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Characterization of the Activation Pathway of Phosphoramidate Triester Prodrugs of Stavudine and Zidovudine

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ABSTRACT

The phosphoramidate triester prodrugs of anti-human HIV 2′,3′-dideoxynucleoside analogs (ddN) represent a convenient approach to bypass the first phosphorylation to ddN 5′-monophosphate (ddNMP), resulting in an improved formation of ddN 5′-triphosphate and, hence, higher antiviral efficacy. Although phosphoramidate derivatization markedly increases the anti-HIV activity of 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) in both wild-type and thymidine kinase-deficient CEM cells, the concept is far less successful for the 3′-azido-2′,3′-dideoxythymidine (AZT) triesters. We now investigated the metabolism of triester prodrugs of d4T and AZT using pure enzymes or different biological media. The efficiency of the first activation step, mediated by carboxylesterases, consists of the formation of the amino acyl ddNMP metabolite. The efficiency of this step was shown to be dependent on the amino acid, alkyl ester, and ddN moiety. Triesters that showed no conversion to the amino acyl ddNMP accumulated as the phenyl-containing intermediate and had poor, if any, anti-HIV activity. In contrast to the relative stability of the triesters in human serum, carboxylesterase-mediated cleavage of the prodrugs was found to be remarkably high in mouse serum. The subsequent conversion of the amino acyl ddNMP metabolite to ddNMP or ddN was highest in rat liver cytosolic enzyme preparations. Although L-alaninyl-d4TMP was efficiently converted to d4TMP, the main metabolite formed from L-alaninyl-AZTMP was the free nucleoside (AZT), thus explaining why d4T prodrugs, but not AZT prodrugs, retain anti-HIV activity in HIV-infected thymidine kinase-deficient cell cultures. The rat liver phosphoramidase responsible for the formation of ddNMP was shown to be distinct from creatine kinase, alkaline phosphatase, and phosphodiesterase.

2′,3′-Dideoxynucleoside analogs (ddN) that are active against HIV [i.e., zidovudine (AZT), stavudine (d4T), didanosine, zalcitabine, and lamivudine] must be converted after cell penetration to their corresponding 5′-triphosphate metabolites to act as inhibitors of HIV reverse transcriptase (Balzarini and De Clercq, 1999). However, for several ddNs, the first phosphorylation catalyzed by cellular kinases [i.e., thymidine kinase (TK) in the case of d4T and AZT] is the rate-limiting step that determines the eventual antiviral activity. In vitro studies on the metabolism of ddN in tumor cell lines or mitogen-stimulated lymphocytes may not fully reflect the in vivo situation. According to the findings of Jacobsson et al. (1995), the TK activity in peripheral blood lymphocytes from HIV-infected persons is about 3-fold lower than that seen in seronegative individuals. In addition, the in vitro and ex vivo data of Antonelli et al. (1996) strongly suggest that long-term treatment with ddN may result in a reduction of TK activity and, hence, reduced phosphorylation efficiency of the lymphocytes. Circumvention of this initial activation step is possible by the design of membrane-soluble prodrugs that deliver directly the ddN 5′-monophosphate (ddNMP) into the HIV-infected cells. Among the several types of nucleotide prodrugs that have already been synthesized, a series of phosphoramidate triesters have emerged as highly promising antiviral agents (Farrow et al., 1990; Valette et al., 1996; Winter et al., 1996; Balzarini et al., 1997; Meier et al., 1997). These triesters consist of a ddNMP for which the phosphate is linked, on one side, to a lipophilic (aryl) group and, on the other side, to an amino acid moiety, via a phosphoramidate (P—N) bond. The L-alaninyl-d4TMP phosphotriester **2** (Fig. 1) can be considered the prototype compound of the phosphoramidate prodrug concept (Balzarini et al., 1996a; McGuigan et al., 1996). Our previous metabolism studies with radiolabeled **2** in human lymphocyte CEM cells revealed that this phosphoramidate triester is

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ABBREVIATIONS: ddN, 2′,3′-dideoxynucleoside analog; ddNMP, 2′,3′-dideoxynucleoside 5′-monophosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; PMSF, phenylmethylsulfonyl fluoride; AZT, 3′-azido-2′,3′-dideoxythymidine; CC_{50}, 50% cytotoxic concentration; HIV, human immunodeficiency virus; IM, intermediate metabolite; AAM, amino acyl metabolite.

able to deliver d4TMP intracellularly (Balzarini et al., 1996a). Consequently, the independence of this prodrug from cellular TK resulted in a markedly improved antiviral activity in TK-deficient cells (CEM/TK$^-$) compared with the parent nucleoside d4T (Balzarini et al., 1996b; McGuigan et al., 1996).

The phosphoramidate prodrug technology has been used for the synthesis of a series of closely related amino acyl aryloxyphosphoramidate triester derivatives of d4T, AZT, and lamivudine (Devine et al., 1990; McGuigan et al., 1993; Valette et al., 1996). The antiviral activity of these nucleoside prodrugs is determined by the different structural parts of the molecule (i.e., the nature of nucleoside, the amino acid, and the alkyl group). However, it is not fully understood how the antiviral data can be correlated to the intracellular decomposition pathway followed by the phosphoramidate derivatives.

Several data indicate that the first step in the activation pathway consists of carboxylesterase-mediated hydrolysis of the carboxylic ester function in the amino acid part (McGuigan et al., 1998; Naesens et al., 1998). This esterase cleavage is thought to be followed by an intramolecular nucleophilic attack of the phosphorus by the carboxyl group with spontaneous elimination of phenol after transient formation of a five-membered cyclic intermediate (Fig. 1). This is followed by the conversion of the ddNMP amino acyl metabolite (AAM) to free ddNMP. It has not been clarified whether cleavage of the P—N bond is predominantly catalyzed by one or more less specific phosphatases (that normally use phosphate esters as a substrate) or by a distinct and specific phosphoramidase (Holzer et al., 1962; Holzer et al., 1966; Fernley, 1971; Snyder and Wilson, 1972; Kelly et al., 1975; Nishino et al., 1994). Phosphoramidases that catalyze the hydrolysis of phosphoramidate compounds have been described in mammalian cells and bacteria (Singer and Fruton, 1957; Stevens-Clark et al., 1968; Parvin and Smith, 1969; Kuba et al., 1994; Müller, 1995; Abraham et al., 1996) and have been characterized in more detail by Shabarova and coworkers (Shabarova, 1970; Ledneva et al., 1967, 1970, 1971; Dudkin et al., 1971a,b; McIntee et al., 1997).

We now investigated the activation pathway of a series of phosphoramidate prodrugs of d4TMP and AZTMP in different biological media (i.e., CEM cell extracts, human serum, mouse serum, and rat liver). The purpose of this study was to reveal the influence of the nature of nucleoside, amino acid, and alkyl moiety on the conversion of the triester to ddNMP, with the aim of optimizing the design of new phosphoramidate derivatives.

Materials and Methods

Cells and Viruses. Wild-type CEM cells (CEM/0) were obtained from the American Type Culture Collection (Rockville, MD). The TK-deficient cell line (CEM/TK$^-$) was kindly provided by Prof. Staffan Eriksson (Swedish University of Agricultural Sciences, Uppsala, Sweden) and Prof. Anna Karlsson (Karolinska Institute, Stockholm, Sweden). CEM/0 and CEM/TK$^-$ cells were grown in 75-cm^2 flasks in RPMI 1640 medium (GIBCO, Paisley, Scotland) supplemented with 10% FCS (GIBCO), 2 mM glutamine (GIBCO), and 0.075% sodium bicarbonate (GIBCO). HIV-1 (strain III$_B$) was a generous gift from Dr. R. C. Gallo (at that time at the National Cancer Institute, Bethesda, MD). HIV-2 (strain ROD) was kindly provided by Dr. L. Montagnier (Pasteur Institute, Paris, France).

Enzymes. Pig liver carboxylesterase (E.C. 3.1.1.1), 5′-nucleotidase (E.C. 3.1.3.5, from *Crotalus adamanteus*), and phosphodiesterase I Type VI (E.C. 3.1.4.1, from *C. adamanteus*) were purchased from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase

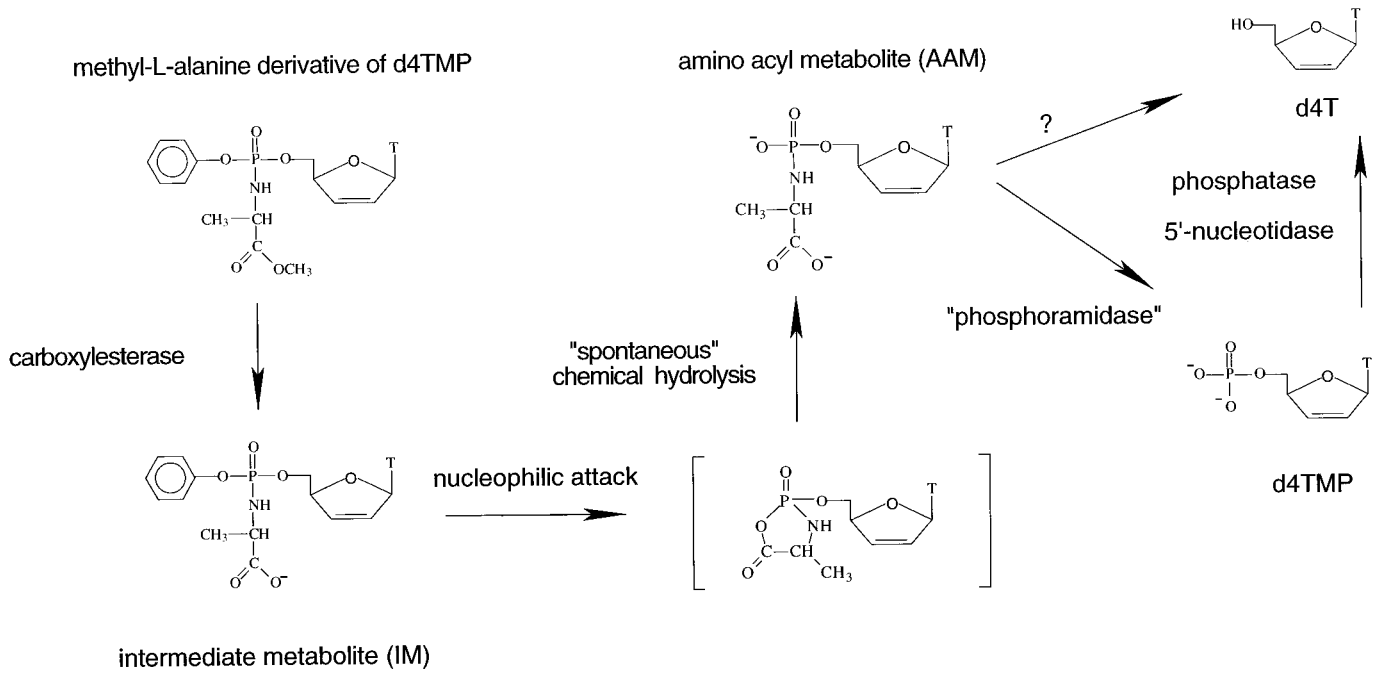


Fig. 1. Proposed activation pathway of the phosphoramidate esters of ddNMP. Shown is the prototype compound **2**, the methyl-L-alanine derivative of d4TMP. After carboxylesterase-mediated cleavage of the carboxylester in the amino acid part, an IM is formed, which is spontaneously converted to the AAM (in this example, L-alaninyl-d4TMP) through intramolecular nucleophilic attack of the phosphorus by the carboxylester. The AAM is then metabolized to ddNMP (in this specific case, d4TMP), through phosphoramidase activity. Further phosphorylation of ddNMP gives the active metabolite ddNTP. Alternatively, free ddN can be formed during hydrolysis of ddNMP by phosphatases or 5′-nucleotidase.

(E.C. 3.1.3.1, from calf intestine) and creatine phosphokinase (E.C. 2.7.3.2, from rabbit muscle) were obtained from Boehringer Mannheim Gmbh (Mannheim, Germany) and SERVA Feinbiochemica (Heidelberg, Germany), respectively.

Anti-HIV Assays. CEM/0 or CEM/TK$^-$ cells were suspended at 250,000 cells/ml cell culture medium and infected with approximately 100 $CCID_{50}$ (1 $CCID_{50}$ is the 50% cell culture infective dose) of HIV-1 or HIV-2. Then, 100 μl of the infected cell suspension was added to the wells of a 96-well microtiter plate containing 100 μl of an appropriate dilution of the test compounds (250, 100, 20, 4, 0.8, 0.16, 0.032, 0.006, and 0.001 μM).

The inhibitory effect of the test compounds on HIV-induced syncytium formation in CEM cells was examined microscopically on day 4 postinfection as described previously (Balzarini et al., 1991). The 50% effective concentration (EC_{50}) was determined as the compound concentration required to inhibit syncytium formation by 50%. In parallel, the cytopathic activity of the compounds was determined in mock-infected cells. The 50% cytopathic concentration (CC_{50}) was defined as the compound concentration that causes 50% reduction in cell proliferation, as determined by automated cell counting in a Coulter Counter (Harpenden, Hertz, UK) on day 4 after the addition of test compound.

Preparation of Crude CEM Cell Extract. To prepare concentrated CEM cell extracts, CEM/0 cells were grown in 2-liter spinner flasks, with the frequent addition of fresh culture medium to ensure exponential growth. When a total amount of 10^9 cells was reached (i.e., 5×10^5 cells/ml), the suspension was centrifuged for 10 min (1200 rpm, 4°C), and the supernatant was withdrawn. The cell pellet was resuspended in PBS and recentrifuged. Then, the cell pellet was resuspended in a small volume of PBS to obtain 10^8 cells/ml. After cell lysis by sonication (3×20 s) and centrifugation to remove cells debris (25,000*g*, 4°C, 10 min), the supernatant was collected. When not used immediately, the cell extracts were stored at −80°C.

Rat Liver Enzyme Preparation. Buffer A consisted of 50 mM Tris · HCl (pH 7.4) supplemented with 0.25 M sucrose and 1 mM EDTA; buffer B consisted of buffer A with 10 mM β-mercaptoethanol; buffer C consisted of buffer B without EDTA; and the corresponding buffers without sucrose were designated A′, B′, and C′.

Two-months-old male WISTAR rats were sacrificed by cervical dislocation, and livers were removed. All subsequent steps were performed at 4°C. Livers were weighed, cut with scissors, and minced in either buffer A or B (10 ml/g wet tissue) in a glass homogenizer (model Potter S; Braun, Melsungen, Germany).

The homogenate was sonicated (3×20 s) and centrifuged at 12,500*g* for 10 min in a Pegasus 65 ultracentrifuge (MSE, Crawley, UK), and the resulting sediment (with gross debris, nuclei, and mitochondria) was discarded. The supernatant was further centrifuged at 105,000*g* for 60 min to sediment the microsomal/lysosomal fraction. The microsomal pellet was resuspended in buffer A (preparation **1**) or in buffer B (preparation **2**). In addition, the supernatant containing the cytosolic enzymes was treated with 320 g/liter ammonium sulfate (32% final concentration), and the mixtures were stirred at 4°C for 15 min.

After centrifugation (20 min, 20,000*g*) and collection of the supernatants, 100 g/liter ammonium sulfate (42% final concentration) was added, and the suspensions were stirred at 4°C for 10 min. After centrifugation (20 min, 20,000*g*), the pellets without and with β-mercaptoethanol were resuspended in 15 ml of buffer A′ and buffer B′, respectively. These preparations, called **3** and **4**, were dialysed overnight against buffer A′ and buffer B′, respectively. Aliquots were stored at −80°C until further use.

A different cytosolic enzyme preparation **5** was obtained by the same method as described for preparations **3** and **4**, with the exception that buffer C was used during liver homogenization and centrifugation and that the final enzyme extract was prepared in buffer C′. Finally, preparation **6** was made with the same procedure as **5**, except that the 60-min centrifugation (105,000*g*) was omitted. The pellet was resuspended in buffer C′, dialyzed overnight against the same buffer, and stored in aliquots at −80°C.

Enzymatic Hydrolysis of Phosphoramidate Prodrugs with Pig Liver Carboxylesterase. The 200-μl reaction mixture was made up in 50 mM Tris · HCl buffer (pH 7.4). The final concentration of the prodrug was 200 μM. The reaction was initiated by the addition of the enzyme. For each determination, control samples lacking either enzyme or prodrug were included. For inhibition assays, pig liver carboxylesterase (16 U/ml) was preincubated for 20 min in buffer containing phenylmethylsulfonyl fluoride (PMSF) before addition of the prodrugs. After 2- or 16-h incubation at 37°C, the reaction was stopped by the addition of 300 μl of ice-cold methanol. After 20 min, the precipitate was removed by centrifugation, and the supernatant was subjected to HPLC analysis. All assays were performed in triplicate.

Inhibition of Phosphoramidase Activity by Iodobenzene. Partially purified liver enzyme extract was preincubated with several concentrations of iodobenzene (1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 μM) for 10 min at 37°C. Then, substrate (L-alaninyl-d4TMP) was added to the reaction mixture at 2 mM. After overnight incubation (i.e., ~16 h), the remaining substrate and the reaction products (i.e., d4TMP, d4T) were quantified by HPLC analysis.

Incubation of Phosphoramidate Prodrugs in CEM Cell Extract, Human Serum, and Mouse Serum. Stock solutions of the compounds at a concentration of 50 or 100 mM and prepared in DMSO were diluted in Tris · HCl buffer (50 mM; pH 7.4) to obtain a working stock at 1.4 mM.

The 200-μl incubation mixture contained 160 μl of biological medium, 20 μl of stock solution in Tris · HCl buffer (final prodrug concentration, 200 μM), and 20 μl of $MgCl_2$ plus β-mercaptoethanol (both at a final concentration of 10 mM). For the inhibition studies, the biological media were preincubated for 30 min at 37°C with 10 mM PMSF before the addition of prodrug. At the end of the incubation period (0 min, 2 h, or 16 h), the samples were put on ice and deproteinized with ice-cold methanol (300 μl) for 20 min. Then, samples were centrifuged for 5 min at 15,000*g*, and the supernatant was analyzed by HPLC. When not assayed immediately, the extracts were stored at −20°C. All experiments were performed in duplicate.

Enzymatic Preparation of L-Alaninyl-d4TMP and L-Alaninyl-AZTMP. L-Alaninyl-d4TMP triester **2** and L-alaninyl-AZTMP triester **14** were incubated during 4 days in Tris · HCl buffer (pH 7.4) containing 100 U/ml pig liver carboxylesterase. Fresh enzyme was added daily, and complete conversion to L-alaninyl-ddNMP was verified by HPLC.

Next, 1-ml aliquots were loaded onto C18 Sep-Pak cartridges (Waters Associates, Milford, MA) preactivated with 2 ml of methanol and 4 ml of water. L-Alaninyl-d4TMP and L-alaninyl-AZTMP were adsorbed by the cartridge while most impurities remained in the solvent. After washing with 0.5 ml of distilled water, the L-alaninyl-ddNMP derivatives were eluted with 2 ml of methanol. The eluates were pooled and evaporated to dryness in vacuo, after which the residues were resuspended in water.

