SATE Pronucleotide Approaches: An Overview

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Abstract: This review depicts *in vitro* and *in vivo* results obtained with nucleotide prodrugs (pronucleotides) bearing *S*-acyl-2-thioethyl (SATE) groups as esterase-labile phosphate protections. New developments are illustrated by the design of mononucleoside mixed phosphoester derivatives leading to the selective intracellular delivery of the corresponding 5'-mononucleotide through two different enzyme-mediated activation steps.

Key words: Nucleotide, prodrug, antiviral, phosphotriester, phosphoramidate, esterase, phosphoamidase, bioconversion.

1. INTRODUCTION

The need to mask the two charged phosphate oxygen's of a 5'-mononucleotide to obtain neutral and lipophilic prodrugs (pronucleotides) led us to study, more than ten years ago, mononucleoside phosphotriesters bearing phosphate protecting groups. To provide the intracellular delivery of a 5'-mononucleotide from its prodrug, the design of such phosphate protecting groups was based on general considerations related to enzymatic and chemical stabilities of phosphotriester derivatives. Because of phosphotriesters are uncommon in nature, specific enzymes (*i.e.* phosphotriesterases) that may cleave them have not been identified in mammalian cells. Consequently, the hydrolysis of simple dialkyl phosphotriesters depends exclusively on chemical mechanisms. In physiological conditions, this hydrolysis is particularly slow and can occur through nucleophilic attacks on phosphorus atom or on α-tetrahedral carbon atom of the alkyl ester group according to the Hard Soft Acids Bases (HSAB) concept [1, 2]. Mechanistic studies of *O*,*S*-ethylene phosphorothioate derivatives hydrolysis (Fig. **1**) illustrate both processes [3-5]. Briefly, *S*-phosphorylated derivatives of 2-mercaptoethanol undergo solvolysis with almost exclusive P-S cleavage leading to ethylene sulphide elimination in neutral conditions. This result can be explained by preferential nucleophilic attack of the hydroxyl function, as "hard" base, on the phosphorus atom as "hard" acid. An intramolecular ligand exchange (pseudo rotation) in the resulting five-coordinate intermediate occurs, placing the sulphur atom into an apical leaving position. The greater leaving ability of the alkylthio ligand and the weakness of the P-S bond (due to less efficient $d\pi$-$p\pi$ bonding) compared to the oxy analogs are cited as factors involved in the fast and selective cleavage of *O,S*-phosphorothioate esters. Finally, the 2-mercaptoethyl phosphoesters spontaneously decompose through nucleophilic attack of the mercaptide anion ("soft" base) on the tetrahedral carbon ("soft" acid) giving rise to the formation of the phosphate function.

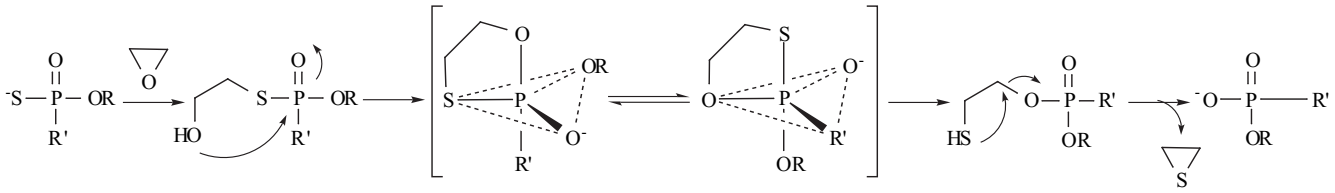

Fig. (1). Hydrolysis of *O,S*-ethylene phosphorothioate derivatives.

We used the selectivity of this nucleophilic process in designing new phosphate protections incorporating a thioethyl chain, the sulphur atom of the thiol function being involved in various enzyme-labile bonds (Fig. **2**) [6-10]. Among them, the *S*-acyl-2-thioethyl (SATE) groups have been extensively studied as efficient esterase-mediated transient phosphate protections for bioactive mononucleotide or phosphonate analogs. Part of this work has already been reviewed [11-14]. The present paper would highlight some *in vitro* results obtained with bis(SATE) phosphotriester derivatives and new developments resulting from their *in vivo* evaluation.

2. MONONUCLEOSIDE BIS(SATE) PHOSPHOTRIESTERS

2.1. *In Vitro* Antiviral Activity

The SATE protection is formally constituted by two-component masking groups and belongs to the double

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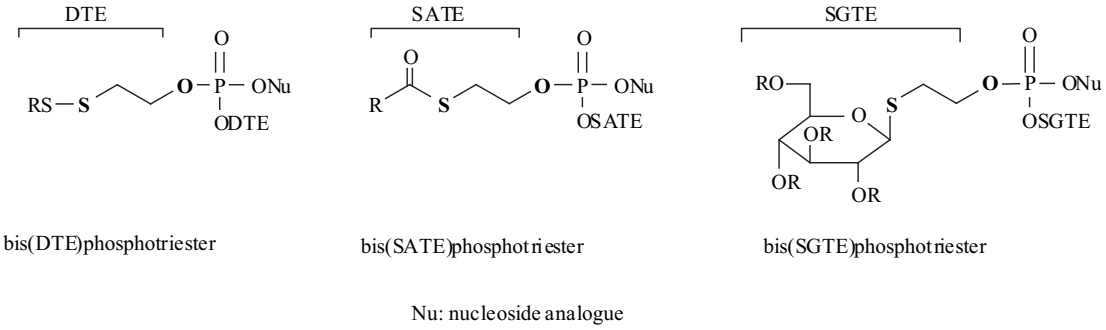


Fig. (2). General structures of phosphotriesters bearing phosphate protections characterised by the presence of a thioethyl chain. DTE : *S*-(2-alkylsulfidyl)-2-thioethyl [6]; SATE: *S*-acyl-2-thioethyl [7]; SGTE: *S*-glucosyl-2-thioethyl [8-10].

prodrug concept [15-18]. Initially investigated in cell extracts [7, 19], metabolism studies with radiolabeled compounds [20, 21] revealed that the decomposition pathway of bis(SATE) pronucleotides requires, in a first step, an esterase-mediated hydrolysis giving rise to an unstable phosphotriester which decomposes spontaneously (Fig. **3**). The resulting SATE phosphodiester was then able to lead to the corresponding 5'-mononucleotide after a similar process. In addition, we demonstrated that SATE groups exhibit a relative stability in extracellular media (culture medium, human serum) associated with a rapid decomposition in all examined cell lines. This requirement is a key factor for prodrugs designed to release selectively the parent mononucleotides inside cells [14]. Moreover, kinetics associated to the monophosphorylated form delivery can be controlled by varying the acyl moiety of the SATE group.

Initially investigated with 3'-azido-3'-deoxythymidine (AZT) as nucleoside model [7, 22], the SATE approach has been applied to anti-human immunodeficiency virus (anti-HIV) nucleoside analogs that are limited by the first phosphorylation step, through a dependence on kinase-mediated phosphorylation, or by a rate limiting step in the anabolism pathway [23-28]. In all cases, *in vitro* evaluation of the corresponding bis(SATE) phosphotriester derivatives shows increased antiviral activities compared to the parent nucleosides. For example, the SATE pronucleotides of 2',3'-dideoxyadenosine (ddA) exhibit a very potent antiretroviral effect in human lymphoblastoid cells (CEM-SS, MT-4), stimulated and unstimulated primary cultured human cell lines such as peripheral blood mononuclear (PBM) cells or monocyte-derived macrophages [23-25, 27]. Table 1 illustrates the efficiency of this approach through the evaluation of several bis(SATE) phosphotriesters of ddA, which differ by the nature of the acyl residues, in PBM cells infected with HIV-1 or simian immunodeficiency virus (SIV). SIV, which is genetically and morphologically similar to HIV, produces an immunodeficiency disease and associated dysfunction in macaques providing the most complete experimental model for HIV disease in humans. The tested compounds proved to be superior to the parent nucleoside, with 50% effective concentration (EC_{50}) values in nanomolar range. Moreover, their cytotoxicity was substantially lower than for ddA. Measured in terms of selectivity index (SI), these bis(SATE) phosphotriesters emerged as very potent and selective inhibitors of HIV and SIV replications *in vitro*. Thus, applied to an antiviral nucleoside analogue which is hampered at the first phosphorylation step, the bis(SATE) pronucleotide approach leads to an enhanced *in vitro* antiviral efficiency.

