-specific, with genotypes C and D more resistant than genotypes A and B. (26,54,55). Other factors that improve the chances of a good therapeutic response are (i) high serum ALT levels, (ii) no cirrhosis, (iii) no co-infection with HIV or hepatitis D virus (HDV), and (iv) age (56,57).

Discrepancies in data of different clinical trials are usually due to different schedules employed and the different viral genotypes and patient populations included in the studies. Interestingly, a higher rate of HBsAg seroconversion was achieved after 24 weeks of therapy with IFN-α than in a 48–52-week course with NAs. In addition, IFN-α administration for more than 24 weeks may improve the extent and durability of the response in CHB. However, side effects and poor tolerance to IFN-α administration limits its prolonged use (9). The most common IFN-α-related adverse effect is serious depression, particularly in patients with a previous history. Other side effects may include fatigue, weight loss, headaches, myalgias, neurological disturbances such as paresthesia and impaired concentration, influenza-like symptoms, hair loss, leukopenia, thrombocytopenia, thyroid disorders, autoimmune disorders and rashes. These adverse phenomena result in low patient compliance and, often, in dose reduction or premature cessation of the treatment. Due to its short half-life time *in vivo*, this agent was replaced by the PEG-modified counterpart.

Pegylated Interferon (PEG IFN-α2a)

In 2005, the FDA approved the use of PEG IFN-α2a for the treatment of CHB (Table I). It was developed by grafting a single branched bis-monomethoxy poly(ethylene glycol) (PEG) chain to a recombinant IFN-α2a molecule. This modification changes the biodistribution of the drug without affecting its activity; i.e., clearance of interferon by the kidneys is reduced. Also, a longer half-life results in more stable plasma concentrations and smaller distribution volumes. Finally, the number of injections was reduced from thrice to once weekly, improving patient compliance. Drug pharmakocinetics depend on the size and attachment location of the PEG moiety.

Clinical trials have shown that PEG IFN-α2a was more effective than conventional IFN-α and LMV in the treatment of both HBeAg-positive and -negative CHB (6,58). On the other hand, coadministartion of PEG IFN-α2a with LMV did not result in any additional sustained antiviral and clinical response compared to PEG IFN-α2a monotherapy, precluding the implementation of a combined therapy. A very recent double-blind clinical trial indicated that the rate of HBeAg seroconversion was 27% and 32% at weeks 48 and 72, respectively, these extents being higher than those shown by NAs (59). Adverse effects are similar to those described for conventional IFN-α2b. Regretfully, the extremely high cost of interferon pharmacotherapy precludes its implementation mainly in developing countries and less affluent populations.

Thymosin alpha-1 (Tα1, Thymalfasin)

Tα1 is an IMP of 28 amino acids derived from thymosin fraction 5 (60). *In vitro*, it promotes a Th1-type immune response as well as T-cell differentiation and maturation,

enhances the production of different cytokines (e.g., IFN-α, IL-2, and IL-3) and increases NK-cell activity and expression of class I histocompatibility antigens (61). Remarkably, randomized clinical trials showed significantly higher sustained response rates when compared to controls and no significant side effects (60). The virological, biochemical (e.g., normalization of ALT levels) and complete response (simultaneous biochemical and virological response) increased gradually after therapy cessation (52). Conversely, the benefit of Tα1 in the treatment of CHB was not significant at the end of the therapy. As opposed to IFN-αTα1, treatment is relatively free of adverse effects. Tα1 has been approved in many developing countries (e.g., Argentina) as a second-line drug (60), though due to the controversial clinical effectiveness, it has not been appproved yet by the US FDA.

A comparative summary of viral, biochemical and histological rates in the course of the infection with NAs and IMPs is presented in Table II.

DRUG DELIVERY AND TARGETING STRATEGIES AND NOVEL ANTI-HBV DRUG CANDIDATES

As described above, most of the commercially available anti-HBV agents are NAs. However, the efficacy of such drugs is limited for the long-term palliation of HBV replication, and it is further challenged by the emergence of drug-resistance mutants. The efficacy of anti-HBV drugs substantially depends on their pharmacokinetics, in particular their distribution and accumulation in the liver. One attractive strategy to improve the activity of anti-HBV agents is to target them into HBV-infected cells by DDS that are recognized by receptors on the surface of the hepatocyte. Therefore, strategies that direct the drug to its site of action—liver parenchyma—may increase the effectiveness of the drug and decrease its potential side effects in other non-target organs (62).

One of the first and more extensively strategies pursued is the design of prodrugs and macromolecular carriers bearing sugar moieties that are recognizable by asialoprotein (ASP) receptor-positive hepatocytes (63,64). Fiume *et al.* reported on the conjugation of the antiviral analogue adenine arabinoside monophosphate (ara-AMP) to lactosaminated human serum albumin (L-HSA) (65). Clinical assays indicated that the administration of the prodrug to HBV-infected patients for 28 days leads to an antiviral activity similar to that of the free drug, though without any clinical adverse effect such as neurotoxicity.

