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# Current Status of Anti-HBV Chemotherapy

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In the past decade, significant progress has been achieved in the battle against hepatitis B virus. In addition to the immunomodulating agents such as interferon-α and thymosin, many novel antiviral agents have been discovered, among which nucleoside analogues are the mainstay. New-generation compounds such as 3TC and famciclovir have shown promise in the treatment of patients chronically infected by this virus, and are on the line for approval. However, viral rebound after cessation of therapy still remains a major problem. Additionally, the reports on the drug resistance to these antiviral agents suggest that combination therapy will be the eventual strategy (Bartholomew *et al.*, 1997; Tipples *et al.*, 1996). Therefore, developments of safe and effective antiviral agents which do not cross-resist with currently available antiviral drugs are still much needed.

**Key words :** Hepatitis B virus, Immunomodulationg agents, Chemotherapy, Nucleoside analogues, 3TC, Famciclovir, Resistance

## INTRODUCTION

Currently, over 350 million people worldwide are estimated as the chronic carriers of hepatitis B virus (HBV), which comprise about 5% of the world population, most of whom live in Asia and Africa. In the U.S., the number of this virus carriers has reached more than one million with about 10,000 new infections occurring annually, which ranks the third in the reported illness behind venereal disease and chickenpox. Although known as a blood-borne disease, transmission of HBV by unprotected sex or body fluid contact is also very common in developed countries whereas mother to infant spread accounts for most of the cases in endemic areas, such as Asia.

HBV infection is responsible for both acute and chronic hepatitis. Acute HBV infection can be variable, with most individuals showing no obvious clinical sign of disease. Generally, at the end of incubation period, a flu-like illness, such as fever, fatigue and malaise occurs followed by, in some cases, jaundice. Fulminant hepatitis is a severe, but rare form of acute infection. On the other hand, 2 to 10% of the individuals who are infected by HBV will become chronic, which occurs much more likely in infants (>90% of neonatal exposures result in chronic infection). On an average, 25 to 40% of the chronic carriers will slowly develop liver cirrhosis and primary hepatocellular carcinoma (HCC), which are the major causes of morbidity and mortality.

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### Hepatitis B virus

Hepatitis B virus belongs to the family of *hepadnaviridae* which comprises several animal viruses, including human HBV, woodchuck hepatitis (WHV), ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV). These viruses share common features such as the genome organization, mode of replication and similar tropism for hepatocytes (Tiollais *et al.*, 1985).

HBV produces several different types of virus-related particles, including: (1) 42~47 nm spheres, known as The Dane particles; (2) 20 nm spheres, generally present in 10,000 to 1,000,000 fold excess over the Dane particles, and (3) smaller amounts of 20 nm filaments. All these particles contain a common hepatitis B surface antigen (HBsAg). Only the Dane particles are infectious and contain the viral core and viral nucleic acid. The smaller spheres and filaments contain only HBsAg and host cell derived lipids. Although not infectious, they are highly immunogenic.

HBV has a small genome which is composed of circular, partially double-stranded DNA (Miller *et al.*, 1989) The minus strand of viral DNA is 3.2 kb in size and the plus strand is shorter and variable in size (1.8 to 2.7 kb). The viral genome is very compact and contains 4 overlapping open reading frames (ORFs) in the minus strand, which allows the viral genome to

encode 50% more information than it would otherwise. ORF *P* covers 75% of the viral genome and encodes the viral DNA polymerase/reverse transcriptase. ORF *C* encodes the structural protein of the nucleocapsid (HBcAg) whereas ORF *S* encodes the viral surface glycoprotein of HBsAg. A poorly understood regulatory protein was encoded by ORF *X*, and the function of this protein is generally thought to be a trans-activator of viral and cellular gene expression.

The protein product of ORF *P* shares similarity with the reverse transcriptase (RT) of the retroviruses in that it has both the reverse transcriptase and RNase H activities. Amino acid sequence alignments of the hepadnaviruses *P* proteins with those of the retroviruses indicated significant homologues within the functional domains, for example, the YMDD (tyr-met-asp-asp) motif within the catalytic site of reverse transcriptase. However, hepatitis has no protease or integrase activites since HBV does not integrate into host chromosomes and it does not carry out proteolytic maturations of its proteins.

## HBV life-cycle

The diagram of HBV life cycle is shown in Fig. 1. The initial events (attachment, and entry) remain poorly understood, and the cellular receptor for HBV is unknown. After initial virus entry, the viral core particle is translocated into the nucleus of host cell, and the viral DNA is then repaired and matured, giving rise to a covalently closed circular DNA (cccDNA or supercoiled DNA). The cccDNA remains episomal and serves as a template for cellular RNA polymerase II, giving rise to several viral RNA transcripts. The largest of these RNAs serves as both the mRNA for the viral polymerase and the pregenomic RNA (pregRNA), which is slightly larger than genomic size, and is packaged into viral particles. At the same time, the smaller RNA transcripts are translated into the viral structural proteins. The synthesis of viral DNA is accomplished with the reverse transcription of the preg-

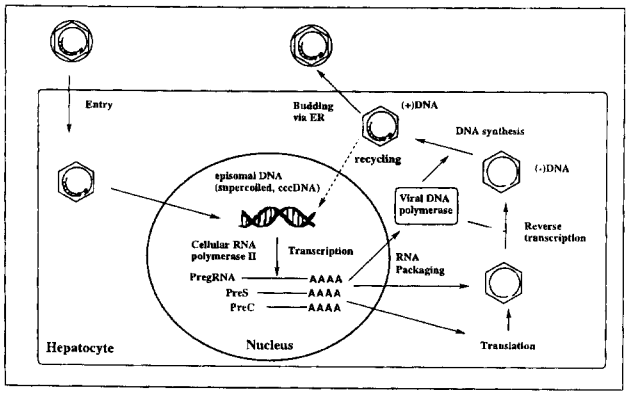



**Fig. 1.** Life cycle of hepatitis B virus.

RNA to the minus strand DNA by viral polymerase, followed by the synthesis of a shorter plus strand DNA to give a partially double stranded viral DNA. This newly synthesized viral DNA can be utilized as a resource for the cccDNA or functions as the viral nucleic acids in the matured virions budding out from the host cells.

## HBV vaccines

HBV represents the first example of a successful recombinant vaccine for a human infectious disease. The original HBV vaccine was prepared from the viral envelope protein (hepatitis B surface antigen HBsAg) that was purified from the plasma of chronically infected individuals, and it was licensed in the U.S. in 1981 (Purcell and Gerin, 1978). Although safe and highly efficacious, the plasma-derived vaccine was replaced with a recombinant vaccine prepared in yeast, in part because the principal source of HBsAg-positive plasma from the manufacture of vaccine was from the same population that subsequently was at the highest risk of contracting AIDS. However, since HBV is the only example of a successful vaccine against a mucosal virus, it has important implications, perhaps, in the control of human immunodeficiency virus (HIV) infection.