Chromatographic Conditions. The phosphoramidate prodrugs of d4TMP and AZTMP and their metabolites were quantified by HPLC. A Superspher 100 RP-18 endcapped column (250×4 mm, 5 μm; Merck, Darmstadt, Germany) fit into a LiChroCART cartridge (Merck) and protected with a LiChrospher 100 RP-18 guard column (Merck) was used. The chromatographic system consisted of a Waters 600E Pump, a Waters 717 plus Autosampler, and a Waters 996 photodiode array detector and was controlled by Millennium software.

The solvent system consisted of acetonitrile (HPLC grade; Fluka, Buchs, Switzerland) and two buffers: buffer A containing 2.5 mM ammonium dihydrogen phosphate plus 5 mM tetrabutylammonium hydrogen sulfate in water (pH 3.5), and buffer B containing 75 mM ammonium dihydrogen phosphate plus 5 mM tetrabutylammonium hydrogen sulfate in water (pH 3.5). The column was equilibrated with 100% buffer A. The samples were separated at a flow rate of 1

ml/min by a linear gradient from 100% A to 97% B plus 3% acetonitrile (0–30 min) and then to 30% buffer B plus 70% acetonitrile (30–60 min).

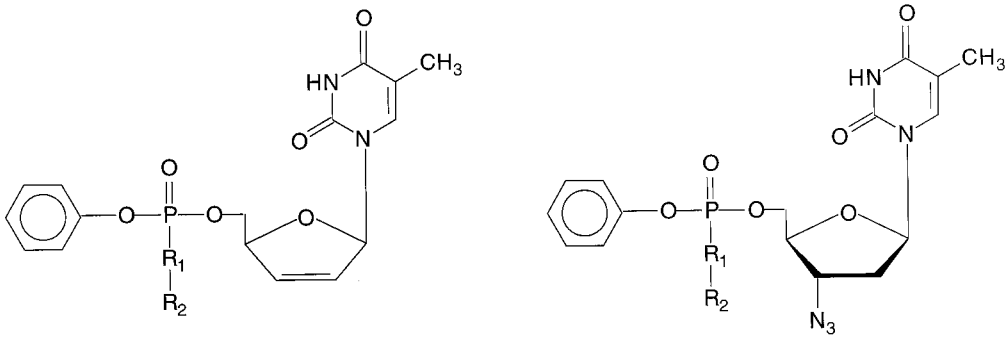
The last conditions were maintained isocratically (60–70 min), followed by a linear gradient to 100% buffer A (70–75 min), and ended by a reequilibration step (75–90 min). The peaks were identified based on comparison with synthetic standards. The retention times for the phosphoramidate prodrugs of d4TMP and AZTMP were in the range of 56 to 64 min. For compound **2**, the retention times were 56, 52, 49, 31, and 29 min, for the prodrug, the intermediate metabolite (IM), the AAM, d4TMP, and d4T, respectively. For compound **14**, the retention times for the prodrug, the IM, the AAM, AZTMP, and AZT were 59, 57, 52, 49, and 47 min, respectively. The other prodrugs and their corresponding metabolites had similar elution patterns.

In addition, the samples were analyzed on an anion exchange Partisphere SAX column (5 μm, 4.6 $\times$ 125 mm; Whatman) to quantify d4TMP and AZTMP and to allow better identification of AAM and IM. The column was equilibrated with 50% buffer A (5 mM ammonium dihydrogen phosphate, pH 5.0) plus 50% water.

The samples were separated at a flow rate of 2 ml/min by the following gradient: 50% buffer A and 50% water (0–5 min), linear gradient to 90% buffer A plus 10% buffer B (0.25 M ammonium dihydrogen phosphate, pH 5.0; 5–20 min), then isocratic conditions (20–25 min), followed by a linear gradient to 50% buffer A, plus 50% water (25–45 min), and finally reequilibration during 13 min. Under these conditions, the retention times for L-alaninyl-d4TMP, L-alaninyl-AZTMP, d4TMP, and AZTMP were 21, 20, 15, and 13 min, respectively.

Results

Anti-HIV Activity in CEM/0 and CEM/TK$^-$ Cells. A selection of phosphoramidate triester derivatives of d4TMP and AZTMP, carrying different amino acids (Fig. 2), were evaluated for their antiviral activity against HIV-1 and HIV-2 in CEM/0 and CEM/TK$^-$ cells (Table 1). For both the d4TMP and AZTMP phosphoramidate derivatives, L-alanine was shown to be the preferred amino acid. Among the d4TMP triesters, the L-alaninyl derivatives **1**, **2**, and **3**, carrying different ester moieties on the alanine part, ranked among the most active compounds, with the EC_{50} value against HIV-1 and HIV-2 in CEM/0 cells being 0.02 to 0.08 μM. Modification of the amino acid moiety resulted in partial or virtually complete loss of antiviral activity compared with the L-alaninyl prodrug derivative. Relatively small structural changes of the amino acids had a marked effect on the eventual antiviral activity. For instance, the L-alanine compound **2** is 40-, >3000-, and 80-fold more active than the corresponding D-alanine **5**, β-alanine **12**, or glycine **6** prodrugs. As a rule



Compound	Amino acid [R1]	Ester [R2]
d4TMP derivatives		
[1]	L-alanine	benzyl
[2]	L-alanine	methyl
[3]	L-alanine	ethyl
[4]	methyl-L-aspartic acid	methyl
[5]	D-alanine	methyl
[6]	glycine	methyl
[7]	L-leucine	methyl
[8]	L-valine	methyl
[9]	L-methionine	methyl
[10]	L-phenylalanine	methyl
[11]	methyl-L-glutamic acid	methyl
[12]	ß-alanine	ethyl

Compound	Amino acid [R1]	Ester [R2]
AZTMP derivatives		
[13]	glycine	methyl
[14]	L-alanine	methyl
[15]	L-leucine	methyl
[16]	D-alanine	methyl
[17]	L-phenylalanine	methyl
[18]	methyl-L-glutamic acid	methyl

Fig. 2. Chemical structures of the aryloxyphosphoramidate esters of d4TMP (left) and AZTMP (right).

for all of the d4TMP triesters tested, the anti-HIV activity was not markedly different in CEM/0 and CEM/TK$^-$ cells. By contrast, the anti-HIV activity of the triesters of AZTMP was significantly lower in CEM/TK$^-$ than CEM/0 cells, and the effect of the different amino acids on the eventual antiviral activity of the AZTMP prodrugs was less pronounced than that observed for the d4TMP prodrugs. From these data, it can be concluded that the antiviral activity of the phosphoramidate prodrug derivatives is strongly dependent on the nature of the nucleoside moiety (d4T or AZT) and the amino acid substituent.

Conversion by Esterase, CEM Cell Extract, Human Serum, or Mouse Serum. The metabolism of the phosphoramidate triesters of d4TMP and AZTMP was studied using pig liver carboxylesterase (E.C. 3.1.1.1) and different biological media (i.e., CEM cell extract, human serum, and mouse serum). The results are shown in Table 2.

After overnight incubation with pig liver carboxylesterase (16 U/ml) at pH 7.4, complete conversion of the d4TMP prodrugs to the corresponding AAM was obtained with the compounds containing L-alanine (**1**, **2**, and **3**), methyl-L-aspartic acid (**4**), D-alanine (**5**), glycine (**6**), and L-phenylalanine (**10**). No formation of the AAM was observed with the L-valine derivative **8** (prodrug kept 100% intact) and the β-alanine derivative **12**, in which case an IM accumulated. Finally, an incomplete conversion of the triester to AAM was observed with the compounds containing L-leucine (**7**), L-methionine (**9**), and methyl-L-glutamic acid (**11**). For these three compounds, a mixture was obtained containing intact prodrug, AAM, and/or IM.

Similar to what was seen with the d4T prodrugs, complete conversion to AAM was obtained for the AZT derivatives containing L-alanine (**14**), D-alanine (**16**), L-phenylalanine (**17**), and glycine (**13**). The AZT derivatives containing L-leucine (**15**) and methyl-L-glutamic acid (**18**) showed an incomplete conversion to AAM, giving a mixture of AAM and IM. For these two compounds, formation of AAM was found to be somewhat less efficient than that for the corresponding d4T compounds.

Next, we determined the carboxylesterase-mediated metabolism of the d4T and AZT prodrugs in different biological media (i.e., CEM cell extract, human serum, and mouse serum). As can be seen in Table 2, the relative conversion patterns in these biological media were fairly comparable to those observed for pig liver carboxylesterase. In all cases, **8** was found to be fully stable. For **12**, no AAM was formed, due to the stability of the IM. For the other compounds studied, the conversion to AAM was most pronounced in mouse serum and least efficient in human serum, whereas an intermediate enzyme activity was present in CEM cell extract. In all three media, the L-alanine derivatives of d4TMP and AZTMP (**2** and **14**, respectively) were among the best converters to their AAMs. For all d4T prodrugs except for **11** and **12**, no accumulation of the IM was seen.

Interestingly, a few AZTMP prodrugs showed partial accumulation of the IM (i.e., **15** and **17**) that was not observed for their corresponding d4TMP derivatives. This is presumably due to a higher chemical stability of the IM.

Finally, when the pig liver carboxylesterase or mouse serum was preincubated during 30 min in the presence of the serine protease inhibitor PMSF (final concentration, 10 mM), which is known to be also an inhibitor of carboxylesterase (Shao and Mitra, 1994), followed by the addition of prototype compounds of the AZTMP prodrug or d4TMP prodrug and overnight incubation, the formation of their AAM was inhibited by more than 90%.