Tishiharu and Takahashi designed an N-glycosylated human IFN-β that showed stronger anti-HBV activity than free IFN-α or IFN-β in asialoprotein receptor-positive human hepatocytes transfected with an HBV secreting construct (66). The stronger activity was supported by the induction of the 2′-5′ oligoadenylate synthetase (an indicator of IFN activity) at much higher levels than the pristine interferon. Also, the conjugate reduced viremia in HBV-transfected athymic nude mice as opposed to the unmodified IMP. In a similar approach, 9-β-D-arabinofuranosyladenine 5′-monophosphate (ara-AMP) was targeted to the liver by conjugation with galactosylated albumin (67,68).

Conjugates of antiviral drug candidates with glycosylated poly-L-lysine instead of albumin have been also synthesized and tested (69–72). For example, Fiume *et al.* conjugated ara-AMP to galactosylated-poly-L-lysine and compared the inhibition of DNA synthesis in liver, intestine and bone marrow with that of the free drug and a galactose-free L-lysine conjugate (70). Findings indicated that the galatosylated conjugate selectively inhibits DNA synthesis in liver. The main advantages of poly-L-lysine over albumin are (i) production by synthetic methods, (ii) availability in a wide range of molecular weights and (iii) large number of side-chain functional groups to couple the drug. Also, it can be administered intramuscularly, unlike albumin conjugates that require i.v. infusion (72). On the other hand, poly-L-lysine has been reported as strongly cytotoxic and immunogenic (73), the cytotoxicity being reduced by glycosylation (70).

Other research groups used arabinogalactan or glycosylated lipoprotein carriers (74) or prodrugs that release the active drug based on the hepatic metabolism (75–77). For example, Erion *et al.* described a series of phosphate and phosphonate prodrugs (HepDirect prodrugs) that result in liver-targeted drug delivery following a cytochrome P450-catalyzed oxidative cleavage reaction in the hepatocytes (75–77). Poor intracellular conversion of NAs to the active phosphorylated counterpart stems from the narrow substrate specificity of the nucleoside kinase, the enzyme that catalyzes the initial phosphorylation to the generation of the monophosphate derivative (75). To overcome this limitation, several prodrugs were designed. However, extracellular hydrolysis leads to monoacid intermediates that are poorly absorbed by liver cells (75). In contrast, prodrugs of 5′-monophosphates of vidarabine, LMV and cytarabine as well as the phosphonic acid adefovir are stable in plasma and tissues, while they are cleaved upon exposure to rat liver homogenates (75). An LMV prodrug was administered intravenously to fasted rats (60.2 mg/kg, 30 mg/kg of LMV equivalents) and the liver and plasma concentrations of LMV-triphosphate and LMV, respectively, were monitored and compared to that of rats treated with LMV (230 mg/kg) (75). The prodrug produced 7.7-fold greater triphosphate levels and an 11.3-fold greater liver exposure than the free drug (Fig. 5). It is worth stressing that the dose of the prodrug was 7.7-fold lower than that of the free

Table II Advantages and Drawbacks of the Treatment of CHB with IMPs and NAs Approved by the US FDA

	IFN-α	PEG-IFN-α	LMV	ADV	ETV	LdT	TDF
Properties	Recombinant cytokine	Recombinant cytokine grafted to PEG	Nucleoside analogue	Nucleotide analogue	Nucleoside analogue	Nucleoside analogue	Nucleotide analogue
Mechanism of action	Antiviral, immunomodulator and antiproliferator	Antiviral, immunomodulator and antiproliferator	Inhibitor of viral polymerase	Inhibitor of viral polymerase	Inhibitor of viral polymerase	Inhibitor of viral polymerase	Inhibitor of viral polymerase
Route	Subcutaneous	Subcutaneous	Oral	Oral	Oral	Oral	Oral
Side effects at licensed doses	Many	Many	Negligible	Negligible	Negligible	Negligible	Negligible
Contra-indications	Numerous	Numerous	Uncommon	Uncommon	Uncommon	Uncommon	Uncommon
Drug resistance (naïve patients)	None	None	1st year: 24%	1st year: 0	1st year: 0	1st year: 3%	1st year: 0%
			2nd year: 42%	2nd year: 3%	2nd year: <1%	2ndyear:20%	2nd year: 0%
			3rd year: 53%	3rd year: 11%	3rd year: <1%	3rd year: -	3rd year: -
			4th year: 70%	4th year: 18%	4th year: <1%	4th year: -	4th year: -
Drug resistance (patients LMV-resistant)	None	None	N.a.	1st year: 18% 2nd year: 25%	1st year: 1% 2nd year: 9%	N.a.	N.A.
				3rd year: -	3rd year: 15%		
				4th year: -	4th year: 39%		
Undetectable HBV DNA (HBeAg-positive patients)	12%*	28%*	62%**	13%**	67%**	60%***	76%**
Undetectable HBV DNA (HBeAg-negative patients)	18%*	43%*	85%**	63%**	90%**	88%***	93%**
HBeAg seroconversion	25%*	33%*	20%**	16%**	21%**	23%***	19%**
HBsAg seroconversion (HBeAg-positive patients)	2–8%*	3–17%*	0%**	0%**	0%**	0%***	1%**
HBsAg seroconversion (HBeAg-negative patients)	2–8%*	3–17%*	1%**	0%**	0%**	0%***	0%**
ALT normalization (HBeAg-positive patients)	18%*	41%*	62%**	54%**	68%**	77%***	68%**
ALT normalization (HBeAg-negative patients)	33%*	59%*	73%**	77%**	78%**	74%***	76%**
[1]Histological improvement (HBeAg-positive patients)	N.A.	41%*	52%**	68%**	72%**	69%***	74%**
[1]Histological improvement (HBeAg-negative patients)	N.A.	48%*	60%**	69%**	70%**	69%***	72%**