Although vaccine can help to prevent the spread of hepatitis B virus, it is not useful for those who have been infected by this virus. Millions of patients worldwide still suffer from this disease and the number of new infections continues to increase. Therefore, there is an urgent need to develop safe and effective anti-HBV drugs.

## Current status of anti-HBV chemotherapy

The lack of an *in vitro* tissue culture system to propagate the hepatitis B virus has hampered the molecular biology studies as well as the screening of antiviral compounds. In 1987, Sells *et al.* (1987) reported the establishment of an *in vitro* cell system, in which a human hepatoblastoma cell line (HepG2) is transfected with a plasmid carrying the hepatitis B virus genome. The cell line was designated as HepG2 2.2.15 cells, and it can constantly produce the HBV specific components, including the infectious Dane particles, HBsAg and HBeAg (Sells *et al.*, 1988). The validity of this system has been demonstrated since the virions produced by these cells can cause HBV infections in chimpanzees (Acs *et al.*, 1987). Generally, the HBV specific proteins (HBsAg or HBeAg) can be assayed by solid phase radioimmunoassay (RIA) and the viral DNA by southern-blot hybridization. Recently, polymerase chain reaction (PCR) technology has also been applied in the quantitation of viral DNA level (Wu *et al.*, 1994).

Several other *in vitro* cell cultures have also been reported, including HB611 (Sureau *et al.*, 1986; Tsurimoto *et al.*, 1987) and duck hepatocytes (Tuttleman *et al.*, 1986). It was found that antiviral compounds display different antiviral potencies in different cell lines, probably due to the different metabolic rates (Kitos *et al.*, 1991). However, HepG2 2.2.15 cell system is the most commonly used cell lines for *in vitro* screenings of anti-HBV compounds. The establishment of experimental animal models (ducks, and woodchucks) has also greatly facilitated the *in vivo* drug studies.

## Interferons

Interferon α is the only drug with demonstrable efficacy against HBV infection, and was approved by FDA for the treatment of chronic HBV infections in 1991. Three preparations have been available to the public, including Roferon A (α-2a), Intron A (α-2b) and Welferon α.

The interferons are a family of related proteins, including four major groups: α, β, ω and γ. As a group, they display a variety of properties that include antiviral, immunomodulatory and antiproliferative effects. Interferons are induced in response to virus infections or to double stranded RNA (dsRNA), and they interfere with several stages of the viral life cycle by activating a number of enzymes including 2',5'-oligoadenylate synthetases (2'-5'A synthetases), and dsRNA dependent protein kinases. Although the actual mechanism of action of interferon α in chronic HBV infections is not known, it has been demostrated to inhibit HBV replication and prolonged therapy can lead to a remission of the disease. Large international multicenter trials have shown that treatment of patients with interferon α results in HBeAg negative and HBV DNA negative in 37% and 34% of the patients, as compared with 13.5% and 24.9% of the untreated patients, respectively (Ryff, 1993). However, the therapy is effective in only 30 to 50% of patients, although the response rate is higher (50 to 60%) in carriers with higher baseline serum aminotransferase and lcwer level of HBV DNA (Korenman *et al.*, 1991). Additionally, relapse has often been observed after discontinuation of the drug. Other limitations of interferon ??include various side effects, relatively high expense and administration by injection.

## Thymosin

Thymosin is a thymic extract that mediates a variety of immunological effects, including augmentation of suppressor T-cell activity and *in vitro* B-cell synthesis of IgG. The peptide preparations of thymosin have been evaluated in small clinical trials with patients of chronic HBV infection. Results from the 6-month studies indicated the higher clearance of HBV (86%) than that of placebo (20%) with no toxicity noted (Mutchinck *et al.*, 1991).

Recently, several multi-center phase III trials with a synthetic analogue, thymosin alpha 1 (Zadaxin) have been conducted (Chien and Liaw, 1995). Thymosin alpha 1 is a 28-amino acid synthetic hormone analogue produced by the conventional solid-phase peptide synthesis and can act as an immune stimulant. In a clinical trial conducted in the People's Republic of China with a total of 150 chronic HBV patients, thymosin alpha 1 was reported to be safe and effective in treatment of chronic HBV infection. The results indicated that thymosin alpha 1 has marked effect on HBV DNA level as a monotherapy (66.7%), whereas combined with interferon α, it could affect both HBV DNA and HBeAg significantly (61.1%). These encouraging results have led to the approval of Zadaxin in several eastern Asian countries including the People's Republic of China, Singapore and the Philippines. However, additional studies are likely to be required for approval of thymosin alpha 1 in the U.S. and Europe and other countries with stricter regulatory authorities.

In addition to thymosin, several other cytokines have been reported to have anti-HBV effects. Transient inhibition of HBV DNA polymerase has been observed in patients treated with interleukin-2 (IL-2) (Bruch *et al.*, 1993), whereas the increased level of tumor necrosis factor (TNF) and IL-1-β in patients with elimination of HBV after interferon α treatment suggested that these agents may contribute to the anti-HBV effects (Guidotti *et al.*, 1994; Sheron *et al.*, 1990).

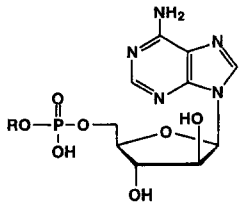
## Nucleoside analogues

Nucleosides represent ones of the most abundant resources for discovering antiviral agents. Many of the nucleoside analogues have been studied or are currently undergoing studies as anti-HBV agents.

### Ara-A and derivatives

Adenine arabinoside (araA, Vidarabine) is a potent inhibitor of HBV DNA polymerase. Its 5'-monophosphate (ara-AMP), a water soluble congener, can be administered intramuscularly. Ara-AMP was the first nucleotide extensively studied for treating chronic HBV infections in humans (Jacyna and Thomas, 1990). Suppression of HBV DNA has been demonstrated and clearance of HBeAg in 33% of the patients has been found in controlled trials. Usually, 8 weeks of treatment is required to effect the loss of HBeAg and HBV DNA, but in many cases, serious neurotoxicity is seen after 4 weeks.

In order to reduce the dose-related side effects, many hepatotropic targeting strategies have been developed.




R = H
R = Lac-HSA
R = Lac-poly-L-lysine

**Fig. 2.** Structures of ara-A and derivatives.

The conjugate of ara-AMP with lactosaminated human serum albumin (Lac-HSA) (Fiume *et al.*, 1981), a galactosyl terminating neoglycoprotein that binds to the Ashwell's receptor (Ashwell and Harford, 1982) and selectively penetrates into hepatocytes by receptor mediated endocytosis, was reported to inhibit virus replication in woodchucks with chronic WHV (Ponzetto *et al.*, 1991), and in patients with HBV infection (Hoofnagle *et al.*, 1984). In these studies, marked antiviral activity was observed at daily doses 3 to 6 times less than the free drug without producing any clinical side effects. Another approach (Fiume *et al.*, 1995), in which the lactosaminated poly-L-lysine (Lac-poly-L-lysine) was conjugated with ara-AMP, has also been reported to show comparable effect with the advantage of a better patient compliance, namely, the IM administration. However, the future status of araA analogues as a single agent therapy is uncertain, but it maybe useful in combination therapy.