As shown in Table 2, the stability of the L-alanine derivatives of d4T on incubation in CEM cell extracts and in human serum was also found to depend on the alkyl moiety, with the conversion rate to AAM in human serum being 80, 62, and 23% for the benzyl **1**, methyl **2**, and ethyl **3** derivatives, respectively. We therefore extended the stability studies in

TABLE 1
Anti-HIV activity of phosphoramidate triesters of d4TMP and AZTMP in wild-type and TK-deficient CEM cells

Number	Amino Acid	Ester	Anti-HIV Activity[a]			Cytotoxicity[b]
			EC_{50} HIV-1 in CEM/0	EC_{50} HIV-2 in CEM/0	EC_{50} HIV-2 in CEM/TK$^-$	CC_{50} in CEM/0
				μM		
d4T-MP derivatives						
1	L-Alanine	Benzyl	0.016	0.016	0.060	25
2	L-Alanine	Methyl	0.075	0.075	0.075	100
3	L-Alanine	Ethyl	0.10	0.070	0.070	55
4	Methyl-L-aspartic acid	Methyl	0.55	0.65	0.33	209
5	D-Alanine	Methyl	3.0	2.0	2.5	>250
6	Glycine	Methyl	6.0	6.0	7.0	>250
7	L-Leucine	Methyl	1.1	2.2	0.40	>250
8	L-Valine	Methyl	12.5	12.5	4.0	>250
9	L-Methionine	Methyl	0.60	0.80	0.34	>250
10	L-Phenylalanine	Methyl	0.80	1.4	0.33	216
11	Methyl-L-glutamic acid	Methyl	8.0	5.3	1.6	>250
12	β-Alanine	Ethyl	250	>250	>250	250
AZT-MP derivatives						
13	Glycine	Methyl	0.097	0.23	>100	>100
14	L-Alanine	Methyl	0.055	0.070	3.3	50
15	L-Leucine	Methyl	0.097	0.16	10	(208)[c]
16	D-Alanine	Methyl	0.80	0.50	100	>250
17	L-Phenylalanine	Methyl	0.14	0.21	13	(205)[c]
18	Methyl-L-glutamic acid	Methyl	1.5	1.1	175	>250

[a] The antiviral activity against HIV-1 or HIV-2 was determined in wild-type (CEM/0) or TK-deficient (CEM/TK$^-$) cells and expressed as EC_{50}, or compound concentration that results in a 50% inhibition of virus-induced cytopathicity.
[b] The cytopathic activity of the compounds was determined in CEM/0 cells and expressed as CC_{50}, or compound concentration that suppresses the cell proliferation by 50%.
[c] Cytotoxicity of the compounds was determined in MT4 cells.

human serum to a large series of L-alaninyl-d4TMP prodrug derivatives, with variations in the alkyl moiety (Fig. 3). To better discriminate between the compounds, the incubation was performed during 3 h (instead of overnight as in the previous studies). The two most striking extremes in stability were the compounds containing phenyl (least stable) and *tert*-butyl (most stable). Compared with the prototype compound **2**, which showed an intermediate stability in human serum, the ethyl derivative was more stable, whereas the benzyl compound was less stable. These data were confirmed in studies with pig liver carboxylesterase: the percentages of prodrug left after 10 min incubation were 1.5, 31, 66, 92, and 98% for the derivatives containing phenyl, benzyl, methyl, ethyl, and *tert*-butyl, respectively.

Optimization of Rat Liver Enzyme Preparation. Incubation of the prodrugs in CEM cell extract, human serum, or mouse serum resulted in only trace levels of ddNMP (d4TMP or AZTMP) or free ddN (d4T or AZT). In addition, no ddN or ddNMP was detected after incubation with pig liver carboxylesterase. These data suggest that a distinct enzyme is involved in the cleavage of the phosphoramidate linkage of the AAM ddNMP to ddNMP.

To obtain a partially purified enzyme preparation that is able to convert the AAM to ddNMP (or ddN), we used rat liver as the enzyme source because it is known that liver is rich with hydrolytic enzymes, including amidases (Ledneva et al., 1967, 1970; Shabarova, 1970). Our procedure was based on the method of Khandwala and Smith (1967). The enzyme was partially purified by ammonium sulfate precipitation (the fraction between 32 and 42% was used). The purification was about 50-fold. The enzymatic activity was determined by monitoring the formation of d4TMP from L-alaninyl-d4TMP. The L-alaninyl-d4TMP source was obtained from high amounts of **2**, exposed to pig liver carboxylesterase. After an extraction and cleaning step with C18 cartridge columns, we obtained an AAM yield of 95% and a compound purity of 98%, as assessed by HPLC.

Six different procedures for partial purification of the rat liver phosphoramidase were performed (see *Materials and Methods*). The phosphoramidase activity in these enzyme fractions was determined from the percentage of L-alaninyl-d4TMP converted to d4TMP plus d4T. The values for the different enzyme preparations were normalized for an equal amount of total protein. Preparations **1** and **2**, corresponding to the microsomal fraction of the liver cells, showed a weak phosphoramidase activity (1.6 and 2.4% conversion, respectively). In contrast, a much higher phosphoramidase activity (6.8% conversion to ddNMP plus ddN) was present in preparation **3**, obtained through ammonium sulfate precipitation of the cytosolic fraction. This enzymatic activity could be further increased to 23.1% by the addition of 10 mM β-mercaptoethanol during preparation (**4**), whereas EDTA had no influence (preparation **5**; 25% conversion). However, the highest phosphoramidase activity was recovered from rat liver by omitting the centrifugation step at 105,000*g*, thus considerably shortening the preparation time. This preparation **6**, with an enzyme activity of 80%, was stored in aliquots at −80°C and was routinely used in the incubation studies with the phosphoramidate derivatives.

Metabolism of AAM in a Rat Liver Enzyme Preparation. Table 3 shows the metabolism of the triester prodrugs of d4T or AZT after overnight incubation in the rat liver enzyme preparation **6**. The conversion of the prodrugs to d4TMP was most pronounced for the derivatives containing L-alanine, followed (in decreasing order) by methyl-L-aspartic acid, glycine, and D-alanine.

TABLE 2
Metabolism of phosphoramidate triesters of d4TMP or AZTMP after overnight incubation with pig liver carboxylesterase, human serum, CEM cell extract, and mouse serum

Number	Amino Acid/Ester	Intact Prodrug and Its Metabolism after Incubation with:[a]											
		Carboxylesterase			Human Serum			CEM Extract			Mouse Serum		
		Intact Prodrug	IM	AAM	Intact Prodrug	IM	AAM	Intact Prodrug	IM	AAM	Intact Prodrug	IM	AAM
		%											
d4TMP derivatives													
1	L-Alanine/Bz	0	0	100	20	0	80	8	0	92	0	0	100
2	L-Alanine/Me	0	0	100	36	0	62[b]	14	0	84[b]	0	0	98[b]
3	L-Alanine/Et	0	0	100	76	0	23[b]	26	0	72[b]	0	0	98[b]
4	Methyl-L-aspartic acid/Me	0	0	100	18	0	82	0	0	99[b]	0	0	99[b]
5	D-Alanine/Me	0	0	100	66	0	34	58	0	42	9	0	91
6	Glycine/Me	0	0	100	55	0	45	49	0	51	0	0	100
7	L-Leucine/Me	13	0	87	79	0	21	52	0	48	20	0	80
8	L-Valine/Me	100	0	0	100	0	0	100	0	0	100	0	0
9	L-Methionine/Me	11	0	89	40	0	60	33	0	67	15	0	85
10	L-Phenylalanine/Me	0	0	100	46	0	54	37	0	63	18	0	82
11	Methyl-L-glutamic acid/Me	0	35	65	38	34	28	25	40	35	0	25	75
12	β-Alanine/Et	0	100	0	92	8	0	74	26	0	0	100	0
AZTMP derivatives													
13	Glycine/Me	0	0	100	52	0	48	ND	ND	ND	0	0	100
14	L-Alanine/Me	0	0	100	42	0	58	8	0	90[b]	0	0	98[b]
15	L-Leucine/Me	0	70	30	53	34	13	24	60	16	7	76	16[b]
16	D-Alanine/Me	0	0	100	54	0	45[b]	54	0	45[b]	0	0	98[b]
17	L-Phenylalanine/Me	0	0	100	42	40	18	38	43	19	29	31	40
18	Methyl-L-glutamic acid/Me	0	51	49	32	45	23	12	39	49	9	37	54

[a] The phosphoramidate derivatives of d4TMP or AZTMP were incubated overnight with carboxylesterase or with biological media, after which the remaining intact prodrug and the IM and AAM following metabolites were determined by HPLC.
[b] Due to the minor formation of ddNMP + ddN (≤2%), the total of intact prodrug + IM + AAM was not 100%.
N.D., not determined.

For these prodrugs, metabolism to d4T was low because the d4T levels were 8- to 32-fold lower than those measured for d4T-MP. In contrast, no d4T or d4TMP was formed after incubation of **7**, **8**, **9**, **10**, **11**, and **12** under our experimental conditions.

Interestingly, d4TMP formation from the benzyl derivative **1** was found to be considerably lower than that from the ethyl **3** or methyl **2** derivatives, although the AAM was in all three cases L-alaninyl-d4TMP. This suggested that the benzyl alcohol that was released from **1** on carboxylesterase-mediated formation of the AAM inhibited the further conversion to d4TMP. To check this hypothesis, an experiment was performed in which **2** or L-alaninyl-d4TMP was incubated with rat liver enzyme preparation in the presence of 10 μM benzyl alcohol. No formation of d4TMP was formed from either **2** or L-alaninyl-d4TMP. In contrast, 10 μM ethanol or methanol had no effect on the conversion of the AAM to ddNMP.

