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Biolabile constructs for pronucleotide design ☆

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Abstract

After summarising the in vitro and in vivo results obtained with nucleotide prodrugs (pronucleotides) bearing two *S*-acyl-2-thioethyl (SATE) groups as esterase-labile phosphate protections, we will describe recent work on mononucleoside mixed phosphoester derivatives. These new series of biolabile constructs were designed to lead to the selective intracellular delivery of the corresponding 5′-mononucleotide through different enzyme-mediated activation steps.

Keywords: Mononucleotide; Prodrug; Phosphotriester; Phosphoramidate; Phosphorothiolate; Antiviral

1. Introduction

In an attempt to improve the therapeutic potential of nucleoside analogues, various mononucleotide prodrugs (pronucleotides), which would be expected to revert back to the corresponding 5′-mononucleotides intracellularly, have been reported during the last decade [1–4]. The difficulty of such approach lies in the fact that transformations involved in the delivery of the 5′-mononucleotide from its corresponding prodrug should be performed selectively inside the target cells. Two main approaches have been developed requiring either structural modifications or introduction of transient groups (Fig. 1). Regarding the first strategy, a specific enzymatic system is needed to perform bioconversion of the structurally modified phosphorylated precursor. This strategy could be illustrated by the design of nucleoside fluorophosphate [5,6] or phosphoramidate [7,8] derivatives. In the second strategy, the hydrolysis of the phosphate-masking group bond requires a difference between the hydrolytic rates in the extra- and intracellular media, and involves either a specific chemical or an enzyme-mediated process. The necessity to mask the two charged phosphate oxygen's of the 5′-mononucleotide in order to obtain neutral and lipophilic prodrugs has led firstly to the development of various mononucleoside phosphotriesters [4,9]. As phosphotriesterase activity has not yet been isolated in mammalian cells, the hydrolysis of simple dialkyl phosphotriesters depends exclusively on chemical mechanisms. In neutral or acidic conditions, the hydrolysis of such phosphotriester derivatives proceeds through C–O bond cleavage following a typical S_N2 process, the dealkylation rate decreasing with the increase of size and bulk of the alkyl group [10]. 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 [11,12]. However, studies of the pH rate-product profile for the aqueous hydrolysis of *O*,*S*-ethylene phosphorothioates demonstrated that *O*-phosphorylated

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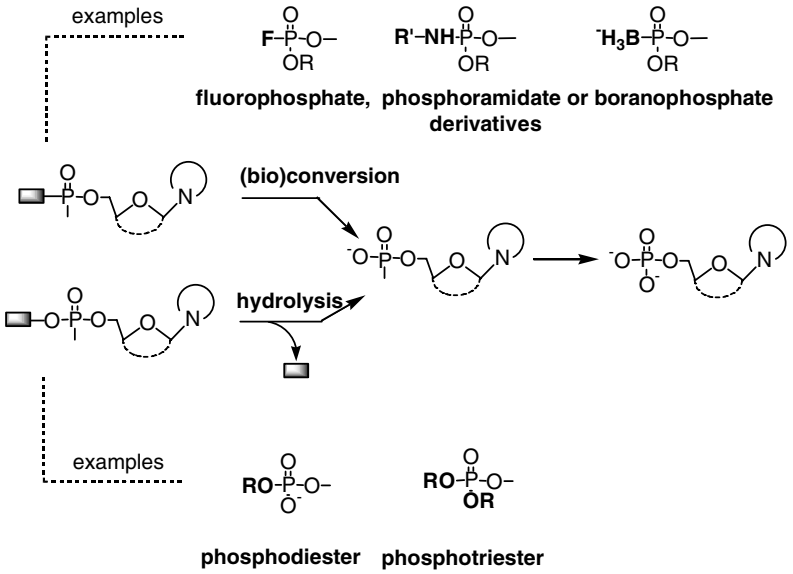

Fig. 1. Main prodrug approaches using structural modifications or masking groups.

derivatives of 2-mercaptoethanol (resulting to exclusive P–S bond cleavage) would be able to eliminate ethylene sulphide in neutral conditions (Fig. 2) [13–15]. Pseudorotation is required to bring the sulphur atom into an apical leaving position. Thus, the greater leaving ability of the thioalkyl ligand, the weakness of the P–S bond (due to less efficient dπ–pπ bonding) compared to the oxy analogues were cited as factors involved in the fast and selective cleavage of *O*,*S*-phosphorothioate esters. It is noteworthy that the selectivity of this process has been used as a chemical procedure for sequencing oligonucleotide phosphorothioates [16].

For our concern, we exploited the selectivity of this spontaneous process to design biolabile phosphate protections incorporating a thioethyl chain and where the sulphur atom of the thiol function is involved in an enzyme-labile bond (double prodrug concept, Fig. 3) [17–19]. Briefly, the first hydrolytic step is the cleavage of the sulphur bond via an enzymatic activity (preferentially intracellular) resulting in the formation of an unstable 2-mercaptoethyl ester, giving rise in a second step through a spontaneous process to the release of the corresponding phosphate function. Our work on this topic has started with symmetrical (two identical protecting groups) mononucleoside phosphotriesters where the thiol function of the thioethyl chain is engaged in a thioester link resulting in a biolabile phosphate protection called SATE (*S*-acyl-2-thioethyl) group [18].

