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Prodrug approaches to improving the oral absorption of antiviral nucleotide analogues

Larryn W Peterson[1] and **Charles E McKenna**[†,2]

[1]PhD candidate, University of Southern California, Department of Chemistry, Los Angeles, CA 90089-0744, USA

[2]Professor of Chemistry, University of Southern California, Department of Chemistry, Los Angeles, CA 90089-0744, USA

Abstract

Nucleotide analogues have been well accepted as therapeutic agents active against a number of viruses. However, their use as antiviral agents is limited by the need for phosphorylation by endogenous enzymes, and if the analogue is orally administered, by low bioavailability due to the presence of an ionizable diacid group. To circumvent these limitations, a number of prodrug approaches have been proposed. The ideal prodrug achieves delivery of a parent drug by attachment of a non-toxic moiety that is stable during transport and delivery, but is readily cleaved to release the parent drug once at the target. Here, a brief overview of several promising prodrug strategies currently under development is given.

Keywords

antiviral agents; oral bioavailability; prodrugs; pronucleotides

1. Introduction

Of the approximately 40 antiviral drugs formally licensed for use, half are nucleoside or nucleotide analogues [1]. Nucleoside drugs *per se* must usually be phosphorylated to the 5′-mono-, 5′-di-, and finally, 5′-triphosphate by intracellular or viral kinases [2] in order to inhibit their therapeutic targets. This requirement limits efficacy, as phosphorylation to the monophosphate by endogenous kinases is slow and typically is the rate-limiting step in human cells [3,4].

The administration of a nucleoside drug as its monophosphate (NMP) is a well-known approach to overcoming this obstacle [3,5]. However, this entails a penalty in the form of decreased membrane permeability. Nucleotide analogues contain an ionizable –O-P(O)$(OH)_2$ group that exists chiefly as a dianion at physiological pH, resulting in low oral bioavailability [5]. In addition, if a NMP succeeds in crossing the intestinal membrane, it

[†]Author for correspondence: Tel: +1 213 740 7007; Fax: +1 213 740 0930; mckenna@usc.edu.

Declaration of interest
LP and CM are co-inventors on a patent related to a portion of the work discussed in this review.

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then becomes a potential substrate for phosphohydrolases (phosphatases and 5′-nucleotidases), which remove the phosphate group [6]. The use of a nucleoside phosphonate –CH_2-P(O)$(OH)_2$ circumvents dephosphorylation, but decreased transport remains an obstacle.

Formulation strategies [7–11] to overcome these limitations are beyond the scope of this short review, which has as its focus an alternative approach: prodrug modification of nucleotide drugs. Promoieties can be attached at a number of positions on an NMP or nucleotide analogue [12,13]. However, the introduction of promoieties at the phosphorus (–[O,CH_2]-P(O)(X)(Y) where X,Y = OR, OR′, NHR″) directly addresses the problem of blocking P-OH ionization *in vivo*. The attachment of a well-designed promoiety increases delivery of the drug to its target, provided that its biochemical and physical properties – including lipophilicity, site-specificity and chemical stability – are conducive to this end [5,13,14].

A prodrug must be stable under delivery conditions [3,5], but it must be capable of conversion to its active parent drug *in vivo* [5], at a rate consistent with pharmacological efficacy. The prodrug and metabolized promoiety/promoieties should have low acute and chronic toxicity [5]. Control of these and other crucial properties, such as aqueous solubility and lipophilicity, remains a key challenge in the development of an effective prodrug.

Esterification with pivaloyloxymethyl (POM), *p*-acyloxybenzyl (PAOB), or isopropyloxy–carbonyloxymethyl (POC) groups has been reviewed extensively [3,5,6,13,15,16] and will not be addressed here. Also, of recent interest, but omitted from this discussion is the approach of Hostetler *et al.* to improve the oral bioavailability of certain antiviral phosphonate drugs by esterification with an ether lipid ester that mimics the natural lipid lysophosphatidylcholine, thus potentially delivering the prodrug within the cell intact [4,17,18]. Our review will examine the prodrug approaches represented by the structures in Figure 1.

2. Phosphoramidate ‘ProTide’ approach

McGuigan has introduced prodrugs (‘ProTides’) based on an amino acid ester promoiety, attached to the drug (as a aryl monophosphate or phosphonate) via a P-N bond, applying this approach to: 4′-azidouridine [19], 4′-azidoadenosine [20], 2′,3′-dideoxyuridine (ddU) [21], carbocyclic L-d4A (L-Cd4A) [22], stauvidine (d4T) [23], 9-[2-(phosphonomethoxy) ethyl]adenine (PMEA) [24], 3′-azidothymidine (AZT) [25], abacavir (ABC) [26] and tenofovir (PMPA, 9-[(*R*)-2-(phosphonomethoxy)propyl]adenine) [27].

