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ANTI-HIV PRONUCLEOTIDES: DECOMPOSITION PATHWAYS AND CORRELATION WITH BIOLOGICAL ACTIVITIES

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Abstract: The purpose of the present study was to compare the decomposition pathways in CEM cell extracts of various phenyl phosphoramidate derivatives of AZT. In addition, the structures of their metabolites were identified. Correlations with their anti-HIV activities in a thymidine kinase deficient (TK^-) CEM cell line have been established with a rationale of designing phosphoramidate pronucleotides capable of delivering intracellularly their respective 5'-nucleoside monophosphate derivatives.

Pronucleotides are neutral nucleotide prodrugs which were designed to overcome the first phosphorylating step of nucleoside analogues: after cell uptake, these compounds are able to deliver the nucleoside 5'-monophosphate (NuMP)[1]. Among the most established pronucleotide series are the phenyl phosphoramidate diester derivatives, introduced by McGuigan[2, 3] and the SATE phosphotriesters[4] (Figure 1).

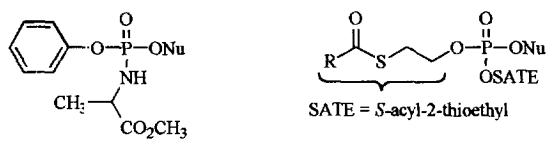

Figure 1. *Two Types of Pronucleotides*

Using AZT or d4T as a nucleoside moiety, such pronucleotides have shown anti-HIV activity on TK^- cell lines[2, 3, 5-7] whereas the parent nucleoside was inactive in these cell lines, further demonstrating the NuMP delivery inside the cells.

Following preliminary stability studies[8, 9] using some phenyl phosphoramidate pronucleotides, this work was undertaken to determine their precise decomposition pathway with the rationale of understanding the design of new optimized pronucleotides.

In cell extract[9] as well as in intact cells[8, 10] the decomposition pathway of the phosphoramidate pronucleotide **1** (Nu= isoddA, d4T) has been shown to proceed through the L-alaninyl metabolite **2** (Scheme 1).

Formation of **2** was proposed to be mediated through an esterase cleavage (Path A) followed by an internal nucleophilic substitution with the elimination of phenol[9]. An alternative or competitive decomposition pathway (Path B) was also envisaged[8, 10], the initial activation step being dependent on water nucleophilic attack on the PV atom with phenol elimination followed by an esterase catalyzed

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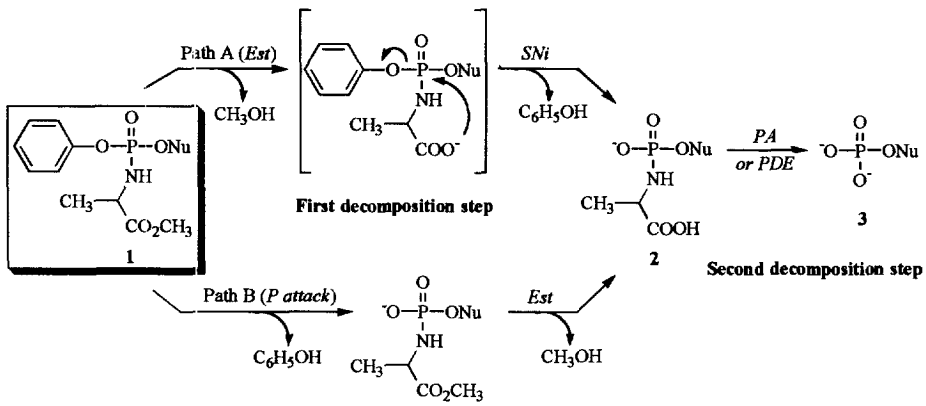
deesterification. Subsequent conversion of 2 to NuMP 3 was suggested to proceed through phosphoamidase[11, 12] or phosphodiesterase[8, 9] activation.



(Est: Esterase, PA: Phosphoamidase, PDE: Phosphodiesterase)

Scheme 1. *Proposed mechanism for the decomposition pathways for a nucleoside phenyl phosphoramidate diester.*

Surprisingly, the modification of the amino acid in 1 led to less active or inactive compounds in TK$^-$ cell lines[3, 13, 14]. Such variation in the antiviral activity may be related to the first or the second decomposition step described in Scheme 1. We first decided to study comparatively the decomposition pathways of some closely related aminoacid phenyl phosphoramidate diester derivatives of AZT: the L-alanine derivative 4 as reference compound, the glycine 5 and the ß-alanine 6 derivatives. Due to its lipophilicity, AZT was selected as nucleoside for the ease of the decomposition analysis using the HPLC/MS coupling technique.

