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Nucleotide analogs as novel anti-hepatitis B virus agents

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During the past decade, nucleotide analogs have emerged as novel antiviral agents against hepatitis B virus. Adefovir dipivoxil, a prototype phosphonate analog, has been approved for chronic hepatitis B virus therapy, and additional phosphonate analogs and di- and tri-nucleotides are under development. Several innovative prodrug derivatizations have also been reported to improve the oral bioavailability of nucleotide analogs, which usually carry a negative charge.

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Current Opinion in Pharmacology 2005, 5:520–528

This review comes from a themed issue on
New technologies
Edited by Patrick Iversen

Available online 8th August 2005

1471-4892/\$ – see front matter

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DOI 10.1016/j.coph.2005.04.019

Introduction

The design of selective, specific and non-toxic antiviral agents has presented extraordinary challenges compared with the design of other antimicrobial agents. This is primarily because, in contrast to bacteria and parasites that are equipped with their own metabolic machinery for reproduction and growth, viruses rely on the host cellular machinery for replication and propagation. Consequently, there are few virus-specific molecular targets that are amenable to antiviral intervention. In addition, drug resistance to antiviral agents is a major problem as many viruses mutate rapidly under the selective pressure of antiviral therapy. Not surprisingly, antiviral chemotherapy has lagged considerably behind antibacterial chemotherapy. In recent times, however, and largely owing to the AIDS epidemic and advances in molecular virology, the structure and function of a few virus-specific molecular targets such as proteases and helicases have been revealed. Furthermore, in the past decade, a repertoire of discovery tools including structure- and mechan-

ism-based drug design, as well as technologies such as antisense [1], ribozymes [2] and RNA interference [3,4], has provided novel strategies for the design of antiviral agents against new molecular targets. Notwithstanding these advances, progress in the discovery of new antiviral therapeutics has remained slow, and current antiviral chemotherapy is heavily dependent on a 'cocktail' of antiviral agents. New antiviral agents, with unique mechanisms of action, that feed into the 'cocktail pipeline' are urgently needed given the propensity of viruses to mutate rapidly under the selective pressure of chemotherapy.

An estimated 400 million people are chronically infected with hepatitis B virus (HBV), which is one of the ten leading killer diseases worldwide [5]. Approximately 25% of such patients develop liver cirrhosis and hepatocellular carcinoma, resulting in a million deaths each year [6]. Furthermore, all chronic carriers suffer from significant disease-associated malaise during their lifetime. In the US alone, there are about 1.25 million adults and children with chronic HBV infection [7,8].

Interferon α , an immunomodulator, and lamivudine (3'-thiacytidine, 3TC), a nucleoside analog that inhibits viral reverse transcriptase, were the first drugs approved for the treatment of HBV [9]. Although daily subcutaneous injection of interferon α reduces viral load in a small percentage of patients, it is associated with significant adverse effects [8,9]. Although 3TC administration suppresses viral load in 96% of patients, viral replication rebound upon cessation of therapy [10,11] and rapid emergence of resistance represent major problems [12,13]. Most recently, entecavir, a nucleoside analog, has been approved for HBV therapy [14].

Following the historical success with nucleoside antivirals, medicinal chemists have continued to design nucleoside analogs that target viral HBV polymerases as anti-HBV agents. In general, all nucleosides need to be phosphorylated to nucleoside mono-, di- and tri-phosphates before they become inhibitors of HBV polymerase. Although several nucleoside analogs are under development, potential emergence of resistance and occurrence of adverse events, including mitochondrial toxicity [15,16], are major issues that need to be considered during long-term anti-HBV therapy. Thus both monotherapy and combination therapy with nucleosides can induce mitochondrial DNA depletion, resulting in mitochondrial toxicity. An excellent review [17] covers the recent advancements in the nucleoside field. In the

past decade, nucleotide analogs have emerged as an alternative and new class of anti-HBV agents and are the focus of this review.

Nucleotide analogs as anti-HBV agents

Historically, nucleotide analogs, as phosphorylated derivatives of nucleosides, were not considered favorably as antiviral agents as they are negatively charged and believed impermeable to cells [18]. Surprisingly, a few nucleotide analogs have been discovered in the past decade with potent anti-HBV activity. Adefovir (ADV) dipivoxil, the first prototype nucleotide analog, has been approved recently for HBV therapy [19].