[1] Histologic response defined as ≥2-point decrease in Knodell Necro-inflammmatory Score from baseline with no worsening of the Knodell Fibrosis Score. *After 24 weeks of treatment. **After 48 weeks of treatment. ***After 52 weeks of treatment.

N.A.: not available.

N.a.: not applicable.

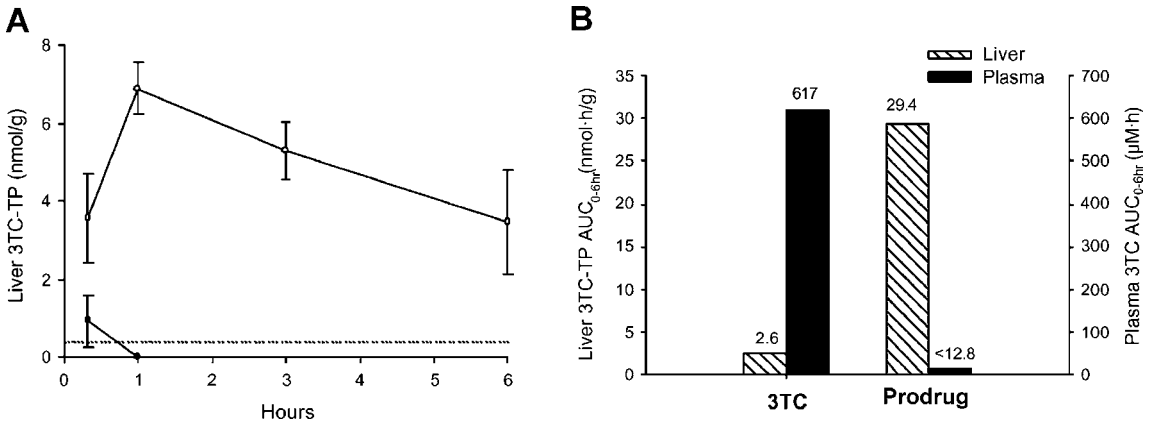

Fig. 5 (**A**) Liver concentration of LMV triphosphate (3TC-TP) after treating male rats ($n = 4$/group) with 3TC (filled circles) (230 mg/kg, iv) and a prodrug (hollow circles) (30 mg/kg of 3TC equivalents, iv). Limit of detection (dashed line) was 0.5 nmol/g. (**B**) Liver 3TC-TP area-under-the-curve until 6 h (AUC0-6 h) and plasma 3TC AUC0-6 h for 3TC and prodrug-treated rats (adapted from Ref. 75 with permission from the American Chemical Society).

counterpart. Conversely, pristine LMV showed much higher plasma concentrations than the targeted one.

More recently, Chimalakonda *et al.* synthesized and characterized a conjugate of LMV with dextran (~25 kDa) for selective delivery to the liver (62). Dextrans are glucose polymers used as molecular carriers for the delivery of drugs to different tissues. It was reported that plasma kinetics and tissue distribution of dextran carriers are dependent on their molecular weight, 20–70 kDa dextrans showing a high degree of selectivity for the liver (62). However, unmodified dextrans accumulate in parenchymal and non-parenchymal hepatic cells indistinctly (78,79).