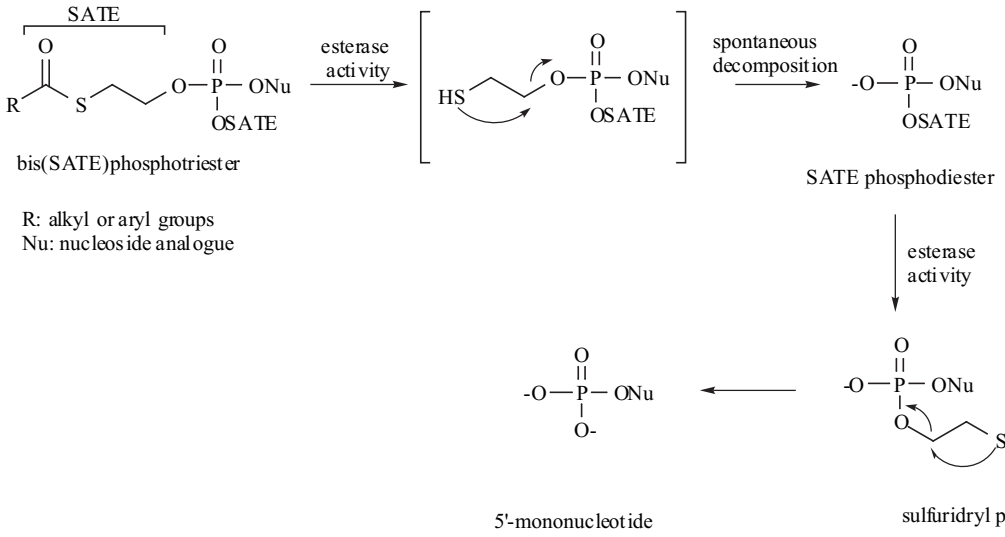
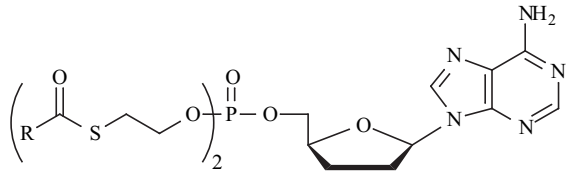


Fig. (3). Decomposition pathway of mononucleoside bis(SATE) phosphotriester derivatives in cell extracts as well as in intact cells [7, 19, 20].

Table 1. Antiviral Activity of bis(SATE) Phosphotriesters of ddA Compared to the Parent Nucleoside in PBM Cells Infected with HIV-1 or SIV (Unpublished Data, Aubertin, A.-M.)



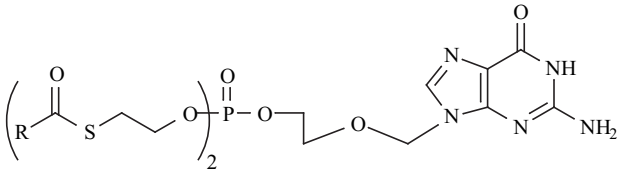
R = CH_3, bis(MeSATE) ddAMP
R = $(CH_3)_2CH$, bis(*i*PrSATE) ddAMP
R = $(CH_3)_3C$, bis(*t*BuSATE) ddAMP

	HIV-1-Lai			SIVmac251		
	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]
ddA	90	22	240	240	>10	na[d]
bis(MeSATE)ddAMP	0.2	2.9	14,500	1.9	0.700	370
bis(*i*PrSATE)ddAMP	4.6	3.2	700	0.04	0.245	6,125
bis(*t*BuSATE)ddAMP	1	1.6	1,600	0.0005	0.140	280,000

[a]EC_{50}, 50% effective concentration or concentration required to inhibit the replication of HIV-1 by 50%. [b]CC_{50}, 50% cytotoxic concentration or concentration required to reduce the viability of uninfected cells by 50%. [c]selectivity index, ratio CC_{50}/EC_{50}. [d]"na" indicates that the SI is not available.

Investigation of the SATE biolabile phosphate protections was also extended to the 5'-mononucleotide of acyclovir [9-(2-hydroxyethoxymethyl) guanine, ACV]. This nucleoside analogue is currently used in the treatment of herpes simplex (HSV) and varicella-zoster (VZV) virus infections. Specificity for the herpes virus-induced thymidine kinase accounts for the wide therapeutic index of ACV but limits its activity spectrum, excluding important pathogens such as human hepatitis B virus (HBV), which does not encode a thymidine kinase. Consequently, the efficacy of ACV in treating patients with chronic HBV is uncertain, although its 5'-triphosphate (ACVTP) is a relatively good inhibitor of HBV DNA polymerase. In order to exploit the potency of ACVTP as anti-HBV agent, two bis(SATE) phosphotriester derivatives of ACV have been evaluated for their inhibitory effects on the replication of HBV (Table 2) in human HBV DNA-transfected human hepatoblastoma-derived liver Hep-G2 cells (2.2.15 cells) as well as in primary duck hepatocyte cultures infected with the duck hepatitis B virus (DHBV) [29, 30]. The DHBV is an available model largely used for the screening and the evaluation of new anti-HBV agents both *in vitro* and *in vivo* in chronically infected animals. In contrast to ACV, the corresponding bis(SATE) phosphotriesters exhibited a potent and selective *in vitro* anti-HBV activity in these experiments, proving that the use of such pronucleotide approach also permits to extend the antiviral spectrum of particular nucleoside analogue. Compared to the established 2.2.15 cell line, the greater activity in primary cells, in which a more dynamic replication occurs and high virus yields are achieved rapidly, may reflect an increased conversion of these derivatives to their phosphorylated forms.

Table 2. Antiviral Activity of bis(SATE) Phosphotriesters of ACV Compared to the Parent Nucleoside in Human HBV DNA-Transfected 2.2.15 Cells and Primary duck Hepatocyte Cultures Infected with DHBV [29, 30]



R = CH_3, bis(MeSATE) ACVMP
R = $(CH_3)_3C$, bis(*t*BuSATE) ACVMP

	Human HBV (2.2.15 cells)			Duck HBV (hepatocytes)		
	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
ACV	111	631	na[d]	0.1	>2000	>20,000
bis(MeSATE)ACVMP	0.7	987	1,400	0.3	5	166
bis(*t*BuSATE)ACVMP	0.2	1,593	7,900	0.0006	120	200,000

[a-d]See Table 1 footnotes.

Differences in the *in vitro* antiviral activity of bis(SATE) pronucleotides (Tables 1 and 2) were associated with multiple factors including lipophilicity, decomposition kinetics (in culture medium and inside cells) of the prodrug, as well as particular metabolism of the parent nucleoside [14]. In cell culture experiments, comparative evaluation of pronucleotides bearing different SATE groups generally showed that, phosphotriesters incorporating the lipophilic and enzymatically resistant *S*-pivaloyl-2-thioethyl (*t*BuSATE) group exhibit similar or superior antiviral effects than the more labile *S*-acetyl-2-thioethyl (MeSATE) phosphate protection.

2.2. Toxicity

Recently reviewed [31], recurrent question is related to toxicity of small molecules released at the time of the decomposition of bipartite protecting groups (Fig. **3**). Regarding this, the enzymatic activation of the SATE protections produces carboxylic acids and episulfide. Carboxylic acids could be easily managed by normal pH-homeostatic mechanisms of cells [32]. Whereas, not much is known about the episulfide behaviour in biological media and, to our knowledge, no appropriate literature data has been reported about its potential cytotoxicity. As hypothesis, this electrophilic side product may be converted to a conjugate with glutathione. In mammalian cells, this abundant non-protein thiol serves both as nucleophile and as reducing agent, and plays a crucial role in the detoxification and repair of cellular injury.