A nucleotide (e.g. adenosine 5'-monophosphate, structure 1; see Figure 1) is the phosphorylated derivative of a nucleoside, and consists of three molecular fragments: the sugar, heterocyclic nucleobase and the phosphate. DNA and RNA, the fundamental building blocks of life, are biopolymers built from nucleotide units. In addition, nucleotides perform diverse biological functions: as an

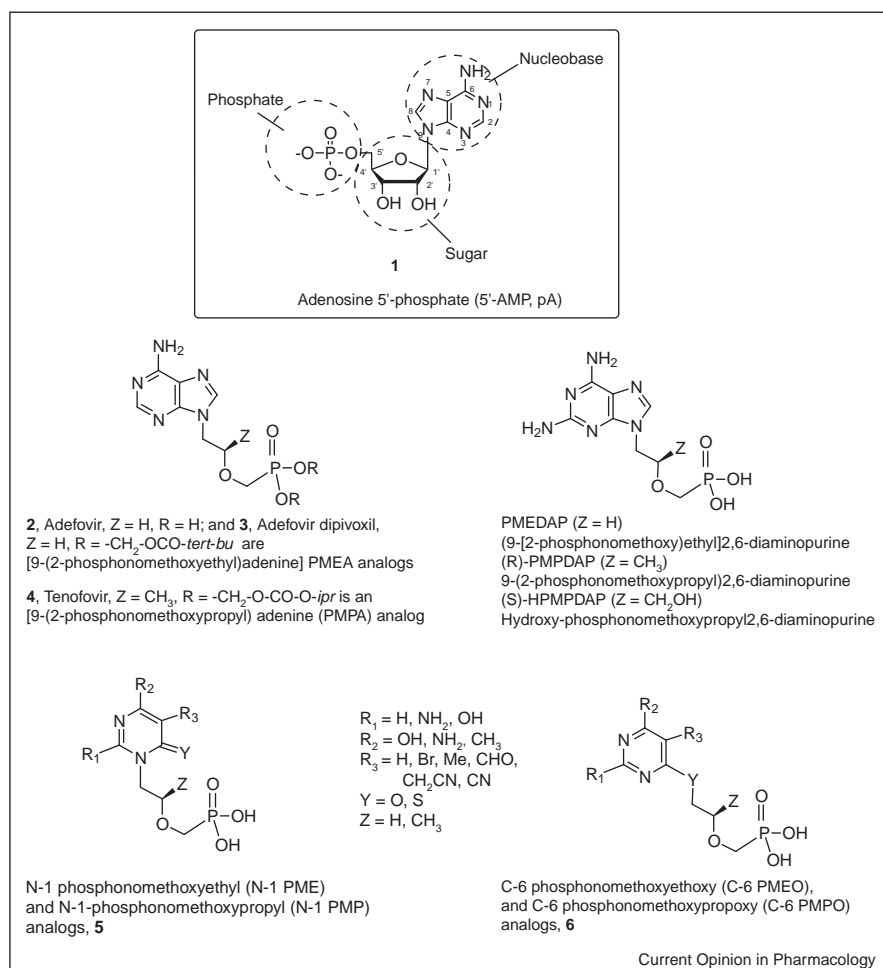
energy pool in the form of di- and tri-phosphates, as 'second hormonal messengers' in the form of cyclic nucleotides, and as essential coenzymes and cofactors for enzyme function.

In a nucleotide structure, the sugar moiety — a deoxyribose (in DNA) or a ribose (in RNA) — is in a cyclic furanoside form linked to one of the nucleobases via a β -glycosidic configuration, resulting in the four nucleosides: adenosine, guanosine, cytidine, and thymidine (uridine in RNA). The family of anti-HBV nucleotides reviewed herein broadly encompasses all nucleotide structural analogs, including the isosteres of sugar, phosphate and nucleobase, as well as their prodrug derivatives.

Acyclic nucleoside phosphonate analogs and their prodrug derivatives

Acyclic nucleoside phosphonate analogs (Figure 1), of which 2 (ADV; 9-(2-phosphonomethoxyethyl) adenine [PMEA]) is the prototype, are structurally related to nucleoside 5'-monophosphates and are characterized by

Figure 1



Structures of nucleotides and nucleotide analogs.

