Expert Opinion

Novel delivery methods for treatment of viral hepatitis: an update

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Viral hepatitis represents the most common cause of chronic liver disease worldwide. Currently approved therapies for chronic hepatitis B include IFN, an immune modulator, and nucleoside analogues lamivudine and adefovir. For chronic hepatitis C, a combination of pegylated IFN-α and ribavirin represents the standard treatment. However, currently available treatments for both these viruses are effective only in a limited number of patients, are costly, prolonged, associated with significant side effects and require a substantial commitment from the patients and healthcare providers. A number of novel antiviral treatments, together with strategies to enhance the response to current therapies, are being explored at present. For all new therapies, as well as for improving existing treatments, selective delivery of medications into liver cells would be desirable to enhance antiviral activity and avoid systemic side effects. New achievements in the field of drug and gene delivery against chronic hepatitis to the liver are reviewed here.

Keywords: asialoglycoprotein receptors, chronic viral hepatitis, drug delivery, hydrodynamic method, liver, viral vectors

Expert Opin. Drug Deliv. (2005) 2(4):707-717

1. Introduction

1.1 Chronic viral hepatitis

Viral hepatitis is a major global health problem. Despite a slightly declining incidence of acute viral hepatitis in recent years, chronic viral hepatitis still remains the most common cause of chronic liver disease throughout the world. Although a vaccine is available for hepatitis B virus (HBV) that can prevent new infections, it is estimated that there are ~ 350 million chronic carriers of HBV [101]. Of the chronically infected patients, ~ 15 – 40% will develop cirrhosis, liver failure or hepatocellular carcinoma [1]. HBV infection accounts for 500,000 – 1.2 million deaths each year and represents the tenth leading cause of death worldwide [1]. Chronic hepatitis C virus (HCV) infection affects > 170 million people globally, of which ~ 20% will progress to cirrhosis, end-stage liver disease and hepatocelullar carcinoma. Worldwide, the incidence of hepatocellular carcinoma has increased, and is now the fifth most frequent cancer in the world, killing 300,000 – 500,000 people each year [1]. Chronic liver disease and cirrhosis account for ~ 400,000 hospitalisations and almost 30,000 deaths annually in the US [2].

1.2 Hepatitis B virus

HBV is a member of the hepadna group of enveloped DNA viruses. Although the mechanism of viral entry into the cell is unknown, it is thought to be a receptor-mediated process that eventually leads to the uncoating of the viral capsid and transport of the released open circular viral genome into the nucleus. In the nucleus, DNA repair enzymes complete the viral plus and minus strands, generating a covalently closed circular (ccc) DNA molecule that serves as the template for

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viral transcription. The HBV viral genome is divided into four domains, which are transcribed into four overlapping transcripts that are exported into the cytoplasm, where the viral proteins are translated, and viral particle assembly and HBV viral genome replication occur [3]. The transcripts encode viral polymerase, core and precore proteins, envelope proteins (including the hepatitis B surface antigen) and X protein, and serve as the pregenomic RNA template. The precore protein is eventually processed in the endoplasmic reticulum (ER) and secreted as hepatitis Be antigen. The envelope proteins are inserted into the ER membrane, and released as either free subviral envelope particles or as part of infectious virions (Dane particles). The core and polymerase proteins assemble around the viral RNA to form HBV RNA-containing capsids. Inside the capsids, the first strand of viral DNA is synthesised by reverse transcription of the RNA, which then serves as the template for the synthesis of the second strand of DNA.

1.2.1 Treatment

Currently approved therapy for the treatment of HBV in the US includes IFN, an immune modulator, and nucleoside analogues, such as lamivudine and adefovir. Combination therapy has the potential of additive or synergistic antiviral effects with a decreased rate of resistance; however, available data on the combinations of IFN with lamivudine or adefovir with lamivudine showed little or no added benefit compared with monotherapy [4]. Thus, new treatments that would have more potent antiviral effects, less toxicity and minimal or no risk of resistance, and would be capable of inducing a sustained response after a finite duration of treatment, are needed. There are several new approaches to the treatment of chronic hepatitis B [5]. New antiviral agents being explored include inhibitors of reverse transcriptase/DNA polymerase and inhibitors of virus entry and core assembly. Immunomodulatory therapy is exploring the use of cytokines to stimulate T helper cell type 1 response and developing therapeutic vaccines (against cytolytic T-lymphocyte [CTL] epitopes, DNA vaccines and peptide vaccines with more potent adjuvants) and monoclonal antibodies. Molecular therapies under development include antisense oligonucleotides, small interfering RNA (siRNA), ribozymes and dominant-negative mutant core proteins. These agents act by disrupting the life cycle of HBV. The widespread use of antisense oligodeoxynucleotides and siRNA therapy, however, has been hampered by an inability to achieve the required intracellular concentrations, which is largely a result of their instability in complex protein mixtures, in addition to a lack of cell-specific delivery systems.