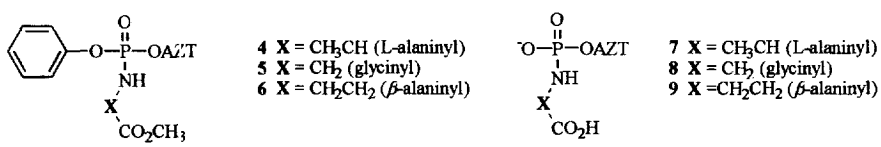


Figure 2. *Structure of the studied amino acid phenyl phosphoramidates of AZT and of their expected metabolites*

Compounds 4, 5 (Figure 2) were synthesized as previously described by McGuigan[15], compound 6 was obtained using a similar procedure. The syntheses of the corresponding phosphoramidate monoesters 7, 8 and 9 (Figure 2) were performed following a previously described procedure [9]. All the compounds were fully characterized using ^{1}H, ^{13}C, ^{31}P NMR, mass spectroscopy and HPLC.

Compounds 4, 5, 6 were incubated in a CEM cell extract and their kinetics of decomposition as well as the identification of their metabolites were performed using HPLC/MS coupling technique. Table 1 reports the half-life ($T_{1/2}$) of these compounds and the structure of their first metabolite detected in CEM cell extracts. The anti-HIV activity of compounds 4-6 is presented in Table 2.

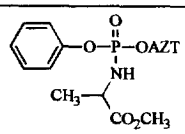
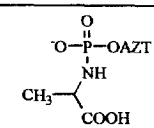
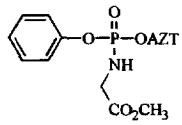
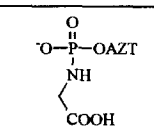
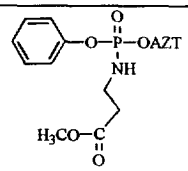
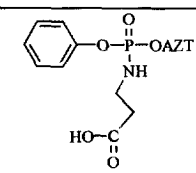
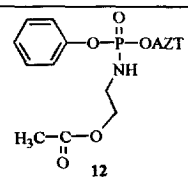
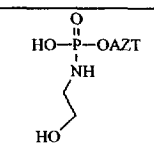
Starting compound	$t_{1/2}$ of decomposition	First observed metabolite
 4	2.5 h	 7
 5	21 h	 8
 6	>8 days	 10
 12	1.5 h	 13

Table 1. *Half-lives of the phosphoramidates derivatives 4, 5, 6, 12 and structure of their first observed metabolites in CEM cell extract (compounds studied at 5.10^{-5}M initial concentration).*

The L-alaninyl phosphoramidate pronucleotide **4** is rapidly degraded ($t_{1/2}$ = 2.5 h) to the intermediate **7**, identified by co-elution with an authentic sample. The initial activation step was esterase mediated since **4** is very stable upon incubation in water, or phosphate buffer (pH=7.0)[16] as well as in heat inactivated cell extract, precluding any nucleophilic attack on the PV or on the C atom of the carbonyl function. In addition, **4** was demonstrated to be a substrate for pig liver esterase (PLE) (see below). Therefore, from these observations pathway B (Scheme 1) can be excluded.

The glycine analogue **5** led to the formation of the expected metabolite **8**, however its rate of formation was slower than that of metabolite **7** from **4**. At this stage, one can assume that either **5** is not a good substrate for esterase or that the rate of the intramolecular mechanism is slower than that involved in the formation of **4**. These data may in part explain why **5** is less active than the reference compound **4** (Table 2).

Unexpectedly, the β-alanine derivative **6** led with only 5 % decomposition rate in 24 hours to the formation of a stable metabolite **10**. This compound was identified by HPLC/MS coupling experiments ($[M-H]^-$ = 493) and was shown to be different from the expected phosphoramidate monoester metabolite **9** (Figure 2). As **6** did not possess an anti-HIV effect in CEM TK^- cell line (Table 2), this may indicate that **6**

is a poor substrate for esterase and that the subsequent intramolecular mechanism does not occur since **10** is stable. In fact, the formation of a six membered ring intermediate (β-alanine derivative) was shown to be less thermodynamically favorable than a five-membered ring one (alanine and glycine derivatives). Therefore, the inactivity of **6** may due to inefficiency of the first decomposition step (Scheme 1).

Using pig liver esterase (PLE, 300 units/μmole, pH= 8.0) as a model for esterase activation, we observed that compounds **4, 5, 6** also led to the formation of the same metabolites **7, 8, 10,** respectively, as detected in cell extract. Moreover, the formation of another metabolite **11** identified by HPLC/MS was observed from **5** (Figure 3) but not from **4**. This may show that the rate of phenol elimination is dependent on the nature of the amino acid moiety. Formation of **11** as an intermediate metabolite also confirms that **5** is decomposed through pathway A (Scheme 1).