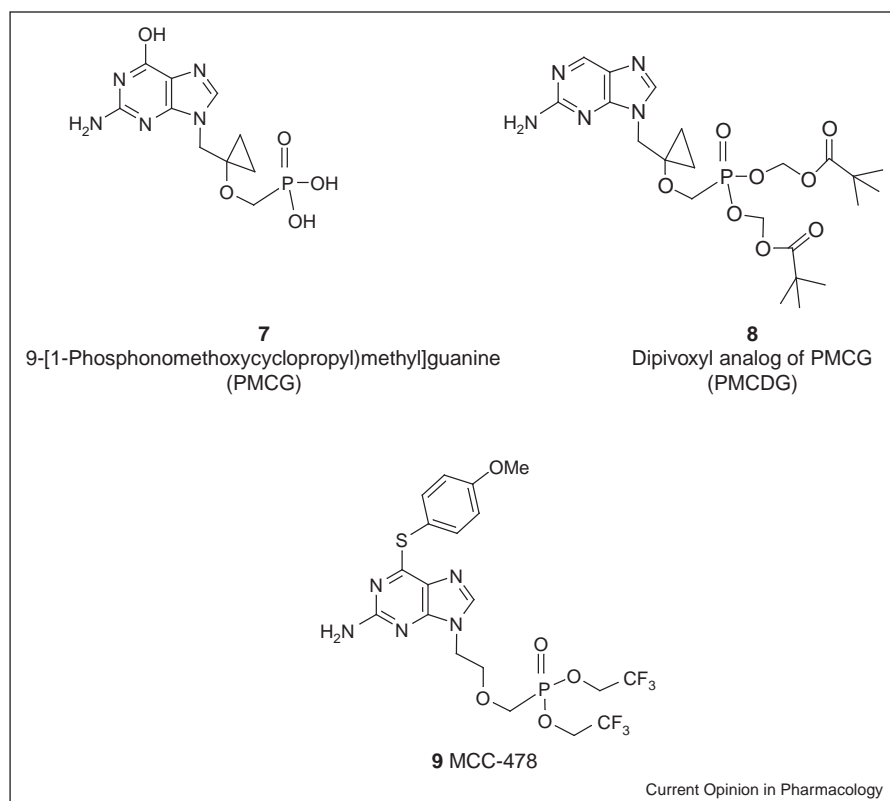
the presence of a phosphonylethyl ether group [O-CH₂-P(O)(OH)₂] in place of phosphate [CH₂-O-P(O)(OH)₂] [20]. The phosphonate analogs are structurally unique because the presence of the phosphonylethyl ether group renders them resistant to the action of phosphatases; they are substrate for kinases so that further phosphorylation of the phosphonate can occur; and the presence of the ether oxygen in the phosphonate moiety provides a binding site for target enzymes such as kinases and polymerases [20]. Indeed, the antiviral activity of ADV is manifested through inhibition of viral reverse transcriptase by the corresponding diphosphorylated metabolite. Several clinical trials of ADV dipivoxil (**3**), the orally bioavailable prodrug of **2**, has been conducted and antiviral efficacy, as well as significantly improved virologic and biochemical parameters, has been reported both for HbeAg-negative and HbeAg-positive patients [19]. The prodrug **3** undergoes rapid enzymatic hydrolysis by non-specific esterases, yielding **2** during or following the absorption process in the gastrointestinal tract. The parent drug (**2**) does not undergo significant metabolism, and is rapidly eliminated unchanged following administration. However, renal tubular nephropathy is a serious and important dose-limiting toxicity associated with **3** [21,12]. Nephrotoxicity is believed to occur as a result of accumulation of **2** in renal proximal tubules through

tubular resorption, and is mediated by the human organic anion transporter enzyme [22]. Recently, tenofovir disoproxil fumarate (**4**), the hydroxymethyl counterpart of **2**, was also found to be efficacious in patients with 3TC-resistant and wild-type HBV co-infected with HIV [23].