Dextran modification with galactose and mannose leads to selective captation by hepatocytes and Kupffer cells, respectively. Also, different electric charges and chemical modifications may alter the biodsitribution; i.e., positively-charged dextrans are taken up more effectively than neutral and negatively-charged counterparts (80,81). The LMV-dextran conjugate (>99% purity) was synthesized through a succinate linker and contained 6.5 mg LMV/100 mg conjugate (62). The prodrug was stable under acid conditions (pH 4.4), while it underwent hydrolysis at pH 7.4. The presence of hepatic lysosomes in the medium induced the release of free drug. A single 5 mg/kg dose of free and conjugated LMV was administered i.v., and the plasma concentration followed up. The free drug could not be detected after 3 h, while the concentration of the pro-drug remained several-fold higher over the time of the study. More interestingly, the hepatic concentrations of the drug were not detectable 15 min after injection (Fig. 6A), and area-under-the-curve (AUC) values were >50-fold higher for LMV-dextran (Fig. 6B). Furthermore, the slow release of LMV due to enzymatic hydrolysis was observed selectively in liver (Fig. 6A). Finally, the drug and the conjugate were not detected in any organ other than

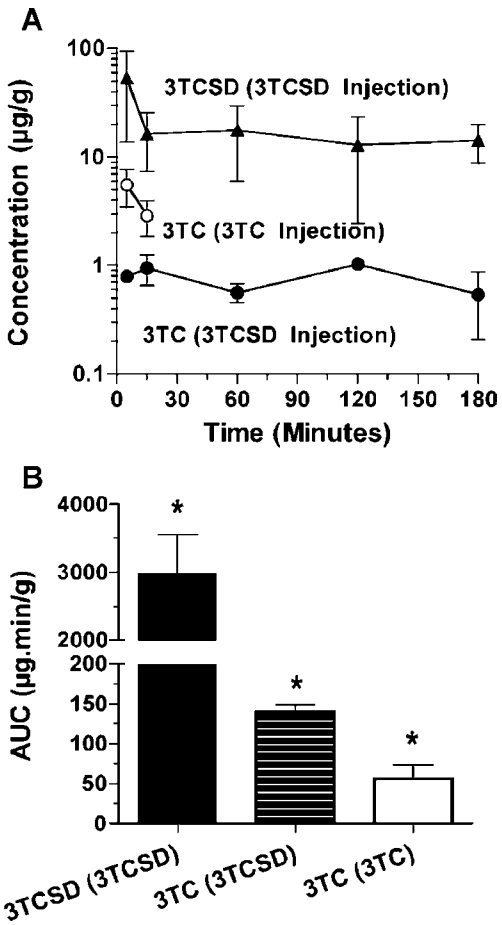

Fig. 6 Liver concentration–time courses (**A**) and AUC values (**B**) of LMV (3TC) and/or dextran-conjugated LMV (3TCSD) after i.v. administration of single 5 mg/kg doses (3TC equivalent) of 3TC or 3TCSD to rats ($n = 3$). * indicates statistically significant differences from the other two groups (reproduced from Ref. 62 with permission from the American Chemical Society).

kidneys, the availability of LMV being similar for both the free and the conjugated drug.

Biessen *et al.* designed divalent and trivalent cluster glycosides with affinity for the asialoglycoprotein receptor to target the antiviral nucleoside 9-(2-phosphonylmethoxyethyl)adenine (PMEA) to hepatocytes, while accumulation in other tissues was significantly reduced (82). The hepatic uptake of the prodrug was >10-fold higher than that of the free drug (52% and 62% *versus* 4.8% of the injected dose, respectively). These data indicated that more than 90% of the drug was taken up by the liver parenchyma. Once in the liver, prodrugs were converted into the respective active agent in the lysosomes and translocated into the cytoplasm. The antiviral activity *in vitro* was enhanced 5- and 52-fold for the divalent and the trivalent glycosides, respectively.

Since targeting to the liver does not necessarily imply hepatocyte targeting, as Kupffer cells display phagocytosis capability (83,84), fine tuning the chemical modification of the carriers might be required to improve the accumulation in one cell type over the other. Moreover, accumulation in liver parenchyma does not imply that the prodrug is converted into the active agent. All these aspects need to be thoroughly addressed to elucidate the potential of a specific drug delivery strategy.

High-density lipoprotein (HDL) represents another promising carrier for hepatic targeting (85). The endogenous HDL takes up cholesterol and phospholipids from peripheral tissues and delivers them to hepatocytes via the apolipoprotein A-I (apoAI), which is the major lipoprotein component in HDL. ApoAI binds to the scavenger receptor, class B, type I (SR.BI) expressed on the surface of hepatocytes, followed by endocytosis and selective translocation of cholesteryl esters and phospholipids into the hepatocyte cytosol (86–88). For example, Feng and co-workers developed a recombinant HDL (rHDL)-acyclovir palmitate complex and evaluated the anti-HBV *in vitro* (89). They elegantly showed that a concentration of 0.0022 μmol/mL inhibited 20% of HBV. To attain the same level of inhibition with acyclovir palmitate- and acyclovir-loaded liposomes and free acyclovir, 20-, 40-, and 200-fold concentrations, respectively, were needed. Biodistribution studies following i.v. administration showed that 71.2% of the dose was recovered in the liver, 10.2% in plasma, and 18.6% in the rest of the body, at 30 min (Fig. 7). Altogether, these results indicated that rHDL-acyclovir palmitate complex displays strong liver-targeting properties (89).