The potential toxicity of SATE groups and their degradation products was evaluated by a large number of experimental assays. Incubation of carboxylic acids and episulfide at about 200 μM does not affect the proliferation of various cell lines, including proliferating human T-cells and primary cell cultures. Comparison of a bis(MeSATE) phosphotriester of AZT to the parent nucleoside in normal human bone marrow progenitor cells indicates that the SATE pro-moiety, as well as its degradation products, do not induce additional toxicity [33]. The mutagenic potential of the bis(MeSATE) derivative of ddA (Table 2) has been evaluated by the Ames test [34]. At all concentrations studied (10 to 2500 μg per plate), the pronucleotide is not mutagenic for the five *S. typhimurium* strains tested even in the presence of metabolic activation (unpublished data, Vian, L.). Finally, acute toxicity study of this compound was also carried in cynomolgus monkeys (unpublished data, Dormont, D. and Clayette, P.). The pronucleotide was subcutaneously administered, three times daily for five days, at three concentrations (0.6, 3 and 15 mg/kg/day). Detailed hematological and serum biochemistry parameters have been determined, neither clinical symptom nor behaviour trouble traducing toxicity in animals were observed.

Nevertheless, illustrated by the *in vitro* evaluation of the bis(SATE) phosphotriesters of ddA and ACV (Tables 1 and

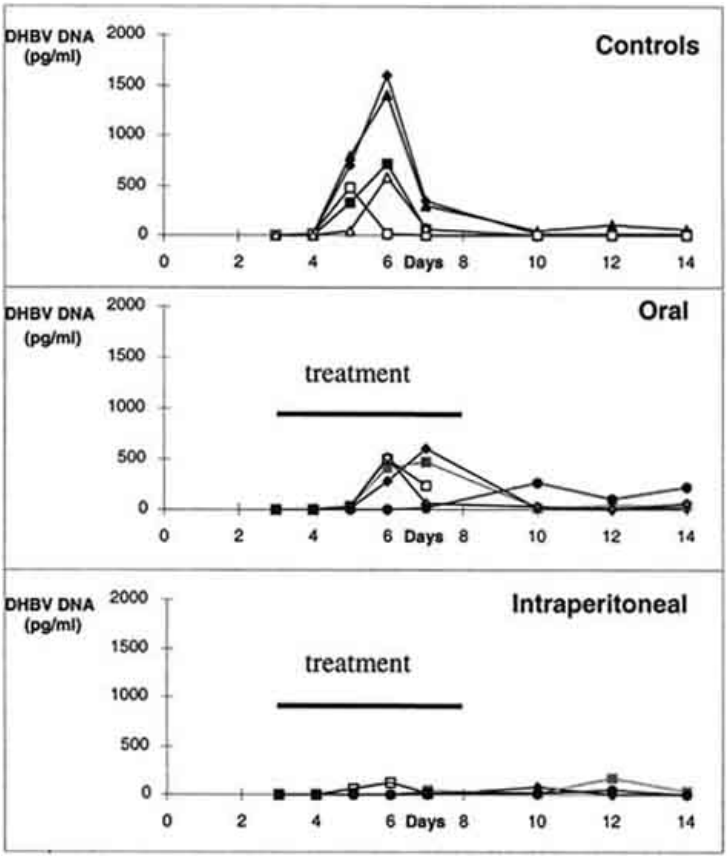


Fig. (4). *In vivo* antiviral activity bis(*t*BuSATE) phosphotriester of ACV in DHBV infected ducks [30]. Ducklings were inoculated with a standard DHBV inoculum 2 days post hatching and then treated at day 3 post-inoculation for 5 days either by oral route (10mg/kg/day) or by intraperitoneal route (5mg/kg/day). Five animals were used in controls groups and each treatment group.

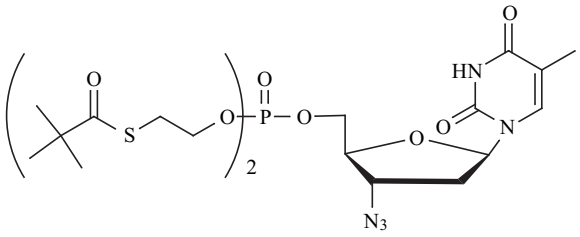
2), an increase of the toxicity can be observed compared to the parent nucleosides. This cytotoxicity often reflects the intracellular accumulation of the phosphorylated forms, which possibly interact with host cellular enzymes. This raises particularly the importance of the selectivity of corresponding 5'-triphosphate derivatives for viral polymerases [33].

2.3. *In Vivo* Studies

Given the potent and selective effects of various mononucleoside bis(SATE) phosphotriesters in cell culture experiments, we decided to investigate their *in vivo* efficacy. In this respect, the *t*BuSATE group has been selected with regard to its relative resistance to esterase hydrolysis [12]. Thus, the corresponding bis(*t*BuSATE) pronucleotide of ACV (Table 2) was evaluated in HBV-infected ducklings [30]. Expressed in terms of molar effective drug concentration, this pronucleotide proved to be superior to ACV after intraperitoneal and oral administrations (Fig. **4**). Nevertheless, the antiviral superiority is less evident *in vivo* than *in vitro* due to the fact that ACV induces an almost complete inhibition of DHBV replication in this animal model.

This result led us to investigate the pharmacokinetics and metabolism of the bis(*t*BuSATE) phosphotriester derivative of AZT in mice [35]. Drug concentrations were measured, using an HPLC methodology, in peripheral red blood cells, brain and lymph nodes that are important targets for combating HIV infection. Striking differences were found between intravenous (i.v.) and oral (p.o.) administrations. Following i.v. administration, the prodrug was rapidly metabolised to yield the corresponding 5'-mononucleotide (AZTMP) and AZT in blood. Despite this limitation, the bis(*t*BuSATE) phosphotriester of AZT resided in mice with a longer mean residence time and terminal half-life. This could be explained by the fact that, although the total body clearance of the prodrug was high, mainly resulting from a fast metabolism, its steady-state volume of distribution was large. It is likely that prodrug distributed rapidly into tissues with a lower metabolising ability. As a result, i.v. administration of prodrug produced significantly greater brain and lymph nodes exposure to AZT. High concentrations of this pronucleotide and AZTMP were present in brain, indicating its ability to penetrate the blood-brain barrier and to be converted into the corresponding 5'-mononucleotide (Fig. **5A**). Finally, in contrast to i.v. administration of AZT in mice, where no AZTMP was detected in peripheral blood cells, the 5'-mononucleotide was observed inside this main blood cell population. Moreover, concentrations of AZTMP were higher than in plasma (Fig. **5B**), showing the ability of such kind of pronucleotides to deliver selectively the corresponding 5'-mononucleotide *in vivo*.

Following p.o. administration, no intact prodrug was observed in mice blood probably due to an extensive metabolism in the gastrointestinal tract and/or liver rich in esterases. This result was corroborated by pharmacokinetic



bis(*t*BuSATE)AZTMP

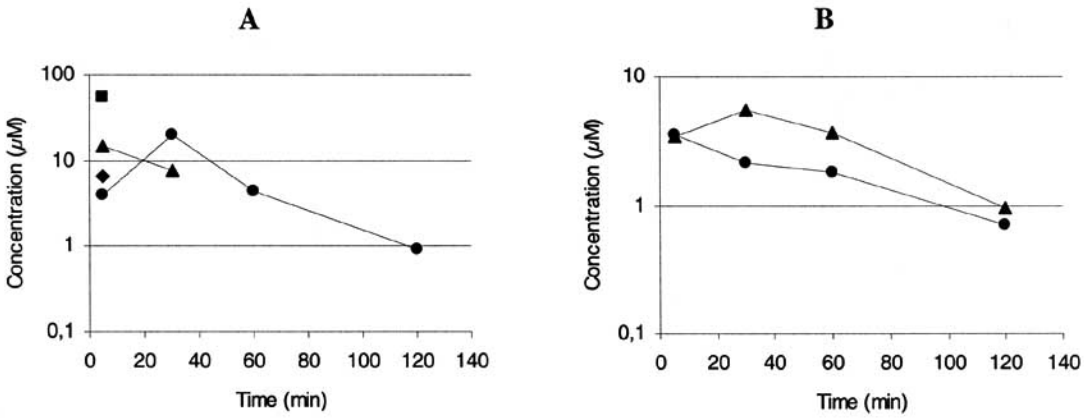
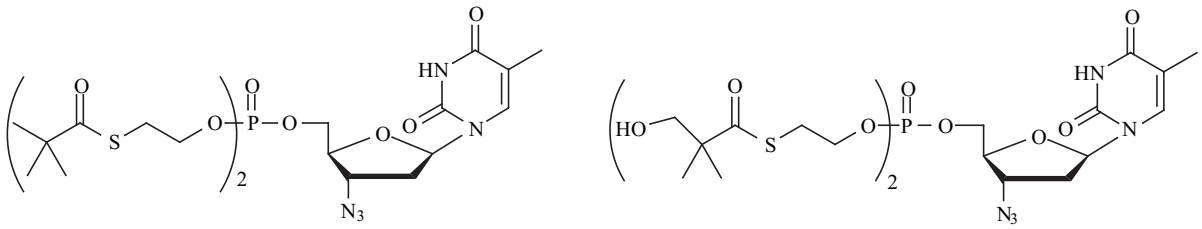