It is interesting to note that most anti-HBV PMEAs analogs such as **2** are based on the adenosine molecular framework. However, several additional phosphonylethyl analogs (**5** and **6**) with a pyrimidine nucleobase have been synthesized recently and evaluated for anti-HBV activity [24**] (Figure 1). The prototype phosphonylethyl analog with pyrimidine nucleobase had *in vitro* anti-HBV activity against both wild-type and 3TC-resistant variants, which was comparable to that of PMEAs. Similarly, the guanosine phosphonate analog 9-(2-phosphonomethoxy cyclopropyl)methylguanine (**7**) is a novel cyclopropyl derivative [25] (Figure 2), which is reported to be more potent than ADV. The corresponding orally bioavailable pivaloyl ester prodrug (**8**) has been synthesized and demonstrated to show excellent efficacy in a woodchuck model of HBV [25].

Another guanosine phosphonate analog, MCC-478 (**9**; Figure 2), is reported to be 80-fold more potent than 3TC in an *in vitro* HBV assay [26]. In addition, the

Figure 2



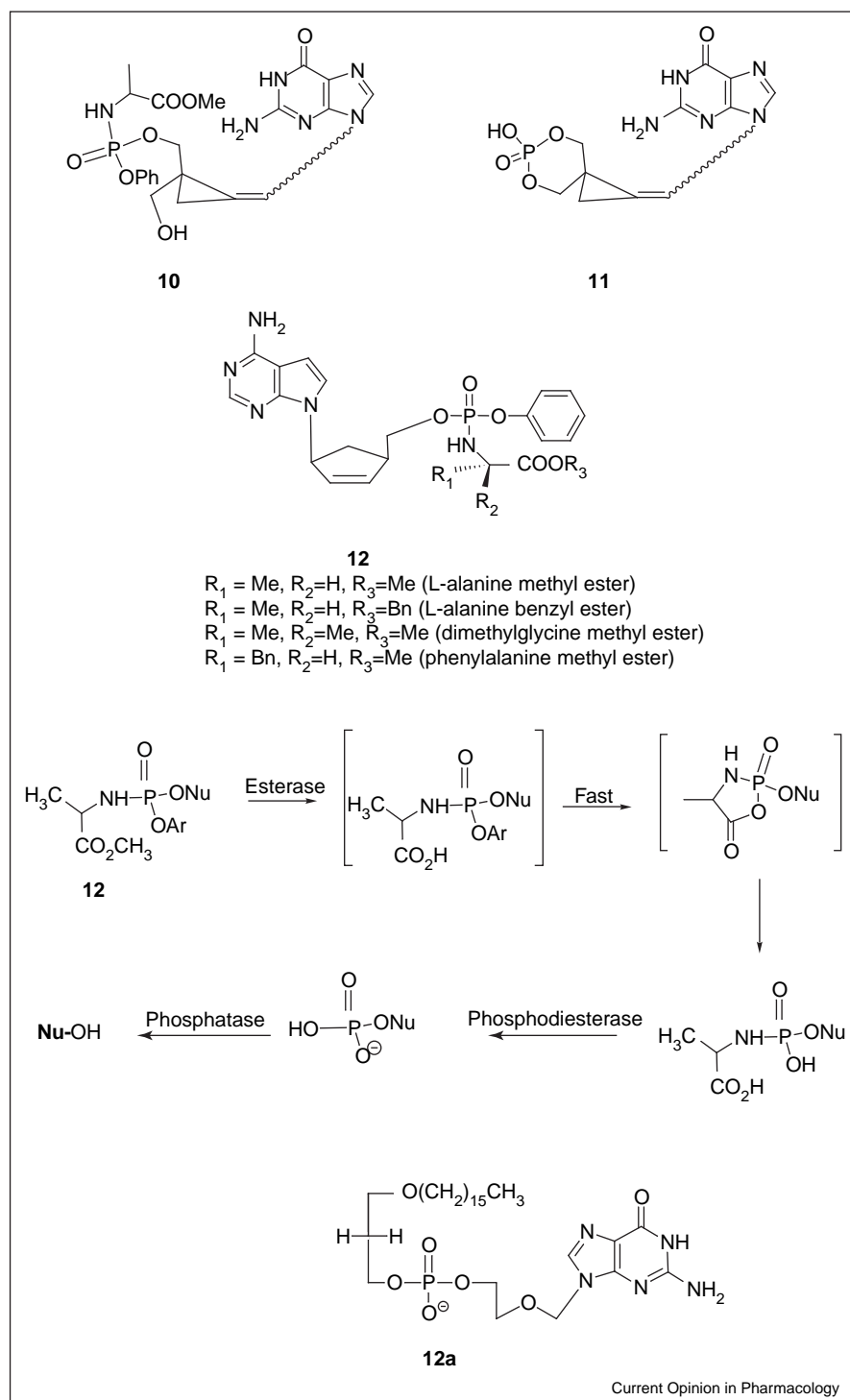
Structure of guanosine phosphonate analogs.