2. Summary on the bis(SATE) approach

It is not our intention to give an overview on the results obtained with bis(SATE) pronucleotides (Fig. 4) in order to overcome mononucleotide delivery drawbacks, these data can be gleaned in the literature [9,20,21]. The corresponding phosphotriester derivatives have been synthesised either using P^{III} (phosphoramidite,...) or P^{V} (phosphodiester, phosphomonoester,...) intermediates. The decomposition pathway of bis(SATE) pronucleotides were investigated both in cell extracts as well as in intact cells using metabolism studies with radiolabeled compounds [18,22]. As expected, it involves firstly an esterase-mediated hydrolysis giving rise to an unstable phosphotriester which decomposes spontaneously (Fig. 4). The resulting SATE phosphodiester is then able to lead to the corresponding 5′-mononucleotide after a similar process. In addition, it was demonstrated that SATE groups exhibit a relative stability in extracellular media (culture medium, human serum) associated with a

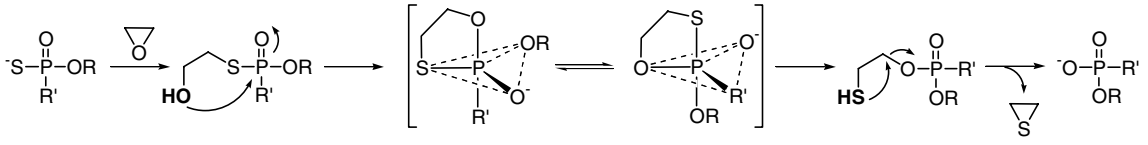

Fig. 2. Hydrolysis of *O*,*S*-ethylene phosphorothioate derivatives.

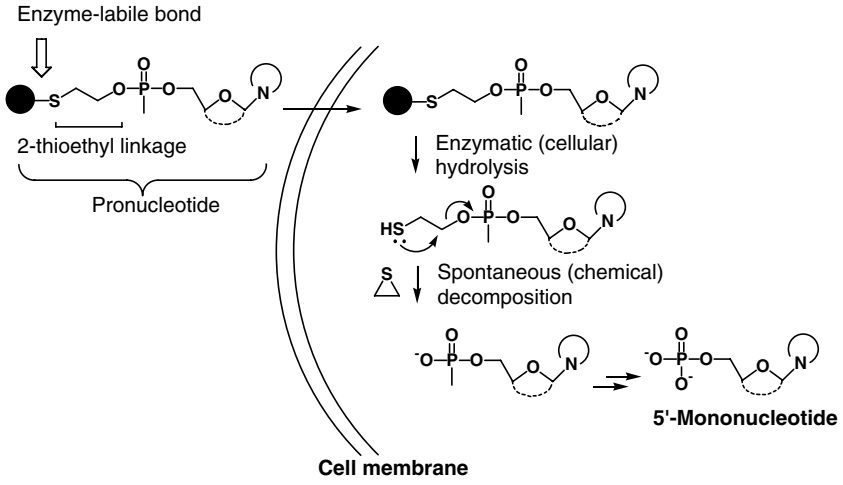

Fig. 3. Principle of the double prodrug concept using a thioethyl linkage.

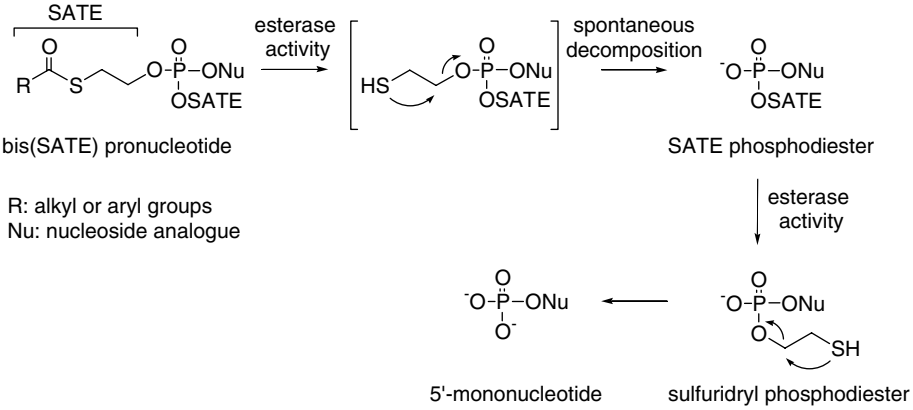

Fig. 4. Decomposition pathway of bis(SATE) pronucleotides in cell extracts and in intact cells.

rapid decomposition in all examined cell lines, this selectivity being a key factor in order to release the parent mononucleotides inside cells. Furthermore, we shown that kinetics associated to the monophosphorylated form delivery can be controlled by varying the nature of the acyl moiety of the SATE group (R residue, Fig. 4) [18].

