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Rational Design of a New Series of Pronucleotide

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Abstract—A new pronucleotide series is described involving a two-step degradation process mediated by, respectively, carboxylesterase and phosphoramidase. Taking AZT as nucleosidyl moiety, it is shown that most of the compounds inhibit HIV replication in TK$^-$ cell line, which proves 5′-AZTMP delivery.

The pronucleotide[1] approach is now a well established strategy allowing the intracellular delivering of 5′-nucleotide (NuMP). Structurally, all the pronucleotide series which have been shown to in vitro deliver the NuMP are neutral phosphotriesters because of cell uptake. Among the various established series (bis POM, cyclosal, bisSATE, phenyl methylester-alanyl-phosphoramidate, etc.),[2–4] we would like to focus on the two last ones and propose a new type of pronucleotides which could be more suitable for in vivo assays.

Pronucleotides such as neutral P^V derivatives, must be stable enough in culture medium (for in vitro experiments) or in serum (for in vivo assays), but should be selectively decomposed into corresponding NuMP once inside the cell. As no phosphotriesterase activity has been identified in eukaryotic cells, the two-step decomposition process must be mediated by a cellular enzyme. The decomposition mechanism of the two well established approaches follows this point (Scheme 1).

In the McGuigan approach the pronucleotide **1** is first activated by a carboxylesterase (CE) to give rise to the phosphoramidate monoester **2** which is then hydrolyzed by a phosphoramidase to the corresponding NuMP (Scheme 1).

Noteworthy in cell extracts and intact cells the intermediate **2** seems quite stable. In addition, this approach is limited to esters of α-amino acid phosphoramidate derivatives bearing a good leaving group (i.e., phenol).

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For the bisSATE approach, the phosphotriester derivative **3** is first decomposed by a carboxylesterase and leads to the formation of the monoSATE phosphodiester **4**, which is then hydrolyzed to the NuMP through the same process.[5] Thus, in both series, the first activation is controlled by carboxylesterase and differs by the second step activating enzyme such as phosphoramidase or carboxylesterase.

Phosphoramidase is not a well known enzyme; it was reported by Shabarova[6] about 40 years ago, isolated from some animal tissue[7,8] and from cell-free extracts of *Escherchia coli*[9] and *D. discoideum*.[10] This enzyme is present in CEM cells.[11] It was also reported in Shabarova's review[6] that this enzymatic system is able to hydrolyze the P–N bond formed between a 5′-nucleotide and an α-amino acid as well as with various alkylamines.

Therefore, the following rational: assuming there is phosphoramidase activity in cells, it should be possible to deliver 5′-nucleotidyl alkylamine monoester inside the cells by using one SATE moiety[12] as phosphate protecting group (Scheme 2).

To explore this approach, we decided to synthesize various SATE alkylamino 5′-nucleotidyl derivatives of AZT, to study their decomposition in CEM cell extracts and to evaluate their activity in CEM TK$^-$ cell lines.

Therefore, we first considered a series of seven alkylamines with pK_a values between 4.68 and 11.25 (Scheme 3). As pointed out by Shabarova[6] and others,[13,14] it was suggested that two factors influence the rate of hydrolysis: the bulk and the basicity of the amine moiety.

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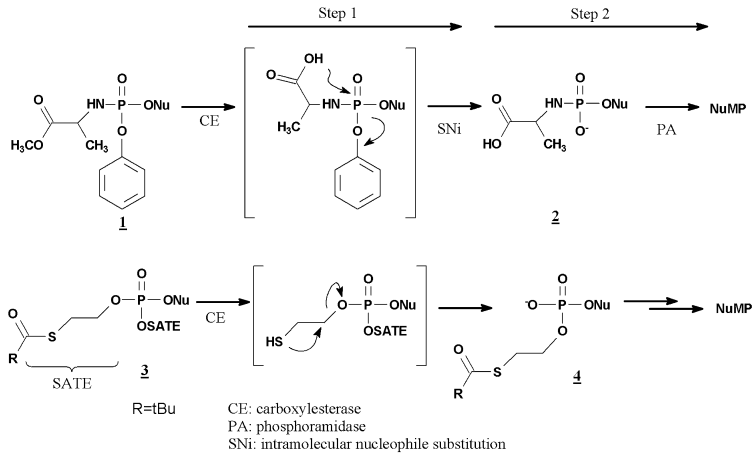


Scheme 1. Decomposition pathways of the phenyl methylester-alanyl-phosphoramidate and the bisSATE phosphotriester derivatives.

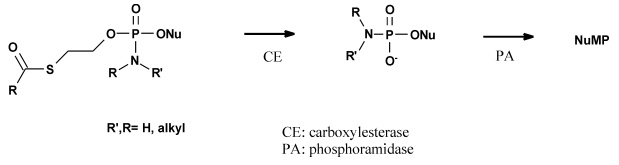
The synthesis of the various phosphoramidate diesters was performed by coupling the *t*BuSATE hydrogenophosphonate diester of AZT with the alkyl amines[15] (Scheme 4). In addition the corresponding phosphoramidate monoesters were obtained by direct coupling of AZTMP with the selected alkylamines (Scheme 4) as references for the study of the decomposition pathway of the AZT phosphoramidate diesters.

We then decided to determine the kinetic and decomposition pathway of **5**–**11** in CEM-SS cell extracts using the on-line HPLC method already published.[16] Half lives of the compounds were determined for the two successive decomposition steps and the metabolites were identified by MS coupling and/or coinjection with authentic synthesized samples. Noteworthy, in cell extract the first half life value reflects the affinity of the phosphoramidate diester for carboxyesterase (SATE elimination) and the second one the affinity of the phosphoramidate monoester for the phosphoramidase.