## Ribavirin

Ribavirin (Virazole), 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide, is a nucleoside analogue that was first synthesized in 1970s (Sidewell *et al.*, 1972). It has shown a broad-spectrum of activities against both DNA and RNA viruses (Sidewell *et al.*, 1985), including adenoviruses, herpes viruses (HSV), influenza viruses, respiratory syncytial virus (RSV), HIV, hepatitis A, B and C viruses and has been approved by the FDA for the treatment of RSV infections in infants.

Ribavirin exerts its action after intracellular phosphorylation to mono-, di- and triphosphate nucleotides. The precise mode of actions includes perturbation of intracellular nucleoside triphosphate pools, interference with the formation of the 5'-cap structure of viral mRNA by competitive inhibition of both guanyltransferase and methyltransferase capping enzymes, direct inhibition of the viral mRNA polymerase complex and enhancement of macrophage inhibition of viral replication. Ribavirin has been studied in patients with chronic hepatitis B but it was ineffective and too toxic for prolonged usage (Fried *et al.*, 1994).

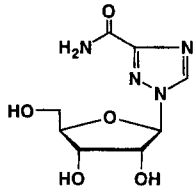



**Fig. 3.** Structure of ribavirin.

## Acyclic nucleosides

Acyclovir (ACV), 9-(2-hydroxyethoxymethyl)guanine, is a synthetic nucleoside analogue in which a linear side chain has been substituted for the cyclic sugar of the naturally occurring guanosine. It is the current medical choice for the treatment of genital herpes and herpes keratitis. In cells infected by herpes viruses, acyclovir can be selectively phosphorylated by the virus-encoded thymidine kinase (TK) to its monophosphate, and transformed by cellular enzymes to the triphosphate, which is then utilized as a substrate by the viral DNA polymerase. Due to the lack of 3'-OH, it functions as a chain terminator to inhibit the viral DNA synthesis.

Acyclovir is a modest inhibitor of HBV DNA polymerase, and showed both *in vitro* and *in vivo* activities in the animal models. However, its efficacy in the human trials is disappointing (Minuk *et al.*, 1992). A prodrug, 6-deoxyacyclovir (Desciclovir), is orally absorbed completely, but appears to have little effect, either. Since the response rate for interferon α therapy is poor, especially for those patients with high HBV DNA levels, the combination usage with acyclovir was explored in the anticipation of lowering the viral load. Although an initial pilot study showed that this combination is efficacious in eradication of HBV DNA (Schalm *et al.*, 1986; Schalm *et al.*, 1986), the results were not sustained in the subsequent controlled study (Berk *et al.*, 1992). However, the concept of reducing the high viral load by interferon and other drugs with good therapeutic index should be further persued.

Ganciclovir (DHPG), 9-[(1,3-dihydroxy-2-propoxy) methyl]guanine, a homologue of acyclovir, is the first antiviral drug to be effective in the treatment of cytomegalovirus (CMV) disease in humans (Spector *et al.*, 1996). Unlike acyclovir, ganciclovir is not an absolute chain terminator, and short subgenomic fragment of CMV DNA continues to be synthesized. The drug's antiviral effects are due to its ability to inhibit the viral DNA synthesis by slowing down the elongation of viral DNA (Martin *et al.*, 1983). In addition to its activity against HSV (Smith *et al.*, 1982), CMV and Epstein-Barr virus (EBV) (Cheng *et al.*, 1983), ganciclovir is also a potent inhibitor of hepadnaviruses, including DHBV (Luscombe *et al.*, 1996) and HBV (Kruining *et al.*, 1995), both *in vitro* and *in vivo*. It was reported that combination of ganciclovir with foscarnet

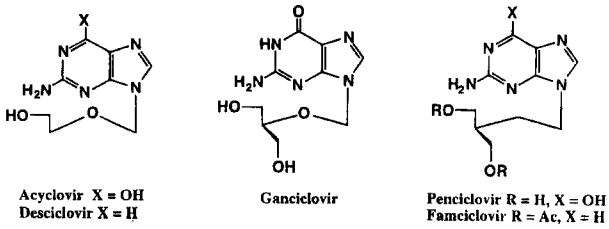



**Fig. 4.** Structures of acyclic nucleosides.

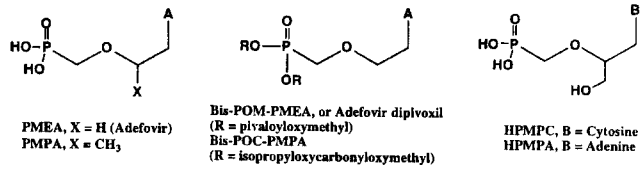



**Fig. 5.** Structures of acyclic nucleoside phosphonate.

(trisodium phosphonoformate) can cause substantial reduction of viremia in primary duck hepatocytes (Civitico *et al.*, 1996) and shows efficacy in controlling the recurrence of HBV infection in the post liver-transplant patients (Gish *et al.*, 1996). However, the drug is too toxic for long-term usage in human because of its bone-marrow toxicity, probably due to its unique inhibition of the human DNA polymerase δ (Isley *et al.*, 1995), which is one of the enzymes responsible for the repair of chromosomal DNA.

Another acyclic nucleoside analogue, penciclovir, 9-(4-hydroxy-4-hydroxymethylbut-1-yl)guanine, which is currently approved for the treatment of herpes zoster infection (Vere Hodge and Cheng, 1993), has also shown potent activity against DHBV and HBV in primary duck hepatocytes (Shaw *et al.*, 1994) and human hepatoblastoma cells 2.2.15 (Korba and Boyd, 1996), respectively. Compared with ganciclovir, which shows a 50% inhibition of DHBV DNA at 4.0 μM, penciclovir is more potent with the same inhibition at 0.7 μM, while its $EC_{90}$ in 2.2.15 cells is 1.6 μM. The diacetyl-6-deoxy derivative, famciclovir, is the oral prodrug (Tsiquaye *et al.*, 1994) of penciclovir, and can be quickly absorbed and sequentially deacetylated in the intestinal wall and liver to yield 6-deoxypenciclovir. The latter can be oxidized by xanthine oxidase in liver to give rise to the parent drug (Perry and Wagstaff, 1995).

A small NIH study tested combination treatment with famciclovir and interferon α in five patients who had previously failed interferon therapy. Famciclovir alone was administered for 4 weeks (500 mg three times daily), followed by famciclovir plus interferon α, 5 million units daily for 12 weeks, followed by 4 weeks of interferon α alone. Two patients responded to the combination, becoming HBV DNA negative and HBeAg negative with normal liver enzyme levels (Marques *et al.*, 1997). Penciclovir/famciclovir are currently undergoing phase III HBV clinical trials in the U.S.