Thus, the SATE approach has been applied to anti-human immunodeficiency virus (anti-HIV) nucleoside analogues 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 [9,21]. In all cases, in vitro evaluation of the corresponding bis(SATE) phosphotriester derivatives shows increased antiviral activities compared to the parent nucleosides. These results prompt us to investigate the efficacy of bis(SATE) pronucleotides in vivo using animal models. In this respect, the *t*-BuSATE group has been selected with regard to its relative resistance to esterase hydrolysis. For example, the bis(*t*-BuSATE) pronucleotide of ACV was evaluated in HBV-infected ducklings [23] and proved to be superior (in terms of molar effective drug concentration) to ACV after intraperitoneal and oral administrations. 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. These preliminary data led us to study the pharmacokinetics and metabolism of corresponding phosphotriesters of AZT [24], radiolabeled derivatives of L-FddC [25] and L-ddA [22], respectively in mice and rhesus monkeys. Striking differences were found between intravenous (i.v.) and oral (p.o.) administrations. Following i.v. administration, the AZT prodrug was rapidly metabolised to yield the corresponding 5′-mononucleotide (AZTMP) and AZT in blood. However, it is likely that this lipophilic prodrug distributed rapidly into tissues with lower metabolising ability, such as brain were high concentrations of the pronucleotide and AZTMP were present indicating its ability to penetrate the blood-brain barrier and to be converted into the corresponding 5′-mononucleotide. 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 studies in rhesus monkeys using the radiolabeled bis(*t*-BuSATE) phosphotriester derivative.

Despite this unquestionable therapeutic interest, a presystemic metabolism, preventing the delivery of the prodrugs in infected cells or tissues, may constitute a limitation to the in vivo development of anti-HIV pronucleotides designed to promote a site-specific delivery by an esterase-mediated activation process. Further researches in the field of pronucleotide approaches has taken up this challenge and several strategies have been envisaged. The first one consists in increasing the enzymatic stability of the esterase-labile phosphate protections by the introduction of various modifications on the *t*-BuSATE group [26–28]. Another possibility is the use of specific enzymatic activation process in order to target cells or tissues according to preferential transport and distribution or to get a selective bioactivation. Attempts illustrating these last strategies will be found in the design of mixed SATE phosphoesters (cf. Section 3).

Thus, in order to answer presystemic metabolism the most interesting results have been obtained with the introduction of polar or ionisable (at physiological pH) functions in the immediate or near vicinity of the ester functionality, the simple one being a chemical modification such as the introduction of a hydroxyl function (HO*t*-BuSATE) in the acyl chain of the *t*-BuSATE [28]. These investigations were based on the fact that carboxylesterases, the most probable candidates for the enzymatic degradation of the SATE phosphotriesters, appeared to have higher reactivity with lipophilic esters than with polar or charged substrates [29]. As consequence, the resulting bis(HO*t*-BuSATE) phosphotriester derivative of AZT shown a marked decrease of its hydrolysis rate in biological media (half-life of 20 h in human serum) compared to the bis(*t*-BuSATE) analogue (half-life of 7.5 h in human serum). Furthermore, it was able to cross in an intact form the Caco-2 cell monolayer, a generally accepted in vitro model for intestinal drug absorption.

If a 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. 4). 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 [22]. 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 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 hydrolysed (or bioconverted) by other enzymatic systems than esterases (Fig. 5).

3. Mixed sate phosphoesters

Three different kinds of mononucleoside mixed SATE phosphoesters were investigated, bearing either an aryl, an amino group or a glucosyl residue, namely SATE aryl phosphotriesters, SATE phosphoramidate diesters and SATE glucosyl phosphorothiolates, respectively (Fig. 5).

3.1. Chemistry

3.1.1. Mononucleoside SATE aryl phosphotriesters

The design of this series is based on literature data 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 [30,31]. Thus, our purpose was to generate intracellularly an aryl phosphodiester by the use of the corresponding SATE phosphorylated precursor. Mononucleoside *t*-BuSATE phosphotriesters of AZT incorporating either phenyl or L-tyrosinyl groups (Fig. 6) have been studied as first models [32]. 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 [33,34].

A synthetic route to the target compounds is presented in Fig. 7. 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 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 hydrolysed 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.

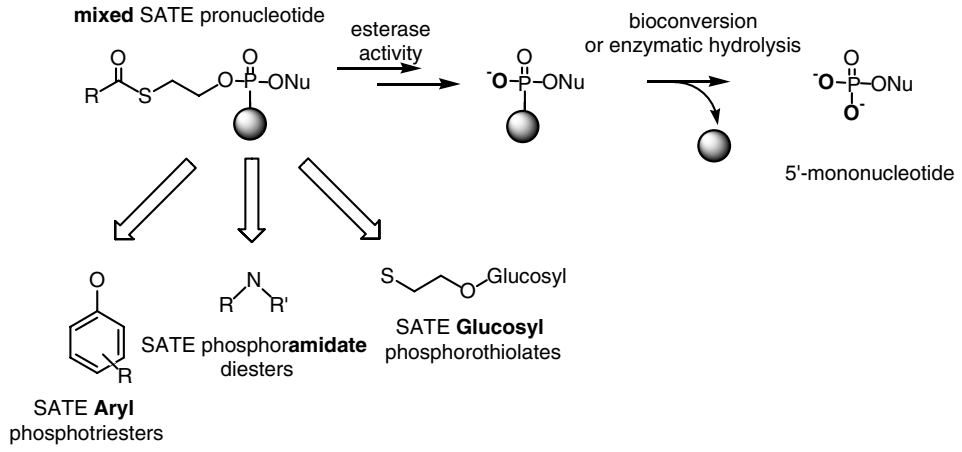

Fig. 5. Mixed SATE pronucleotide approach.

