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Original article

Design, synthesis and biological evaluation of phosphorodiamidate prodrugs of antiviral and anticancer nucleosides

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ABSTRACT

We herein report the application of the phosphorodiamidate phosphate prodrug approach to a series of thirteen nucleoside analogs with antiviral or anticancer activity. Twenty-five symmetrical phosphorodiamidates were synthesized, bearing esterified L-Alanine (and in one case D-Alanine) in the prodrug moiety, each as single stereoisomer. The presence of an achiral phosphorus represents a potential advantage over the phosphoramidate ProTide approach, where diastereoisomeric mixtures are routinely obtained, and different biological profiles may be expected from the diastereoisomers. Optimization of the synthetic pathway allowed us to identify two general methods depending on the particular nucleoside analogs. All the compounds were biologically evaluated in antiviral and anticancer assays and several showed improvement of activity compared to their parent nucleosides, as in the case of ddA, d4T, abacavir and acyclovir against HIV-1 and/or HIV-2. The biological results were supported by metabolism studies with carboxypeptidase Y monitored by ^{31}P NMR to investigate their bioactivation. This work further validates the phosphorodiamidate approach as a monophosphate prodrug motif with broad application in the antiviral and anticancer fields.

1. Introduction

Nucleoside analogs (NAs) play a pivotal role in antiviral and anticancer therapy [1,2]. They are structurally related to the natural nucleosides bearing modifications at the base and/or at the sugar moieties, which, in most of the cases, confer selectivity *versus* the desired targets [2]. NAs are prodrugs and they need to be converted into their active species, which usually consists of their 5′-triphosphate form [3]. Their bioactivation pathway often involves three consecutive phosphorylation steps starting from the parent nucleoside, which is converted to its mono-, di- and finally triphosphate form. Some NAs, such as abacavir or famciclovir for instance, require also additional bioactivation steps in order to display their biological activity [4,5]. The first step of phosphorylation is usually considered to be the rate-limiting step in the bioactivation of NAs, with few exceptions, such as zidovudine for which the second phosphorylation may be rate limiting [6]. After long-term treatment with NAs the activity of the nucleoside kinases involved in the first step of bioactivation may be decreased, therefore leading to drug resistance onset [7]. Several monophosphate prodrug strategies are currently under investigations to overcome these issues [8], including the phosphoramidate ProTide approach developed in our group [9–11]. More recently the phosphorodiamidate technology applied to 6-*O*-alkyl-2′-*C*-methylguanosine was reported by us as a new promising approach for the delivery of monophosphates inside the cell. Several stability studies on the 6-*O*-alkyl-2′-*C*-methylguanosine diamidates have shown a good stability profile under different conditions such as acid (pH = 2) and mild basic buffer (pH = 8.5–11), and in human serum [12]. This novel prodrug approach has been validated both *in vitro* and *in vivo* and some compounds are already under consideration for clinical studies [12]. Similarly, several acyclic nucleoside

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phosphonate diamidate prodrugs showed a better biological profile compared to the parent compounds [13]. In this approach two amino acid esters are introduced on the monophosphate moiety in order to mask the negative charges. As also in the case of the phosphoramidate diester approach of Wagner and colleagues [14], the phosphorus in the symmetrical diamidate prodrug is achiral, thus avoiding the presence of diastereoisomeric mixtures as in the case of the phosphoramidate ProTide derivatives. In fact, it has been reported how two diastereoisomers may interact differently with the enzymes involved in the bioactivation pathway, thus leading to different biological profiles [15]. Moreover, the diamidate motif bears non-toxic and natural promoieties and obviate the need for a phenyl or naphthyl moiety. The putative bioactivation pathway of diamidate prodrugs, depicted in Scheme 1, is similar to the one reported for ProTides. The first step (**a**) may be mediated by an esterase or a carboxypeptidase-type enzyme, which is responsible for the cleavage of one of the two esters. This mechanism has been already described and supported by enzymatic experiments using ^{31}P NMR [12]. The second step (**b**) involves an intramolecular attack of the carboxylate anion to the phosphorus with elimination of the second amino acid and formation of a five-membered ring (mixed anhydride intermediate). Spontaneous hydrolysis (**c**) of the cycle then leads to the formation of an intermediate bearing two negative charges. Finally, for the last step (**d**), a phosphoramidase-type enzyme cleaves the P–N bond to form the NA monophosphate.

We were keen to probe the scope of this new diamidate prodrug motif across several therapeutic arenas and for a broad range of NAs. In this context, we herein applied the diamidate approach to NAs with either antiviral or anticancer activity, and the novel prodrug compounds were evaluated for their biological activities. The NAs considered for this study are: 6-*O*-ethyl-2′-deoxy-2′-α-fluoro-2′-β-*C*-methylguanosine (**1**), stavudine (d4T, **2**), 2′,3′-dideoxyadenosine (ddA, **3**), zidovudine (AZT, **4**), lamivudine (3TC, **5**), *N*-acetyl-lamivudine (*N*-acetyl-3TC, **6**), 4′-azidouridine (4′-AzU, **7**), 4′-azidocytidine (4′-AzC, **8**), ribavirin (RBV, **9**), acyclovir (ACV, **10**), abacavir (ABC, **11**), the bicyclic nucleoside analog **12** (BCNA, also known as Cf1743) and acadesine (AICA, **13**) (Fig. 1).