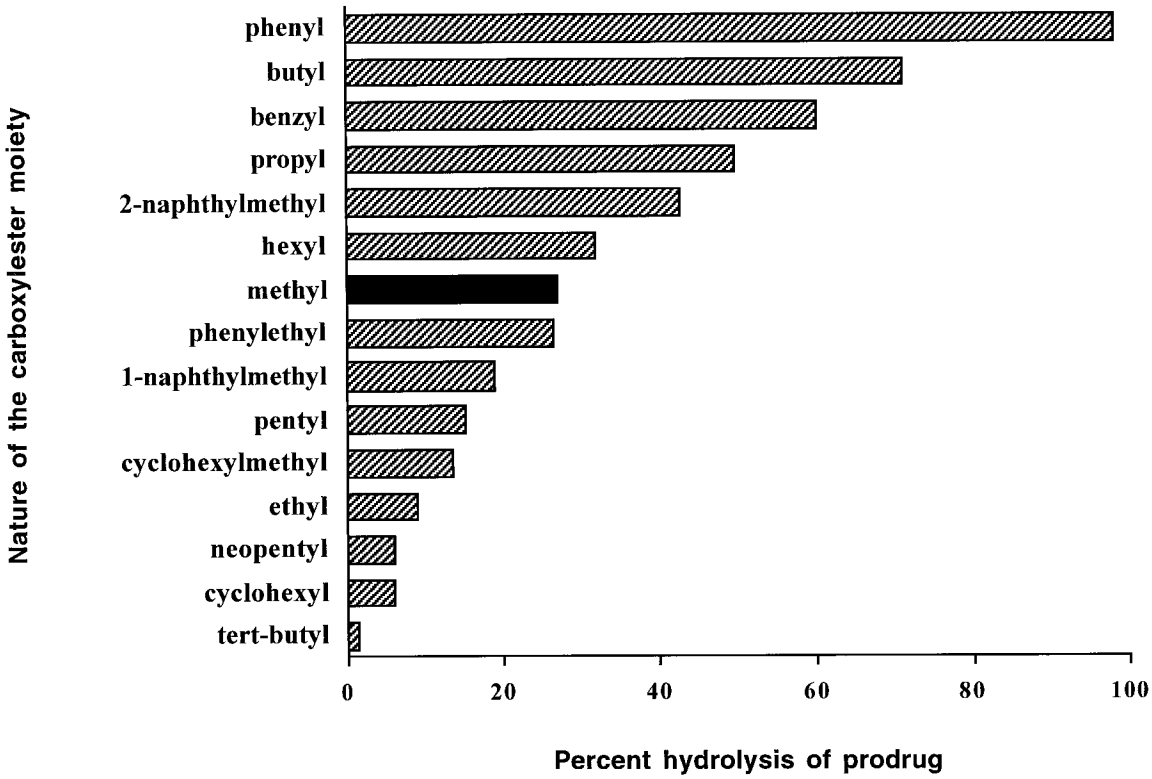


Fig. 3. Enzymatic stability of phosphoramidate triesters of d4TMP as a function of the carboxylester moiety. The compounds were incubated in human serum during 3 h, after which the remaining d4TMP prodrug was determined by HPLC. The black column represents the prototype compound **2**.

TABLE 3
Conversion of phosphoramidate triesters after overnight incubation in rat liver enzyme preparation

Number	Amino Acid/Ester	Percentage of Total[a]				
		Intact Prodrug	IM	AAM	ddNMP	ddN
d4TMP derivatives						
1	L-Alanine/Bz	0	0	92	8	0
2	L-Alanine/Me	0	0	29	67	4
3	L-Alanine/Et	0	0	48	46	6
4	Methyl-L-aspartic acid/Me	0	0	67	32	1
5	D-Alanine/Me	5	0	93	2	0
6	Glycine/Me	0	0	94	6	0
7	L-Leucine/Me	18	0	82	0	0
8	L-Valine/Me	100	0	0	0	0
9	L-Methionine/Me	15	0	85	0	0
10	L-Phenylalanine/Me	12	0	88	0	0
11	Methyl-L-glutamic acid/Me	0	21	79	0	0
12	β-Alanine/Et	0	100	0	0	0
AZTMP derivatives						
13	Glycine/Me	0	0	91	3	6
14	L-Alanine/Me	0	0	84	6	10
15	L-Leucine/Me	9	21	68	0	2
16	D-Alanine/Me	0	0	84	7	9
17	L-Phenylalanine/Me	23	12	64	0	1
18	Methyl-L-glutamic acid/Me	7	25	67.8	0	0.2

[a] The prodrug derivatives of d4TMP or AZTMP were incubated overnight with the rat liver enzyme preparation, after which the remaining intact prodrug and the following metabolites were determined by HPLC: IM; d4TMP or AZTMP (ddNMP); and free d4T or AZT (ddN).

From a comparison of compounds **5** and **2**, it is clear that formation of d4TMP from the D-alaninyl-d4TMP metabolite is much less efficient than that from the L-alaninyl-d4TMP metabolite. This is in sharp contrast to what was seen for the corresponding AZT prodrugs **16** and **14** because for these compounds, the total rate of conversion to AZTMP plus AZT was 16%. In fact, a clear difference was visible between the L-alaninyl derivatives of d4T **2** and AZT **14**, with the total conversion to ddN plus ddNMP being 71 and 16%, respectively. Finally, all the AZT prodrugs tested showed conversion to free AZT, whereas for the d4T prodrugs, formation of the free nucleoside was much less pronounced or not detectable under our assay conditions.

Conversion of AAMs to d4TMP or AZTMP. Pure L-alaninyl-d4TMP and L-alaninyl-AZTMP (prepared from **2** and **14** by carboxylesterase-mediated hydrolysis) were incubated overnight in the rat liver enzyme preparation. The marked differences in their metabolism are clearly depicted in Fig. 4. After 2-h incubation, 20% of L-alaninyl-d4TMP was converted, with the main metabolite being d4TMP, whereas the d4T formation was negligible at this time point. After 16 h, only 20% of L-alaninyl-d4TMP was left, 59% was present as d4TMP, and 21% was present as d4T.

In contrast, conversion of L-alaninyl-AZTMP to AZTMP was markedly slower and much less efficient: after 16 h, 84% of L-alaninyl-AZTMP had remained intact, and the percentage of AZTMP and AZT was 6 and 10%, respectively.

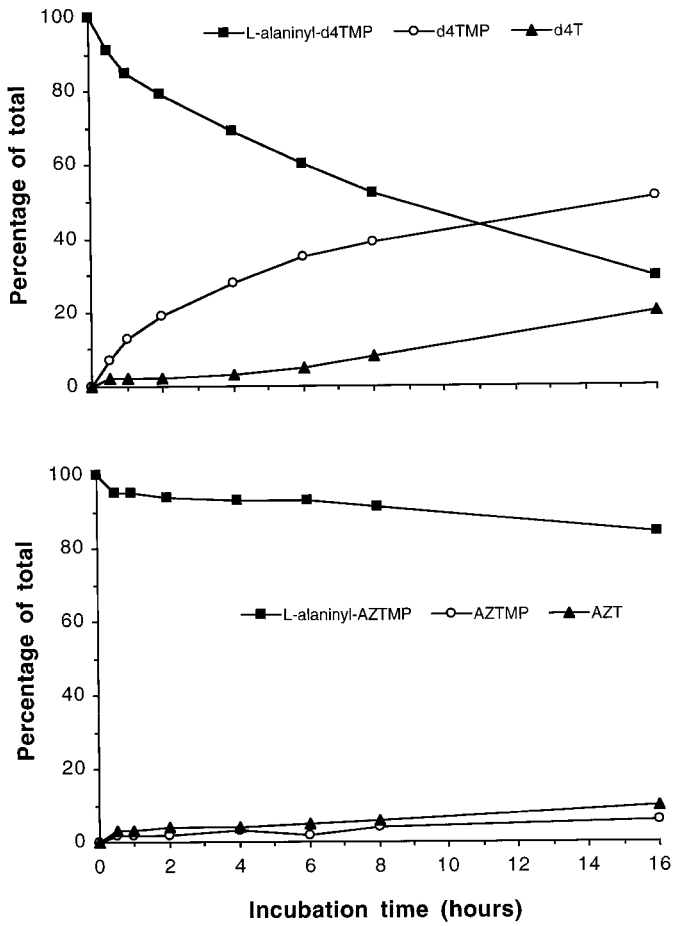


Fig. 4. Metabolism of the AAM compounds L-alaninyl-d4TMP (top) and L-alaninyl-AZTMP (bottom) by the rat liver enzyme preparation. At different time points during incubation, HPLC analysis was performed to determine the remaining AAM compound (■) and the metabolites ddNMP (○) and free ddN (▲).

To study the enzymatic stability of the ddNMPs under these experimental conditions, an overnight incubation of AZTMP and d4TMP was performed in the rat liver preparation. Enzymatic hydrolysis to free ddN was found to be much more pronounced for AZTMP than for d4TMP. For instance, the percentage hydrolysis of d4TMP was 6 and 35% after 1- and 16-h incubation, respectively, whereas for AZTMP, the percentage hydrolysis was 24 and 70% after 1- and 16-h incubation, respectively.