1.3 Hepatitis C virus

HCV is an enveloped RNA virus of *Hepacivirus* genus in the *Flaviviridae* family. The mechanism of HCV entry into the cell is still poorly understood, although CD81 and low-density lipoprotein may potentially play a role as cellular receptors. After binding to the cellular receptor, the lipid envelope dissociates, and the HCV RNA genome and the structural proteins are delivered into the cytoplasm. Once inside the cell, the HCV genome uses the host ribosomes to translate viral RNA into viral proteins. The HCV genome is a 9.5-kb, single-stranded, positive-sense RNA with a single coding region and two flanking non-coding regions [6]. The coding region contains genetic information for three structural proteins (core, envelope 1 and envelope 2) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Both groups of proteins have been implicated in modulating the host immune response and carcinogenesis. The flanking non-coding regions (the 5′- and 3′-untranslated regions [5′- and 3′-UTR]) regulate translation and replication of the virus. Translation of the genomic RNA is initiated by a highly conserved secondary structure within the 5′-UTR, called the internal ribosome entry site, which permits engagement of the host ribosome. Because the 5′-UTR sequence is highly conserved among HCV species, it represents a logical target for designing antiviral molecular therapies. Translation proceeds in the 5′ to 3′ direction, producing a single viral polyprotein that is subsequently cleaved into individual viral proteins by action of the host signal peptidase and the viral proteases, NS2–3 and NS3–4 serine protease. Thus, the NS3 is an attractive target for antiviral therapy, and specific inhibitors of its substrate-binding site are being designed. Post-translational viral replication occurs after the non-structural protein NS5B (the viral polymerase) copies the original infecting viral RNA via a negative-strand intermediate, while the NS3 protein unravels these growing RNA strands to allow their packaging with viral particles containing core and envelope proteins. Packaged virus is then exported extracellularly [7]. Six major HCV genotypes and > 100 subtypes have been identified throughout the world on the basis of molecular homologies of the conserved and non-conserved regions. Furthermore, several distinct but closely related HCV sequences can co-exist within each infected individual. These are referred to as quasi-species and reflect the high replication rate of the virus and the lack of a proofreading capacity of the viral RNA-dependent RNA polymerase. The heterogeneity of HCV species causes many problems in the therapy and development of an effective HCV vaccine.

1.3.1 Treatment

Currently approved therapy for chronic HCV includes a combination of pegylated IFN-α and ribavirin [8,9]. However, the treatment is effective in only 50% of patients, costly, prolonged (6 – 12 months), not suitable for all patients, associated with significant morbidity and requires a substantial commitment from the patients and healthcare providers. As increasing numbers of chronic hepatitis C patients are treated, the numbers of non-responders to current therapies will also rise, resulting in a larger group of patients with limited alternative therapeutic options.

A number of novel antihepatitis C treatments are currently being explored, together with several strategies to enhance the

response to current therapies [10]. For IFNs, strategies for specialised delivery include the use of disposable infusion pumps, controlled release formulations for parenteral use (including implantable sustained release devices), conjugation with albumin, encapsulation and polyaminoacid-based oral delivery systems. A number of ribavirin-like drugs such as levovirin and viramidine are being studied. Viral enzyme inhibitors to NS3 protease, NS3 helicase and NS5B polymerase are in preclinical or early clinical (Phase I) trials. A number of antisense oligonucleotides and ribozymes against HCV have been developed, two of which are in early clinical study [11].

For all new therapies, as well as for improving existing treatments for viral hepatitis, selective delivery of medications into liver cells, instead of their general distribution to all the tissues and organs in the body, would be desirable in order to enhance the antiviral activity and avoid systemic side effects. This review will summarise novel methods for the delivery of antiviral drugs and expression vectors containing elements that inhibit hepatitis viral replications.

2. Novel delivery methods

2.1 Delivery by liver-specific asialoglycoprotein receptors

One approach to liver-specific drug targeting is to couple the drugs to galactosyl-terminating glycoproteins (asialoglycoproteins). The presence of abundant high-affinity asialoglycoprotein receptors (ASGPR) on the plasma membranes of mammalian liver cells allow for efficient uptake of asialoglycoproteins. Liver-specific delivery of small molecules, as well as large molecules such as nucleic acids, has been accomplished by this approach. For this purpose, natural asialoglycoprotein ligands, or other molecules to which terminal galactose residues are chemically linked, may be used. Examples of the latter include lactosylated lipids, lactosylated human albumin and lactosylated polyamino acids.

2.1.1 *Natural asialoglycoprotein carriers*

Natural asialoglycoproteins, such as asialofetuin and asialoorosomucoid, were modified so that the carrier contained, not only a liver cell recognition component, but also a nucleic acid binding component. Such carrier molecules allowed for targeted delivery of DNA or RNA molecules to hepatic ASGPR [12] *in vitro* and *in vivo* [13]. The low efficiency of transgene expression was improved by the incorporation of endosomolytic or fusogenic peptides into the liver-specific carrier. Incorporation of adenovirus [14] or vesicular stomatitis virus G peptide [15] or listeriolysin O protein [16] to an asialoorosomucoid-polylysine carrier resulted in a 10 to 100-fold increase in transgene expression.