Miao *et al.* studied the anti-HBV activity of ADV-loaded solid lipid nanoparticles (SLN) prepared by solvent diffusion method in water (90). The entrapment efficiency was 16.7%, and the drug loading was 3.9%. The inhibitory performance of encapsulated ADV on HBsAg, HBeAg and viral DNA levels was significantly higher than that of the free drug.

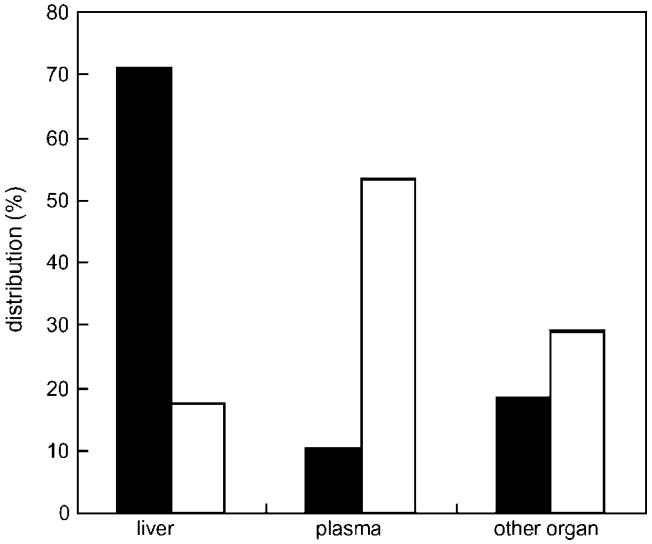

Fig. 7 Distribution of free acyclovir palmitate (white bars) and rHDL–acyclovir palmitate (black bars) in rats (reproduced from Ref. 89 with permission from Elsevier).

Envelope HBV proteins (e.g., recombinant large-protein, L-protein) that form hollow virus-like nanoparticles (~80 nm), are so-called bionanocapsules (BNC), and display special affinity for human hepatocytes have also been investigated. This property was shown to be especially noticeable for the large HBsAg that contains the sequence QLDPAF; this amino acidic portion seems to be the hepatocyte binding region (91). To enhance the stability of the BNC, the density of cystein residues of the S domain of the protein needs to be minimized. Otherwise, the nanostructures aggregate due to random disulfide bridging when stored for a long period at 4°C (92).

Overall, these studies support the general concept that targeted delivery of antiviral drugs to the liver potentially increases the efficacy of these drugs in the treatment of viral liver infections while, at the same time, decreasing their toxic effects in other tissues and organs due to systemic exposure. However, the choice of the carrier and the targeting functional group need optimization to reduce carrier-related side effects, such as increased alkaline phosphatase levels seen with lactosylated human serum albumin, or to mitigate drawbacks associated with variability in the density and the affinity found with the asialoglycoprotein receptors (62). The former is an indication of acute toxicity, while the latter could suggest a long-term range adaptative process. On the other hand, regardless of the viremia and histologic improvement, these drugs do not eradicate a key player in the perpetuation of the infection: the cccDNA. Consequently, new anti-HBV drug candidates continue to be thoroughly investigated. Among them are zinc finger proteins (ZFPs) (12), antisense oligonucleotides (93), nitazoxanide and second generation thiazolides (94), nosiheptide (85), small interfering

RNA (siRNA) (95–102), phenopropenamides and other non-nucleoside agents (103,104). Woginin, ellagic acid, artemisinin and artesunate, chryophanol β-D-glucoside, saikosaponine C, protostane triterpenes and bicyclol are natural products that were also shown to be active against HBV (105,106). The investigation of novel agents implies also the appearance of unknown technological drawbacks that need to be overcome even in the context of preliminary *in vitro* assays. The present section will briefly describe the different drug candidates that are being evaluated as potential antivirals and the different strategies to improve their physicochemical (e.g., solubility) and pharmacokinetic features towards their biological evaluation.

Zinc Finger Proteins (ZFPs)

ZFPs are Cys2His2 DNA-binding proteins that can be designed to target novel DNA sequences with high specificity and affinity. Each Zn finger is approximately 30 aminoacids in length and is composed of two beta sheets and an alpha helix that are coordinated by a zinc ion (12,107,108). The alpha helix lies within the major groove of double-stranded DNA and makes specific contact with 3 bp of DNA. By stringing zinc fingers in tandem, a unique DNA sequence of 18 bp can be specifically recognized. As mentioned above, NAs can decrease virus production by inhibiting the viral polymerase. However, complete clearance by these drugs is not common because of the persistence of nuclear cccDNA. To target cccDNA, six different ZFPs that bind sequences in the DHBV enhancer region were designed; DHBV is a model virus for HBV (12). These authors have shown that ZFPs may have a dual effect: (i) inhibition of enhancer activity on core and small surface promoters and (ii) steric hindrance of RNA polymerase across the enhancer, resulting in a reduction of stable complete transcripts. ZFPs as therapeutics may be delivered either in protein form or through gene delivery. As protein, ZFPs may be encapsulated within liposomes, nanoparticles, or synthetic polymers. Liposomes can be taken up by a number of different cell types, including monocytes/macrophages, spleen cells and liver cells (12). In addition, liposome pegylation enhances the uptake by the spleen and liver over the uptake by phagocytic cells (109). As gene therapy, ZFPs may be delivered by using replication-incompetent adenoviruses and cationic liposomes (12).

