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Anti-HBV nucleotide prodrug analogs: Synthesis, bioreversibility, and cytotoxicity studies

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Abstract—Several pronucleotide analogs of the model anti-HBV dinucleotide 3′-dA-$U_{2'OMe}$ have been synthesized and evaluated for stability, bioreversibility and cytotoxicity. These studies have helped identify potential candidates for further evaluation.

The concept of using chemically modified drugs as prodrug analogs is an established paradigm in the pharmaceutical development of a number of different drugs.[1] The prodrug strategies permit transient modification of the physicochemical properties of the drug in order to: (a) improve chemical stability, (b) alter aqueous solubility, (c) improve bioavailability, (d) target specific tissues, (e) facilitate synergistic drug combinations, (f) overcome first-pass metabolic effects, (g) serve as lipophilic carrier for hydrophilic drugs, and (h) serve as a chemical depot for sustained drug delivery.[2]

Prodrug strategies are also being applied in the case of oligonucleotides (18–30 mers), which are being developed as potentially novel class of therapeutic agents using technologies such as aptamers,[3] antisense,[4a,b] ribozymes,[4c] RNA interference,[4d,e,f] and immunostimulation.[4g] Being highly charged, large molecular weight compounds, oligonucleotides have unfavorable physicochemical attributes for cell permeation by passive diffusion. Consequently, the design of prodrug analogs of oligonucleotides has mainly focused on the partial masking of their negatively charged backbone by bioreversible, lipophilic groups. Several such analogs have been synthesized and bioreversibility has been demonstrated in vitro.[5] However, there are no reports of in vivo oral bioavailability studies of oligonucleotide prodrugs. The lack of oral, transdermal, and other non-invasive, patient-compliant delivery systems, coupled with inefficient cellular permeability, represents a significant hurdle in the therapeutic advancement of these molecules. Shorter chain oligonucleotides (less than 8-mers) with lesser number of charges and smaller molecular weight compared to 20-mer oligonucleotides represent a promising class of novel molecules with potential therapeutic and diagnostic properties.[6] Indeed, recent reports[7–10] suggest that mono-, di-, tri-, and short chain oligonucleotides possess significant biological activity that can be exploited for therapeutic applications. In this context, we have recently reported that certain di-, and tri-nucleoside phosphorothioate (PS) and phosphoramidate analogs exhibit potent anti-HBV activity in vitro and in vivo.[9] Although dimer and trimer PS analogs are negatively charged small molecules, studies of ^{35}S-labeled compounds in rats have revealed that these compounds are not orally bioavailable (unpublished observations). The lack of oral bioavailability may be due to a number of factors including: (a) the acidic environment in the stomach that causes substantial degradation of the nucleotide, (b) the negative charge on the backbone that suppresses permeation of the nucleotide through the intestinal mucosal barrier, and (c) the presence of various digestive enzymes in the GI tract that degrade the compound. Given that both longer and shorter chain oligonucleotides are not orally bioavailable, it appeared to us that in the case of smaller nucleotide class of compounds, charge rather than the size of the compound may be a more important factor in determining bioavailability and that masking the negative charge on the backbone may potentially provide orally bioavailable nucleotide compounds.

Keywords: Anti-HBV nucleotide; Prodrug; Bioreversibility; Cytotoxicity; Stability; Dinucleotide.
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To develop orally bioavailable analogs of di-, and trinucleotides, we carried out the synthesis and evaluation of a number of *S*-functionalized, uncharged pronucleotide derivatives of a model dinucleotide. Our design of pronucleotide derivatives was based upon the ability of a target enzyme to unmask a latent functionality to reveal the parent nucleotide in vivo. This paper summarizes our results on the design, synthesis, stability, bioreversibility, and cytotoxicity studies of various analogs.

The general structure of dinucleotide derivatives **1**–**3** and the expected mechanism for their esterase-mediated conversion to the parent dimer **4** are depicted in Figure 1 and include: (a) *S*-(acyloxyalkyl)thiophosphate analogs (**1**). The acyloxyalkyl analogs,[2b] exemplified by the antibiotics pivampicillin, bacampicillin, as well as, the anti-HBV agent adefovir dipivoxil[11] are clinically used, orally bioavailable ester prodrug analogs. In vivo, the conversion of the prodrug to the parent molecule is believed to occur via esterase-mediated hydrolysis in plasma and/or liver, with concomitant liberation of formaldehyde and carboxylic acid. Guided by this clinical precedence and our previous studies,[5b] we designed the pivaloyl ester pronucleotide analog **6a** of the anti-HBV PS-dimer, 3′-dA-$U_{2'OMe}$ **5** (Fig. 2). (b) The *S*-(acyloxyaryl)thiophosphate analogs (**2**). The acyloxyaryl analogs of daunorubicin, doxorubicin, phosphorodiamide mustard, acivicin, and PEG-daunorubicin conjugate are well known[2b] and have been extensively evaluated in vitro and in vivo. Although a reactive methylene quinone intermediate is transiently released upon hydrolysis of these prodrugs, rapid capture of a water molecule by the semi-quinone intermediate results in