The original approach involved preparation of simple alkyloxy phosphoramidates (Figure 2A(1)), but has evolved into aryloxy phosphoramide pronucleotides with distinct structure–activity relationship detail [28]. Phosphorodiamidates (Figure 2A(2)) were also prepared, but no biological benefit versus the phosphoramidates was observed and synthetic yields were lower [28]. Interestingly, analogues linked through an oxygen resulted in a significant decrease in antiviral activity [29], possibly because the nucleoside monophosphate was not released from the diester intermediate [28]. Diaryl pronucleotides (Figure 2A(3)) were not

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active in kinase-deficient cells [30], due to poor intracellular delivery of the NMP or possibly chemical instability of the diaryl masking groups. Overall, aryloxy phosphoramidates (Figure 2A(4)) appear to hold the most promise for delivery of the therapeutic agent.

Aryloxy phosphoramidates were designed to release the NMP intracellularly via both chemical and enzymatic mechanisms (Figure 2B). The first step in the activation process is cleavage of the amino acid ester by a carboxyesterase [28] to afford 6 (Figure 2B), although a thorough investigation by Venkatachalam and coworkers found that activation by a lipase or protease is also possible and that these enzymes have different specificities for the substituent on the aryl group, the amino acid and the stereochemistry at the phosphorus [31]. Subsequent nucleophilic attack at the phosphorus by the carboxyl group releases the aryloxy group, forming a transient cyclic diester, which is hydrolyzed to form the amino acyl metabolite (AAM, Figure 2B(7)) [28]. In the final step, the amino acid moiety is cleaved by a phosphoramidase to release the nucleoside monophosphate (Figure 2B(8)) and an amino acid [28].

McGuigan and coworkers have thoroughly studied the aryloxy phosphoramidates of d4T and have been able to gain extensive structure–activity relationship insight. In general, the methyl, ethyl and benzyl esters lead to potent activity, while bulkier esters (t-butyl and isopropyl) are significantly less active than the methyl ester [32], most likely due to the increased stability to enzymatic hydrolysis [20]. A quantitative structure–activity relationship (QSAR) study on the variation of amino acid esters further described the most potent esters as those with considerable lipophilicity slightly removed from the ester bond [33]. In a separate report, it was found that the conversion of AAM to NMP was inhibited when benzyl alcohol was released [34]. This inhibition was not observed when ethanol or methanol was released [34].

Although there are some exceptions depending on the nature of the drug used, the most successful pronucleotides contain L-alanine as the amino acid [34,35]. Exchange of L-alanine for either glycine or L-leucine reduced the antiviral activity 70- and 13-fold, respectively [36]. When L-valine was used, the antiviral activity was reduced 147-fold, and furthermore, 100% of the intact prodrug was recovered when it was exposed to pig liver carboxyesterases [34]. When D-alanine was substituted for L-alanine, the potency was decreased 35-fold [34]. The exact reason for the preference for alanine remains unknown. When the achiral amino acid analogue α,α-dimethylglycine was prepared, the antiviral activity was only reduced three-fold [36], which illustrates the fact that natural amino acids are not essential for activity. However, when such amino acids were used, a preference for α-amino acids was observed [34]. The β-amino acid phosphoramidates showed efficient ester cleavage, but no phenyl loss was detected, and the AAM was not observed [34]. This suggests a possible entropy barrier that increases with chain length.

Studies to determine the optimal aryloxy group were also performed [37]. The greatest activity was achieved when the aryl group had a *p*-Cl substituent, and generally for aryl groups that function as mildly electron-withdrawing, lipophilic substituents [37]. The potential for toxicity of the released phenol was not discussed. A naphthyl group was also

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reported to be an effective aryl moiety for delivering anticancer agents [38], and this activity is most likely transferable to antiviral agents.

To obtain successful intracellular delivery of NMP, the pronucleotides need to be resistant to hydrolysis during the absorption and distribution process. The chemical stability of the pronucleotides was studied, and all exhibited satisfying stability over the range of pHs studied (2.0 – 7.4) [39]. The aryloxy phosphoramidates were significantly less resistant to decomposition in plasma or cell extracts, indicative of the need for enzymatic activation [39]. After overnight exposure to pig liver carboxyesterases, CEM cell extract, human serum and mouse serum, AAM was formed from a majority of the L-amino acid-containing pronucleotides [34]. When carbocyclic adenosine phosphoramidates were evaluated in intestinal and liver S9 homogenates, some of the most antivirally potent analogues exhibited complete decomposition over 1 h in intestinal homogenate [22], but isopropyl and t-butyl esters on the amino acid increased intestinal stability [22]. Similarly, D-alanine and glycine exhibited the highest intestinal stability [22], which highlights the complexity in obtaining a structure–activity relationship. Although the usage of this aryloxy phosphoramidate prodrug approach with nucleotide analogues containing a phosphonate may be more difficult due to decreased chemical stability [40], an example of successful application to tenofovir has been described [27].

