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Dedicated to Professor David Shugar on the occasion of his 80th birthday

New insights regarding the potential of the pronucleotide approach in antiviral chemotherapy*

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The rationale for a pronucleotide approach based on the use of phosphotriesters which incorporate enzyme-mediated bio-labile protection is discussed in detail. Among the studied bio-labile phosphate protecting groups, the *S*-acyl-2-thioethyl (SATE) groups appeared the most promising as exemplified in cell culture systems in the case of the pronucleotides of 3′-azido-3′-deoxythymidine, 2′,3′-didehydro--3′-deoxythymidine, 2′,3′-dideoxyadenosine and acyclovir. *In vivo* implementations of such bis(SATE) pronucleotides have been planned for future animal studies.

Currently, nucleoside analogues play an important role in antiviral chemotherapy [1], and among the only sixteen compounds which have been approved in the U.S.A. to be licensed as antiviral drugs eleven have a nucleosidic structure [2, 3]. Due to their structural analogy with natural purine and pyrimidine nucleosides, one can assume that the mode of action of these analogues is based on a disruption of the biosynthesis of viral nucleic acids. This implies that viruses possess or encode some enzymes (like DNA and RNA polymerases, or reverse transcriptase) which are different from those of the host cells. Thus, if one considers the mode of action of antiviral nucleoside analogues, it is noteworthy that these compounds mainly exert their effects after intracellular conversion to the corresponding triphosphorylated form (Scheme 1). The first metabolic step involves the transformation to a nucleoside monophosphate, which is then phosphorylated further to the di- and triphosphate by cellular kinases. And the triphosphate, by inhibiting the viral polymerase or following its in-

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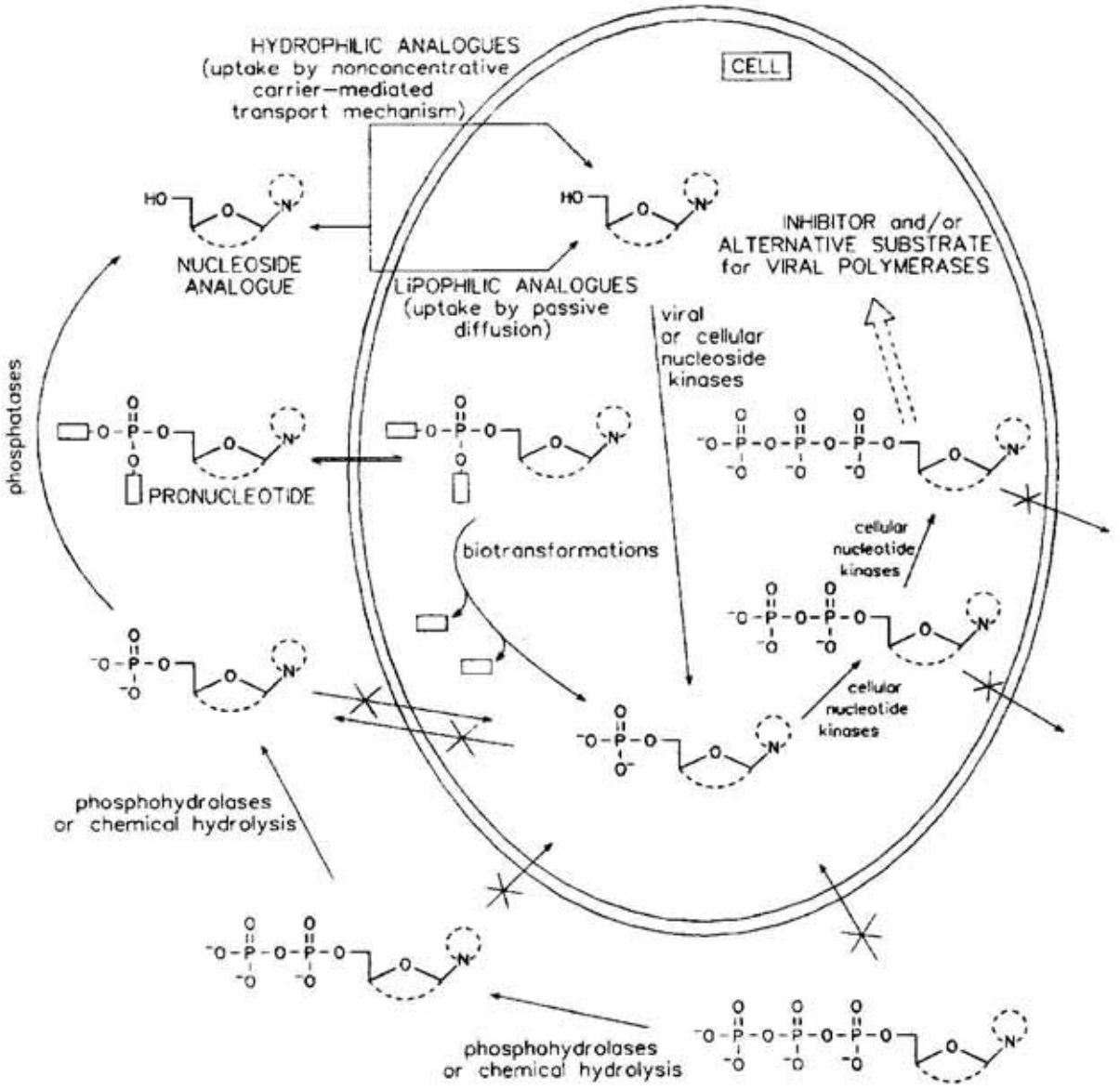
Abbreviations: AZT, 3′-azido-3′-deoxythymidine; CEM, and CEM-SS, human T-lymphoblastoid cell lines; CFU-GM, colony forming units of granulocytes and monocytes-macrophages; CMV, cytomegalovirus; D4T, 2′,3′-didehydro-3′-deoxythy- midine; DD, 2′,3′-dideoxy-derivative of a given nucleoside or nucleotide; DTE, dithioethyl group; DB, 1,8-diazabicyclo(5.4.0)undecene-7-ene; EC, effective concentration; HBV, hepatitis B virus; HPMPC, (*S*)-1-(3-hydroxy-2-(phos- phonylmethoxy)-propylcytosine; IC, inhibitory concentration; MSNT, mesitylene sulfonyl nitrotriazole; NGF, nerve growth factor; PBM, peripheral blood mononuclear cells; PMEA, 9-(2-phosphonometho- xyethyl)adenine; POM, (pivaloyloxy)methyl group; SATE, *S*-acyl-2-thioethyl group; THF, tetrahy- drofurane; TK, thymidine kinase.