Different synthetic conditions were necessary depending on solubility and reactivity issues of the parent nucleosides, and a total of twenty-five diamidates were synthesized. Based on the previous work published on ProTides and diamidates, we selected L-alanine (L-Ala) as the amino acid of choice with benzyl and 2,2-dimethylpropyl as preferred ester moieties. For some derivatives, methyl and cyclohexyl esters were considered and, in one case, D-alanine (D-Ala) was used as the amino acid moiety.

2. Results and discussion

2.1. Chemistry

At first, we applied our previously reported successful methodology for the synthesis of anti-HCV 6-*O*-alkyl-2′-*C*-methylguanosine 5′-phosphorodiamidates [12] to 6-*O*-ethyl-2′-deoxy-2′-α-fluoro-2′-β-*C*-methylguanosine **1**, d4T **2**, ddA **3**, AZT **4**, and 3TC **5**. This procedure, called method A in this paper, is represented in Scheme 2.

In this strategy, the unprotected nucleoside dissolved in THF was treated with phosphorus oxychloride (1 equivalent) in the presence of triethylamine (1 equivalent) to generate a phosphorodichloridate intermediate (^{31}P NMR signal at ~7–8 ppm), which was not isolated. Then, an excess of the appropriate amino acid ester as *p*-toluene sulfonate (*p*-TSA) salt (usually 5 equivalents) and triethylamine (5–10 equivalents) were added, leading after stirring at room temperature for 18–20 h to the desired phosphorodiamidates **14**–**21** (^{31}P NMR signals at δ_P ~ 11–14 ppm).

Interestingly, in the case of 3TC (**5**) both 5′-OH and 4-NH_2 functional groups reacted yielding the tetradiamidate derivative **22**, and the *N*-4-diamidate derivative **23** (Scheme 3).

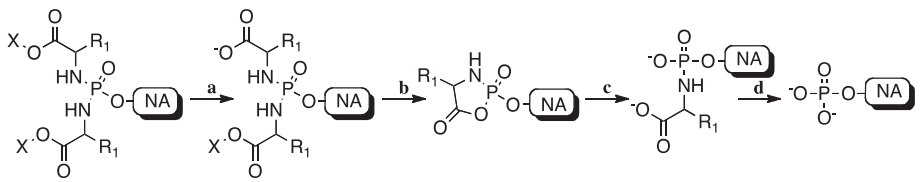
However, when applied to the other NAs reported in this work, this method was not successful, probably due to a lack of solubility in the organic solvent used. Based on these findings, a modification of a synthetic approach reported by Yoshikawa et al. for the synthesis of monophosphate species was used [16]. This same method was previously reported for the synthesis of some phosphorodiamidates [17].

This second strategy, named method B, was used for the synthesis of diamidates of 3TC (**5**), *N*-acetyl 3TC (**6**), 4′-AzU (**7**), 4′-AzC (**8**), RBV (**9**), ACV (**10**), ABC (**11**), Cf1743 (**12**), and AICA (**13**) (Scheme 4).

The reaction was carried out using either trimethylphosphate or triethylphosphate and $POCl_3$ to afford the corresponding intermediate dichlorophosphates following its formation by ^{31}P NMR (δ_P = ~7). The addition of an excess of the appropriate amino acid ester salts in the presence of DIPEA or TEA yielded the desired phosphorodiamidates **24**–**40**.

2.2. Biological results

The arylphosphoramidate ProTide technology as a monophosphate prodrug approach has proven to be a powerful tool in terms of enhancing the biological activity, by-passing certain mechanisms of resistance, as well as extending the biological profile of several NAs. Moreover, the increased lipophilicity of these prodrugs may also lead to a better delivery inside the cells by passive diffusion, thus favoring a greater uptake of the drug. For instance, it has been reported how the application of the ProTide approach greatly enhanced the antiviral activity of the anti-hepatitis C virus (HCV) agent 2-amino-6-methoxy-9-(2-*C*-methyl-β-D-ribofuranosyl) purine, [11] overcame the mechanisms of resistance for 5-fluoro-2′-deoxyuridine [18] and extended the biological profile of BVdU and ACV from antiherpetic to anticancer and anti-human immunodeficiency virus (HIV) agents, respectively [19,20]. In a similar context, the phosphorodiamidates reported here were tested *versus* different cancer cell lines as well as *versus* a range of viruses with the aim to investigate the full potential of this phosphate prodrug moiety. To probe the delivery of the monophosphate inside the cells, thus by-passing the first step of phosphorylation, thymidine kinase-deficient (TK^-) mutant cancer cells and herpes virus strains were also used.



Scheme 1. Putative bioactivation pathway of diamidate prodrugs: **a**) enzyme-mediated ester hydrolysis; **b**) spontaneous intracellular displacement; **c**) spontaneous hydrolysis; **d**) enzyme-mediated P–N bond cleavage.