A variety of acyclic nucleoside phosphonate analogues have been investigated in the treatment of HIV infections and accompanying opportunistic infections (HSV-1, HSV-2 and CMV). HPMPC (Cidofovir) exhibits potent *in vitro* and *in vivo* activities against a broad-spectrum of herpes viruses including CMV (De Clercq *et al.*, 1987), and has been recently approved for the treatment of CMV retinitis in AIDS patients (Hitchcock *et al.*, 1996). HPMPA was reported to exhibit anti-HBV activity in both duck hepatocytes and HepG2 2.2.15 cells with an $EC_{50}$ of 1.2 μM (Yokota *et al.*, 1990; Yokota *et al.*, 1991). PMEA (Adefovir) is effective against both herpes viruses and retroviruses and is currently undergoing phase II clinical trials against HIV. Since the replicative cycle of hepadnaviruses resembles to some extent that of the retroviruses, PMEA has also been evaluated against HBV.

It was found that PMEA (Adefovir) exhibits potent inhibitory effect on HBV replication and viral antigen production in both of two human hepatoma cell lines transfected with HBV (HepG2 2.2.15 and HB611) with $IC_{50}$ of 0.7 μM and 1.2 μM, respectively (Heijtink *et al.*, 1993). PMEA also shows an inhibition to DHBV in primary duck hepatocytes ($IC_{50}$ 0.2 μM) as well as in ducks (Nicoll *et al.*, 1996). The orally available prodrug of PMEA, bis-POM-PMEA (Adefovir dipivoxil, GS-0840), has been designed to improve the bioavailability (6 to 30%), and is currently undergoing phase II clinical trials agaist HBV in Great Britain. Although the cytotoxicity was increased somewhat relative to adefovir, adefovir dipivoxil was still about 3-fold more selective than the parent compound. Adefovir dipivoxil was reported to be more active than adefovir in inhibiting the replication of HSV-2 in Vero cell culture. The $IC_{50}$ value for adefovir dipivoxil and adefovir were reported to be 0.6 μM and 119 μM, respectively. More recently, another congener, PMPA has also been reported to exhibit potent antiretroviral activity. The oral prodrug, bis-POC-PMPA (bis-isopropyloxycarbonyloxy-methyl-PMPA) was designed to reduce the side effects due to pivaloate generated from POM (Fridland *et al.*, 1997).

## Carbocyclic nucleosides

The carbocyclic analogue of 2'-deoxyguanosine (2'-CDG) is the first carbocyclic nucleoside which was reported to exhibit potent inhibition to HBV replication (Price *et al.*, 1989). In 2.2.15 cells, 2'-CDG showed a 50% inhibition to HBV DNA polymerase activity at 5 ng/mL, whereas at 25 ng/mL, the complete disappearance of HBV replication was observed.

In the HepG2 cells, 2'-CDG was found to be converted to its triphosphate, which can be efficiently incorporated into HBV DNA, although the exact enzy-

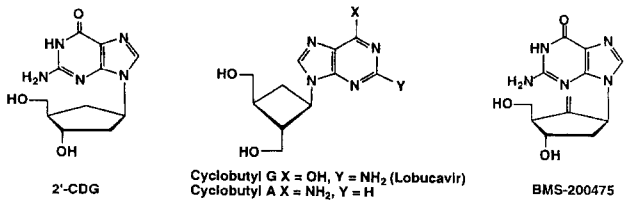



**Fig. 6.** Structures of anti-HBV carbocyclic nucleosides.

mes responsible for its phosphorylation were not clear. The triphosphate of 2'-CDG is a competitive inhibitor of dGTP for both HBV DNA polymerase and eukaryotic DNA polymerase δ, and the Ki of 2'-CDGTP for the viral enzyme is 6 times lower than the latter enzyme (Price *et al.*, 1992). 2'-CDG was also reported to induce a prolonged inhibition to DHBV DNA synthesis in primary duck hepatocyte cultures and in the liver (Fourel *et al.*, 1994). However, 2'-CDG was found to be toxic with a 50% inhibition of cell growth (HepG 2 2.2.15 cells) at 32 μM (Jansen *et al.*, 1993). Therefore, it is not suitable for the long-term usage in humans, yet it is commonly used as a reference for *in vitro* evaluation of antiviral compounds (Wu *et al.*, 1994).

Several other carbocyclic nucleosides have also been reported to exhibit anti-HBV activities. For example, administration of cyclobutyl G (Lobucavir) or cyclobutyl A at 70 mg/kg led to a rapid reduction of DHBV DNA to undetectable levels in serum, and in only 1 of 4 animals did DHBV DNA become detectable again within 10 days after stopping the drug (Dusheiko, 1995). Lobucavir (LBV) is a deoxyguanine nucleoside analogue with the broad-spectrum antiviral activity. LBV was previously shown to inhibit herpes simplex virus (HSV) DNA polymerase after phosphorylation by the HSV thymidine kinase. LBV inhibited HCMV DNA synthesis to a degree comparable to that of ganciclovir (GCV), a drug known to target the viral DNA polymerase. The expression of late proteins and RNA, dependent on viral DNA synthesis, was also inhibited by LBV. Immediate-early and early HCMV gene expression was unaffected, suggesting that LBV acts temporally coincident with HCMV DNA synthesis and not through cytotoxicity. *In vitro,* the triphosphate of LBV was a potent inhibitor of HCMV DNA polymerase with a K*i* of 5 nM. LBV was phosphorylated to its triphosphate form intracellularly in both infected and uninfected cells, with phosphorylated metabolite levels two- to threefold higher in infected cells (Tenny *et al.*, 1997).

Most recently, a novel carbocyclic nucleoside, BMS-200475, has been reported to exhibit potent anti-HBV activity (2.2.15 cells) with an $EC_{50}$ of 3 nM (Bisacchi *et al.*, 1997), which represents one of the most potent anti-HBV nucleosides discovered so far. BMS-200475 has an excellent selectivity index (> 8,000) in 2.2.15 cells with an $IC_{50}$ of 30 μM. *In vitro* biochemical studies indicated that BMS-200475 can be efficiently phosphorylated by cellular enzymes to its triphosphate, which is a potent inhibitor of HBV DNA polymerase, inhibiting both priming and elongation steps of HBV DNA replication (Colonno *et al.*, 1997). Nineteen chronically-infected woodchucks were treated orally once a day for 8 weeks at 0.5 mg/kg of BMS-200475. Serum WHV DNA levels became undetectable in all animals within 1~5 weeks. Thirteen animals were placed at a dosing regiment of 0.5 mg/kg of BMS-200475 given orally once a week. Twelve of 13 animals have undetectable EWHV DNA serum levels in 24 weeks, 16 weeks after daily dosing was halted. This study is still ongoing. The maintenance of suppressed serum WHV DNA levels in the woodchucks by weekly BMS-200475 dosing suggests that less frequent dosing with this potent nucleoside may be of value in the therapy of hepatitis B infection in men (Clark *et al.*, 1997). BMS-200475 is currently undergoing phase II clinical trials as an anti-HBV agent.