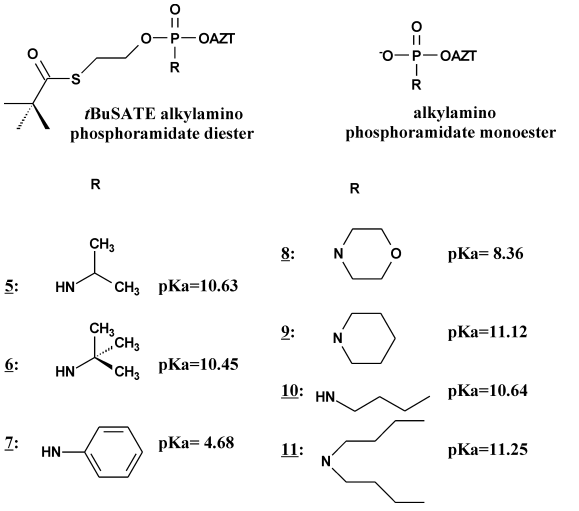
The results given in Table 1 show that the CE *t*BuSATE group elimination is rapid ($0.96 < t_{1/2} < 2.1$ h) compared to the phosphoramidase hydrolysis ($72 < t_{1/2} < 696$ h). At this stage we do not know if this last statement is reliable, indeed nothing is known about the PA stability, and partial denaturation of such an enzyme may have occurred during cell extract preparation.

The stability of compounds **5**–**11** was also evaluated in culture medium containing 10% of heat inactivated fœtal calf serum (Table 1). All these compounds proved to be much more stable in the culture media than in cell extract. Therefore this new pronucleotide series could be considered as a potential candidate for in vitro NuMP intracellular delivery. Furthermore, the partition coefficients, expressed as log P, were determined for compounds **5**–**11** and are reported in Table 1.

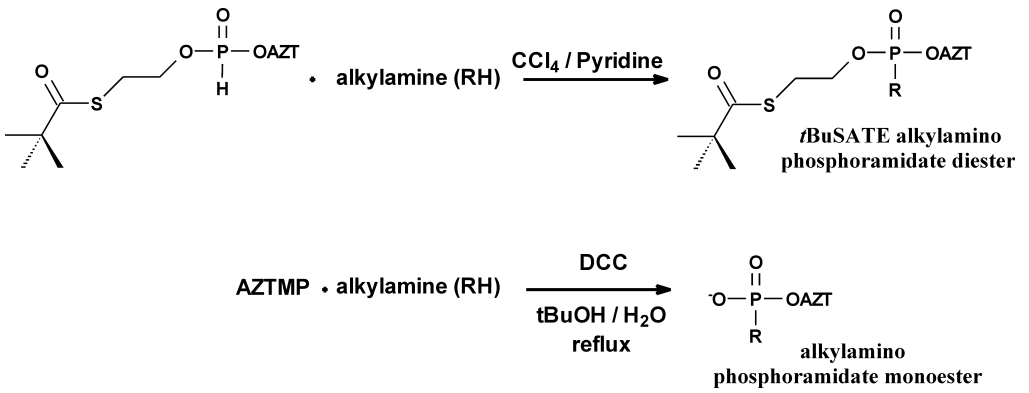
Compounds **5**–**11** were evaluated for their ability to inhibit HIV replication in TK$^-$ CEM-SS cell line. As shown in Table 1, most of the compounds present an EC_{50} in the micromolar range ($0.63 < EC_{50} < 5.2$ μM) except for **7** and **11**. The absence of anti-HIV activity for these two compounds may be related to the poor affinity of the corresponding phosphoramidate monoesters for the phosphoramidase. The enzymatic hydrolysis mechanism is not known but seems to involve an *N*-protonation step,[6] hence the importance of the pK_a of the amine that may be related to the lack of activity of **7** (aniline p$K_a = 4.68$). Furthermore, the substrate ability of the hindered amine moiety to the binding site of the phosphoramidase should also be considered and may explain why compound **11** is inactive. This observation has been confirmed in other pronucleotide series incorporating di-*n*-butylamine and also with other bulky amines even if presenting appropriate pK_a (data not shown).



Scheme 2. Expected decomposition pattern.



Scheme 3. Akylamines selected and phosphoramidate diester and monoester derivatives.



Scheme 4. Synthesis of phosphoramidate diesters and monoesters.

Table 1. Log P measured, kinetic and activity results[a]

	Log P measured[17]	$t_{1/2}$ (h) culture medium	$t_{1/2}$ (h) Human serum	$t_{1/2}$ (h) Cell extract		TK$^-$ CEM-SS (μM)	
				Step 1	Step 2	EC_{50}	CC_{50}
5	1.91	200	10	1.2	72	0.63	>100
6	2.45	350	23	1.2	96	1.5	>100
7	2.55	100	23	1.4	Stable[a]	>10	>10
8	1.86	385	23	2.1	696	2.0	>100
9	2.77	ND	ND	1.2	168	5.2	>100
10	2.67	165	29	1.3	144	1.2	>100
11	4.00	136	23	0.96	528	>10	>10
AZT	—	—	—	—		>100	>100

ND, not determined.
[a]No decomposition observed up to 96 h.

In conclusion, one can say that the monoSATE phosphoramidate approach allows delivery of AZTMP inside the cells when the amine moiety presents appropriate pK_a values and that a suitable affinity of the corresponding phosphoramidate monoester for the enzyme is reached. In addition, their important stability in human serum as well as their lipophilicity, expressed by their log P, may open the way to in vivo evaluation of such pronucleotides.

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