bis-trifluoroethyl ester moiety reportedly confers oral bioavailability to the parent molecule. It is interesting to note that, in addition to MC-478, the hydrolysis products derived from **9** and its *O*-demethylated metabolite also exhibited significant anti-HBV activity.

Cyclic nucleoside phosphate analogs and their prodrug derivatives

The *Z* and *E*-2,2-bis-(hydroxymethyl)methylene cyclopropane derivatives, **10** and **11** (Figure 3), are a novel group of nucleotide analogs with broad-spectrum antiviral

Figure 3



Phosphoramidate and phosphoglyceryl prodrugs of nucleotides.

activity, including anti-HBV activity [27]. The pronucleotide phenylmethyl phosphor-L-alaninate (**10**) and cyclic phosphonate (**11**) are reported to be active anti-HBV agents with EC_{50} s of 4.1 μ M and 0.8 μ M, respectively.

Nucleoside analogs as nucleotide prodrugs

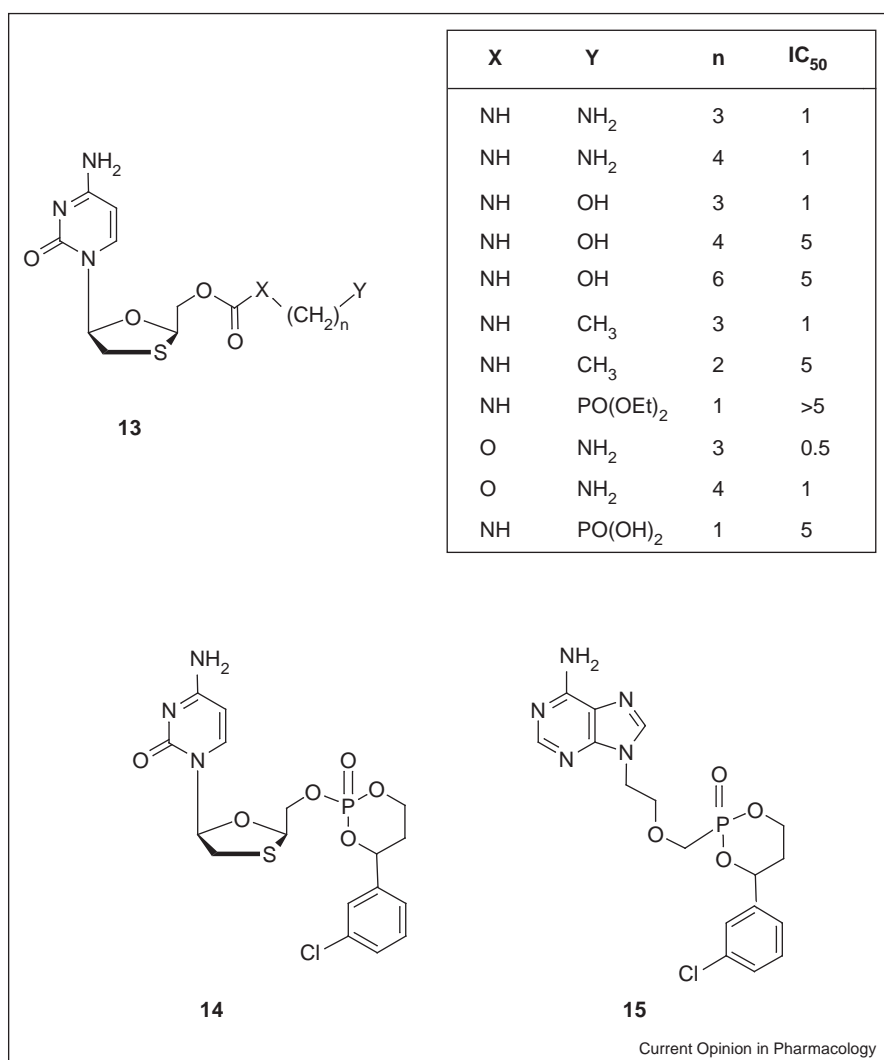
Interestingly, prodrug strategies have been employed to improve the potency of moderately active nucleoside anti-HBV agents (Figure 3). For example, although the racemic carbocyclic 2',3'-dideoxy-2',3'-didehydro-7-deazaadenosine was only moderately active (EC_{50} of 2 μ M), the corresponding amino acid methyl ester derivative (**12**) provided a 40-fold enhancement of potency (EC_{50} of 0.05–0.1 μ M) [28**]. It was proposed that the amino acid carbomethoxy phosphodiester moiety in **12** (Figure 3) facilitated the intracellular delivery of nucleotides [29]. Thus, once inside the cell, a combination of esterase-, phosphodiesterase- and phosphatase-mediated hydroly-