Fig. (5). (A) Distribution of the bis(*t*BuSATE)phosphotriester of AZT (square), AZTMP (diamond) and AZT (circle) in brain following i.v. administration compared to the parent nucleoside (triangle). (B) Concentrations of AZTMP in plasma (circle) and peripheral red blood cells (triangle) following i.v. administration of the bis(*t*BuSATE)phosphotriester of AZT [35].



bis(*t*BuSATE)AZTMP

$t_{1/2}$ (human serum): 7.5 h

log P (octanol/water): 4.0

aqueous solubility: 0.031 g/L (25 °C)

bis(HO*t*BuSATE)AZTMP

$t_{1/2}$ (human serum): 20 h

log P (octanol/water): 1.5

aqueous solubility: 2.6 g/L (25 °C)

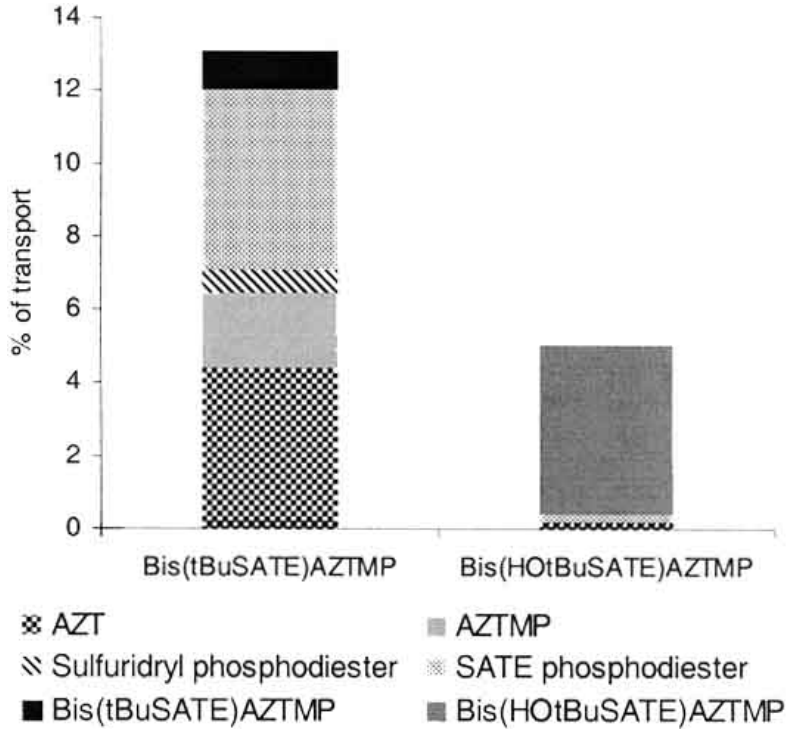


Fig. (6). Total cumulative transport of bis(*t*BuSATE)phosphotriester of AZT and its corresponding hydroxylated analogue across Caco-2 monolayer (2h incubation) in presence of Gelucire 44/14 (0.125% w/v). Transport is expressed as percentage of the initial amount (100μM) of tested phosphotriesters [38].

studies in rhesus monkeys using another radiolabelled bis(*t*BuSATE) phosphotriester derivative [21].

In order to answer presystemic metabolism, various modifications have been introduced on the *t*BuSATE group. Attempts illustrating this strategy could be found in the increase of the thioalkyl chain length [36] as well as the introduction of a methyl group at the Cα-position [37]. Probably, the most interesting results have been found with the introduction of a polar function in the near vicinity of the ester functionality [38]. Thus, a simple chemical modification such as the introduction of a hydroxyl function in the acyl chain of the *t*BuSATE moiety (HO*t*BuSATE, Fig. **6**), leads to a marked decrease of the hydrolysis rate of the resulting phosphotriester in biological media. This result is in agreement with the fact that carboxylesterases, the most probable candidates for the enzymatic degradation of the SATE phosphotriesters, appear to have higher reactivity with lipophilic esters than with polar or charged substrates [39, 40]. As a consequence, the resulting bis(HO*t*BuSATE) phosphotriester derivative of AZT (Fig. **6**) is able to cross in intact form the Caco-2 cell monolayer, a generally accepted *in vitro* model for intestinal drug absorption [41], even if the presence of polar functions affects the lipophilicity of the prodrug and its membrane diffusion properties.

If relative enzymatic stability is required to envisage a site-specific delivery of the 5'-mononucleotide from symmetrical phosphotriesters, this may also preclude the required bioconversion. This ambiguity is related to the specific decomposition process of mononucleoside bis(SATE) phosphotriesters (Fig. **3**). The removal of the second SATE masking group could be considered as a rate-limiting step in the general process leading to the delivery of the 5'-mononucleotide. Indeed, the second activation step proceeds more slowly due to the proximity of the phosphate negative charge and the enzyme active site. The charge has also an important second effect in reducing the rate of the intramolecular nucleophilic substitution, which leads to the C-O bond fission of the phosphate ester. Consequently, an

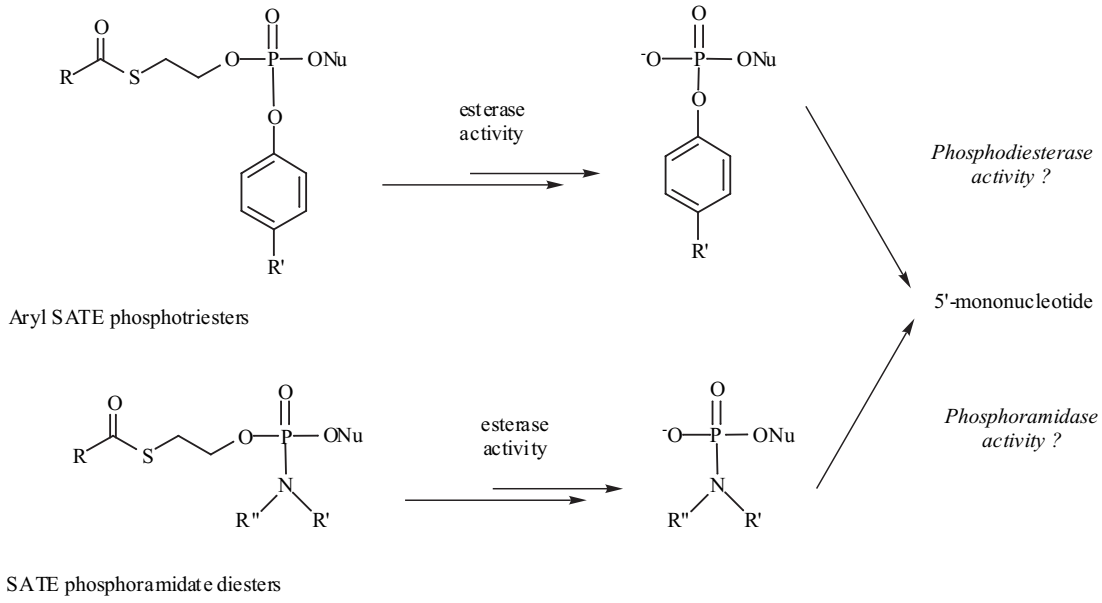


Fig. (7). General structure and expected decomposition processes of two series of mononucleoside mixed SATE phosphoesters.

increased stability of the SATE group may result to inappropriate kinetic parameters related to the 5'-monucleotide delivery.