Nitazoxanide

Nitazoxanide ([2-[5-nitro-1,3-thiazol-2-yl)carbamoyl]phenyl] acetate) was the first thiazolide licenced in the USA for the treatment of *Cryptosporidium parvum* and *Giardia lamblia* infections in immunocompetent adults and children in 2002 (110). Recent data have shown an eventual role as therapy for influenza virus infection, chronic hepatitis C virus (HCV) infection and CHB (94). As an antiviral, nitazoxanide belongs to the family of the immunomodulators. Its mechanism of action would be through activation of the PKR (111). Reports suggested that both nitazoxanide, and its metabolite, tizoxanide, have a potent inhibitory activity against HBV (112), and in combination with other antiviral agents, such as LMV or ADV, also showed synergistic effects (113). Thiazolides have a very favorable toxicity profile with a very low incidence of mild gastrointestinal side effects. Second generation molecules in controlled release formulations are in development (94). However, the extremely low aqueous solubility of this drug (8 μg/mL) might preclude any biological evaluation and might demand the development of an appropriate carrier.

Nosiheptide

Nosiheptide is a poorly-water-soluble natural polypeptide antibiotic that has been shown to inhibit hepatitis B virus DNA, HBsAg and HBeAg secreted by an HBV-transfected cell line (HepG2.2.15) (114). To improve the solubility, Cai *et al.* prepared nosiheptide-loaded liposomes by means of sodium deoxycholate dialysis and sonication, and evaluated the inhibition of HBsAg and HBeAg in HepG2.2.15 (115). Liposomes were stable over 2 years at −0°C. Drug concentrations that inhibited HBsAg by 46.9%, 55.4%, 65% and secreted HBeAg by 15.1%, 36.2%, 36.8% were 1.25, 2.5 and 5.0 μg/mL, respectively. More recently, Feng *et al.* reported on the construction of a recombinant HDL-nosiheptide complex comprising ApoAI, phosphatidylcholine, and the nosiheptide to target this anti-HBV agent to hepatocytes and overcome the poor distribution in liver *in vivo* (85). Complexes displayed a high loading efficiency (>80%) and a small diameter (30 nm). The drug accumulated mainly in the liver 30 min after i.v. injection in male Wistar rats. Moreover, the effective concentration to attain 50% virus inhibition in HepG2.2.15 was 0.63 mg/mL, this value being 40 and 200 times lower than nosiheptide-loaded liposomes and the free drug, respectively.

Small Interfering RNA (siRNA)

RNA interference (RNAi) is a natural conserved process by which double-stranded siRNA induces sequence-specific, post-transcriptional gene silencing by binding to its complementary mRNA and triggering its elimination. It is an evolutionary mechanism for protecting the genome against invasion by mobile genetic elements such as transposons and viruses (95). Potent knockdown of a gene of interest with high sequence specificity makes RNAi a powerful tool for studying gene function and for treating a variety of diseases. RNAi can specially inhibit the function of any chosen target gene and has shown antiviral effect against

HBV, HCV and HIV (96). Since HBV makes extensive use of ORFs within the DNA genome, multiple HBV RNAs will make the virus susceptible to RNAi. According to this, Xin *et al.* showed that a combination of siRNAs was more effective than each individual siRNA in (i) inhibiting antigen expression and viral replication and (ii) significantly suppressing HBV cccDNA amplification (99). A main drawback is that, as previously depicted, HBV genome is prone to mute, and mutation strains escape silencing by RNAi. Thus, strategies that can effectively overcome viral mutation by combining siRNAs which can simultaneously target multiple sites of HBV gene should be developed (96). It was also demonstrated that inhibition of HBV replication by siRNAs may also enhance the antitumor immune response, since the inhibition of HBV expression by RNAi led to up-regulation of MHC class I-related molecule A (MICA); MICA is a ligand of the NKG2D receptor expressed on NK cells, CD8+ T-cells, gammadelta T-cells and some myeloid cells (101). However, the poor siRNA stability *in vivo*, the low cellular uptake and the limited selectivity still remain crucial hurdles towards its clinical implementation. Chemical modifications can be used to enhance their stability, prevent them from triggering an immune response, control their pharmacokinetic profiles and reduce nonspecific effects without affecting their biological activity (95). In addition, bioconjugation of one or both strands of siRNA with lipids and biodegradable polymers, such as poly(β-amino esters), is often desirable to (i) further increase their thermodynamic and nuclease activity, (ii) improve the biodistribution and pharmacokinetic profile, (iii) target them to specific cell types, and (iv) decrease their immunoactivation (95). The sequence design of siRNA molecules is also important to improve their efficacy as well as to reduce the potential for off-target effects (gene silencing effect caused by siRNAs in non-target mRNAs through the RNAi mechanism) and activation of the immune system (91).