tic processes results in the conversion of the amino acid ester to the nucleoside [30]. However, an alternate hypothesis for the mechanism of bioconversion and antiviral activity has also been presented for the corresponding phosphomonoester amidates [31].

Another exciting strategy for enhancing efficacy and oral bioavailability of nucleosides is through the use of glyceryl ester or phospholipid analogs (Figure 3). Thus, 1-*O*-octadecyl-sn-glycero-3-phospho acyclovir (a nucleotide analog; **12a**) was found to be a potent inhibitor of HBV in the woodchuck, whereas the parent nucleoside acyclovir was inactive [32].

Other important pronucleotide strategies that have been applied broadly in the antiviral field include the *S*-acyl-2-thioethyl derivatives of nucleotides [33] and the salicylic acid-derived or cycloSal analogs [34].

Figure 4



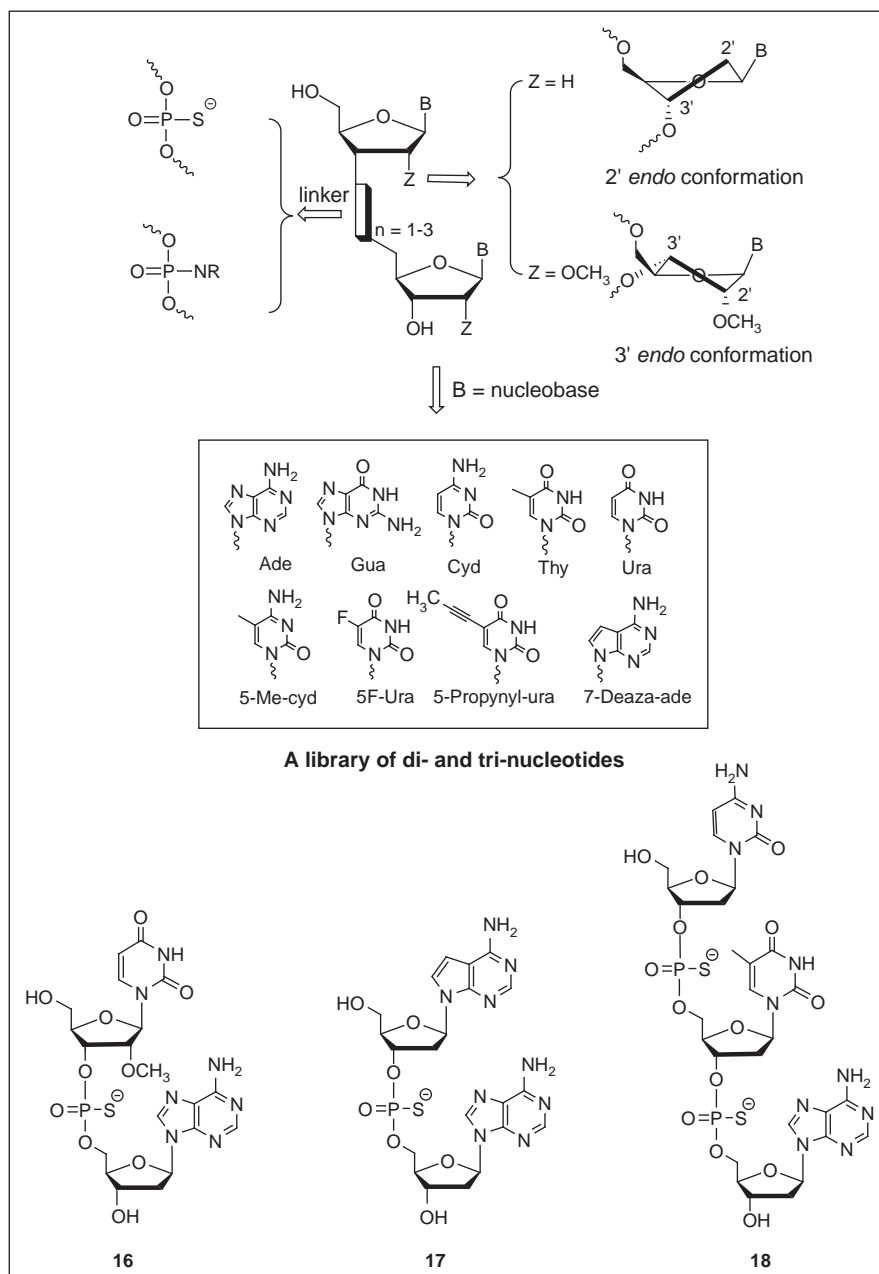
New prodrug analogs of 3-TC and adefovir.