Thus, we decided to evaluate the potentialities of new series of mixed pronucleotides incorporating only one SATE chain and a different second group linked to the phosphorus atom, which will be hydrolyzed (or bioconverted) by other enzymatic systems than esterases.

3. MONONUCLEOSIDE MIXED SATE PHOSPHOESTERS

We investigated two different kinds of mononucleoside mixed SATE phosphoesters bearing either an aryl or an amino group, namely aryl SATE phosphotriesters and SATE phosphoramidate diesters (Fig. **7**), respectively.

3.1. Mononucleoside Aryl SATE Phosphotriesters

Design of this series is based on literature data [42-44] which show that nucleotide phosphodiesterases, a family of ubiquitous enzymes, have good affinity for aromatic substrates and are able to selectively hydrolyse aryl phosphodiester derivatives into their corresponding phosphomonoesters [44, 45]. Thus, our purpose was to generate intracellularly an aryl phosphodiester by the use of the corresponding SATE phosphorylated precursor (Fig. **7**). Mononucleoside *t*BuSATE phosphotriesters of AZT incorporating either phenyl or L-tyrosinyl groups (Fig. **8**) have been studied as first models [46]. We pursued our investigations in this series by varying the nature of the aryl substituent in order to determine the influence of ionisable, polar or lipophilic functions in regards to anti-HIV activities and kinetic decomposition parameters [47, 48].

3.1.1. Synthesis

A simple retro synthetic route to the target compounds is presented in Figure **9**. Briefly, the mixed phosphotriester derivatives are obtained by *in situ* oxidation of the corresponding phosphite triesters. The phosphoramidite agents bearing the two different phosphate protections consist in subsequent coupling of the *t*BuSATE chain and

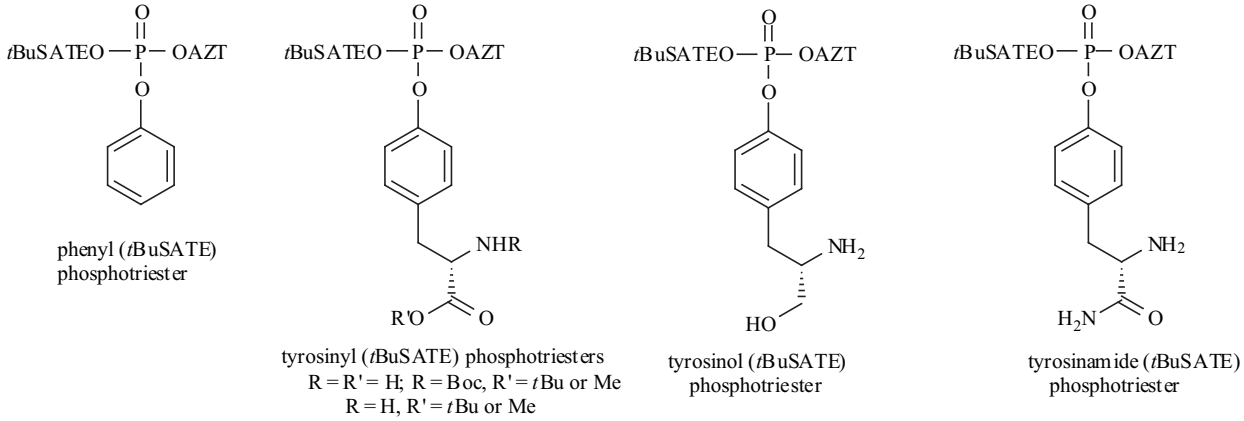


Fig. (8). Selected structures of mononucleoside aryl SATE phosphotriesters.

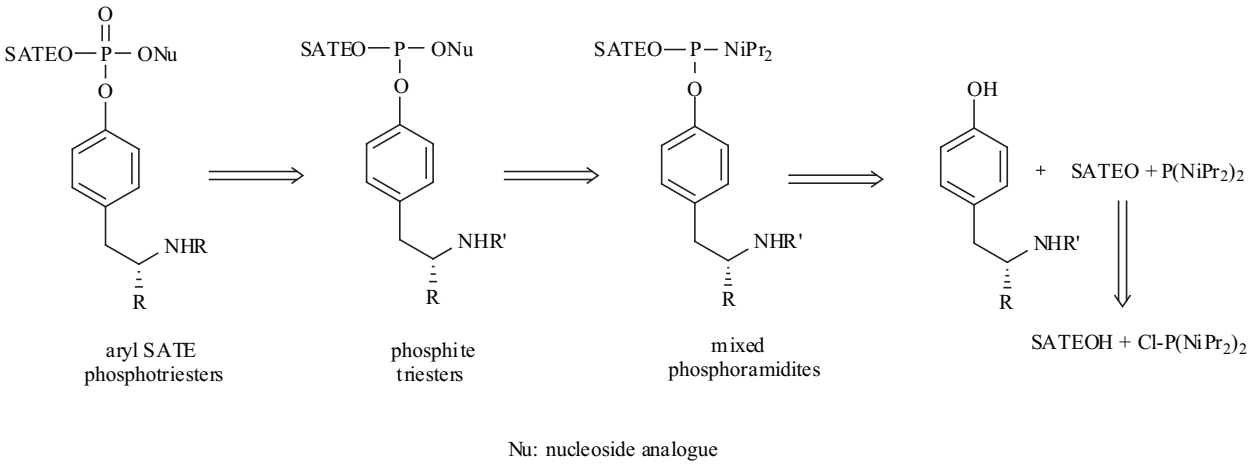


Fig. (9). General retrosynthetic route to mononucleoside aryl SATE phosphotriesters.

then, the appropriate aryl residue on the commercially available bis(diisopropylamino) chlorophosphine.

The choice of the protecting groups of the aryl residues should be done thoughtfully. Indeed, their cleavage conditions have to be compatible with the stability of the final derivatives, which possess base and nucleophile sensitive functions (thioester, phosphotriester). Consequently, we used acid-labile protecting groups (*tert*-butyloxycarbonyl, *tert*-butylester and isopropylidene) and/or groups susceptible to be hydrolyzed by an enzymatic system during biological studies (*i.e.* acetyl group, methyl or *tert*-butyl esters), in order to keep the integrity of the final compounds.

3.1.2. Antiviral Activity

In human T_4-lymphoblastoid CEM-SS and MT-4 cells, all the aryl (*t*BuSATE) phosphotriesters displayed anti-retroviral activity that is comparable to AZT. In contrast to