Antisense DNA oligodeoxynucleotides are effective antiviral agents but are not efficiently taken up by cells because of the overall negative charge and, once inside the cell, they are susceptible to intracellular degradation. Binding antisense oligodeoxynucleotides to asialofetuin-poly-L-lysine and asialoorosomucoid-poly-L-lysine carriers reduced their intracellular degradation and increased their uptake into liver cells by > 20-fold *in vitro* [17,18]. Antisense oligonucleotide against the woodchuck hepatitis virus (WHV) delivered by asialoorosomucoid-polylysine carriers to WHV-infected woodchucks significantly decreased viraemia by 5 to 10-fold [19]. Antisense molecules bound to asialoglyprotein carriers have also been effective in the inhibition of HCV-related gene expression in cell culture [20].

2.1.2 *Liposomes and cationic lipid carriers*

Liposomes and cationic lipids used to deliver genes to cells could also be modified to deliver antiviral drugs such as nucleoside analogues, IFN and antisense oligonucleotides specifically into hepatocytes. Antiviral nucleosides, such as dideoxycytidine (ddC) [21] and dideoxyguanosine (ddG) [22], when linked to dioleoylphosphate (a phospholipid), formed lipophilic prodrugs that had antiviral activities against HBV in culture and *in vivo* in WHV-infected woodchucks with significantly reduced toxicity when compared with the administration of free nucleoside. The combination of lipophilic nucleoside prodrug with lactosylated low-density lipoprotein (LDL) [23] or synthetic high-density lipoprotein (HDL) [24] resulted in lipid particles that were selectively taken up by parenchymal liver cells via the ASGPR. This approach provides an effective delivery method for the targeting of many drugs to hepatocytes for various indications (e.g., prostaglandin E_1 incorporated into galactosylated liposomes is now being investigated for the treatment of fulminant hepatitis [25]).

Conjugation of 9-(2-phosphonylmethoxyethyl)adenine (PMEA or adefovir), a potent inhibitor of HBV replication, to fatty acid formed a lipophilic prodrug. The prodrug readily associated with lactosylated HDL to produce a stable product, which was taken up specifically by hepatocytes [26] with the release of active adefovir and resultant inhibition of HBV DNA synthesis in a HBV-producing cell culture model [27]. Adefovir could also be targeted to hepatocytes by direct conjugation to bi- and trivalent cluster glycosides, K(GN)(2) and K(2)(GN)3, with high affinity for ASGPR [28]. Conjugation improved liver uptake of adefovir by > 10-fold compared with the free drug, and 90% of uptake could be attributed to parenchymal cells. Accumulation of the adefovir prodrugs in extrahepatic tissues was substantially reduced. After cellular uptake, both adefovir conjugates were converted into the parent drug in lysosomes and the released adefovir was rapidly translocated into the cytosol. Its antiviral activity *in vitro* was significantly enhanced when compared with treatment of cells with the free drug alone.

Asialofetuin-bearing liposomes encapsulating IFN-γ were bound and internalised by a human hepatoma cell line (Hep G2 cells), selectively through ASGPR, and inhibited HBV replication in cells transfected with HBV DNA [29]. In a separate study, the activity of targeted liposomal IFN-α was tested *in vitro* on cultured Chang liver cells by measuring

metallothionein gene expression [30]. Metallothionein gene expression was induced in all treated cells, but the levels of mRNA varied among groups treated with different liposomal formulations of IFN-α and were lower than those induced by free IFN.

Conjugation of antisense oligonucleotides to cholesterol or *bis*-cholesterol enabled the molecules to be associated with lactosylated LDL, which resulted in their specific uptake by liver cells [31]. Then, coupling of antisense oligodeoxynucleotides to bile acids (natural hepatic ligands) allowed for their targeting to liver cells. Lehman *et al.* showed that bile acid modified antisense oligonucleotides directed against HCV RNA had enhanced lipophilic properties and formed stable duplex target DNA and RNA strands [32].

2.1.3 Lactosaminated serum albumin

When conjugated to lactosaminated serum albumin, adenine arabinoside 5-monophosphate (ara-AMP or vidarabine), a nucleoside analogue, was selectively taken up by liver cells and was effective in inhibiting viral DNA synthesis in the liver [33-35]. Radiolabelled ara-AMP–lactosylated albumin conjugate intravenously injected into mice infected with *Ectromelia hepatitis* virus was selectively delivered to the liver, with only small quantities taken up by spleen, bone marrow, intestine and brain cells. The conjugate was stable in the bloodstream, where only a small percentage of the bound drug was enzymatically released from the carrier. In the liver, the drug was released in a pharmacologically active form, and showed its efficacy through the inhibition of viral DNA synthesis without affecting cellular DNA synthesis in the intestine and bone marrow, and without noticeable toxicity or immunogenicity even after repeated injections. Ara-AMP– or acyclovir–lactosylated human serum albumin conjugate injected intravenously into WHV-infected woodchucks reduced circulating WHV DNA levels, and the doses required to achieve the effect were significantly lower than those of the free drugs [36].