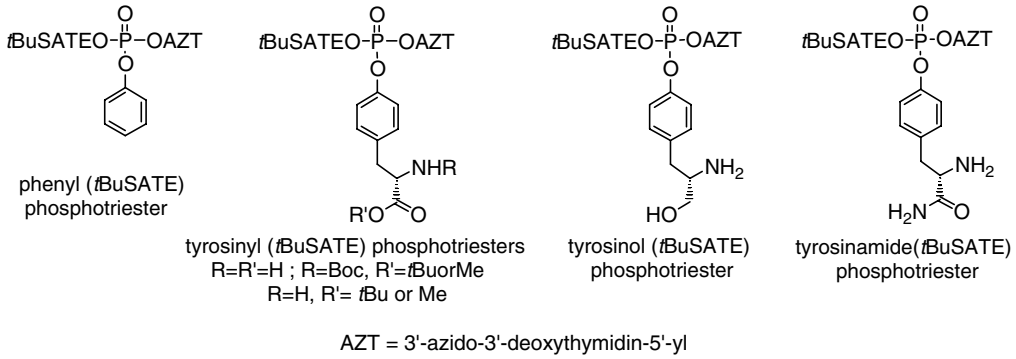

Fig. 6. Selected structures of SATE aryl phosphotriesters.

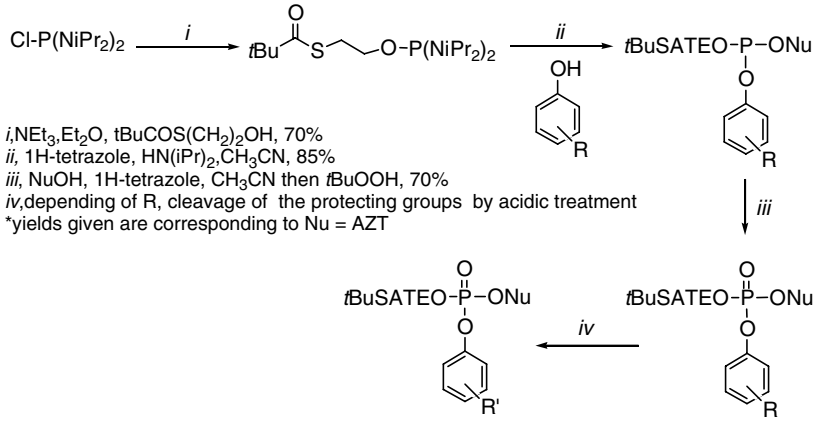

Fig. 7. General synthetic pathway to mononucleoside SATE Aryl phosphotriesters.

3.1.2. Mononucleoside SATE phosphoramidate diesters

A well-known promising pronucleotide series is represented by mononucleoside aryl phosphoramidate diesters, containing methyl esterified amino acids [35,36]. The delivery of the desired 5′-mononucleotide from such kind of entities, combines different decomposition mechanisms (i.e., enzymatic and chemical) as well as bioactivation and bioconversion processes. The final step of the conversion process results in the bioconversion of a mononucleoside phosphoramidate monoester into the corresponding 5′-mononucleotide. Thus, few studies have suggested that the cleavage of the P–N bond may be catalysed by phosphodiesterase or phosphoamidase activities [37,38]. Phosphoamidase activity has been described in mammalian cells and isolated from various sources [39]. 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 [38]. We decided to exploit this enzymatic activity in the design of a new series of mixed SATE phosphoesters, namely mononucleoside SATE phosphoramidate diesters (Fig. 5). At the opposite of mononucleoside aryl phosphoramidate diesters, such a 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 a phosphoamidase activity.

A large number of *t*-BuSATE phosphoramidate diesters of AZT (Fig. 8) bearing various methyl-esterified amino acids, as well as aliphatic and aromatic amino residues have been synthesised and studied [40,41].

Among the methods described for the preparation of nucleoside phosphoramidate diesters [42–44], we used a general procedure (Fig. 9) based on a convenient hydrogenphosphonate approach [45] involving oxidation of an *H*-phosphonate diester precursor with carbon tetrachloride followed by in situ coupling with an excess of the corresponding amines. Firstly, the *t*-BuSATE precursor could either be phosphitylated with bis(*N*,*N*-diisopropylamino) chlorophosphine or salicylchlorophosphite reagent yielding respectively the corresponding phosphorobisamidite or the cyclic phosphite intermediates. Both derivatives could be hydrolysed into *H*-phosphonate monoester according to traditional procedures [45]. The latter was then condensed with the nucleoside analogue (AZT) in presence of pivaloyl chloride as condensing agent leading to the mononucleoside *H*-phosphonate diester as a diastereoisomeric mixture (1/1). Finally, oxidative coupling [44] of *H*-phosphonate diester with amines has produced the desired phosphoramidate diesters in yields of 20–80% as diastereoisomeric mixtures after purification by silica gel column chromatography. In the case of amino acid methyl esters

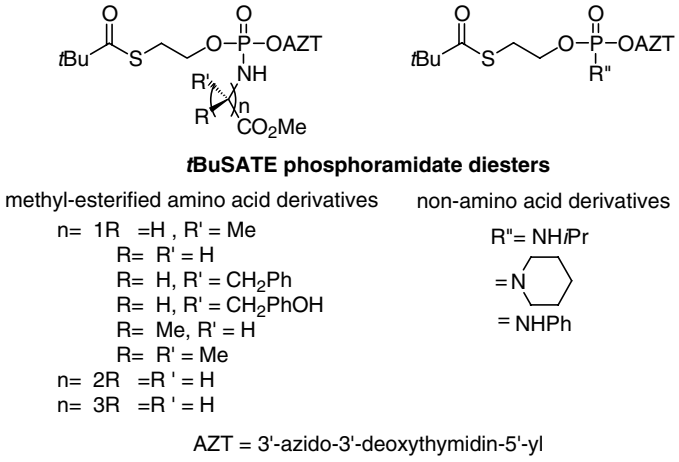

Fig. 8. Selected structures of SATE phosphoramidate diesters.