To further determine whether the enzyme responsible for conversion of AAM to ddNMP had phosphoramidase activity, the effect of iodobenzene, which is structurally closely related to the phosphoramidase inhibitor iodosobenzene (Singer and Fruton, 1957), was determined. A complete inhibition of L-alaninyl-d4TMP conversion to d4TMP in the rat liver preparation was achieved with iodobenzene at a concentration of 1 μM (Fig. 5). At lower concentrations, iodobenzene inhibited the phosphoramidase activity in a concentration-dependent manner, with a 50% inhibitory concentration of 10 nM (defined as the concentration at which the formation of d4TMP plus d4T was inhibited by 50%). It should be mentioned that iodobenzene was inactive as an inhibitor of phosphodiesterase and carboxylesterase at 1 mM (data not shown).

In addition, we examined the effect of phosphocreatine on the metabolism of L-alaninyl-d4TMP during overnight incubation of L-alaninyl-d4TMP with our partially purified rat liver phosphoramidate preparation. Phosphocreatine at a concentration of 10 mM caused a partial inhibition (about 50%) of d4TMP formation. On the other hand, the effect of **2** or L-alaninyl-d4TMP on the creatine phosphokinase-dependent metabolism of phosphocreatine was also determined. Creatine phosphokinase catalyzes the reversible transfer of a phosphate group from phosphocreatine to ADP to form creatine and ATP. An inhibition assay was performed in which phosphocreatine and ADP (both at 1 mM) were incubated with creatine phosphokinase (6 U/ml) in the presence of **2** (2 mM) or L-alaninyl-d4TMP (2 mM). Neither phosphoramidate compound had any influence on ATP formation (as determined by HPLC).

Finally, we investigated the metabolism of **2**, **14**, and their AAMs by different commercial enzymes, namely, phosphodiesterase I (type VI), alkaline phosphatase, and 5′-nucleotidase. Enzymatic hydrolysis of the AAM was observed only with phosphodiesterase I (0.5 U/ml). After overnight incubation, 40% of L-alaninyl-d4TMP was metabolized, giving 36% d4TMP and 4% d4T, whereas L-alaninyl-AZTMP was 67% converted, giving 14% AZTMP and 53% AZT. This phosphodiesterase activity was not inhibited by 10 mM iodobenzene. Phosphodiesterase I was also able to hydrolyze d4TMP and AZTMP, giving 19 and 100% of d4T and AZT, respectively, pointing to contaminating phosphatase in the enzyme preparation. Alkaline phosphatase (10 U/ml) and 5′-nucleotidase (6 U/ml) caused a complete hydrolysis of d4TMP and AZTMP to the free nucleoside.

Physicochemical Properties of Rat Liver Phosphoramidase. The partially purified rat liver enzyme was found to display a markedly enhanced activity on the addition of $MgCl_2$ (10 mM), and this cofactor was routinely used in phosphoramidase assays.

The rat liver phosphoramidase showed an optimal enzymatic activity at pH 7.4 that was 8-fold reduced at pH 5.4 and 9.4. Metal-chelating agents such as EDTA had no effect on the phosphoramidase activity of the rat liver enzyme preparation. By using ultrafiltration membranes with different molecular mass cut-off values (from 10 to 100 kDa), we determined that the highest phosphoramidase activity was present in the rat liver enzyme fraction with a molecular mass ranging from 50 to 100 kDa.

Discussion

The phosphoramidate derivatives of ddN were designed to act as membrane-soluble nucleotide prodrugs that enable intracellular delivery of the ddNMP, thus bypassing the first activation step by cellular kinases (TK in the case of d4T and AZT; McGuigan et al., 1996). The ddNMP is then further phosphorylated to ddN 5′-triphosphate, the active metabolite that inhibits HIV reverse transcriptase (Balzarini et al., 1998; Balzarini and De Clercq, 1999). In this study, we focused on a series of phosphoramidate triesters of d4TMP and AZTMP, with variations in the amino acid moiety and the attached alkyl group. The antiviral activity in HIV-infected CEM cells was found to be determined by three structural parameters: the nature of the nucleoside (d4T or AZT), the amino acid moiety, and the carboxyl ester group. Most importantly, the phosphoramidate derivatives of d4TMP were found to be equally active in wild-type and TK-deficient CEM/TK$^-$ cells, thus proving that the TK bypass concept is fully successful with these d4T prodrugs. This is in sharp contrast to the failure of the AZTMP triesters to afford pronounced antiviral activity in CEM/TK$^-$ cells. In addition, depending on the amino acid moiety, large differences were seen in the antivirally effective EC_{50} values of the d4TMP triesters, with L-alanine being the preferred amino acid.

To better understand the structure-activity relationship of the phosphoramidate derivatives of d4TMP and AZTMP, we performed a detailed study on their metabolism, using purified enzymes as well as crude and partially purified enzyme preparations. Several groups have suggested that the activation is initiated by the carboxylesterase-mediated hydrolysis of the carboxyl ester in the amino acid part (Fig. 1; Valette et al., 1996; Winter et al., 1996; McGuigan et al., 1998). This esterase cleavage is followed by a nucleophilic attack of the phosphorus by the carboxyl group, with elimination of phenol after the formation of a five-membered cyclic intermediate. The resulting AAM is then converted to ddNMP, which is either further phosphorylated to ddNTP by kinases or cleaved to free ddN by phosphatases and/or nucleotidases.

We first concentrated on the carboxylesterase-dependent formation of the AAM. Our previous studies on the metabolism of radiolabeled **2** in intact cells have pointed to the intermediary formation of a key metabolite (AAM), which markedly accumulates intracellularly (Balzarini et al., 1996a). We now performed direct incubations of several phosphoramidate derivatives of d4TMP and AZTMP with high amounts of pig liver carboxylesterase. AAM was very efficiently formed from the L-alanine-containing triesters of d4TMP or AZTMP. The L-valine- and β-alanine-containing d4TMP derivatives did not convert to AAM. This is consistent with the low or marginal antiviral activity of these two compounds. Qualitatively, a similar pattern for AAM formation was observed when the triesters were incubated in biological media (i.e., human serum, CEM cell extract, or mouse serum). Overall, the conversion to AAM proved to be highest in mouse serum, lowest in human serum, and intermediate in CEM cell extract. These data are in agreement with the ubiquitous presence of carboxylesterases in mammalian tissues, albeit at enzyme levels that are highly dependent on tissue type and species (Robbi and Beaufay, 1983; Hosokawa et al., 1990).

Some triesters showed a significant conversion to a metabolite, of which the retention time on HPLC was between those of the triester and the AAM. We hypothesize that this is the IM that is formed after hydrolysis of the carboxyl ester in the amino acid moiety and that may be assumed to have a high chemical instability (Fig. 1). The nature of the side chain of the amino acid and the nature of the sugar moiety, in particular the azido group at the 3′-position in the case of AZT, may play an essential role in the formation of the AAM through the hypothetical cyclic intermediate. The only excep-

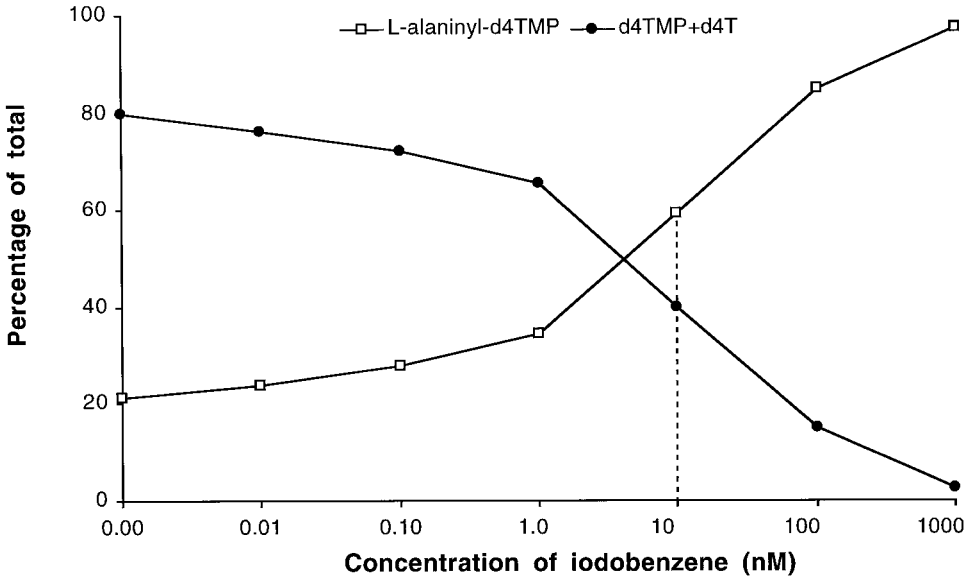


Fig. 5. Inhibitory effect of iodobenzene on the metabolism of L-alaninyl-d4TMP by rat liver phosphoramidase. L-Alaninyl-d4TMP was incubated with the rat liver enzyme preparation in the presence of different concentrations of iodobenzene. Metabolism was followed by HPLC analysis of the remaining alaninyl-d4TMP (□) and of formation of the metabolites (d4TMP plus d4T; ●). The dashed line represents the IC_{50} value, defined as the iodobenzene concentration at which the conversion of L-alaninyl-d4TMP to d4TMP plus d4T is inhibited by 50%.

tion seen here was the β-alanine triester of d4TMP, of which the IM proved fully stable. The most logical explanation is that due to the extra carbon in the β-alanine chain, this IM is unable to form a six-membered cyclic intermediate to allow further conversion to AAM.

To obtain optimal delivery of the ddNMP inside the target cells (i.e., the HIV-infected lymphocytes), an efficient conversion rate by carboxylesterase may be considered as favorable. However, in the in vivo situation, the prodrugs can reach the target cells only if they are resistant to hydrolysis by extracellular carboxylesterases (such as in serum). If not, partial conversion of the prodrugs to AAM would result in a lower cell penetration and, hence, reduced antiviral response. Thus, a compromise must be reached between the extracellular stability of the prodrugs and their conversion to the AAM once they have been taken up intracellularly.