corporation into the nucleic acid of the virus is the entity actually responsible for the biological effect of the parent drug.

From the mode of action of nucleoside analogues, two problems arise [4]. The first is related to the usually observed more or less detrimental concomitant toxicity, which is inversely proportional to the selectivity of the interaction of the nucleoside analogue triphosphate with the viral polymerase *versus* the cellular polymerases. The second problem concerns the efficiency of the three phosphorylation steps. In this regard, ideally the ratio of the intracellular concentration of the nucleoside analogue triphosphate and the corresponding natural nucleoside triphosphate must be as high as possible. In most cases, it is the first phosphorylation reaction which is rate-limiting.

Since the vital steps in the mode of action of nucleoside analogues lie in their conversion into the corresponding phosphorylated forms, one question could be asked, "why is it that the nucleoside, generally inactive by itself, is always administered as a drug"? In other words, "why not administer the phosphorylated forms of the nucleoside analogue"? The answer is simple: only nucleosides are able to enter cells easily, either following a transporter-mediated mechanism or by passive diffusion. On the other hand, nucleotides owing to their negative charges cannot penetrate cell membranes. The



Scheme 1. Mode of action of antiviral analogues.

phosphoric anhydride bonds are quickly hydrolyzed and the resulting monophosphate derivative is re-converted to the parent nucleoside by many non-specific serum phosphatases (Scheme 1). These considerations explain why many attempts in several laboratories have been undertaken in order to increase the delivery of nucleotide analogues into cells. Two attempts seem to be promising: the use of liposomes [5] and the pronucleotide approach.

THE PRONUCLEOTIDE APPROACH

Since it is generally accepted that the intracellular transport of intact nucleotides is hindered by the negative charges on the phosphate group, the design of most prodrugs involved a reduction in the total charge through modifications of the phosphate. The resulting prodrugs were expected to be sufficiently lipophilic to be able to enter into the cells, wherein they would undergo biotransformations to generate the free nucleotides [6–8]. Since the aim of the pronucleotide approach is the intracellular delivery of nucleoside analogue phosphorylated forms, the consequences will be to bypass the often encountered problem of the inefficient phosphorylation steps in the mode of action of nucleoside analogues and to overcome some resistance phenomena resulting from kinase mutations. As regards other expected results, we can suggest a possible increase of stability towards some catabolic reactions, an improvement of oral bioavailability, and a modulation of some pharmacokinetic parameters and the tissue distribution of the parent drug.