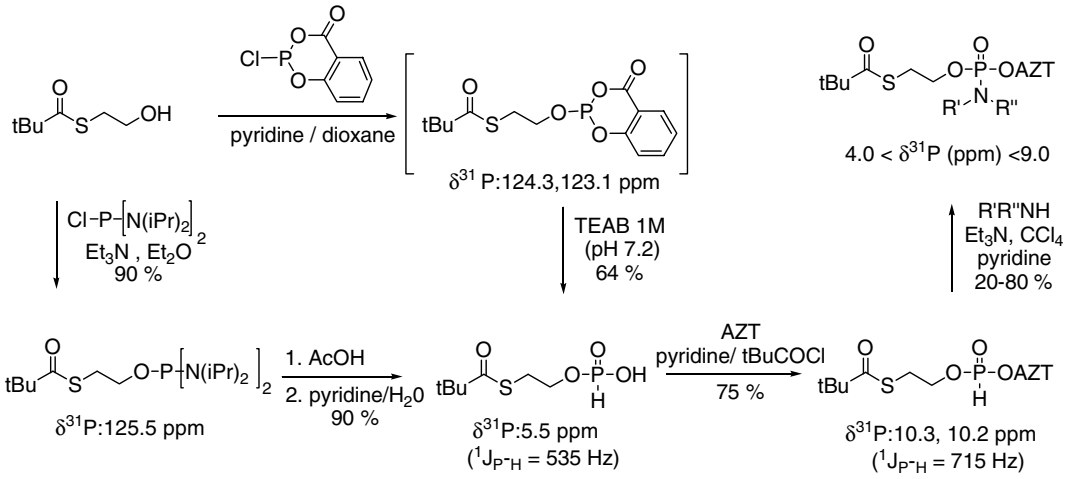

Fig. 9. General synthetic pathway to mononucleoside SATE phosphoramidate diesters.

commercially available as hydrochloride salts, addition of triethylamine in the medium was required to initiate nucleophilic attack of amino function on the putative in situ formed phosphorus (V) intermediate namely phosphorochloridate.

3.1.3. Mononucleoside SATE glucosyl phosphorothiolates

The third series of mixed SATE phosphoesters, we would like to report as potential pronucleotides concerns SATE glucosyl phosphorothiolate derivatives. The design of these novel entities is based on previous works of our group on this topic (Fig. 10) [19,46]. Thus, we previously demonstrated that mononucleoside phosphotriester derivatives incorporating two β-glucopyranosidyl moieties associated to the phosphorus atom through the 2-thioethyl linkage (i.e., bis(SGTE) phosphotriesters, Fig. 10) cannot be considered as mononucleotide prodrugs. These data were related to the lack of affinity of such derivatives towards target-enzymes (i.e., glucosidases) probably due to the presence of the sulphur atom in the anomeric position of the sugar. In this respect, we decided to evaluate the potentiality of an isomer model of the SATE group, namely isoSATE, where the sulphur and the oxygen atoms of the linkage have been exchanged [47]. The resulting mononucleoside phosphorothiolate derivatives bearing

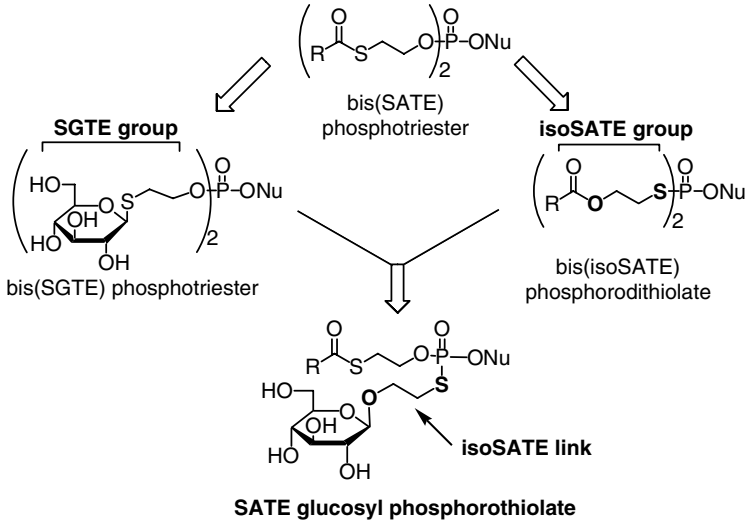

Fig. 10. Structures of symmetrical mononucleotides (bis phosphoesters) allowing the design of novel mixed biolabile constructs.