Ara-AMP–lactosylated serum albumin conjugate was used in several small pilot clinical trials for chronic hepatitis B in Italy. Daily infusion of the conjugate at 34 – 53 mg/kg (ara-AMP 1.5 – 2.3 mg/kg) resulted in decreased HBV DNA replication in all patients, at a dose that was three- to sixfold lower than the dose of the free drug, without observed adverse effects, but HBV DNA rose again after drug administration was stopped [37,38]. One patient developed antibodies to the conjugate. Significant increases in alkaline phosphatase and platelet counts, with a decrease in the number of erythrocytes, haematocrit and haemoglobin levels, were observed among patients but neurotoxic side effects typically associated with the use of the free ara-AMP were absent. Wang *et al.* reported similar results in 10 patients with chronic hepatitis B [39]. Infusion of ara-AMP–lactosylated albumin conjugate resulted in the same antiviral activity as infusion with the free drug, but the same effect was achieved with a significantly lower dose of active substance.

2.1.4 Lactosamined polylysine

Lactosamined poly-L-lysine was used as a liver-specific carrier for delivering nucleic acids and small antiviral nucleoside analogues to hepatocytes because lactosylation rendered it liver specific, and the lysine molecules allowed for chemical linkages to biological molecules. When ara-AMP, initially coupled to small molecular weight lactosaminated poly-L-lysine, was administered to mice by an intramuscular route, it was selectively taken up by the liver, had no acute toxicity, did not induce antibodies and the conjugate was highly soluble in water [40]. High molecular mass lactosaminated poly-L-lysine allowed for a greater concentration of ara-AMP, ribavirin and azidothymidine to be incoporated and had low renal elimination compared with antiviral-low molecular weight lactosylated polylysine conjugates [41]. Ara-AMP–high molecular weight lactosylated polylysine conjugates did not produce acute toxicity or antibodies in mice after repeated injections. Liver concentration of the conjugated antiviral drug was three times greater than in the kidney, spleen and intestine, compared with unconjugated free ara-AMP, where the amounts of radioactivity in the liver, spleen and kidney were similar [42]. When used in WHV-infected woodchucks, the conjugate markedly decreased viraemia at a dose of 4.2 and 7 mg/kg/day (equal to 1.5 and 2.5 mg/kg of ara-AMP), which was significantly lower than the dose required for the free drug treatment (5 mg/kg) [42]. This carrier was further improved for clinical use, and the optimal molecular weight was established to be between 45,000 and 65,000 Da, which guaranteed the high solubility required for the intramuscular route of administration and low renal elimination of the drug [43]. Conjugate preparations with these properties, when intramuscularly administered to WHV-infected woodchucks for 37 days at a dose of 5.8 mg/kg/day, exerted a strong antiviral activity. These preparations were devoid of acute toxicity when tested in rats and caused no toxic effects when administered at doses 10-fold higher than those active in woodchucks.

Ribavirin can also be covalently bound to a lactosylated polylysine carrier at high concentration [44]. The resulting conjugate was very soluble in 0.9% NaCl, stable in the blood and selectively taken up by liver cells when administered intramuscularly to mice. The concentration of ribavirin–lactosylated polylysine conjugate was low in kidneys, indicating that the conjugate was lost through the kidney only in small quantities. The antiviral activity of the conjugate was studied in mice infected with a strain of murine hepatitis virus known to be sensitive to ribavirin. Coupled ribavirin, intramuscularly injected, inhibited viral replication in the liver at a daily dose two- to threefold lower than that of the free drug. In mice injected with a tritiated ribavirin conjugate, the ratios between the levels of radioactivity in liver and erythrocytes were 2.2- or 4.7-fold higher than in animals given free ribavirin [45].

Another group of antiviral drugs, antisense oligonucleotides, were also targeted to hepatocytes *in vitro* using galactosylated poly-L-lysine as a drug carrier. A 16-mer phosphorothioate analogue of the antisense oligonucleotide directed against the HBV

U5-like region was linked with galactosylated poly-L-lysine [46]. A 2:1 molar ratio of the carrier to antisense oligonucleotide optimised the complex formation. Fluorescent histochemistry indicated that the conjugate had selective affinity to the rat liver tissues. The efficacy of the conjugate was tested on an HBV producing liver cell line (Hep G2 2.2.15 cells), where it showed more specific inhibition of HBV gene expression than was seen with the free antisense oligonucleotide, and significantly depressed viral replication.

2.1.5 Polysaccharide delivery vehicles

2.1.5.1 Arabinogalactan

Ara-AMP has also been successfully conjugated to arabinogalactan, a naturally occurring plant polysaccharide [47]. Daily injections of the conjugate into WHV-infected woodchucks decreased serum levels of WHV DNA at a dose of 3 mg/kg of ara-AMP, which was fivefold lower than the dose of the free drug required for the same effect.