## 2',3'-Dideoxy nucleosides

2',3'-Dideoxynucleosides represent the most fruitful class of compounds as anti-HIV agents. Several analogues, including AZT, ddI, ddC, d4T, and 3TC have been approved by FDA for the treatment of HIV infections. Since the replication strategy of HBV resembles that of the retroviruses, in particular, the reverse transcription, many 2',3'-dideoxynucleoside RT inhibitors have been studied as potential anti-HBV agents. AZT, the first approved anti- HIV agent, was found to be ineffective against HBV in the cell culture assays (2.2.15 cells). Although ddA showed a significant inhibitory effect on DHBV replication in ducklings (Martin *et al.*, 1988), the clinical trial with the parent drug ddI indicated that it's not effective against chronic hepatitis B virus in humans (Fried *et al.*, 1992). In a cell culture study, the anti-HBV activities of several 2', 3'-dideoxynucleosides were compared (Korba and Milman, 1991). It was found that ddC exhibits a significant inhibitory effect on the replication of HBV DNA. Another analogue, ddG also shows significant antiviral effect at the same level. Although the 2,6-diaminopurine derivative is less potent *in vitro,* rapid clearance of viral DNA has been observed after administration to Peking ducks at a dose of 10 mg/kg i.m.

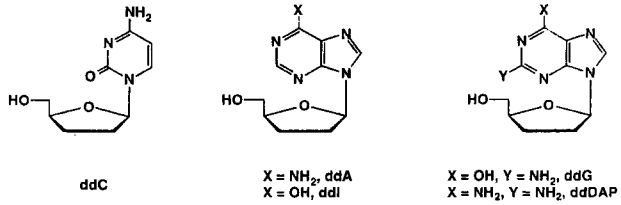



**Fig. 7.** Structures of anti-HBV 2',3'-dideoxynucleosides-I.

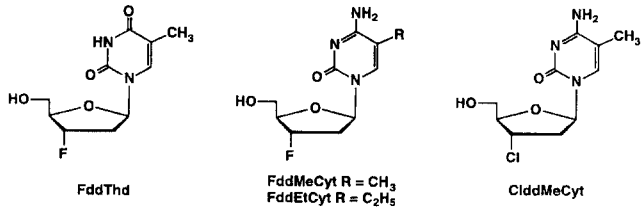



**Fig. 8.** Structures of anti-HBV 2',3'-dideoxynucleosides-II.

twice daily (Lee *et al.*, 1989).

Several other 2',3'-dideoxynucleosides have been tested for their potential antiviral activities *in vitro* using the HepG2 2.2.15 cells. It was found that 2',3'-dideoxy-3'-fluorothymidine (FddThd), 2',3'-dideoxy-3'-fluoro-5-methylcytidine (FddMeCyt), 2',3'-dideoxy-3'-fluoro-5-ethylcytidine and other analogues display cytostatic activities at concentrations between 0.54 (FddMeCyt) and 3.93 μM (2',3'-dideoxy-3'-fluoro-5-ethylcytidine). Of these compounds, FddThd was the most effective antiviral agent with more than 90% reduction of HBV DNA synthesis was measured at 0.03 μM. The most potent antiviral agents among the cytidine analogues tested *in vitro* were FddMeCyt (more than 90% reduction of HBV DNA synthesis at 0.1 μM) and CldMeCyt (0.1 μM). FddThd and FddMeCyt were also effective against DHBV *in vivo.* Administrations of FddThd and FddMeCyt to ducks infected with DHBV for 12 days blocked the virus production. Termination of treatment with FddThd of infected animals led to reappearance of the virus in serum, though at lower levels (Matthes *et al.*, 1992).

Despite the potent anti-HBV activity shown by the 2',3'-dideoxynucleosides, long- term usage of these compounds as therapeutic anti-HBV drugs is prohibited since treatment of AIDS patients with these nucleosides (AZT, ddC, ddI, d4T and 3TC) have been associated with various clinical toxicities including bone-marrow suppression, myopathy, peripheral neuropathy and pancreatitis. The phase II clinical trials with FddThd were called off due to the death of two HIV-1 infected patients. The underlying mechanism for these toxicities was proposed to be the inhibition of mitochondrial DNA (mtDNA) synthesis by these nucleosides (Chen *et al.*, 1991).

Studies in cell cultures indicated that the delayed toxicity is due to the inhibition of DNA polymerase γ, the enzyme responsible for mtDNA synthesis. For example, ddC was found to be phosphorylated to ddCTP by the sequential action of deoxycytidine kinase, cytosine nucleoside monophosphate kinase, and nucleoside diphosphate kinase (Starnes and Cheng, 1987). Although ddCTP is a poor inhibitor ($K_i$ > 100 μM) of the chromosomal DNA polymerases α, β, and ε, it is a potent inhibitor of the DNA polymerase γ with a $K_i$ of 0.02 μM, which is at the same level as its inhibition to HIV RT ($K_i$ of 0.01 to 0.03 μM). The ddCTP

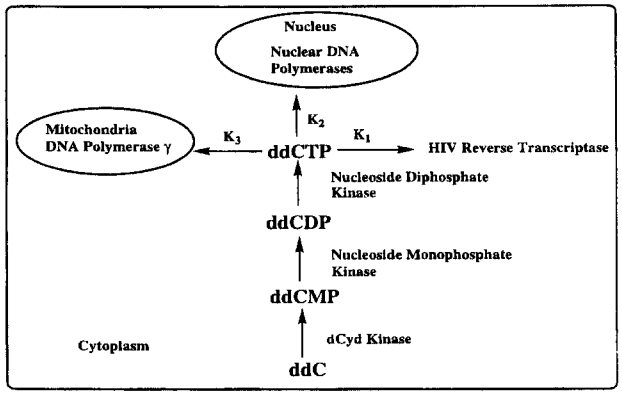



**Fig. 9.** Metabolism and inhibition of mitochondria DNA synthesis by ddC.

formed in cytoplasm can be transported into mitochondria, where it is utilized by DNA polymerase γ as a substrate. Due to the lack of the 3'-OH, extention of DNA chain is terminated and only the removal of the incorporated nucleoside by exonucleases can restore the DNA synthesis. Similar findings have also been observed with ddI and d4T, whereas the toxicity of AZT is likely due to its inhibition of one or more of the chromosomal DNA polymerases, since its $K_i$ for DNA polymerase γ is at 1 μM.

## 2'-Fluoro-arabinofuranosylpyrimidine nucleosides

The 2'-fluoro-substituted arabinofuranosylpyrimidine nucleosides have shown potent *in vitro* and *in vivo* activities against medically important herpes viruses (Watanabe *et al.*, 1979; Watanabe *et al.*, 1984; Watanabe *et al.*, 1983). FIAC and FIAU are preferentially phosphorylated in HSV-1 infected cells via the virus encoded thymidine kinase (Kreis *et al.*, 1982). Consequently, these compounds are more effective as inhibitors of herpes viruses which are $TK^+$ than viruses which are deficient in TK activity (Lopez *et al.*, 1980).