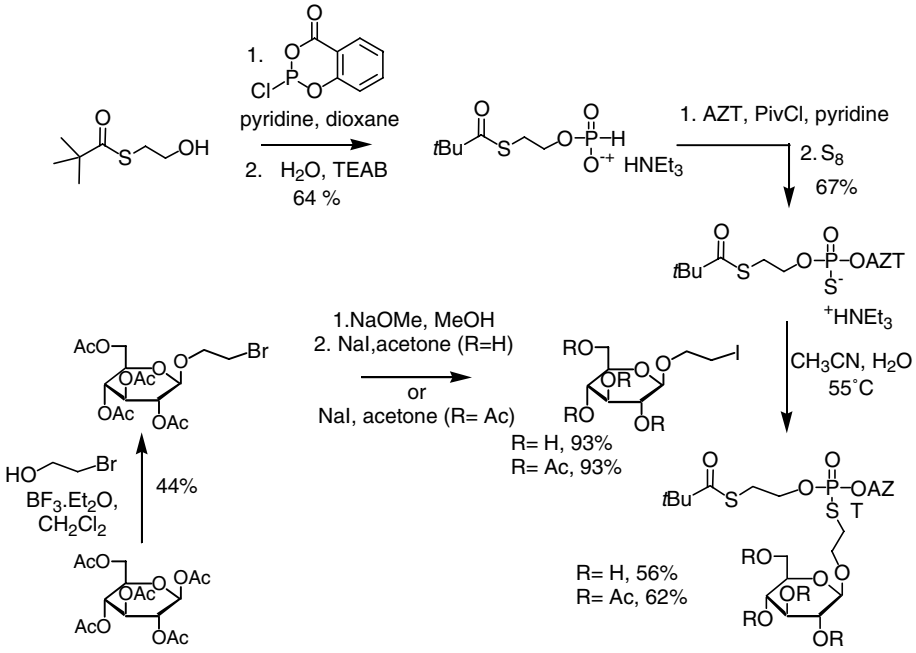

Fig. 11. Synthetic pathway to SATE glucosyl phosphorothiolate derivatives.

two *O*-acyl-2-oxyethyl groups (bis(isoSATE) phosphorodithiolates, Fig. 10) allowed the efficient intracellular delivery of their parent mononucleotides.

On the basis of the results obtained in the SGTE and isoSATE series, we decided to combine both structural modifications by studying phosphorothiolate derivatives incorporating two different biolabile phosphate protections: a SATE group and a glucosyl residue linked to the phosphorus atom through an isoSATE linkage (Fig. 10). The synthesis of such a kind of phosphorothiolate derivatives combined two common strategies involving P^{III} and P^{V} intermediates (Fig. 11), and was carried out using AZT as first nucleosidic model. On one hand, the *H*-phosphonate monoester of the SATE precursor was obtained from the thioester using salicylchlorophosphite as phosphitylating agent. Condensation of this first phosphorylated intermediate with AZT, in presence of pivaloyl chloride, led to the corresponding *H*-phosphonate diester which was in situ oxidised, using elemental sulphur, into the phosphorothioate (diastereoisomeric mixture, 1/1). On the other hand, boron trifluoride etherate-induced glycosylation of 2-bromoethanol using commercially available 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose as donor and gave rise to the corresponding β-glucopyranoside [48,49]. Only, the β-anomer was isolated showing that, in these conditions, glycosylation was more rapid than anomerisation. Then, from the bromoglucoside halogen exchange by sodium iodide afforded the 2-iodoethyl-β-D-glucosides (peracylated residue and deprotected analogue where the acetyl groups have been removed using methanolic sodium methoxyde). Subsequent coupling of the phosphorothioate diester with 2-iodoethyl-β-D-glucopyranosides leads to the corresponding phosphorothiolate derivatives, as diastereoisomeric mixtures (1/1).

3.2. Biological data

All phosphoester derivatives of the three series of potential 5′-mononucleotide prodrugs were evaluated for their inhibitory effects on the replication of HIV-1 in CEM-SS, MT-4 and in a thymidine-kinase deficient cell line (CEM/TK^-), in comparison to the parent nucleoside AZT. In CEM-SS and MT-4 cell lines, the tested compounds significantly inhibited the multiplication of HIV-1 with 50% effective concentration (EC_{50}) values similar to those observed for AZT. In CEM/TK^- cells (Figs. 12 and 13), as expected AZT proved to be inactive against HIV-1 replication at a concentration up to 100 μM. In contrast, all the studied derivatives exhibited significant antiviral activity (about 1–10 μM concentration), showing their ability to act as pronucleotides.

For two series out of the three, i.e., SATE aryl phosphotriesters and SATE glucosyl phosphorothiolates (Fig. 12, chart graph) differences were observed between the EC_{50} of one derivative of each family. These two compounds are the *t*-BuSATE (tyrosinyl) phosphotriester (R = R′ = H) and the *t*-BuSATE (glucosyl) phosphorothiolate (R = H) which exhibited EC_{50} values of 29 and 17 μM, respectively. The decrease of activity for these two derivatives may be related to the hydrophilicity induced either by the carboxylate function of tyrosinyl residue, or by the multiple hydroxyl functions of the glucosyl moiety. These polar functions might influence cellular penetration and/or enzymatic decomposition of the prodrugs.