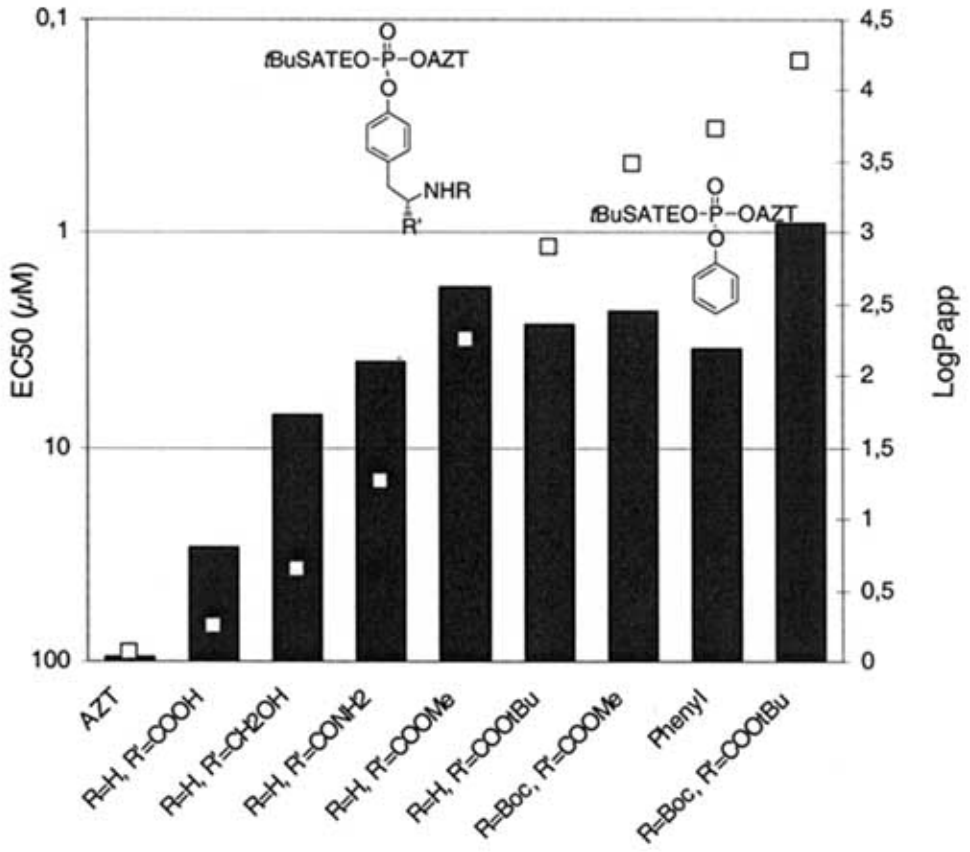


Fig. (10). Anti-HIV activity in CEM/TK$^-$ cell line (chart graph) and apparent partition coefficients (Log P*app*, open square) of selected *t*BuSATE aryl phosphotriesters of AZT compared to the parent nucleoside [47, 50].

the parent nucleoside, most of the tested prodrugs exhibited significant anti-HIV effects in thymidine kinase-deficient (CEM/TK^-) cell line, demonstrating the successful release of the corresponding 5'-mononucleotide inside infected cells. Thus, they can be considered as a new series of pronucleotides.

Comparison of EC_{50} in CEM/TK^- cells and apparent partition coefficients (Fig. **10**) indicates that the presence of a free carboxylate function might be responsible for the loss of antiviral activity of the tyrosinyl (*t*BuSATE) phosphotriester derivative (Fig. **8**, R=R'=H). This is corroborated by the EC_{50} values obtained for other hydrophilic phosphotriesters such as tyrosinol and tyrosinamide derivatives, incorporating polar but not anionic groups in place of the acid function.

3.1.3. Stability Studies

The decomposition pathways and kinetic data of the aryl SATE phosphotriesters of AZT were determined in culture medium, which is the extracellular medium used for

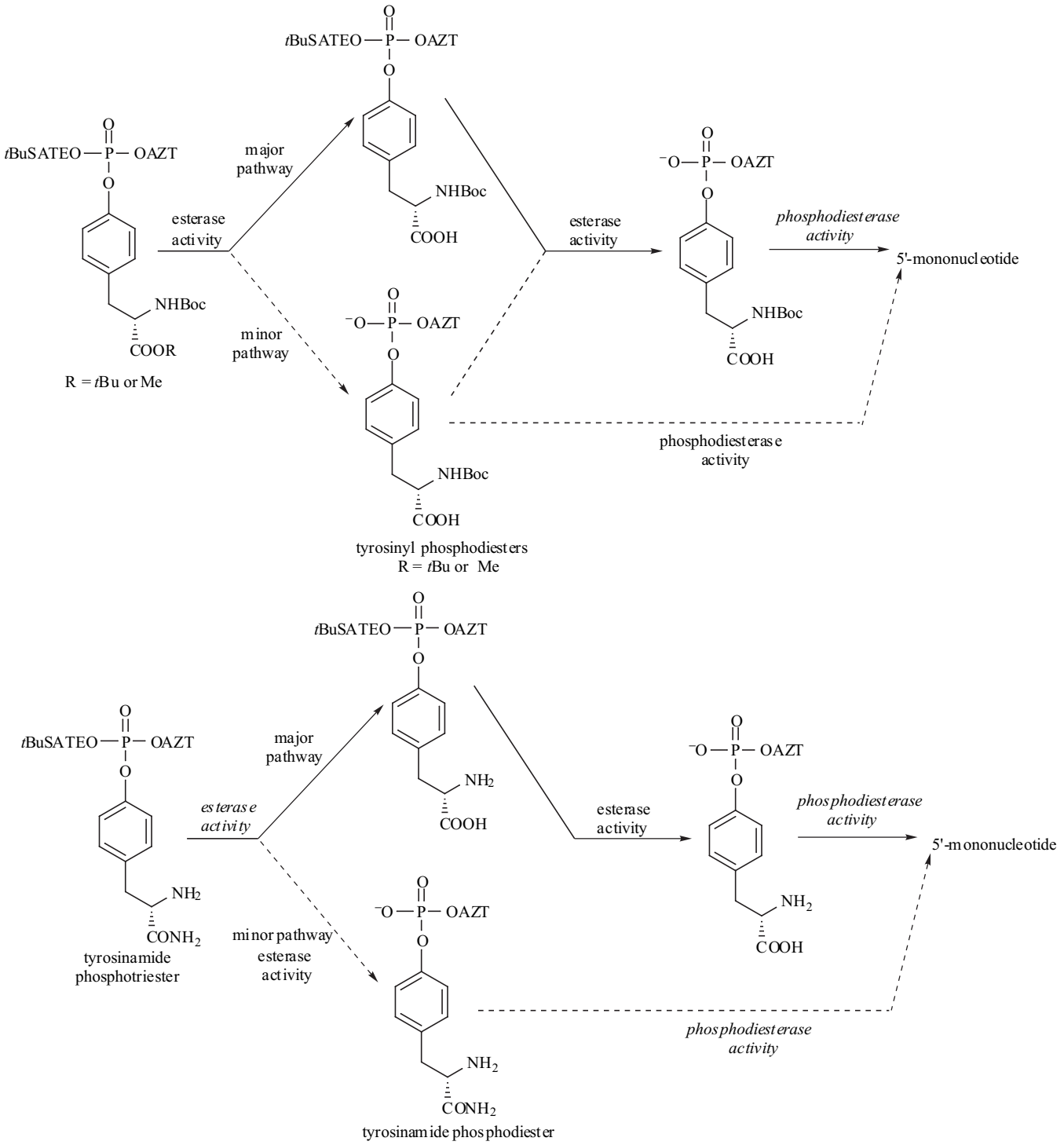


Fig. (11). Proposed decomposition pathways of selected *t*BuSATE aryl phosphotriesters of AZT in total CEM-SS cell extracts [49, 50].

antiviral evaluation in cell culture systems, as well as in total CEM-SS extracts, a mimic for the intracellular medium.

As required, all compounds metabolised very slowly in culture medium (half-life about a week) compared to cell extracts (Table 3). In both media, tyrosinyl and tyrosinol phosphotriester derivatives were bioconverted following a similar decomposition process, which involves in a first step an esterase-mediated activation [49, 50]. Depending to the nature of the aryl substituent, the *t*BuSATE chain loss could also be concomitant with the cleavage of the ester group of tyrosinyl residues (Fig. **11**). Both decomposition pathways gave rise to the formation of the corresponding aryl phosphodiester derivatives. Then, these metabolites were substrate for a second enzymatic activity leading to the delivery of the 5'-mononucleotide. The same experiments were carried out with the tyrosinamide phosphotriester derivative and its behaviour was puzzling (Fig. **11**). A putative amidase activity [51-53] was supposed to be involved in the hydrolysis of the amide bond into a carboxylate function. The last steps of the decomposition proceed via esterase hydrolysis (loss of the *t*BuSATE chain) and then another enzymatic activity (recovery of AZTMP).

In addition, the stability of corresponding aryl phosphodiester intermediates was evaluated in cell extracts either heat-inactivated, in order to confirm that their conversion into AZTMP was not due to chemical process, or pre-incubated with EDTA, which is known as an inhibitor of type I phosphodiesterase [45]. The corresponding kinetic data are in agreement with the fact that decomposition of the studied aryl phosphodiesters is likely to be due to a type I phosphodiesterase activity [50].

In cell culture experiments, the antiviral activity measured for a pronucleotide series is the consequence of a large number of factors such as passive diffusion across cell membranes, chemical and enzymatic stabilities in extra and intracellular media, selectivity of the decomposition pathways into the 5'-mononucleotide and kinetics related to this (bio)conversion. Moreover, this complex picture may also integrate the particular metabolism of the nucleoside analogue from which the prodrug approach is applied. Finally, as previously discussed, the nature of the studied cell line can affect the range of the biological response.