Comprehensive articles of siRNA for the treatment of HBV and delivery strategies have been published elsewhere (95–102). siRNA targeting different ORF could also lead to different efficacy in inhibiting HBV expression and replication. Regarding this point, Fu *et al.* compared the inhibition diversity produced by siRNAs targeting different ORFs and showed that these RNA molecules caused different endpoints on HBV expression and replication (98). siRNA cocktails from ORF C, S, and X with high genotype conservation would ensure greater efficacy and minimize potential viral mutation; X- and C-target siRNAs could significantly inhibit viral DNA load (97). As mentioned above, the development of siRNAs as therapeutic agents strongly depends on the availability of safe and effective intracellular delivery systems, such as poly(β-amino esters) (97), and cationic liposomes, formulated with cationic lipids (102).

Kim *et al.* formulated synthetic siRNA/apolipoprotein A-I/1,2-dioleoyl-3-trimethylammonium-propane complexes against HBV (116). They showed that these nanoparticles are selectively taken up by the liver and can significantly reduce viral protein expression by receptor-mediated endocytosis in one single low dose (< or = 2 mg/kg) (116). In addition, they have persistent antiviral effect for up to 8 days. Nishina *et al.* used alpha-tocopherol as a carrier molecule of siRNA *in vivo*, since it has its own physiological transport pathway to most of the organs (117). Neither induction of IFNs nor other side effects were found. Sato *et al.* designed an antifibrotic therapy for liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone (118). This approach has a therapeutic potential for reversing human liver cirrhosis. Studies carried out by Morrissey *et al.* showed that siRNA targeted to the HBV RNA and incorporated into specialized liposomes to form a stable nucleic-acid-lipid particle (SNAP) enhances persistence of *in vivo* activity and allows the use of lower doses and reduction in dosing frequency (119). Carmona *et al.* produced 5 mol% PEGylated siRNA/cationic liposomes complexes with sizes between 80 and 100 nm (100). The PEG linkage was pH sensitive to enable the pH-triggered release of the genetic material from endosomes. After a single i.v. bolus, siRNA-loaded liposomes accumulated mainly in hepatocytes; PEGylation prevented the uptake by Kupffer cells. Also, no signs of hepatic or renal toxicity were apparent. Cho *et al.* investigated the inhibition of NF-B-inducing kinase (NIK), a protein that promotes HCC, by means of NIK-specific siRNA-loaded cationic liposomes (102). To improve the stability of siRNA *in vivo* and target the asialoglycoprotein receptors in liver cancer cells, the nanocarriers were surface grafted with β-sitosterol glucoside.

Heteroarylpyrimidines (HAP)

In 2003, a new group of compounds that specifically targets the encapsidation step before viral replication occurs has been identified. These compounds belong to the class of HAPs (120). HAPs were more potent than LMV in a cell-based HBV replication assay. The mechanism involves the binding to the core protein and its degradation.

Phenopropenamides

Another group of compounds that would inhibit the encapsidation step are the phenopropenamides (103,104). Their mechanism of action appears to be different from that of the HAPs. These compounds directly inhibit the formation of the nucleocapsid. In the cell-based replication system, the phenopropenamides are not as potent as LMV in inhibiting HBV replication (121). Other non-nucleoside

inhibitors of HBV that inhibit viral replication by interfering at several levels of DNA replication, nucleocapsid assembly, virus maturation, and capsid organization, such as the acyclic pyrimidine nucleosides have been investigated (42).

Antisense Oligonucleotides (asODN)

Antisense oligonucleotides agents that produce their effects through an antisense mechanism offer the possibility of developing highly specific alternatives to traditional pharmacological antagonists, thereby providing a novel class of therapeutic compounds that act at the level of gene expression. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. In the case of antisense RNA, they prevent protein translation of certain mRNA strands by binding to them. Antisense DNA can be used to target a specific, complementary (coding or non-coding) RNA. If binding takes places, this DNA/RNA hybrid can be degraded by the enzyme RNAse-H (122). In order to target asODN to hepatocytes in the treatment of CHB, cationic liposomes were developed as gene carriers co-modified with the ligand of the asialoglycoprotein receptor, β-sitosterol-β-D-glucoside (sito-G) and the nonionic surfactant, Brij 35 (93). Cellular uptake with high transfection efficiency was reported to involve both endocytosis and membrane fusion. Sito-G enhanced receptor-mediated endocytosis, and nonionic surfactant Brij 35 facilitated membrane fusion, the co-modification resulting in the most efficient transfection without enhanced cytotoxicity (93). Shi *et al.* designed asODN-loaded cationic liposomes modified with soybean sterylglucoside (SG) and polyethylene glycol-distearoylphosphatidilethanolamine (PEG-CL) for therapy of CHB (122). Biodistribution studies showed that the liposomes enhanced the accumulation of ODN in the liver and spleen, while decreasing its blood concentration. These authors also demonstrated that SG/PEG-CL-mediated ODN transfer to the liver is an effective gene delivery method for cell-specific targeting of genes in the therapy of HBV infection. SG and PEG-modified cationic liposomes have also proven to be an alternative carrier for hepatocyte-selective drug targeting (123).