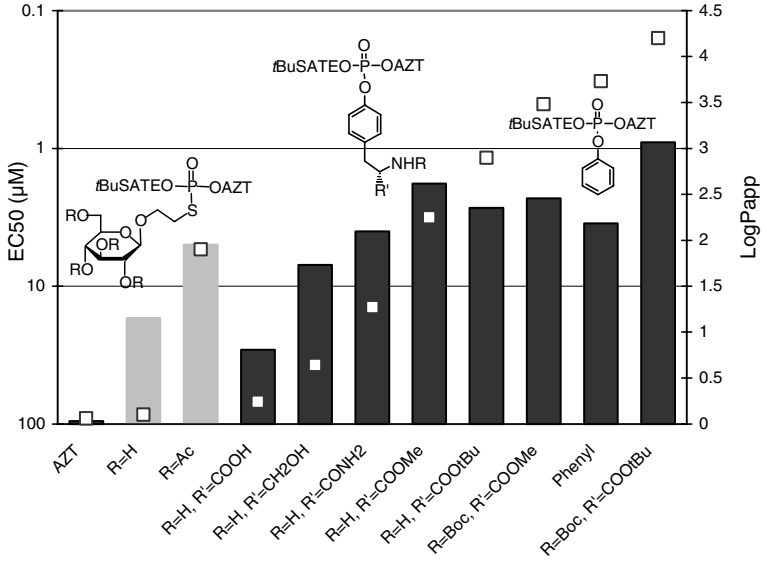

Fig. 12. Anti-HIV activity in CEM/TK^- cell line (chart graph) and apparent partition coefficients (Log P*app*, open square) of selected *t*-BuSATE aryl phosphotriester and *t*-BuSATE glucosyl phosphorothiolate derivatives of AZT compared to the parent nucleoside.

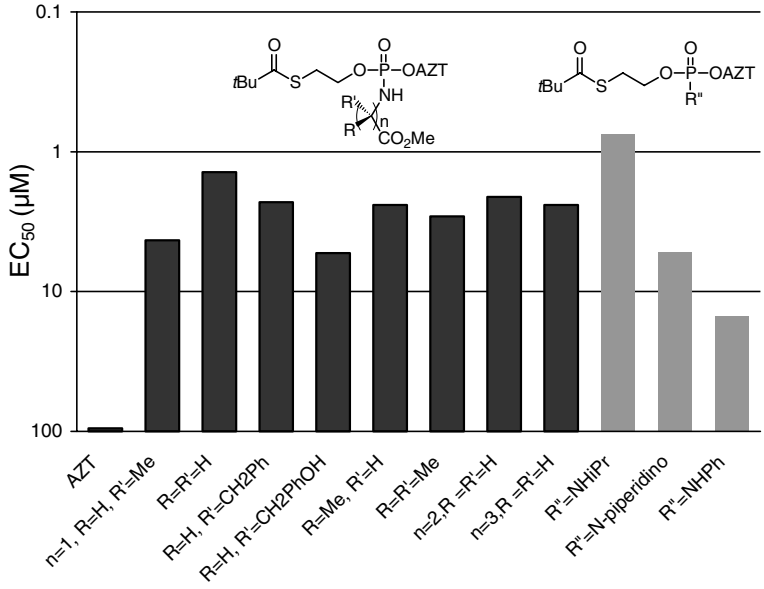

Fig. 13. Anti-HIV activity in CEM-SS/TK $^{-}$ of selected *t*-BuSATE phosphoramidate diesters compared to AZT.

Indeed, comparison of EC_{50} in CEM/TK^{-} cells and apparent partition coefficients (Fig. 12) indicates that the presence of highly hydrophilic groups might be responsible for the loss of antiviral activity. In the *t*-BuSATE aryl phosphotriester series, this is corroborated by the EC_{50} values obtained for other hydrophilic phosphotriesters such as tyrosinol (R = H, R′ = CH_2OH) and tyrosinamide (R = H, R′ = $CONH_2$) derivatives, incorporating polar but not anionic groups in place of the acid function.

Concerning the mixed SATE phosphoramidate series (Fig. 13), comparative evaluation in CEM/TK^{-} cell line shown that the derivative bearing an isopropylamino substituent emerged as the most potent inhibitor with an EC_{50} value at 0.75 μM. Modification of the amino-acid counterpart did not lead to notable variation in antiretroviral activity of the corresponding phosphoramidate diesters. More interesting, the presence of an α-amino acid did not appear as a structural requirement for antiviral activity. This last observation 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.

3.3. Stability studies

Behaviour of novel series of AZT pronucleotideswere investigated in various biological media using an in-house ‘‘online cleaning’’ UV/LC/MS coupling technique [18]. As example, these studies were carried out in culture medium (CM), which is the extracellular medium used for antiviral evaluation in cell culture systems, as well as in total CEM-SS extracts (TCE), a mimic for the intracellular medium. As required, all compounds were metabolised very slowly in culture medium (half-life about a week) compared to cell extracts.

3.3.1. Mononucleoside SATE aryl phosphotriesters

Decomposition pathways and kinetic data were determined in both media (CM and TCE) for the entire series and corresponding results can be gleaned in the literature [33,34]. As example, tyrosinyl and tyrosinol phosphotriester derivatives were bioconverted following a similar decomposition process which involves in a first step an esterase-mediated activation. 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. 14). 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.

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 [50]. 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.