A priori, the pronucleotide approach could apply to dinucleoside monophosphates and to mononucleoside tri-, di- or monophosphates. In the latter case, phosphodiester, phosphonate or phosphotriester derivatives could be envisaged. In fact, as previously reported [8], it appears that the mononucleoside phosphotriester seems to be the most appropriate structure in regard to the pronucleotide approach. Furthermore, to be effective such a mononucleoside phosphotriester pronucleotide approach must fulfill some prerequisites. Firstly, the phosphotriester must be soluble and stable in the extracellular medium, but also lipophilic enough to be able to penetrate cell membranes by passive diffusion. Secondly, it must be intracellularly and selectively converted into its monophosphate. The first step of this conversion must be sufficiently fast in order to avoid any escape of the phosphotriester outside the cell. Removal of the remaining masking group from the resulting phosphodiester can be then carried out either following a similar mechanism as in the first step, or by phosphodiesterases. So, the crucial question is to determine what kind of phosphate protecting groups can be used. The requirement that ideally the first step must be effected more rapidly inside the cell than in the extracellular medium precludes the use of simple alkyl and aryl masking groups which can only be chemically hydrolyzed since no phosphotriesterase activity has been reported as of yet in mammalian cells. On the other hand, selective intracellular transformation of a phosphotriester into a phosphodiester could be envisaged by using bifunctional bio-labile protecting groups, as shown in Scheme 2. Such bifunctional groups are susceptible to attack by cellular enzymes, giving rise to a new phosphotriester which is chemically unstable and which decomposes spontaneously to afford the phosphodiester intermediate. This phosphodiester will be then converted into the desired nucleoside monophosphate.

Bis(POM), bis(DTE) AND bis(SATE) PHOSPHOTRIESTERS

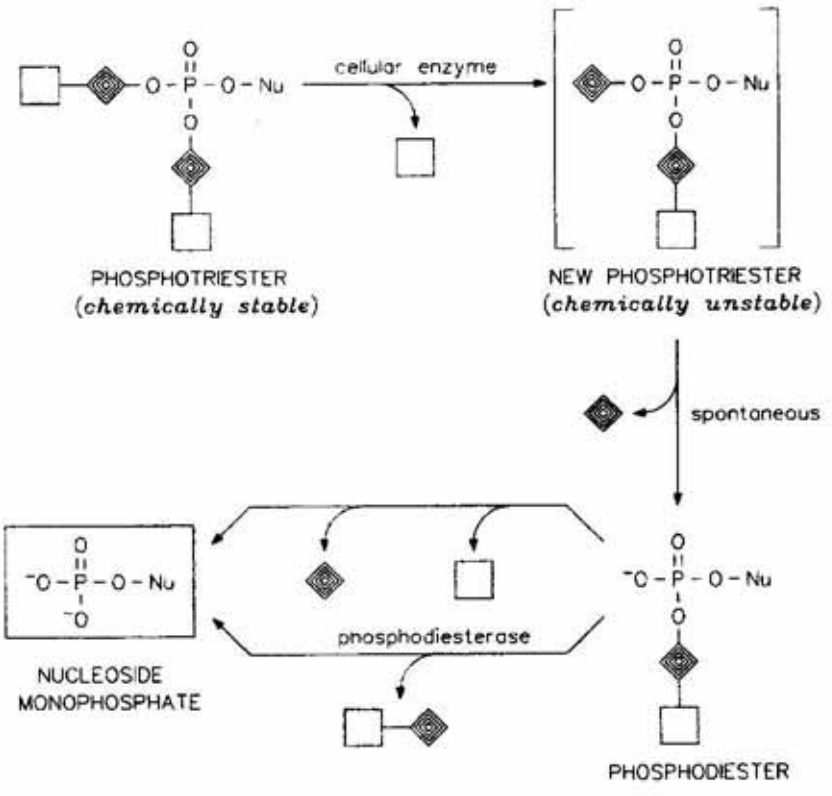
Among the few bio-labile phosphate protecting groups which have been cited in the literature, the acyloxymethyl groups initially introduced by Farquhar, and more particularly the (pivaloyloxy)methyl group (POM), deserve more attention [9]. These groups are cleaved by carboxyesterases to give rise to a hydroxymethyl phosphotriester intermediate, which is unstable and which spontaneously eliminates formaldehyde to afford the phosphodiester. Then, the phosphodiester will be converted into the nucleoside monophosphate following a similar mechanism as for the removal of the first protecting group, or by action of phosphodiesterases [10] (Scheme 3).