The 5'-*O*-carbonate and 5'-*O*-carbamate derivatives (**13**; Figure 4) can be regarded as nucleotide analogs of 3-TC [35]. The 5'-*O*-carbonate analog, a prodrug, is enzymatically hydrolyzed to 3-TC, and its anti-HBV activity is attributable to that of 'free' 3-TC resulting from hydrolysis [35]. However, the carbamate analog, resistant to hydrolytic conver-

sion to 3-TC, is also a potent anti-HBV agent, although it is devoid of reverse transcriptase (RT) inhibitory activity. Its mechanism of action is yet to be delineated.

Recently, a new class of nucleoside phosphate and phosphonate prodrugs (**14** and **15**), designated 'HepDirect'

Figure 5



Discovery of di- and tri-nucleotides as novel anti-HBV agents by combinatorial approach. The di and tri-nucleotide library members were phosphorothioate and phosphoramidate analogs. The phosphorothioate group could potentially participate in electrostatic interactions, whereas the non-ionic phosphoramidates could facilitate delivery into cells. The dominant furanose modification was the substitution of a 2'-OMe group in place of a 2'-hydrogen in the deoxyribofuranoside ring. The 2'-substituent can act as a 'conformational switch' that transforms the furanose ring pucker from the 2'-endo to 3'-endo, thereby affecting the global conformation of the individual library members. The nucleobase modifications included substitution on the heterocycle moiety that provided additional hydrophobicity to the library members. Reproduced with permission from [42].

prodrugs (Figure 4), has been described [36^{*}]. These prodrugs are 4-arylsusbstitued cyclic 1,3 propanyl esters, which are designed to undergo oxidative cleavage via cytochrome P450, an enzyme expressed predominantly in the liver. Both 3-TC and ADV prodrugs (**14**, **15**) were found to be selectively cleaved by liver homogenates to give the corresponding bioactive nucleoside phosphate and phosphonate.

Di- and tri-nucleotide analogs as novel anti-HBV agents

Recently di- and tri-nucleotides have been designed as a novel class of anti-HBV agents using the rationale that structural and functional proteins of viruses can contain nucleotide-binding domains within protein α -helices and β -sheets [37]. It appeared that by combinatorial synthesis of structurally diverse nucleotide compounds (Figure 5) and screening using cell-based anti-HBV assays, antiviral compounds could be identified that modulate virus-specific molecular pathways. In theory, this approach would allow simultaneous functional validation of a target and discovery of a lead structure that modulates the function of the target [38,39].

Accordingly, through a process of iterative synthesis and screening [40–43], three compounds (**16**, **17** and **18**) were identified from a 1000-member library of di- and tri-nucleotide phosphorothioates. These compounds demonstrated consistent and potent anti-HBV activity against both wild-type and 3-TC-resistant HBV strains (Table 1). The compounds **16–18** also displayed a favorable cytotoxicity profile with high CC₅₀ values against Madin Darby bovine kidney (MDBK) cells (800 μ M), human foreskin fibroblasts (300 μ M), human peripheral blood mononuclear cells (>300 μ M), and Vero cells (>1000 μ M) [44^{**}].