In the aryl SATE phosphotriester series, an appropriate lipophilicity of the prodrug associated to a rapid bioconversion process led to potent anti-HIV effects in the CEM/TK$^-$ cell line (Table 3). The release of the 5'-mononucleotide from such pronucleotides is clearly ruled out by the second decomposition step related to the phosphodiesterase affinity for the corresponding aryl phosphodiester metabolites.

These results corroborated data obtained with bis(SATE) phosphotriester derivatives of AZT, showing that an important increase of the enzymatic stability of a pronucleotide is correlated to a decrease of its *in vitro* antiviral activity. Thus, using AZT as nucleosidic model, anti-HIV effect in CEM/TK$^-$ cells seems to be dependent on the fast intracellular release of the 5'-mononucleotide from the prodrug. This fact could be tentatively explained with regard to the specific metabolism of AZT and has been largely discussed in a precedent review [14].

3.2. Mononucleoside SATE Phosphoramidate Diesters

Mononucleoside phenyl phosphoramidate diesters, containing methyl esterified amino acids, constitute another promising pronucleotide series [54, 55]. Starting from such kind of phosphorylated entity, delivery of the corresponding 5'-mononucleotide combines different decomposition mechanisms (*i.e.* enzymatic and chemical) as well as bioactivation and bioconversion processes (Fig. **12**). Initially investigated in cell extracts [28], metabolism studies with a radiolabelled compound [56] revealed that the decomposition pathway of these prodrugs requires, in a first step, an esterase-mediated hydrolysis of the carboxylic ester function of amino acid. This bioactivation is thought to be followed by an intramolecular nucleophilic attack of the carboxylate

Table 3. Compilation Data on the Decomposition Kinetics of Selected aryl SATE Derivatives of AZT (and their Major Metabolites) in Total CEM-SS Cell Extracts, Anti-HIV Activity in CEM/TK$^-$ Cell Line and Apparent Partition Coefficients (log P*app*) [49, 50]

Compound	Esterase activity	Phosphodiesterase activity	$t_{1/2}$ of AZTMP formation	EC_{50} (µM)	logP_{app}
tyrosinyl R=R'=H	1.2 h	25 h	25 h	29	0.25
tyrosinol	2.5 h	43.1 h	42 h	7	0.64
tyrosinamide	23 min[a]	33.5 h	38 h	4	1.27
tyrosinyl R=H, R'=Me	< 2 min[b]	25 h	25 h	1.8	2.25
tyrosinyl R=H, R'=*t*Bu	20 min	3.9 h	5.1 h	2.7	2.90
tyrosinyl R=Boc, R'=Me	< 1 min[b]	5 h	6	2.3	3.48
phenyl	1.2 h	nd[c]	nd[c]	3.5	
tyrosinyl R=Boc, R'=*t*Bu	< 10 min[b]	4.3 h	5.3 h	0.9	4.29

[a] the half-life corresponds to the conversion of the amide function in acid (putative amidase activity). [b] the half-life corresponds to the esterase hydrolysis of the Me or *t*Bu ester function of tyrosinyl residue which precedes the *t*BuSATE loss. [c] not determined because of the slow hydrolysis of the intermediate and low accumulation of metabolites.

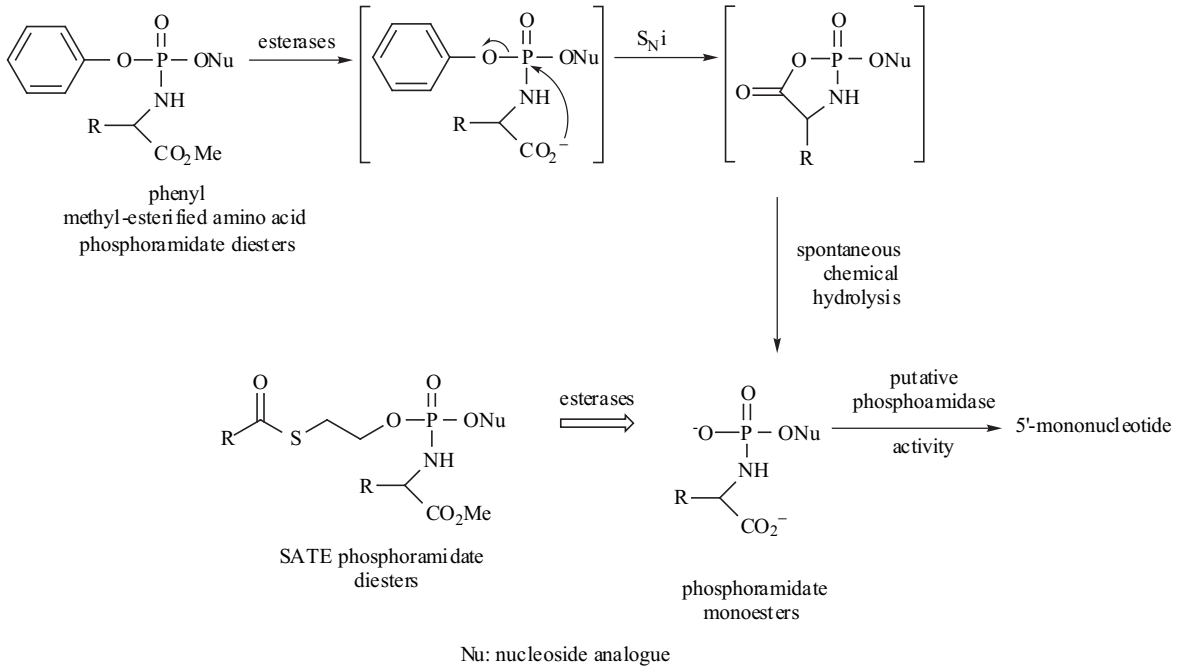


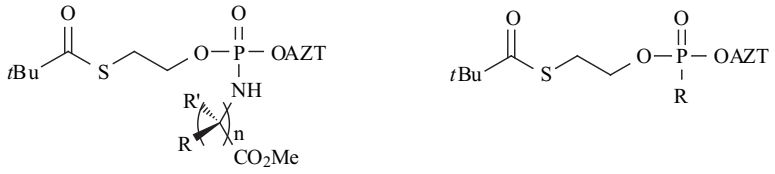
Fig. (12). Proposed decomposition pathways for mononucleoside phenyl phosphoramidate diesters containing methyl-esterified amino acids and the corresponding SATE phosphoramidate diesters.

function to the phosphorus with elimination of the aryl substituent, after transient formation of a five-membered cyclic intermediate. Finally, bioconversion of the resulting mononucleoside phosphoramidate monoester leads to the release of the corresponding 5'-mononucleotide [28, 57, 58]. Several studies have suggested that the cleavage of the P-N bond may be catalysed by phosphodiesterase or phosphoamidase activity [58-61]. Phosphoamidase activity has been described in mammalian cells and isolated from various sources [62-65]. More recently, a partially purified rat liver enzyme, distinct from creatine kinase, alkaline phosphatase and phosphodiesterase, was found to be able to hydrolyse mononucleoside phosphoramidate monoesters [58].

We decided to exploit this enzymatic activity in the design of a new series of mixed SATE phosphoesters, namely mononucleoside SATE phosphoramidate diesters (Fig. **12**). Such new kind of prodrugs would be able to deliver the 5'-mononucleotide by a direct and simple process involving successively an esterase activation followed by phosphoamidase activity.

3.2.1. Synthesis

A large number of *t*BuSATE phosphoramidate diesters of AZT bearing various methyl-esterified amino acids, aliphatic and aromatic amino residues (Fig. **13**) have been synthesized



*t*BuSATE phosphoramidate diesters

methyl-esterified amino acid derivatives

n = 1 R = H, R' = Me
R = H, R' = H
R = H, R' = CH_2Ph
R = H, R' = CH_2PhOH
R = H, R' = $CH_2OTBDMS$
R = H, R' = CH_2OAc
R = Me, R' = H
R = Me, R' = Me
n = 2 R = H, R' = H
n = 3 R = H, R' = H

non-amino acid derivatives

R = NH*i*Pr

= N

= NHPh

AZT = 3'-azido-3'-deoxythymidin-5'-yl

Fig. (13). Selected structures of mononucleoside SATE phosphoramidate diesters.