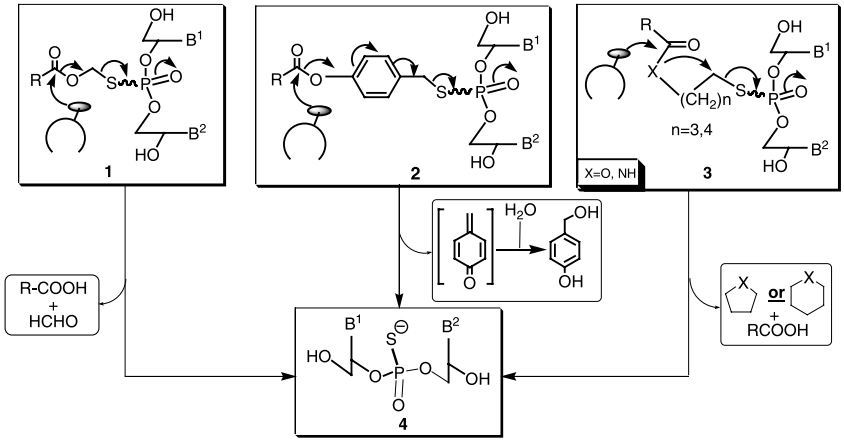
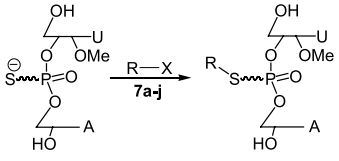


Figure 1. General structure of dinucleotide prodrugs and the proposed mechanism of bioreversibility.



Synthesis of pronucleotides: Representative preparation of pronucleotide **6a.** To a solution of the dinucleotide sodium salt (50 mg, 0.082 mmol) in water (1 mL) was added a solution of iodomethyl pivalate (**7a,** 85 mg, 0.35 mmol) in acetone (2 mL). The reaction was stirred overnight in the dark and concentrated with a few mgs of sodium bisulfite. The crude product was purified by column chromatography and **6a** eluted out in a mixture of DCM/MeOH (90/10). Concentration *in vacuo* gave chromatographically pure white solid. All analogs were prepared using similar procedures. ^{31}P NMR, 28.7, 27.9 δ ppm.

Structure of intermediates

#	R	X
7a[17]		I
7b@		I
7c#		Br
7d@		I
7e *		I
7f$		I
7g%		I
7h*		Br
7i$		I
7j$		I

@ prepared by the reaction of R-OH with CsI/BF_3.Et_2O;[16a] #ROH with $SOBr_2$/DCM; $ROH with $SOCl_2$ /DMF/KI;[16b] %obtained by reaction of di-iodobutane and sodium nicotinate *obtained from commercial sources.

Evaluation of pronucleotides

#	$t_{1/2}$ (min) in serum	$CC_{50(\mu M)}$ Vero	MDBK	HFF
6a	~60*	>1000	>1000	>1000
6b	~30*	>1000	>1000	>1000
6c	n.d.$	> 300	>300	100
6d	%	>1000	>1000	>1000
6e	>60 @	>1000	>1000	n.d
6f	>120@	>1000	>1000	n.d
6g	>120@	>1000	>1000	>1000
6h	~120@	>1000	>1000	>1000
6i	%	>1000	>1000	n.d
6j	%	>1000	>1000	>1000

* Conversion to the parent **5** observed; @ Half-life refers to the hydrolysis of the ester, and further conversion to **5** did not occur; %the pronucleotide remained unchanged even after 24 h in serum; $not determined as the compound was not soluble in DMSO.