Interestingly, the analogs were metabolically stable with no evidence of conversion to mono-, di- or tri-phosphate forms. Therefore, it appears that the observed anti-HBV activity of the analogs is attributable to the intact structure. The compounds **16–18** therefore represent a new and unique class of antiviral agents.

Analog **16–18** were found to be direct inhibitors of viral replication through inhibition of intracellular HBV DNA

replication. However, there is currently no evidence of either intracellular phosphorylation of these compounds or their incorporation into HBV DNA. It is also highly unlikely that the compounds are inhibitors of viral integrase [45], as integration of HBV is not part of the viral replication cycle. From available information, it is conceivable that these molecules cause inhibition of the HBV RT-directed priming step before elongation of the negative (first) HBV DNA strand. This could occur either by direct interaction with the polymerase or by competition for the binding of the 4-base primer sequence to the initiation site for elongation of the HBV negative strand of DNA.

The analogs acted cooperatively in combination with 3-TC, although less promising interactions with ADV were observed with dinucleotides **16** and **17** when compared with the trinucleotide **18**. The mechanisms related to the observed interactions are currently unclear, but might reflect potential differences in intracellular metabolism or mechanisms of activity against HBV. As these compounds are not phosphorylated and do not appear to be inhibitors of HBV polymerase via incorporation, cooperative effects with potent nucleoside analogues would not be surprising.

The dinucleotide analog **16** was evaluated for anti-HBV activity in a transgenic mouse model; transgenic mice were derived from a 1.3.32 line and obtained from Frank Chisari (Scripps Research Institute, LaJolla, CA) [46,47^{*}]. As predicted by its potent anti-HBV activity in cell culture, compound **16** was also active as an anti-HBV agent [47^{*}] in the transgenic mouse model, with a minimal effective dose between 1.6 mg/kg and 0.5 mg/kg, comparable to that of ADV dipivoxil. Unlike ADV, compound **16** does not appear to act as a classical chain terminator based upon Southern blot analysis of liver HBV DNA. Thus, these analogs might have a different mode of action and may provide an opportunity for administration in combination with other chain terminators to achieve synergistic antiviral activity, and to minimize the emergence of resistant strains of virus. Evaluation of these compounds in other HBV animal models and development of orally bioavailable analogs are underway.

Table 1

Relative potency of analogs against wild-type HBV and drug-resistant HBV mutants evaluated using cell-based assays.

Compound	EC ₅₀ against wild-type HBV (μ M)	EC ₉₀ against the M204V mutant (μ M)	EC ₉₀ against the M204I mutant (μ M)	EC ₉₀ against the L180M mutant (μ M)
3TC	0.061	>100	>100	>100
ADV dipivoxil	0.465	9.5	9.0	10
Compound 16 ^a	0.258	9.8	10	12
Compound 17 ^a	0.325	12	11	13
Compound 18 ^a	2.4	ND	ND	ND

^a See Figure 5 for structures. ND, not determined.

Conclusions

Nucleotide analogs represent an exciting class of anti-HBV agents with different mechanism(s) of action compared with the classic nucleoside analogs. Some of these nucleotide analogs have been developed successfully as orally bioavailable prodrugs, and act synergistically with other anti-HBV agents. Successful anti-HBV therapy is dependent upon efficacious combinations of anti-HBV agents which are relatively non-toxic upon longer-term use, overcome viral resistance to chemotherapy, and prevent viral replication rebound following cessation of therapy. Towards this objective, there is genuine optimism that selected nucleotide analogs could become front-line agents to be used in combination with other agents in anti-HBV therapy. Furthermore, the anti-HBV drug discovery strategies described herein could be potentially employed in developing novel antiviral agents against other existing and emerging viruses [48].

Acknowledgements

We are greatly indebted to the National Institutes of Health, for support of our research through NIAID, under a Research Project Cooperative Agreement Grant Award 5 UO1 AI058270. We also thank many scientists and collaborators whose work has been cited for their contribution to our program.

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