The analogues, FIAC, FMAU and FEAU have been shown to inhibit WHV replication in the chronically infected woodchucks (Fourel *et al.*, 1990). To a less extent, FMAU and FIAC demonstrated activity against DHBV in the duck models (Fourel *et al.*, 1992). Another analogue, FIAU, has been shown to decrease the levels of HBV DNA in patients with chronic hepatitis B virus (Fried and Di Bisceglie, 1992). In a cell culture study, FIAC and FIAU were reported to inhibit HBV replication by 90% at concentrations of 34±7 and 24±4 μM, respectively. Although the mechanism of action underlying the anti-HBV activity of these compounds is not well characterized, FIACTP has been shown to inhibit endogenous HBV DNA polymerase activity which suggests that the anti-HBV activity of this class of compounds may be mediated, in part, at the level of viral DNA polymerase (Hanz *et al.*, 1984).

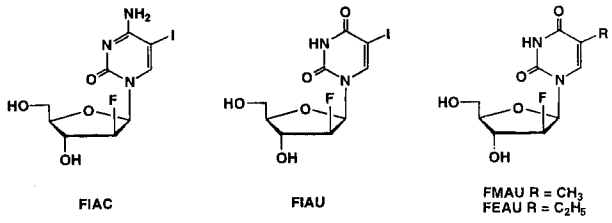



**Fig. 10.** Structures of 2'-fluoro-D-arabinofuranosyl pyrimidines.

However, in a NIH-run clinical trial carried out in the summer of 1993, five of twenty patients died of liver failure and five others were forced to undergo liver-transplants after receiving FIAU for more than one or two months (Maçilwain, 1993; Touchette, 1993). Pathological studies have shown that the drug causes lactic acidosis and microvesicular steatosis, and the toxicity was suggested to result from the dysfunction of mitochondria (Parker and Cheng, 1994).

Studies with cultured cells (HepG2 2.2.15) indicated that FIAU is activated by host cell enzymes including thymidine kinase, thymidylate kinase and pyrimidine diphosphate kinase to FIAUTP, which inhibits the viral DNA polymerase. However, FIAUTP is also efficiently used as a substrate by DNA polymerase γ. Due to the presence of 3'-OH, incorporation of FIAUTP into DNA does not necessarily result in chain termination, although the rate of DNA chain extension is slower. Unlike ddC, treatment of cells with FIAU does not lead to the decline of mtDNA content, therefore, the delayed toxicity of FIAU is likely due to its incorporation into mtDNA, which disrupts the expression of DNA in some manner, resulting in either decreased production of proteins or the production of defective proteins. Since there is no known mechanism available to repair this fraudulent incorporation, the effect is irreversible (Parker and Cheng, 1994; Cui *et al.*, 1995). Furthermore, FIAU can be metabolized *in vivo* by thymidylate synthase to FMAUTP, which has been shown to have severe neurotoxicity in patients (Abbruzzese *et al.*, 1989).

Since mitochondria are the organelles that generate ATP for the metabolic energy of cells, interference of mtDNA function results in the short of energy, which has to be compensated alternatively by glycolysis (Parker and Cheng, 1995). This leads to the increased production of lactic acid, which was observed in both the cultured cells as well as in the liver biopsy of FIAU treated patients.

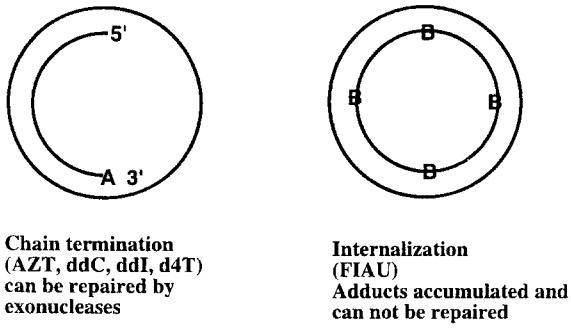



**Fig. 11.** Different mitochondrial toxicities caused by nucleoside analogues.

## Oxathiolane, dioxolane nucleosides and L-nucleosides

In the search of anti-HIV agents, a novel class of nucleosides has been discovered, where the 3'-carbon of the furanose ring is replaced by a hetero-atom (S or O). Originally, (±)-Dioxolane-thymidine and (±)-BCH-189 were reported to exhibit anti-HIV activity by Norbeck *et al.* (1989) and Belleau *et al.*, (Belleau *et al.*, 1997; Soudeyns *et al.*, 1991) respectively. (±)-BCH-189 has also been found to exhibit potent anti-HBV activity in 2.2.15 cells (Doong *et al.*, 1991).

The stereospecific syntheses of the enantiomerically pure isomers of (±)-Dioxolane thymidine and (±)-BCH-189 were first reported by Chu and co-workers (Chu *et al.*, 1991; Beach *et al.*, 1992). Comparison of the antiviral activity indicated that the β-L-isomer (3TC) is more potent and less toxic than the β-D-isomer [(+)-BCH-189] in PBM cells. In 2.2.15 cells, 3TC exhibits a 50% inhibition to viral DNA replication at 0.01 μM without any cytotoxicity, whereas (+)-BCH-189 shows an $EC_{50}$ of 0.5 μM with significant cytotoxicities in a variety of cell lines. A more recent investigation (Cui *et al.*, 1996) on the effect of nucleoside analogues on mitochondrial function in HepG2 cells indicated that 3TC has no effect on either mtDNA synthesis or lactic acid production, while (+)-BCH-189 shows a dose-dependent effect on both mtDNA synthesis and lactic acid production. The lack of mitochondrial toxicity for 3TC was suggested to be due to the the stereospecific uptake of deoxynucleoside triphosphate by the mitochondrial membrane (Parker and Cheng, 1994). In cells, 3TC can be phosphorylated by dCK and other cellular enzymes to its mono-, di- and triphosphate, which functions as a chain terminator of the viral DNA synthesis (Severini *et al.*, 1995). Culture studies using the 2.2.15 cell line have demonstrated that combination treatments with 3TC and either interferon or penciclovir reduced the

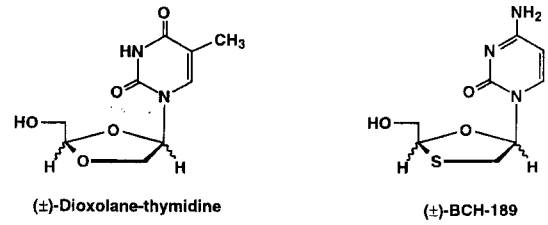



**Fig. 12.** Structures of (±)-dioxolane-thymidine and (±)-BCH-189.

90% effective concentrations of these agents 10 to 30-fold (BE Korba, Antiviral Res. 29:49).

Monotherapy with interferon, 3TC, or famciclovir suppressed WHV viremia in chronically infected woodchucks approximately 10 to 300-fold after 12 to 24 weeks of treatment at doses which are the approximately equivalent (based on metabolic body size, K 3/4) to those used in humans. Combination treatments with 3TC and either interferon or famciclovir reduced WHV viremia by the end of the treatment periods significantly more than the corresponding monotherapies and significantly more than the levels predicted for additive antiviral effects. However, following the withdrawal of treatment, essentially no difference was observed in the rebound of WHV viremia between combination and monotherapies (Korba *et al.*, 1997).