MONOTHERAPY *VERSUS* COMBINED THERAPY

In the past decade, CHB therapy has taken a turn due to the increased availability of novel and effective antiviral agents with potent activity against HBV. Many studies have shown the benefits of single-agent therapy, but the high rate of viral resistance and its association with an impairment of liver histology and progression of disease is a matter of great concern (8). Combined therapy seems to be a highly attractive strategy, though very few studies using this approach have been performed until now. Most of these studies have been combinations of IFN-α+LMV or LMV+ADV. Controlled clinical studies using combinations of IFN-α+LMV have not shown an improvement in seroconversion rates in spite of the higher antiviral effect and the delay in the emergence of HBV mutants resistant to LMV. Moreover, the drawbacks of IFN-α administration limit its use as a combinatory agent (124).

Preliminary short-term controlled clinical studies of combination therapy with NAs displaying a complementary cross-resistance profile and different modes of inhibitory action (e.g. LMV+ADV; ETV+TDF) have not demonstrated superiority to monotherapy in terms of sustained virological response. However, these clinical studies have shown additive or synergistic antiviral effects and a delay in the development of resistance (125,126). On the other hand, combination therapy is indeed useful as a second-line strategy when resistance emerges during monotherapy with NAs. For example, the addition of ADV or TDF is recommended in cases of LMV- or LdT-resistant strains. In cases of HBV mutants harboring ADV-resistance, the switch to TDF and the addition of LMV, ETV or LdT as a combinatory agent has been implemented (126). Combination therapy is also used for preventing HBV infection following liver transplantation (125).

Considering such experiences, guidelines for CHB monotherapy/combined therapy are still under great debate and will require further clinical research. In any event, since the concept of combined antiviral therapy is well established for HIV- and HCV-infected patients, as well as for tuberculosis, these experiences may support the gradual clinical evaluation of combined therapies also in HBV. It is worth mentioning, though, that the combined therapy might seriously affect patient compliance and adherence and demand the design and development of novel fixed dose combinations (FDC) similar to those implemented in HIV and tuberculosis.

PERSPECTIVES

One of the major aims of antiviral therapy against chronic HBV infection is to reduce the high morbidity and mortality related to the disease. Despite the significant progress made in the last few years in the anti-HBV therapy development, there is still a long way to go and too much to be done to achieve the ideal goals for treatment of patients with this chronic disease: (i) potent and long-lasting inhibition of viral replication, (ii) eradication of the pool of cccDNA from the nucleus of infected hepatocytes, (iii) prevention of viral DNA integration into the host genome, and (iv) liver histology improvement. To achieve these goals, it seems crucial to better exploit the potential targets

for therapeutic intervention. In agreement with Zoulim *et al.*, rational targets might also include inhibitors of several steps of HBV viral life cycle (viral attachment and entry, conversion from relaxed-circular DNA to cccDNA, capsid assembly, viral envelopment, and secretion of viral particles) (22). Bearing in mind that viral genome integration also represents an important step in the HCC development, targeting of this event seems worth exploring. In this context, our better understanding of viral pathogenesis is undoubtedly decisive for the immediate development of new strategies for the therapy of CHB. Furthermore, the implementation of liver-targeting DDS strategies in general and nanotechnologies in particular may provide tools to (i) improve the effectiveness and applicability of approved drugs by overcoming or delaying the development of viral resistance, (ii) constrain the appearance of systemic side effects by promoting selective accumulation in the liver, and (iii) increase patient compliance and adherence to therapeutic regimens by reducing the administration frequency (127–129). Also, the discovery and evaluation of new drug candidates will encompass still unknow, technological drawbacks and will demand the design of more appropriate drug carriers to primarily address the biological evaluation *in vitro* and, later on, to enable the evaluation of their performance in preclinical and clinical trials (128). In this context, pharmaceutical sciences appear as a key complementary research field. Finally, ethical questions related to the appropriate access of all the patients (regardless of their socioeconomic status) to these novel drugs and delivery systems are emerging and, in this context, need to be seriously and comprehensively addressed.

ACKNOWLEDGEMENTS

This work was partially supported by the University of Buenos Aires (Grant UBACyT B424). MLC thanks a PhD scholarship of the University of Buenos Aires.

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