2.1.5.2 Pullulan

Currently, human IFN used together with ribavirin is the mainstay of treatment for chronic HCV infection. However, its use is associated with significant side effects. To reduce the side effects, IFN was conjugated to pullulan, a water-soluble polysaccharide [48]. Diethylenetriaminepentaacetic acid (DTPA), a chelating residue, was introduced to pullulan, and, subsequently conjugated to human IFN-β, was conjugated with DTPA–pullulan by mixing in an aqueous solution containing zinc ions. Intravenous injections of the conjugate into mice enhanced induction of an antiviral enzyme, 2′,5′-oligoadenylate synthetase, specifically in the liver, to a significantly greater extent than was enhanced by free IFN-β, and the duration of the enzyme induction was longer with administration of IFN–pullulan conjugate when compared with administration of IFN only.

2.1.6 Synthetic polymer carriers

Hydrophilic *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer was shown to be effective in binding antisense HBV oligonucleotide, and resulted in inhibiting *HBV* gene expression in Hep G2 cells [49]. Covalent attachment of the oligonucleotides to the HPMA copolymers via non-degradable dipeptide Gly-Gly spacers resulted in sequestering of the oligonucleotides in vesicles after internalisation, whereas their conjugation via a lysosomally cleavable tetrapeptide, Gly-Phe-Leu-Gly spacer, resulted in release of the oligonucleotide in the lysosome, and its subsequent translocation into the cytoplasm and nucleus of the cells. The HPMA copolymer–oligonucleotide conjugate possessed antiviral activity, indicating that phosphorothioate oligonucleotides released from the carrier in the lysosome were able to escape into the cytoplasm and remain active. Modification of the HPMA copolymer by covalent attachment of lactose or triantennary galactosyl resulted in targeted delivery of these compounds to hepatocytes [50]. Lactosylated poly-L-glutamic hydrazide was used as a targetable carrier to hepatocytes for prostaglandin E_1 [51]. The novel conjugate was water soluble and, after intravenous injection in mice, showed rapid accumulation in the liver. Its pharmacological activity examined in mice with fulminant hepatitis was promising.

Microparticles of an antihepatotoxic drug, glycyrrhetic acid, were prepared using poly-DL-lactic acid-co-glycolic acid as a drug carrier. Their *in vitro* properties, biodistribution and therapeutic effects against carbon tetrachloride-induced hepatitis in mice were investigated [52]. Glycyrrhetic acid microparticles were mainly distributed to the liver, and in mice with liver damaged by carbon tetrachloride the conjugate significantly suppressed the plasma level of glutamic pyruvic transaminase for almost 6 days, which was significantly longer than the effect of the drug itself.

2.2 Viral delivery

Gene therapy has been used to deliver antisense oligonucleotides, ribozymes and siRNA for the inhibition of hepatitis viral gene expression, by using both viral vectors and non-viral delivery methods. Viral vectors of adenoviruses, adeno-associated viruses (AAV), retroviruses and lentivirus origins have been used to express antisense oligonucleotides, ribozymes and siRNA, and are shown to be effective in the inhibition of *HBV* or *HCV* gene expression in cultured hepatocytes [53-56]. AAV and lentiviral vectors designed for siRNA delivery are commercially available; however, a major drawback of current viral-vector technology is that viral vectors cannot be targeted to specific organs. Examples of the deleterious effects of non-targeting are seen in the use of the latest generation lentiviral and adenoviral vectors, which result in inadvertent transduction of germline and antigen-presenting cells, diminishing the overall efficacy of the viral vectors, increasing the risk of inducing neutralising antibodies against the transgene product and raising additional safety and ethical concerns [57,58].

One way to overcome these obstacles was to modify the viral vectors to target liver cells. The incorporation of adenovirus into a ligand-based DNA carrier system greatly decreased its infectivity towards normally susceptible cells that do not have asialoglycoprotein receptors [14]. However, for cells that have ASGPRs, modified viruses retained their infectivity. Modified viruses enhanced gene expression in liver cells by 13- to 30-fold, when compared with asialoglycoprotein-polylysine DNA complex alone (without the virus), and the number of cells transfected was found to be 200-fold higher than by complex alone. Thus, the addition of the viral component offered enhanced gene expression, and coupling to the asialoglycoprotein–polylysine conjugate delivered transgenes specifically to ASGPR-bearing hepatocytes.

2.3 Nanoparticles

Nanoparticles are colloidal particles composed of either non-biodegradable or biodegradable polymers and are between 10 and 1000 nm in size. Nanoparticles were used to deliver pharmaceutical agents because of the ease and reproducibility of preparations, increased stability of bound drug and their

ability to deliver higher concentrations of drugs to a desired location with reduced side effects. Nanoparticles were successfully used for the delivery of genes and drugs to human hepatocytes, but their use in the delivery of antiviral drugs to the liver has been limited.