3.3.2. Mononucleoside SATE phosphoramidate diesters

Such a kind of derivatives were designed in order to circumvent potential limitations of phenyl phosphoramidite diesters bearing esterified aminoacid, through the use of a less restrictive bioactivation process. In this respect, we previously reported a stability study related to *t*-BuSATE β-alaninyl phosphoramidate diester of

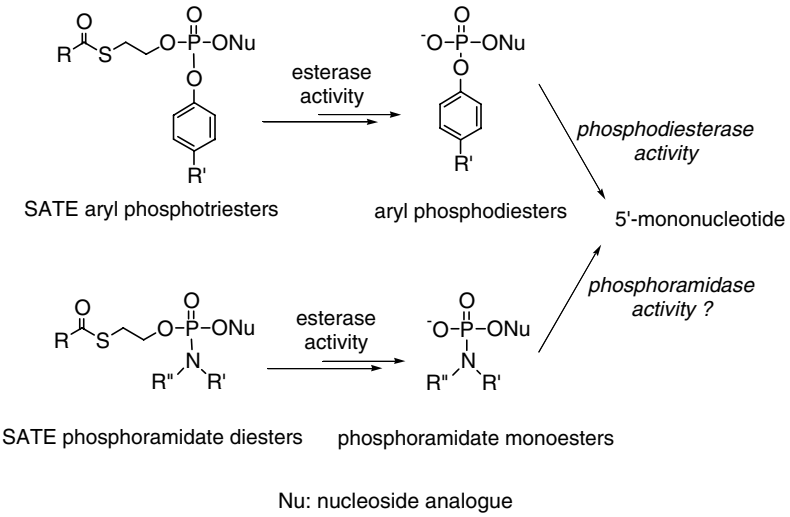

Fig. 14. General decomposition pathways proposed for SATE aryl phosphotriesters and SATE phosphoramidate diesters.

AZT in total CEM-SS cell extracts [51]. This pronucleotide is converted into the corresponding phosphoramidate monoester through esterase activation (Fig. 14), whereas this pivotal metabolite cannot be obtain from any phenyl phosphoramidate analogues, the chain elongation limiting the intramolecular displacement of aryl group by the carboxylate function released after enzymatic activation. In the absence of identification, biological data presented here (cf. Section 3.2) 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 [41]. Illustrated by the absence of anti-HIV activity of the anilinyl derivative in CEM/TK$^-$ cells (Fig. 13), 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.

3.3.3. Mononucleoside SATE glucosyl phosphorothiolates

The expected decomposition pathway of the phosphorothiolate derivatives (Fig. 15) may involved in the first step an esterase activation leading to the loss of the SATE group and formation of glucosyl phosphorothiolatediesters. These intermediates should then be converted into the corresponding 5′-mononucleotide by a glucosidase-mediated cleavage of the anomeric bond followed by the rearrangement process and decomposition mechanism proposed in the bis(isoSATE) pronucleotide series [47]. Preliminary stability studies were performed in order to correlate the antiviral evaluation data with this expected decomposition mechanism and to support our hypothesis on the behaviour of such a kind of prodrugs. Thus, in TCE incubation of these derivatives gave rise to the formation of the corresponding glucosyl phosphorothiolate diester but this metabolite appeared to be stable due to the complete absence of β-glucosidase activity in this medium [46].

Consequently, we decided to synthesise the glucosyl phosphorothiolate diester derivative of AZT (Fig. 15) and to evaluate its substrate properties towards purified sweet almond β-glucosidase (EC 3.2.1.21), an enzyme which catalyses the hydrolysis of a wide range of glucosides, in comparison to the *p*-nitrophenyl β-glucopyranoside (a well known substrate) [52]. Thus, the

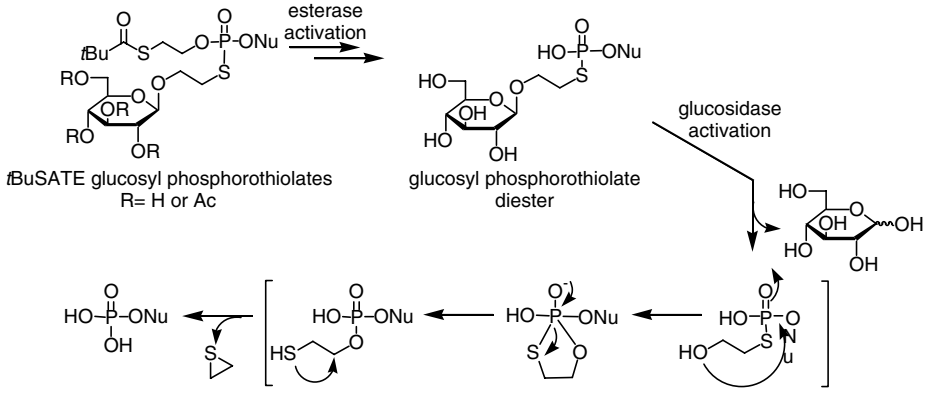

Fig. 15. Hypothetical decomposition pathway for SATE glucosyl phosphorothiolates.

studied metabolite was effectively hydrolysed by the purified enzyme leading to the selective formation of the 5′-mononucleotide of AZT. Compared to the type-substrate, the enzyme exhibited a similar affinity for the phosphorylated derivative, indicating that the aglycon part does not affect the binding.

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 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 analogues 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 different enzymatic systems in their decomposition process. In this respect, mononucleoside SATE aryl phosphotriesters, SATE phosphoramidate diesters and SATE glucosyl phosphorothiolates 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, a phosphoamidase or glucosidase 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 on the aryl or amidate moieties opens the way to the discovery of mononucleotide prodrugs with an adequate balance between 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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