The pharmacokinetics and oral bioavailability of aryloxy phosphoramidates, specifically abacavir phosphoramidates, were examined [35]. When the abacavir methyl alaninyl–phosphoramidate was administered intravenously, the pronucleotide was rapidly cleared from the plasma with a half-life of 7 min [35]. Similar results were observed following oral administration [35]. However, the major metabolite observed was the AAM [35]. Total exposure to the pronucleotide and its active metabolites was reported to approach that estimated for a similar dose of the parent drug, abacavir, resulting in an overall bioavailability of 50% [35]. The epithelial permeability of a series of d4T aryloxy phosphoramidates was evaluated in Caco-2 and MDCK monolayers [41]. The pronucleotides exhibited relatively low permeability, which may be partially explained by their susceptibility to first-pass metabolism in the intestinal epithelial cells and by being substrates of P-gp [41]. In general, this work exemplifies the difficulty in delivering the NMP to the target while avoiding significant metabolism during absorption and distribution. To obtain optimal antiviral activity of each pronucleotide, the fine tuning of each element (amino acid, ester, and aryl moiety) is required.

3. Monoester prodrugs

Amino acid phosphoramidate monoesters designed to release the NMP after a single activation by an endogenous phosphoramidase have been described by Wagner, who has applied this approach to AZT [42,43] and ddA [44], as well as anticancer drugs [45].

After the delivery of AZT monophosphate by a glycoslyated carrier attached through lysine was reported [46], Wagner and coworkers proposed that NMP could be efficiently delivered by non-polar amino acid phosphoramidate monoesters and that the aryl group was not necessary. Furthermore, these phosphoramidate monoesters were stable in cell culture and

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rat and human plasma [42]. A series of these compounds were synthesized containing an amino acid (tryptophan methyl ester [Figure 2C(9)] or phenylalanine methyl ester [Figure 2C(11)]) connected via a P-N bond to the NMP with the other P-OH left as a free acid or esterified to a simple alkyl group [47]. The tryptophan monoester (Figure 2C(9)) exhibited the best antiviral activity, with an eight-fold increase over AZT with no cytotoxicity observed at the levels tested [47]. Further studies have been done to investigate the activation pathway of these pronucleotides and optimize their structures.

The effect of changing the amino acid was studied in peripheral blood mononuclear cells (PBMC) [42]. The best antiviral activity was obtained with the L-alanine methyl ester [42], consistent with McGuigan's ProTides. Furthermore, enhanced activity was observed with the L-tryptophan derivative (Figure 2C(9)) compared with the L-phenylalanine (Figure 2C(11)), L-valine and L-leucine derivatives [42]. When evaluated in CEM cells, the L-alanine and L-phenylalanine derivatives exhibited antiviral activity comparable to AZT [42]. This suggests that a simple structure–activity relationship does not exist. In order to avoid the polar carboxylate formed after interaction of the pronucleotides with carboxyesterases, the amino acid methyl ester was substituted by a methyl amide [42]. The authors reported that this exchange had little effect on the antiviral activity of the tryptophan derivatives, while the phenylalanine methyl amide derivatives exhibited increased potency [42]. However, the methyl amide derivatives exhibited greater *in vitro* and *in vivo* stability [48]. The antiviral activity did not exhibit a strong dependence on the amino acid stereochemistry [42], but the inclusion of the D-isomer versus the L-isomer led to decreased volumes of distribution [48]. Overall, the L-tryptophan methyl amide derivative (Figure 2C(10)) was selected for further studies.

To better understand the differences in potency, Wagner and coworkers investigated the ability of the pronucleotide to deliver NMP intracellularly [42]. The antiviral activity is strongly related to the intracellular levels of nucleoside triphosphate. In both PBMCs and CEM cells, AZT was able to produce higher levels of AZT triphosphate than the pronucleotides [42]. However, when evaluated in CEM cells, the intracellular levels of the tryptophan methyl ester (Figure 2C(9)) and phenylalanine methyl ester (Figure 2C(11)) pronucleotides did not plateau [49]. Therefore, the differences in potency may be derived from the ability of a phosphoramidase to cleave the P-N bond and release the NMP.

The oral bioavailability, disposition and stability of the AZT phosphoramidate monoesters were evaluated in rats [50]. The phosphoramidate monoesters were stable in tissue homogenates, intestinal contents, and rat and human plasma [48,50]. The tryptophan methyl amide derivative (Figure 2C(10)) exhibited the best pharmacokinetic parameters. However, in simulated gastric fluids at pH 2.0, the pronucleotide exhibited a significantly reduced half-life of 5 h, but greater stability as the pH increased [50]. These results are consistent with greater chemical hydrolysis of P-N bonds at lower pH [51]. The pronucleotide was not detected in plasma or urine, which was confirmed in an *in situ* single pass perfusion study where little or no absorption of the pronucleotide in the 120 min perfusion period was detected [50]. AZT was observed in plasma and urine samples accounting for 29.5% of the dose, while 54.3% of the dose was recovered 4 h post-dosing (intravenously) as intact pronucleotide in the bile [50]. These results offer some possible explanations for the zero

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