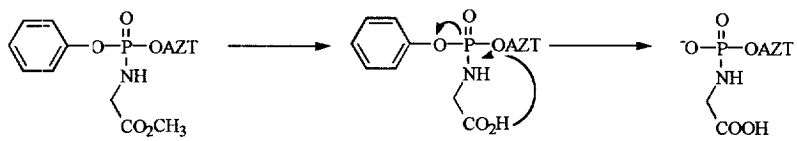


Figure 3. *Decomposition pathway of compound 5 in presence of PLE.*

Similarly to the decomposition mechanism of **4** and **5** in cell extract by an initial esterase activation, followed by an intramolecular mechanism through a five membered ring intermediate, we postulated that the decomposition of the corresponding O-acetylethanolamine amidate derivative **12** could also occur through such mechanism (Figure 4). Therefore, the synthesis of **12** was envisaged in order to evaluate its decomposition pathway as well as its potential biological activity (Table 2). This derivative was obtained according McGuigan procedure using *O*-acetylethanolamine hydrochloride[17] as starting material.

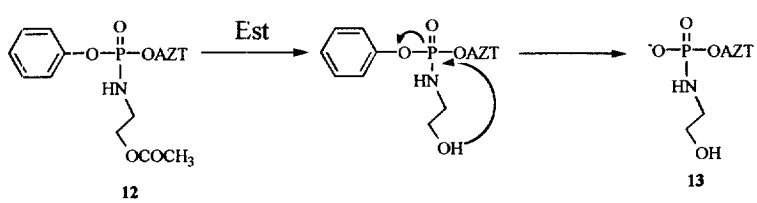


Figure 4. *Decomposition pathway of 12 in cell extract.*

As expected, incubation of **12** in cell extract led to the formation of **13** which was identified by HPLC/MS and by coelution with an authentic standard synthesized following Stawinski's procedure[18]. Compound **12** was cleaved by esterase, followed by an intramolecular elimination of phenol (Figure 4). However, **12** did not present any anti-HIV effect in CEM TK$^-$ cell line (Table 2) indicating that **13** was not further metabolized to AZTMP.

The conversion of the phosphoramidate monoester derivatives **2** into NuMP **3** (second decomposition step: Scheme 1) has been suggested to be mediated by a phosphodiesterase[8, 9] or more likely phosphoamidase[11, 12]. Such phosphoamidases were isolated by Shabarova[19] from bacterial and

mammalian sources and more recently by Kuba from rat liver[20]. However, the activity of these enzymes seems to be strongly dependent on the structure of the phosphoramidate substrate[19]. In CEM cell extracts slight enzymatic activity may be seen as **7** and **8** are slowly decomposed (data not shown) to the corresponding NuMP (see also ref 7).

Therefore compounds **4, 5, 6** and **12** are substrate for esterase, however only **4** and **5** possess an anti-HIV activity in CEM TK$^-$ cells (Table 2). The lack of antiviral activity of **6** and **12** can be explained by low rates of the first or the second decomposition step (Scheme 1).

Compounds	EC_{50}[a]	CC_{50}[b]
AZT	$> 10^{-4}$	$> 10^{-4}$
4	$4.1\ 10^{-6}$	$9.7\ 10^{-5}$
5	$6.3\ 10^{-5}$	$> 10^{-4}$
6	$> 10^{-4}$	$> 10^{-4}$
12	$> 10^{-5}$	$> 10^{-5}$

[a] EC_{50} is the 50% effective concentration (M) or concentration required to inhibit the replication of HIV-1 by 50%.
[b] CC_{50} is the 50% cytotoxic concentration (M) or concentration required to reduce the viability of uninfected cells by 50%.

Table 2. *Anti-HIV activity and cytotoxicity of the phosphoramidates diester derivatives **4-6** and **12** in a thymidine kinase deficient CEM cell line infected with HIV-1.*

From our study, we can conclude that aryl amino acid ester phosphoramidate pronucleotides must fulfill the following requirements to deliver intracellularly the NuMP:

i- the aryl phosphoramidate diester must be a substrate for a cellular esterase to initiate a nucleophile center.

ii- once formed the nucleophile center must attack the PV atom with subsequent phenol elimination through a transitory formation of a five membered ring intermediate [21, 22].

iii- the resulting phosphoramidate monoester must be a substrate for an intracellular enzyme (phosphoamidase?) which selectively delivers intracellularly the NuMP.

Each of the three conditions could be rate limiting for the expression of the activity in TK$^-$ cell lines. The model compound **4** seems to fulfill successively the three above conditions hence explaining its anti-HIV activity. Based on (i) and on its half-life (Table 1), the glycine analogue **5** was less active than **4.** The inability of **6** to deliver NuMP and hence its inactivity (Table 2), could be correlated with (i) (very long $t_{1/2}$) and also with (ii) (unfavourable six membered ring intermediate as **10** is extremely stable). The absence of activity of **12** may be related to point (iii) as it fulfills the two previous conditions.

As the phosphoramidate pronucleotide approach appears to be an attractive strategy for intracellular NuMP delivery, we hope that this work will contribute to the understanding of the mechanism of action of these compounds and will help to design new optimized pronucleotides with a more potent antiviral efficacy.

Acknowledgments:

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