Valaciclovir polybutylcyanoacrylate, a potential anti-HBV agent, was made into nanoparticles and shown to be concentrated in mice liver shortly after intraveneous administration, but its efficacy against HBV has not been proven [59]. Antisense oligonucleotides bound to biodegradable polyalkylcyanoacrylate nanoparticles were shown to be less susceptible to intracellular degradation and had increased cellular uptake in cultured cells [60]. Poly-*N*-p-vinylbenzyl-D-lactonamide coated-poly-L-lactic acid-nanospheres were used for the encapsulation of Z-Asp, a caspase inhibitor, and its delivery to hepatocytes, *in vitro* and *in vivo*, in a mouse model of acute hepatitis [61]. Encapsulation significantly extended the intracellular retention time in hepatocytes, which increased the bioavailability of the caspase inhibitor. The therapeutic effect *in vivo* was temporally controllable by modifying the component of the nanospheres, rescuing mice from lethal hepatic injury.

HBV L particles, surface engineered HBV nanoparticles, were shown to be effective in delivering genes specifically to liver cells [62]. In a mouse xenograft model, intravenous injection of HBV L particles carrying the green fluorescent protein gene resulted in observable fluorescence only in human hepatocellular carcinomas, not in other human carcinomas or mouse tissues. When the gene encoding human clotting factor IX was transferred into the xenograft model using HBV L particles, Factor IX was produced at levels relevant to the treatment of haemophilia B. The limitation of the HBV L particles' drug delivery is their immunogenicity in long-term use after repeated administration. Although there are still significant obstacles for their clinical use, in view of the ease of production, particle stability and liver targetability, HBV L particles may be more promising than conventional liposomes.

2.4 HepDirect prodrugs

HepDirect Prodrugs is a new class of phosphate and phosphonate prodrugs that are cleaved by cytochrome P450 (CYP) 3A to nucleoside analogues and reactive aryl vinyl ketones [63]. The advantage of this form of prodrug delivery is that liver targetability of antiviral nucleoside analogues can be achieved because CYP3A is a parenchymal liver cell-specific enzyme. The other product of CYP3A oxidation, reactive aryl ketones, could be rapidly detoxified by conjugation with liver glutathione and eliminated. Recently, HepDirect prodrugs for adefovir and cytarabine (araC) were tested in mice. In mice given HepDirect adefovir prodrug, the intracellular concentration of adefovir metabolites was 4.5- to 7.5-fold higher in the liver compared with the concentration in the kidney and intestine. Mice given HepDirect prodrug araC had 12.6-fold higher liver cytarabine-5′-triphosphte (araCTP) levels when compared with mice given an equivalent dose of free araC. In the same animals, liver/bone marrow ratio araCTP was > 20-fold and peak plasma araC 45-fold lower, clearly indicating liver targeting. A 5-day mouse safety study with daily intraperitaneal injection of araC prodrug resulted in no decrease in body weight, no change in bone marrow cell numbers and no evidence of liver toxicity as measured by serum liver enzymes and bilirubin levels; thus, HepDirect prodrugs may be useful in the treatment of chronic HBV or HCV liver diseases.

2.5. Physical methods for delivering antiviral drugs and genes to the liver

2.5.1 Hydrodynamic method

Budker *et al.* published the first report of successful naked DNA delivery to the liver following intravascular manipulation [64]. Naked plasmid DNA in hypertonic solutions was injected intraportally in mice whose hepatic veins were transiently occluded, and high levels of luciferase and β-galactosidase gene expression throughout the entire liver were achieved. Plasmid constructs with a variety of reporter genes were then injected into the afferent and efferent vessels of the liver in mice, rats and dogs. Efficient plasmid expression was obtained following delivery via the portal and hepatic vein, and the bile duct. The use of hyperosmotic injection solutions and occlusion of the blood outflow from the liver substantially increased the expression levels. Combining these surgical approaches with improved plasmid vectors resulted in very high levels of foreign gene expression in the liver [65]. Subsequently, the technique was refined by eliminating the surgery. High levels of gene expression in the liver, ≤ 20%, were obtained following rapid tail vein injections of naked plasmid DNA in a volume that was 2.5-fold the blood volume of the mouse [66].

The hydrodynamic method was also successful for introducing siRNA into the liver [67]. When an siRNA derived from firefly luciferase was co-injected with a luciferase-expression plasmid, luciferase siRNAs reduced luciferase expression by an average of 81%. However, this method has limited clinical application in humans because of the risk of rapid injection and the need for the use of excessive volumes of fluid. To reduce the risk, a new catheter-based method for minimally invasive hydrodynamic gene delivery to the isolated rabbit liver was developed [68]. Using a lobar technique, plasmid DNA was delivered hydrodynamically to an isolated hepatic lobe using a balloon catheter to occlude a selected hepatic vein. In a whole organ technique, the entire hepatic venous system was isolated and the DNA solution injected hydrodynamically into the vena cava between two balloons used to block hepatic venous outflow, yielding much higher serum levels of reporter gene when compared with lobar delivery [68]. Very recently, regional hydrodynamic gene delivery via branches of the portal vein was evaluated in a rat model, by using 3-ml volumes (~ 12 ml/kg) of isotonic DNA solution delivered at 24 ml/min to the right lateral lobe (~ 20% of the liver mass). Under these conditions, > 95% of gene delivery