Our studies have revealed that the carboxylester group linked to the amino acid moiety has pronounced influence on the pharmacokinetics of the triester and its associated stability. Indeed, the stability in human serum of L-alaninyl-containing phosphoramidates of d4TMP proved to be highly dependent on the nature of the alkyl ester group, with, for instance, an ethyl providing higher stability than the methyl group. Because an additional concern may be the safety of the alcohol that is released by carboxylesterase, the ethyl derivative may seem preferential over the methyl derivative. Moreover, the more stable ethyl ester derivative showed a slight advantage in antiviral activity.

Next, we investigated the second part of the activation pathway, consisting of the cleavage of AAM to ddNMP or free ddN. Although incubation of the triesters in CEM cell extract, human serum, or mouse serum resulted in limited formation of ddNMP and ddN, this conversion was considerably higher when a rat liver enzyme preparation was used. The metabolism of AAM to ddNMP and ddN was found markedly depending on the amino acid moiety, with L-alanine being the preferred amino acid, thus fully agreeing with the superior antiviral activity of the L-alaninyl-containing phosphoramidate triesters.

Moreover, the d4TMP triesters were found to be superior to the corresponding AZTMP triesters in two aspects: a higher total amount of ddNMP plus ddN release and a markedly higher ratio of ddNMP to ddN (Fig. 6). These results were further confirmed in incubation studies with purified AAM compounds. After overnight incubation, the percentage of AAM left was 20 and 84% for L-alaninyl-d4TMP and L-alaninyl-AZTMP, and the ratio of ddNMP to ddN was 2.4 and 0.6, respectively. The latter result can be explained by the higher sensitivity of AZTMP than d4TMP to nonspecific phosphatases and/or 5′-nucleotidases in this preparation. Similar observations were obtained for the prodrug derivatives of d4TMP (**3** and **4**) and AZTMP (**13** and **16**). These data are fully consistent with the observation that the d4TMP triesters, but not the AZTMP triesters, keep their anti-HIV activity in TK-deficient CEM cells. Clearly, the nature of the nucleoside in the prodrug determines the degree at which the kinase-bypass concept is successful.

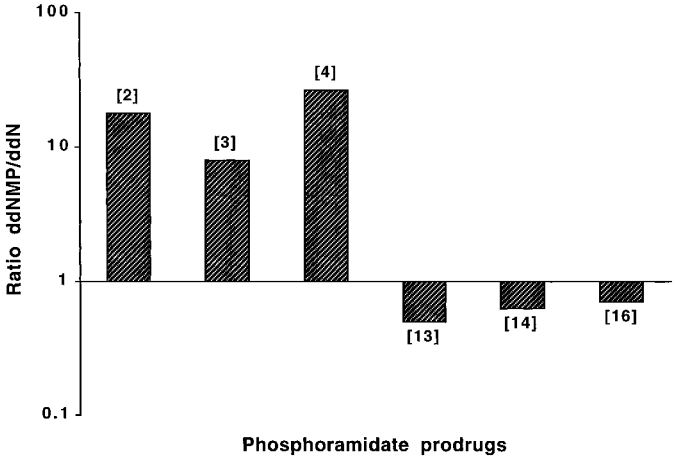


Fig. 6. Formation of ddNMP versus free ddN on overnight incubation with rat liver enzyme preparation for the phosphoramidate triesters of d4TMP (compounds **2**, **3**, and **4**) or AZTMP (compounds **13**, **14**, and **16**). Shown is the ratio of ddNMP (d4TMP or AZTMP) to ddN (d4T or AZT).

The last part of our study was focused on the partial purification and characterization of the enzyme that hydrolyzes the phosphoramidate (P—N) linkage in the AAM. The original definition of phosphoramidase (E.C. 3.9.1.1) as given by Dixon and Webb (1979) refers to an enzyme that is acting on a phosphorus-nitrogen (P—N) bond. However, different enzymes with phosphoramidase activity have been isolated from various sources (i.e., rat liver, spleen, or kidney), and the enzyme has been associated with both microsomal and cytosolic fractions (Holzer et al., 1966; Snyder and Wilson, 1972; Kuba et al., 1994; Nishino et al., 1994). In these studies, the phosphoramidase activity was determined based on release of free phosphate from the P—N substrate. In our studies, we describe an enzyme activity that hydrolyzes a P—N bond with the release of a substituted phosphate (i.e., a phosphate attached to a nucleoside moiety). After fractionation of a rat liver homogenate by centrifugal separation, the different subcellular fractions (mitochondrial, microsomal, and cytosolic) were evaluated for phosphoramidase activity by incubation with L-alaninyl-d4TMP and measurement of the d4TMP formation. The highest enzymatic activity was found in the cytosolic fraction. Reduction in the preparation time and the addition of β-mercaptoethanol in the isolation buffer and magnesium chloride in the incubation mixture resulted in a significantly higher enzyme yield and activity. Such an enzyme activity has been described by Shabarova and coworkers (Ledneva et al., 1967, 1970; Shabarova, 1970, 1971; Dudkin et al., 1971a,b) and recently by McIntee et al. (1997). Shabarova (1970) reported on the discovery of a nucleoside 5′-phosphoramidase in some animal tissues (i.e., rabbit liver). This enzyme hydrolyses the phosphoramide bond to form the nucleotide and the amino acid. Nucleotide 5′-amidates were the most readily hydrolyzed compounds, and the enzyme preparation proved capable of hydrolyzing both L- and D-amino acid derivatives of nucleotides (Shabarova, 1970). McIntee et al. (1997) recently found that the 3-indolyl aminoacyl phosphoramidate prodrugs of AZT and 3′-fluoro-2′,3′-dideoxythymidine were also substrates for phosphoramidase activity in peripheral blood mononuclear cell extracts. In this respect, the 3′-fluoro-2′,3′-dideoxythymidine-MP derivative was a better substrate than the AZTMP derivative. The d4TMP derivative was not included in this study.

The inhibitory effect of iodobenzene on the phosphoramidase activity is similar to that previously reported for the closely related compound iodosobenzene (Singer and Fruton, 1957). At high concentrations, the naturally occurring phos-

phoramidate compound phosphocreatine was shown to be able to partially inhibit the phosphoramidase-mediated hydrolysis of L-alaninyl-d4TMP. However, L-alaninyl-d4TMP proved not to be a substrate for creatine phosphokinase, the enzyme that catalyzes the phosphorylation of creatine. In addition, we incubated the AAM compounds with phosphodiesterase, alkaline phosphatase, and 5′-nucleotidase. Phosphodiesterase I was able to hydrolyze L-alaninyl-d4TMP and L-alaninyl-AZTMP, yet this reaction was not inhibited by iodobenzene. Taken together, these results suggest that the phosphoramidase enzyme in the rat liver fraction that recognizes the phosphoramidate ddNMP prodrugs is distinct from known esterases. We also found that 10 μM benzylalcohol is able to completely block the conversion of L-alaninyl-d4TMP to d4TMP. However, when benzylalcohol is released in the intact cells on conversion of the L-alaninyl-d4TMP prodrug to L-alaninyl-d4TMP, it will immediately be spread over the whole cell content, and it is even expected to diffuse out of the cells to the extracellular medium. Therefore, it is reasonable to assume that the benzylalcohol released from the phosphoramidate prodrug has no chance to efficiently inhibit the phosphoramidase-catalyzed intracellular release of d4TMP. The potent antiviral activity of the benzyl prodrug ester derivative is in agreement with this hypothesis. We are currently planning the isolation of the phosphoramidase enzyme by ion-exchange or affinity chromatography to identify its physicochemical properties, substrate specificity, and physiological role. These insights should help to design new phosphoramidate prodrugs with improved biochemical and therapeutic properties.

In Fig. 1, we proposed as the main metabolic pathway of the prodrugs the release of the alkyl (methyl) ester group by carboxylesterases before the release of the aryl part of the molecule. Indeed, we recently revealed that an α amino acid is necessary for biological activity and consistently results in the formation of the amino acyl diester (McGuigan et al., 1998a). In contrast, β (and longer) amino acids also show efficient ester cleavage but are biologically inert and show no phenyl loss. This strongly implies that 1) the amino acyl liberation is necessary for biological action, 2) an α amino acid is necessary for the phenyl cleavage (by intramolecular catalysis), and 3) phenyl loss proceeds after ester cleavage. Similarly, in a recent report (McGuigan et al., 1998b), we noted that replacement of methyl by *t*-butyl as the carboxylate ester lead to a significant reduction in antiviral potency. This directly correlated with the high stability of the *t*-butyl ester to any esterase-mediated degradation. Because the stability of the phenyl phosphate group per se should be unaffected by such a modification at the carboxyl terminus, the "apparent" stabilization of the phenyl phosphate toward cleavage (and resulting reduction in antiviral potency) can only arise from the carboxylate ester stabilization. Again, these data strongly support the suggestion that carboxyl ester cleavage is a necessary prerequisite for phenyl loss and for eventual antiviral activity.

Acknowledgments

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References

Abraham TW, Kalman TI, McIntee EJ and Wagner CR (1996) Synthesis and biological activity of aromatic amino acid phosphoramidates of 5-fluoro-2′-deoxyuridine and 1-β-arabinofuranosylcytosine: Evidence of phosphoramidase activity. *J Med Chem* **39:**4569–4575.