In addition to its *in vitro* anti-HBV activity, 3TC has also been shown to effectively suppress DHBV in ducks and HBV in chimpanzees (Tyrrell *et al.*, 1993). Among patients with HIV infection and chronic hepatitis B, 3TC rapidly reduced HBV DNA to undetectable levels, with no serious adverse effects at doses 5 to 600 mg per day (Benhamou *et al.*, 1995). In a recent phase II clinical trial, the HBV DNA levels became undetectable in 70% of the patients who received 25 mg dose and 100% of those treated at 100 mg or 300 mg dose (Dienstag *et al.*, 1995). However, relapses have been observed in most of the patients after cessation of therapy, and the results suggested that sustained responses were more likely in patients with initially low HBV DNA levels and high alanine aminotransferase levels. Drug resistances to 3TC have also been reported, and a similar mutation to the 3TC-resistant HIV-RT in HBV viral polymerase (YMDD to YIDD) has been demonstrated both *in vitro* (Fisher *et al.*, 1996) and observed in orthotopic liver-transplant patients who do not response to 3TC treatment (Ling *et al.*, 1996; Tipples *et al.*, 1996). 3TC has been recently approved by FDA for the treatment of AIDS patients in combination with AZT and is undergoing phase III clinical trials as an anti-HBV agents (Dienstag *et al.*, 1995).

The 5-fluoro congener of 3TC, cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-cytosine (FTC), has been shown to exhibit potent anti-HBV activity *in vitro* ($EC_{50}$ 0.01 μM) in the hepatoma cell lines (Schinazi *et al.*, 1992; Furman *et al.*, 1992). Additionally, FTC also showed strong inhibition to the replication of DHBV in vivo in chronically infected ducks (Fourel *et al.*, 1994). Although the D-enantiomer is not as potent as the L-isomer, it does not show significant cytotoxicity, either. Biological studies suggested that the β-L-isomer can be efficiently phosphorylated by dCK as for the β-D-isomer, however, it is not a good substrate for the cytidine deaminase, which degrades the D-isomer at a much higher rate than the L-isomer. This difference between the D and L-isomers towards anabolic and catabolic enzymes results in the enhanced antiviral potency of the L-isomer (Schinazi *et al.*, 1992; Furman *et al.*, 1912). Recently, it was reported that neither the D- or L-isomer nor the racemate of (±)-FTC shows any dose-dependent adverse effect on the mitochondrial function. FTC is currently undergoing phase II clinical trials as an anti-HBV agent.

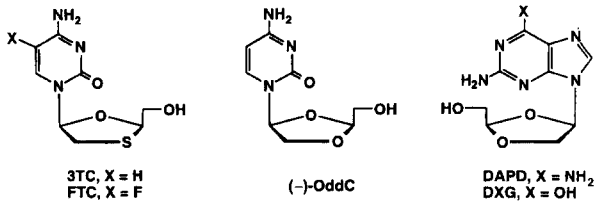



**Fig. 13.** Structures of anti-HBV dioxolane and oxathiolane nucleosides.

Extensive structure-activity relationship studies lead to the discoveries of L-(-)-OddC and DAPD. L-(-)-OddC exhibits extremely potent anti-HIV activity in cell cultures ($EC_{50}$ 2 and 5 nM in PBM and CEM cells, respectively) and anti-HBV activity ($EC_{50}$ 0.5 nM in 2.2.15 cells). However, this compound is also very toxic ($IC_{50}$ 0.26 and 0.10 μM in CEM and Vero cells, respectively) (Kim *et al.*, 1992). Since L-(-)-OddC inhibits the growth of hepatocellular and prostate tumors that are generally difficult to treat, it is currently being developed as an anti-cancer agent (Grove *et al.*, 1995).

DAPD is the 2,6-diaminopurine dioxolane analogue with the sugar moiety in the natural D-configuration. DAPD exhibits potent activity against both HIV (Kim *et al.*, 1993) ($EC_{50}$ 0.03 μM in PBM cells) and HBV (Schinazi *et al.*, 1994) ($EC_{50}$ 0.09 μM in 2.2.15 cells) with a favorable toxicity profile. Animal studies indicated that DAPD is the prodrug of dioxolane-guanine (DXG), and it can be converted to DXG *in vivo* by adenosine deaminase (ADA) (Rajagopalan *et al.*, 1994). Studies in woodchucks indicated a half life of 6.7 h for DAPD after intravenous administration. The oral bioavailability ranges from 3.7% to 8.2% (Rajagopalan *et al.*, 1996). Currently, DAPD is undergoing preclinical studies as an anti-HIV and anti-HBV agent.

The interesting findings that some of the nucleosides with unnatural L-configurations show more potent antiviral activity with lower cytotoxicity than corresponding D-counterparts led to the extensive screening of L-nucleosides as potential antiviral agents. In this regard, several new anti-HBV agents have been discovered.

The L-enantiomer of the anti-HIV drug ddC was reported to exhibit potent anti-HBV activity ($EC_{50}$ 0.01 μM in 2.2.15 cells). Its 5-fluoro congener showed not only potent anti-HBV activity ($EC_{50}$ 0.01 μM) but also potent anti-HIV activity without siginificant toxicity in cell cultures (Gosselin *et al.*, 1994; Lin *et al.*, 1994).

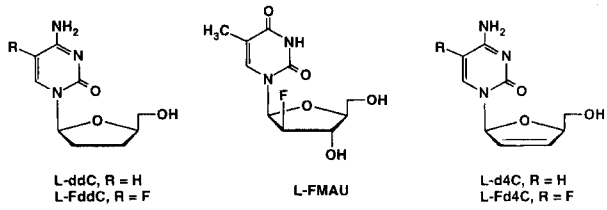



**Fig. 14.** Structures of anti-HBV L-nucleosides.

Although L-FddC does not significantly decrease the mtDNA content, a recent study indicated that it does result in the increased lactic acid production in HepG2 cells. Recently, L-FddC has also been shown to be a strong inhibitor of DHBV both *in vitro* and *in vivo* (Zoulim *et al.*, 1996).

Two other L-nucleosides, namely, L-Fd4C and L-d 4C have also been reported to have potent antiviral activities (Lin *et al.*, 1996). L-Fd4C shows extremely potent anti-HBV activity ($EC_{50}$ 2 nM in 2.2.15 cells) and anti-HIV activity ($EC_{50}$ 0.09 μM in CEM cells), whereas L-d4C is less potent against both viruses (8 nM and 1.0 μM for HBV and HIV, respectively). Both compounds inhibit cell growth at concentrations below 20 μM, although L-Fd4C does not exhibit significant inhibition of mtDNA at 100 μM.