occurred in the targeted right lateral lobe, but outflow obstruction was still essential for successful gene delivery [69]. The hydrodynamic method of introducing genes or vectors expressing antiviral drugs has many benefits, including the high variety of liver vessels that can be used (e.g., portal or hepatic vein or bile duct), the ability to target entire liver or specific lobes, the ability to deliver naked DNA or DNA–polymer complexes and the possibility for a significant portion of hepatocytes to express the transgene from a single injection in small and large animal models. Although these techniques are promising for delivering antiviral gene expression vectors to the liver, and intravenous gene delivery to limb muscle is commercially available, many obstacles still need to be overcome before clinical applicability in the treatment of viral diseases.

2.5.2 *Implants*

Local implantation of a drug-releasing matrix was used to improve the delivery of antiviral therapeutics to the liver [70]. A cylindrical matrix prepared from a polyglycerol ester of fatty acids was used to continuously release recombinant human IFN-α (rHuIFN-α) [71]. After subcutaneous insertion of matrix implants into mice, the activity of 2′,5′-oligoadenylate synthetase (OAS), an indicator of antiviral state, increased in liver extracts and serum, and remained high for > 1 week. Comparing OAS activity between the matrix–implant and multiple injections of the rHuIFN-α on day 7 revealed that continuously released rHuIFN-α from the implant had an effect almost equivalent to that of three or seven injections/week.

3. Conclusions

Current antiviral therapy has many limitations, including significant adverse effects and morbidity. A number of novel antiviral drugs are currently being explored, together with several strategies to enhance the response to present therapies. For many of the new therapies being developed, as well as for improving existing treatments, selective delivery of medication into target cells would be desirable, not only to enable or enhance their antiviral effects, but also to avoid systemic side effects. A number of delivery methods have been investigated for different drugs including the use of ligands as carriers for recognition by cellular receptors, viral vectors and physical methods for drug delivery.

4. Expert opinion

Table 1 summarises methods used to deliver antiviral drugs specifically to the liver cells. In general, targeted delivery of drugs to liver can be divided into four categories:

- delivery to AsGRs on hepatocytes using natural ligands or ligands that have been altered to include galactose terminal molecules
- liposomes
- viruses
- physical methods

All delivery methods to the liver have advantages and disadvantages.

Carriers designed to target ASGPRs can efficiently deliver genes, antisense molecules and novel nucleoside analogues to liver cells with minimal toxicity and lack of humoral immunological response to the carrier molecules. In addition, conjugates with adenine arabinoside monophosphate and ribavirin were stable in the blood, achieved higher concentrations in the liver compared with other organs and showed efficacy in small animal models. However, this method has limited success in delivering genes and antisense molecules in liver cells due to low levels of expression of transgenes and antisense molecules.

Liposomes and other cationic lipids by themselves are not hepatocyte specific; however, when modified by lactosamination, liposomes and other cationic lipids, they can be used to deliver genes, antisense oligonucleotides, nucleoside analogues and IFN specifically to hepatocytes. Antiviral nucleosides, such as ddC, ddG and adefovir, required covalent linkage to phospholipids or fatty acids. Conjugates of adefovir–lithocholic acid and lactosylated-reconstituted HDL appeared to be stable at neutral pH or in plasma but completely released the active drug at lysosomal pH. When administered to rats, the conjugate was mostly distributed to the liver and taken up by ASGPRs on parenchymal cells. In a cell culture model, such conjugates effectively inhibited HBV DNA synthesis. The efficacy of nucleoside analogues bound to liver-specific liposomes has not been demonstrated *in vivo* in animal models or in clinical trials.

A promising novel alternative to obtain liver targeting of antivirals drugs is the chemical modification of antivirals such as adefovir or araC to small prodrugs (HepDirect). The prodrugs are distributed throughout the body but are cleaved to their active form only in the liver by CMP3A, a liver-specific enzyme. Liver specificity and lack of toxicity of the prodrugs have been demonstrated in mice. The effectiveness of this novel class of antiviral agent against chronic HBV or HCV disease has not been tested so far.

Viral vectors of adenovirus, AAV, retrovirus and lentivirus origins have been used to express antisense oligonucleotides, ribozymes and siRNA, and are shown to be effective in the inhibition of *HBV* or *HCV* gene expression *in vitro*. Host immune response to viral vectors, which was a major drawback of this approach, have been addressed by the development of 'gutless' vectors. Although high levels of expression of foreign genes can be achieved, a major drawback of current viral-vector technology is that the vectors cannot be targeted to specific organs. One of many attempts to overcome these obstacles involves the incorporation of viral vector into a ligand-based DNA carrier system, which greatly decreased its infectivity but retained enhanced gene expression. Very

Table 1. Methods for drug/gene delivery, molecules delivered and models in which they were tested.