Figure 2. Synthesis of dinucleotide prodrugs and their biological evaluation.

its conversion to the innocuous benzyl alcohol species (Fig. 1) thereby minimizing any cellular injury. Using this rationale, the analogs **6b–d** (Fig. 2) were designed to evaluate their potential as pronucleotide analogs of **5** including the ester analogs **6b**, and **6c** (which have a long chain alkoxy group that imparts greater lipophilicity to the molecule), as well as, the amide analog **6d**, and (**c**) *S*-alkyl derivatives with a terminal functional group (**3**) are designed such that during enzyme-mediated hydrolytic process, a latent nucleophilic group is uncovered, which is juxtapositioned to attack the electrophilic carbon α to the thiophosphate moiety resulting in the release of the parent dinucleotide. This design is similar to the extensively used 'cyclization-based' and 'trimethyl lock' prodrug concepts,[2,12] and was conceptualized for the pronucleotide analogs **6e–j** of **5** (Fig. 2).

For our studies, we employed the R_p, S_p mixture of the phosphorothioate analog **5**, which was synthesized in large scale (6 mmol of nucleoside-loaded controlled-pore glass (CPG) support) using solid-phase phosphoramidite chemistry,[13] in conjunction with a specially fabricated LOTUS reactor®.[14] The dA-linked CPG support was prepared using our recently discovered ultrafast functionalization and loading process for solid supports.[14] For the sulfurization of the internucleotidic dinucleoside phosphite coupled product, a solution of 3*H*-1,2-benzodithiole-3-one-1,1,-dioxide (0.4 M in dry CH_3CN)[15] was employed. Following processing, chromatographic purification, and lyophilization, the sodium salt of R_p, S_p **5** (~60:40 mixture) was obtained >96% pure, which was characterized by ^{31}P and ^{1}H NMR. The pronucleotide derivatives **6a–j** were synthesized in yields of 50–70% by chemoselective S-alkylation of R_p, S_p-**5** with the corresponding iodo-, or bromo-derivatives **7a–j** in aqueous acetone or methanol, followed by work-up and chromatographic purification. The requisite intermediates **7a–j** were synthesized directly from the corresponding hydroxy compounds[16] or by halogen exchange reaction from the corresponding chloro derivatives (see Fig. 2).[5b] ^{31}P NMR of each pronucleotide analog **6a–j** showed two peaks in the range of δ 28–34 ppm (characteristic of the thiophosphate triester moiety) corresponding to a ~55:45 ratio of the R_p, S_p isomers.

Evaluation of bioreversibility of the pronucleotides was carried out in rabbit serum in phosphate buffer at 37 °C. In order to monitor the hydrolytic conversion of the pronucleotides to the dinucleotide **5**, aliquots of incubate were removed at different time points, processed, and analyzed using reversed-phase HPLC. It was found that the analogs **6a** and **6b** were readily converted to the parent **5** with half-lives ($t_{1/2}$) of 60 and 30 min, respectively. Also, complete conversion of **6a** and **6b** to the parent **5** occurred in ~3 h. The analogs **6a** and **6b** were stable for up to 24 h in phosphate buffer (0.1 M, pH 7.2). Furthermore, there was no evidence of any significant stereodifferentiation or desulfurization during the hydrolysis of the R_p, S_p isomers in the mixture. Interestingly, both **6a** and **6b** were resistant to the hydrolytic action of pig liver esterase (PLE) and bovine chymotrypsin (data not shown), thereby suggesting that the analogs may have significant half-life in the Gl tract that could facilitate oral absorption of the intact pronucleotide. These observations are in contrast to the behavior of the corresponding pronucleotides of R_p, S_p TT-PS dimer where significant stereodifferentiation was noted along with much slower rates of hydrolysis in serum and PLE.[17] It is possible that due to different sugar puckering in 2′-OMe-uridine ($C_{3'}$-endo) compared to a thymidine ($C_{2'}$-endo), the global conformation of **6a** and **6b** may be significantly different from that corresponding to TT dimer pronucleotides. Consequently, the ester groups in **6a** and **6b** may be more favorably poised for attack by the nucleophilic site of the esterases.

Although the ester analogs **6e–g** were hydrolyzed to the corresponding alcohols, and **6h** to the corresponding carboxylic acid, their subsequent conversion to **5** by cyclization did not occur. Also, the amide analogs **6d**, **6i**, and **6j** remained unchanged even after incubation in serum for 24 h.[18] Furthermore, all analogs were stable indefinitely when stored at −20 °C as a lyophilized powder. We next examined the cytotoxicity profile[19] of the pronucleotide derivatives in different cell lines such as MDBK, Vero, and HFF. As shown in Figure 2, most analogs, except for **6c** had $CC_{50} > 1000$ μM in these cell lines demonstrating good safety profile for these compounds.

In conclusion, we have synthesized and evaluated a number of pronucleotides of **5** and the analogs **6a** and **6b** appear to be promising candidates for further evaluation. Further studies are ongoing and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.058.

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