Another L-nucleoside, L-FMAU, was reported by Chu *et al.* as a potent antiviral agent against HBV ($EC_{50}$ 0.1 μM in 2.2.15 cells) (Chu *et al.*, 1995). L-FMAU was found to have low cytotoxicities in a variety of cell lines including MT2, CEM, H1 and 2.2.15 and bone marrow progenitor cells, while the D-counterpart, D-FMAU, is much less active and significantly more toxic. Therefore, the selectivity index for L-FMAU is much higher than that of D-FMAU (2,000 *vs* 25 in 2.2.15 cells). In addition to its potent anti-HBV activity, L-FMAU also exhibits potent anti-EBV activity. The $EC_{90}$ for L-FMAU is at the same level as the current clinical drug, DHPG (5.0 μM in H1 cells), whereas its selectivity is better than the latter (183 *vs* 15). Chronic WHV carrier woodchucks were treated for 4 weeks with daily oral doses of L-FMAU ranging from 0.3 to 10.0 mg/kg, and prompt, dose-related reductions in serum WHV DNA was observed. Four chronic WHV carrier woodchucks were treated with L-FMAU for 12 weeks at an oral dose of 10.0 mg/kg/day. A greater than 1000-fold reduction in serum WHV DNA was observed within 7 days, was decreased 100-fold in L-FMAU-treated woodchucks, but WHsAg remained detectable by ELISA. Hepatic WHV DNA replicative intermediates were decreased greater than 10-fold at the end of treatment, and hepatic expression of WHcAg assessed by immunohistochemistry also was significantly decreased. In one woodchuck, serum WHV DNA returned to the pretreatment level 20 weeks after drug withdrawl while in the other three, serum WHV DNA remained undetectable by dot blot analysis for more than 28 weeks (Peek *et al.*, 1997).

The anti-HBV mechanism of L-FMAU has been reported by Pai *et al.* (Pai *et al.*, 1996) It was found that L-FMAU produced a dose-dependent inhibition to the viral DNA replication in 2.2.15 cells with a 50% inhibitory concentration at 0.1 μM. There was no inhibitory effect on HBV transcription or protein synthesis. Repeated treatment of HepG2 cells with L-FMAU at 1 μM for 9 days did not result in any decrease in the total mtDNA content. Additionally, at concentration of 200 μM, L-FMAU did not adversely affect mitochondrial function as determined by lactic acid production in L-FMAU treated hepatoma cells. L-FMAU was found to be metabolized in cells by the cellular thymidine kinase and deoxycytidine kinase to its monophosphate, and subsequently to the di- and triphosphate by some unknown enzymes. Although the precise mechanism of action of L-FMAU is not clear, a dose-dependent inhibition of HBV DNA synthesis by L-FMAUTP was observed in the DNA polymerase assays with isolated HBV particles, suggesting that inhibition of viral DNA polymerase may account for its anti-HBV activity.

In addition to its *in vitro* anti-HBV activity, L-FMAU also exhibits potent activity against DHBV in primary duck hepatocyte ($EC_{50}$ 0.1 μM). Oral administration of L-FMAU (40 mg/kg/day) for 5 days to Peking ducks congenitally infected with chronic DHBV reduced the viremia level markedly (72% compared to control) without any abnormalities (Zoulim *et al.*, 1996). Furthermore, *in vivo* efficacy studies in woodchucks chronically infected with WHV indicated that L-FMAU completely suppresses the viral DNA during the drug treatment at 10 mg/kg/day, and no virus rebound was observed for 24 weeks after cessation of the drug treatment (Tennant *et al.*, 1997).

The pharmacokinetics of L-FMAU in rats have been reported by Wright *et al.* (Wright *et al.*, 1995). A linear disposition of L-FMAU was observed over the dosage of 10 to 50 mg/kg after intravenous administration. In another study (Wright *et al.*, 1996), the oral bioavailability of L-FMAU was found to range from 59 to 64% after oral administration to rats. The pharmacokinetics study of L-FMAU in woodchucks indicated a terminal phase half life of *ca.* 4.5 h following intravenous administration and a bioavailability of 40% after oral administration (Witcher *et al.*, 1997).

These outstanding features of L-FMAU make it a promising clinical candidate for the treatment of chronic HBV infections.

## Miscellaneous anti-HBV agents

### Foscarnet

Foscarnet, trisodium phosphonoformate (PFA) is an

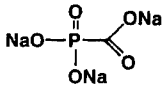



**Fig. 15.** Structure of foscarnet.

organic analogue of inorganic pyrophosphate which inhibits both DNA and RNA polymerases. Foscarnet has shown antiviral activities against a variety of viruses, including HSV-1 and 2, CMV, EBV, HHV-6, HIV, HBV and VZV, and it's currently used in the treatment of CMV diseases (Helgstrand *et al.*, 1978; Wagstaff *et al.*, 1994).

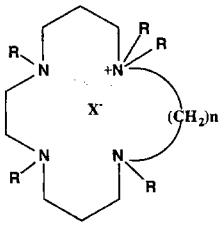
However, due to its non-specific action on DNA and RNA polymerase, it's too toxic for the long-term usage in chronic HBV patients. Studies suggested that better regimens for foscarnet are combination usage with other antiviral drugs such as ganciclovir, which have been shown recently to be effective in controlling the recurrence of HBV infection in post liver-transplant patients.

### *N,N,N',N'',N'''*-pentakis (ω-aminoalkyl) tetraazamacrocycles

The antiviral activity of a new class of N,N,N',N'', N'''-pentakis (ω-aminoalkyl) tetraazamacrocycles was evaluated in primary duck hepatocyte cultures infected with the duck hepatitis B virus (DHBV). Three of the four tested compounds were able to selectively inhibit DHBV replication by acting at an early step of the hepadnanavirus infection but were associated with significant toxicity. Cytotoxicity measurements were based on the estimation of hepatocyte viability after drug treatment by uptake of neutral red dye. Hepatocytes in 24-well tissue culture plates were cultured in medium containing various concentrations of the tested compound with daily changes of medium. Four wells per assay were used. After 9 days of treatment, cell viability was estimated. The $CC_{50}$ was defined as the concentration required to reduce cell viability by 50% (Hantz *et al.*, 1997).

### Antisense oligonucleotides

Antisense oligonucleotides are either DNA or RNA (ribozyme) oligomers which can bind to target sequences, resulting in either the block of transcription or cleavage of the target mRNA. Since the binding strictly follows the Watson-Crick base pairings rule, the action is specific to the mRNA of a viral or tumor proteins.




1a: n = 2; X = TsO⁻ R = -(CH2)5-N(Boc)Bn

1b: n = 2; X = Cl⁻ R = -(CH2)5-N(H)Bn

2a: n = 3; X = TsO⁻ R = -(CH2)5-N(Boc)Bn

2b: n = 3; X = Cl⁻ R = -(CH2)5-N(H)Bn

**Fig. 16.** Structure of *N,N,N',N'',N'''*-pentakis (ω-aminoalkyl) tetraazamacrocycles.

Antisense oligonucleotides (ASON) have been shown to inhibit HBV replication and the production of viral specific proteins *in vitro* in hepatoma cell lines (Korba and Gerin, 1995; Wu and Wu, 1992). Recently, the effective inhibition of DHBV pre-S proteins has also been demostrated *in vivo* (Offensperger *et al.*, 1994). However, due to the large size and highly ionic property, systemic usage of these macromolecules as therapeutic agents remains a problem.

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