Means of delivery	Drug	Model	Ref.
Asialoorosomucoid ± polylysine	DNA and RNA molecules	*In vitro*: HepG2 cells, Huh7 cells, erythroblasts *In vivo*: rat, mouse	[12-16]
Asialoorosomucoid/asialofetuin-polylysine	Antisense oligonucleotides	*In vitro*: HepG2 2.2.15 cells, Huh7 cells, PLC/PRF/5 cells *In vivo*: woodchuck	[17-20]
Liposomes	Dideoxycytidine, dideoxyguanosine	*In vitro*: HepG2 2.2.15 cells *In vivo*: mouse, woodchuck	[21,22]
Liposomes	IFN-α and -β	*In vitro*: Hep-HB107 (HBV transfected HepG2) cells, Chang cells	[29,30]
Lactosylated synthetic HDL	PMEA (adefovir)	*In vitro*: Hep AD38 (HBV transfected HepG2 cells) *In vivo*: rat	[27,28]
Lactosylated LDL	Antisense oligonucleotides	*In vivo*: rat	[31]
Lactosylated serum albumin	Adenine arabinoside-5-monophosphate	*In vivo*: mouse, woodchuck, pilot clinical trials	[33-39]
	Acyclovir	*In vivo*: woodchuck	[36]
Lactosylated polylysine	Adenine arabinoside-5-monophosphate	*In vivo*: mouse, woodchuck	[40-43]
	Acyclovir, ribavirin, azidothmidine	*In vivo*: mouse	[41,44,45]
	Antisense oligonucleotides	*In vitro*: HepG2 2.2.15 cells	[46]
Arabinogalactan	Adenine arabinoside-5-monophosphate	*In vivo*: woodchuck	[47]
Pullulan	IFN-β	*In vivo*: mouse	[48]
HPMA copolymer	Antisense oligonucleotides	*In vitro*: HepG2 cells	[49]
HepDirect prodrugs	PMEA (adefovir) and araC	*In vivo*: mouse	[63]
Retrovirus	Ribozymes, antisense oligonucleotides	*In vitro*: Huh7 cells, HepG2 2.2.15 cells	[53,54]
Recombinant retroviral and adenoviral vectors	Dominant-negative mutants of the hepadnaviral core protein	*In vitro*: LMH chicken hepatoma cells, HepG2 cells	[55]
Adenovirus	siRNA	*In vitro*: HCV producing Huh-7	[56]
Adenovirus incorporated into asialoglycoprotein–polylysine ligand system	DNA complex	*In vitro*: Huh-7 cells	[14]
Nanoparticles	Nucleoside analogues, oligonucleotides, genes, drugs		[59-62]
Hydrodynamic method	Naked DNA, siRNA	*In vivo*: mouse, rat, rabbit, dog	[64-69]
Locally implanted drug-releasing matrix	IFN-α	*In vivo*: mouse	[71]

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HPMA: Hydrophilic *N*-(2-hydroxypropyl)-methacrylamide; LDL: Low-density lipoprotein; LMH: Leghorn strain m; PMEA: 9-(2-phosphonylmethoxyethyl)adenine; siRNA: Small intefering RNA.

recently, a novel gene and drug delivery system, using engineered hepatitis B surface nanoparticles that have no infectivity and are recognised specifically by liver cells, has been described.

Physical approaches of introducing genes to the liver by systemic hydrodynamic transfection can achieve high levels of gene expression in rodents but requires a relatively large delivery volume which makes it unsafe for practice in humans. Recently, hydrodynamic delivery has been improved by a catheter-based method for minimally invasive gene delivery to the isolated liver, which yields high levels of gene expression and has the potential to be developed for clinical practice. There are also attempts to improve delivery of antiviral therapeutics via local implantation to the liver, but without further improvement these methods are still not ready for clinical use. The use of implanted devices or nanoparticles may increase the bioavailability of antiviral drugs but cannot be considered hepatocyte-specific delivery systems. This is an important consideration as hepatocytes are the main site of viral replication.

Although much has been learned about viral hepatitis, especially regarding the mechanisms by which the agents

cause the disease, safe and effective cures remain elusive. Antiviral drugs designed to interfere with vital viral processes often have side effects caused by their interaction with host metabolic function. The delivery of potent compounds to the sites where they will be most effective, while preventing side effects in other tissues and organs, requires targeted delivery. More research will be required to design and develop strategies to allow delivery to specific cell types. Because the liver is the site of the majority of damage and viral replication in viral hepatitis, it is reasonable to seek targeting mechanisms to this organ for future drug development to combat this difficult clinical problem.

Acknowledgements

The authors' research on hepatocyte-specific carriers and novel antiviral agents is supported by grants from the National Institutes of Health DK-42182 (GYW), the Herman Lopata Chair in Hepatitis Research (GYW) and by a grant from the American Liver Foundation (CHW).

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