[66]. These compounds were obtained by a one-pot procedure using a convenient hydrogenphosphonate approach [67] involving the oxidative coupling [68] of an *H*-phosphonate diester precursor [69] with the corresponding amines.

3.2.2. Antiviral Activity

In contrast to AZT, most of the studied derivatives exhibited significant anti-HIV effects in HIV-1 infected CEM/TK$^-$ cells with EC_{50} values at micromolar range (Fig. **14**), proving their ability to act as 5'-mononucleotide prodrugs. In this comparative evaluation, the phosphoramidate diester bearing an isopropylamino substituent emerged as the most potent inhibitor with an EC_{50} value at 0.75 μM. Changing the amino acid part did not lead to notable variation in antiretroviral activity of the corresponding phosphoramidate derivatives in CEM/TK$^-$ cells. More interesting, the presence of an α-amino acid did not appear as a structural requirement for antiviral activity. Thus, compounds with β-alaninyl and amino*iso*butyryl residues were equipotent with the glycinyl derivative whereas the same amino acid modifications in the phenyl phosphoramidate series resulted in the loss of activity [57, 70]. Finally, the anti-HIV evaluation in CEM/TK$^-$ cells of *t*BuSATE phosphoramidate diesters bearing non amino acid substituents clearly demonstrates that the acyl amino acid group is not required to bypass the first phosphorylation step of AZT by the intracellular delivery of its corresponding 5'-mononucleotide.

3.2.3. Stability Studies

The metabolic pathway of mononucleoside phenyl phosphoramidate diesters containing methyl-esterified amino acids (Fig. **12**) has been studied by different approaches. We previously demonstrated that the affinity of the amino acid methyl ester for cellular esterases and the intramolecular cyclisation are prerequisites for the biological activity of this type of prodrugs [57]. In this respect, we previously reported a stability study related to *t*BuSATE β-alaninyl phosphoramidate diester of AZT in total CEM-SS cell extracts [71]. This pronucleotide is transformed into the corresponding phosphoramidate monoester whereas this pivotal metabolite cannot be obtain from the phenyl phosphoramidate analogue, the chain elongation limiting the intramolecular displacement of aryl group by the carboxylate function released after enzymatic activation [57]. Similar results were found through the replacement of L-alaninyl residue by glycinyl [72] or D-alaninyl [73] methyl ester substituents giving rise to a significant decrease of activity in the phenyl phosphoramidate series.

The use of mononucleoside SATE phosphoramidate diesters seems to circumvent these potential limitations by a less restrictive bioactivation process (*i.e.* esterase hydrolysis, Fig. **12**). In the absence of identification, data presented here suggest that the putative enzymatic activity involved in this bioconversion exhibits a poor substrate specificity with regard to the variation of the amino residues in the studied pronucleotides. Illustrated by the absence of anti-HIV

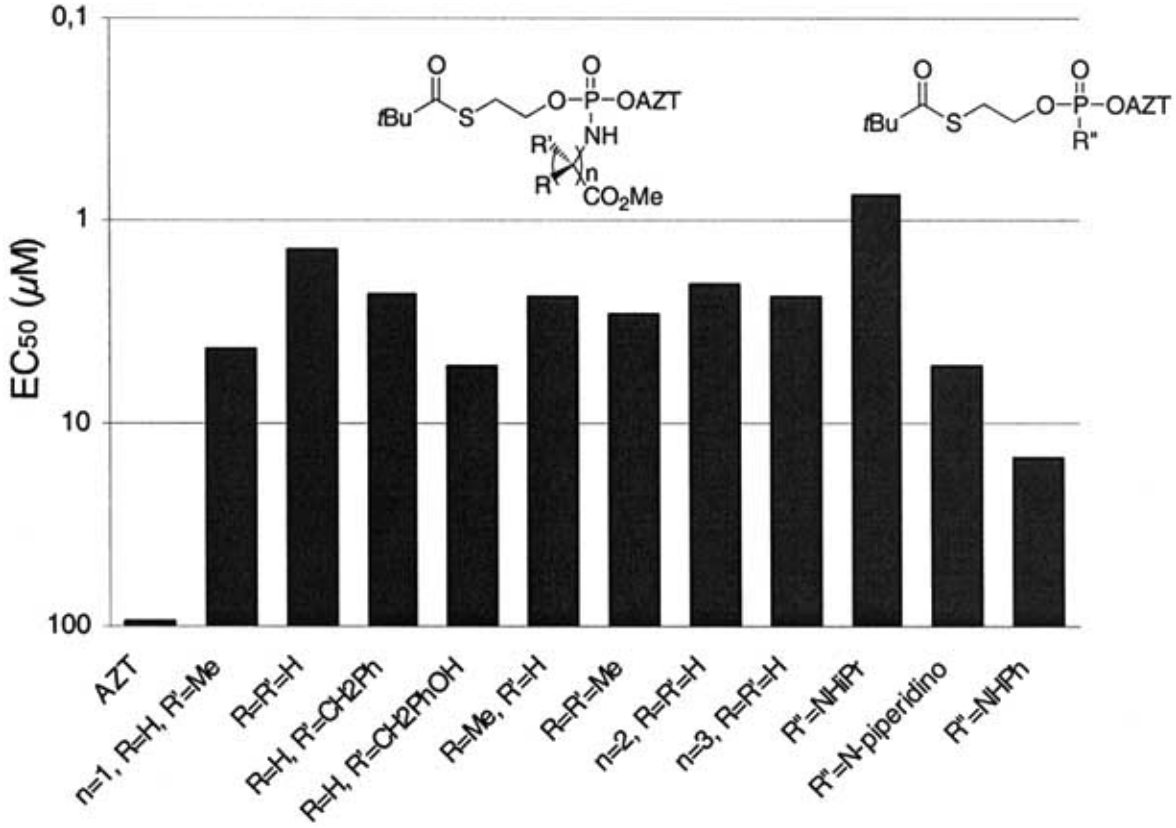


Fig. (14). Anti-HIV activity in CEM-SS/TK$^-$ of selected *t*BuSATE phosphoramidate diesters compared to AZT.

activity of the anilinyl derivative in CEM/TK$^-$ cells (Fig. **14**), the nitrogen atom basicity, more than structural parameters (*i.e.* steric hindrance), seems to be a critical factor in the hydrolysis of the P-N bond. This might be related to N-protonation process during the mechanism of enzymatic hydrolysis [64, 74].

4. CONCLUSION

In the past decades, intensive investigations have provided insights into the nature of chemical and biological requirements associated to the *in vitro* selective delivery of 5'-mononucleotide inside the cells from phosphorylated precursors. Our work in this topic started with mononucleoside symmetrical phosphotriesters bearing SATE groups as biolabile phosphate protection. *In vitro*, the use of such kind of pronucleotides gave rise to increase the activity and to extend the spectrum of antiviral nucleoside analogs hampered at the first metabolisation step, without additional toxicity related to the degradation products of the SATE moiety.

According to our initial pharmacological aim, *i.e.* (selective) delivery of a biological 5'-mononucleotide into infected cells or tissues, *in vivo* studies of several bis(SATE) phosphotriester models led us to investigate mononucleoside mixed SATE phosphoesters involving two different enzymatic systems in their decomposition process. In this respect, mononucleoside aryl SATE phosphotriesters and SATE phosphoramidates diesters proved their ability to act as mononucleotide prodrugs in cell culture experiments. Their proposed decomposition pathways require firstly a common activation step mediated by esterases followed by a phosphodiesterase or a phosphoamidase hydrolysis, respectively.

Associated to an increased enzymatic stability of the SATE groups through the introduction of polar functions, the large number of chemical modifications, which could be envisaged either on the aryl or amidate moieties, opens the way to the discovery of mononucleotide prodrugs with an adequate balance among aqueous solubility, lipophilicity and enzymatic stability in order to envisage further *in vivo* pharmacological studies. Work on this topic is currently in progress in our group.

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