Antonelli G, Turriziani O, Verri A, Narciso P, Ferri F, D'Offizi G and Dianzani F (1996) Long-term exposure to zidovudine affects in vitro and in vivo the efficiency of phosphorylation of thymidine kinase. *AIDS Res Hum Retroviruses* **12:**223–228.

Balzarini J and De Clercq E (1999) Nucleoside and nonnucleoside reverse transcriptase inhibitors active against HIV, in *Textbook of AIDS Medicine* (Merigan TC, Bartlett JG and Bolognesi D eds) pp 815–847, Williams and Wilkins, Baltimore.

Balzarini J, Egberink H, Hartmann K, Cahard D, Vahlenkamp T, Thormar H, De Clercq E and McGuigan C (1996b) Anti-retrovirus specificity and intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine (stavudine) and its 5′-monophosphate triester prodrug So-324. *Mol Pharmacol* **50:**1207–1213.

Balzarini J, Karlsson S, Aquaro A, Perno CF, Cahard D, Naesens L, De Clercq E and McGuigan C (1996a) Mechanism of anti-HIV action of masked alaninyl d4TMP derivatives. *Proc Natl Acad Sci USA* **93:**7295–7299.

Balzarini J, Kruining J, Wedgwood OM, Pannecouque C, Aquaro S, Perno CF, Naesens L, Witvrouw M, Heijtink R, De Clercq E and McGuigan C (1997) Conversion of 2′,3′-dideoxyadenosine (ddA) and 2′,3′-didehydro-2′,3′-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Lett* **410:**324–328.

Balzarini J, Naesens L and De Clercq E (1998) New antivirals: Mechanism of action and resistance development. *Curr Opin Microbiol* **1:**535–546.

Balzarini J, Naesens L, Slachmuylders J, Niphuis H, Rosenberg I, Holy A, Schellekens H and De Clercq E (1991) 9-(2-Phosphonylmethoxyethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication in vitro and simian immunodeficiency virus infection in rhesus monkeys. *AIDS* **5:**21–28.

Devine KG, McGuigan C, O'Connor TJ, Nicholls SR and Kinchington D (1990) Novel phosphate derivatives of zidovudine as anti-HIV compounds. *AIDS* **4:**371–373.

Dixon M and Webb EC (1979) *Enzymes*, 3rd ed. Longman, London.

Dudkin SM, Ledneva RK, Shabarova ZA and Prokofev MA (1971a) Hydrolysis of uridine-5′ N-aryl and N-alkyl phosphoramidates by ribonucleoside-5′ phosphoramidase. *FEBS Lett* **16:**48–50.

Dudkin SM, Ledneva RK, Shabarova ZA and Prokofev MA (1971b) Substrate specificity of ribonucleoside-5′-phosphoamidase studied by a spectrophotometric method. *Dokl Akad Nauk SSSR* **196:**93–95.

Farrow SN, Jones AS, Kumar A, Walker RT, Balzarini J and De Clercq E (1990) Synthesis and biological properties of novel phosphotriesters: A new approach to the introduction of biologically active nucleotides into cells. *J Med Chem* **33:**1400–1406.

Fernley HN (1971) Mammalian alkaline phosphatases, in *The Enzymes* (Boyer PD ed) pp 417–447, Academic Press, New York.

Holzer ME, Burrow DJ and Smith RA (1962) Metabolism of phosphoramidates. I. Enzymatic hydrolysis and transfer reactions. *Biochim Biophys Acta* **56:**491–501.

Holzer ME, Johnson KD and Smith RA (1966) Metabolism of phosphoramidates. III. A microsomal phosphoramidate hexose transfer system. *Biochim Biophys Acta* **122:**232–243.

Hosokawa M, Maki T and Satoh T (1990) Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. *Arch Biochem Biophys* **277:**219–227.

Jacobsson B, Britton S, He Q, Karlsson A and Eriksson S (1995) Decreased thymidine kinase levels in peripheral blood cells from HIV-seropositive individuals: Implications for zidovudine metabolism. *AIDS Res Hum Retroviruses* **11:**805–811.

Kelly SJ, Dardinger DE and Butler LG (1975) Hydrolysis of phosphonate esters catalyzed by 5′-nucleotide phosphodiesterase. *Biochemistry* **14:**4983–4988.

Khandwala PK and Smith RA (1967) Metabolism of phosphoramidates. IV. A phosphoryl transfer dependent on purine nucleosides. *Biochim Biophys Acta* **136:**448–458.

Kuba M, Okizaki T, Ohmori H and Kumon A (1994) Nucleoside monophosphoramidate hydrolase from rat liver: Purification and characterization. *Int J Biochem* **26:**235–245.

Ledneva RK, Preobrazhenskaya NN, Shabarova ZA and Prokofev MA. Enzymic hydrolysis of aryl- and alkylamides of uridine-5′-monophosphate. *Mol Biol* **5:**264–269.

Ledneva RK, Preobrazhenskaya NN, Shinskii NG, Shabarova ZA and Prokofev MA (1970) Analogs of uridine-5′-phosphoamides and their hydrolysis by ribonucleoside-5′-phosphoamidase. *Dokl Akad Nauk SSSR* **193:**1308–1310.

Ledneva RK, Shabarova ZA and Prokofev MA (1967) Enzymic hydrolysis of the phosphoamide bond of nucleotide-(P.fwdarw. N)-peptides. *Dokl Akad Nauk SSSR* **172:**977–980.

McGuigan C, Cahard D, Sheeka HM, De Clercq E and Balzarini J (1996) Phosphoramidate derivatives of d4T with improved anti-HIV efficacy retain full activity in thymidine kinase-deficient cells. *Bioorg Med Chem Lett* **6:**1183–1186.

McGuigan C, Pathirana RN, Balzarini J and De Clercq E (1993) Intracellular delivery of bio-active AZT nucleotides by aryl phosphate derivatives of AZT. *J Med Chem* **36:**1048–1052.

McGuigan C, Sutton PW, Cahard D, Turner K, O'Leary G, Wang Y, Gumbleton M, De Clercq E and Balzarini J (1998b) Synthesis, anti-human immunodeficiency virus activity and esterase lability of some novel carboxylic ester-modified phosphoramidate derivatives of stavudine (d4T). *Antiviral Chem Chemother* **9:**473–479.

McGuigan C, Tsang HW, Sutton PW, De Clercq E and Balzarini J (1998a) Synthesis and anti-HIV activity of some novel chain-extended phosphoramidate derivatives of d4T (stavudine): Esterase hydrolysis as a rapid predictive test for antiviral potency. *Antiviral Chem Chemother* **9:**109–115.

McIntee EJ, Remmel RP, Schinazi RF, Abraham TW and Wagner CR (1997) Probing the mechanism of action and decomposition of amino acid phosphomonoester amidates of antiviral nucleoside prodrugs. *J Med Chem* **40:**3323–3331.

Meier C, Knispel T, De Clercq E and Balzarini J (1997) ADA-bypass by lipophilic

cycloSAL-ddAMP pro-nucleotides: A second example of the efficiency of the cycloSAL-concept. *Bioorg Med Chem Lett* **7:**1577–1582.

Müller HE (1995) Investigations of culture and properties of *Afipia spp. Zbl Bakt* **282:**18–23.

Naesens L, Cahard D, Salgado A, Bidois L and De Clercq E (1998) Metabolism and anti-HIV activity of phosphoramidate derivatives of d4TMP with variation in the amino acid moiety, in *Purine and Pyrimidine Metabolism in Man. IX* (Griesmacher A, Chiba P, Müller MM eds) pp 753–757, Plenum Press, New York.

Nishino M, Tsujimura S, Kuba M and Kumon A (1994) N^{ω}-Phosphoarginine phosphatase from rat renal microsome was alkaline phosphatase. *Arch Biochem Biophys* **312:**101–106.

Parvin R and Smith RA (1969) Phosphoramidates. V. Probable identity of rat liver microsomal glucose 6-phosphatase, phosphoramidase, and phosphoramidate-hexose phosphotranspherase. *Biochemistry* **8:**1748–1755.

Robbi M and Beaufay H (1983) Purification and characterization of various esterases from rat liver. *Eur J Biochem* **137:**293–301.

Shabarova ZA (1970) Synthesis nucleotide-peptides. *Progr Nucleic Acid Res Mol Biol* **10:**145–182.

Shao Z and Mitra AK (1994) Bile salt-fatty acid mixed micelles as nasal absorption promoters. III. Effects on nasal transport and enzymatic degradation of acyclovir prodrugs. *Pharm Res* **11:**243–250.

Singer F and Fruton JS (1957) Some properties of beef spleen phosphoamidase. *J Biol Chem* **229:**111–119.

Snyder SL and Wilson IB (1972) Investigations on the alkaline phosphatase catalyzed hydrolysis of phosphoramidates: Substituent effects and transphosphorylation. *Biochemistry* **11:**3220–3223.

Stevens-Clark JR, Theisen MC, Conklin KA and Smith RA (1968) Phosphoramidates. VI. Purification and characterization of a phosphoryl transfer enzyme from *Escherichia coli*. *J Biol Chem* **243:**4468–4473.

Valette G, Pompon A, Girardet JL, Cappellaci L, Franchetti P, Grifantimi M, Colla La P, Loi AG, Perigaud C, Gosselin G and Imbach JL (1996) Decomposition pathways and in vitro HIV inhibitory effects of IsoddA pronucleotides: Towards a rational approach for intracellular delivery of nucleotide 5′-monophosphates. *J Med Chem* **39:**1981–1990.

Winter H, Maeda Y, Mitsuya H and Zemlicka J (1996) Phosphodiester amidates of allenic nucleoside analogues: Anti-HIV activity and possible mechanism of action. *J Med Chem* **39:**3300–3306.

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