For our part, we conceived recently two other kinds of bio-labile protecting groups, namely the dithioethyl (DTE) and the *S*-acyl-2-thioethyl (SATE) phosphate protecting groups [11]. These DTE and SATE groups are cleaved, re-

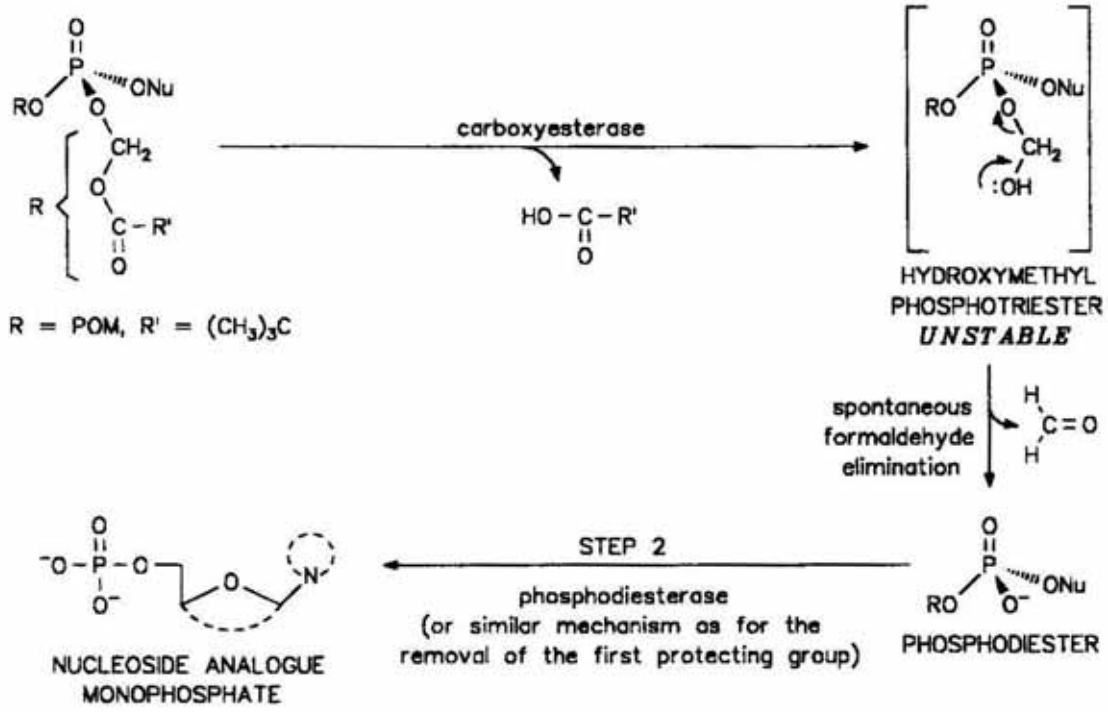
spectively, by reductases and carboxyesterases to give rise to a thioethyl phosphotriester intermediate, which is also unstable and which spontaneously eliminates episulfide to afford the phosphodiester (Scheme 4). As in the case of the POM group, the phosphodiester is subsequently converted into the monophosphate derivative either by following a similar mechanism as for the removal of the first protecting group or by action of phosphodiesterases.

Such a concept of pronucleotides with bio-labile bis(POM), bis(DTE) and bis(SATE) protections was validated using three different nucleoside models, namely: (i) 2',3'-dideoxyuridine (DDU), which is devoid of any anti-viral activity, although its triphosphate is a powerful inhibitor of the human immunodeficiency virus (HIV) reverse transcriptase; (ii) 3'-azido-3'-deoxythymidine (AZT), which is fully inactive in thymidine kinase deficient (TK^-) cells; and (iii) 9-(2-phosphonomethoxyethyl)adenine (PMEA), an acyclic phosphonate analogue which enters cells with difficulty. Thus, it has been shown that such pronucleotides (Scheme 5): in the case of DDU, induce an anti-HIV activity in various cell lines [11–14]; in the case of AZT, induce some activity in TK^- cells [14, 15]; in the case of PMEA, increase the anti-HIV and anti-herpes simplex virus activity of the parent phosphonate in cell culture [14, 15–19] as well as its oral bioavailability in animals [19, 20].

In order to obtain comparative information on the respective bis(POM), bis(DTE) and bis(SATE) phosphate protecting groups, we recently studied in detail, on the basis of the AZT model, both the anti-HIV potency and some pharmacokinetic parameters of the corresponding phosphotriester derivatives [15]. The comparison of the anti-HIV activities was carried out on two CEM cell lines, one of which was thymidine kinase deficient (Table 1). All the evaluated pronucleotides showed significant anti-viral activity in both cell lines. However, in thymidine kinase deficient CEM cells, the bis(methylSATE) phosphotriester was the most efficient compound, with the highest selectivity index. As expected, the nucleoside parent AZT was totally inactive in this TK^- cell line.

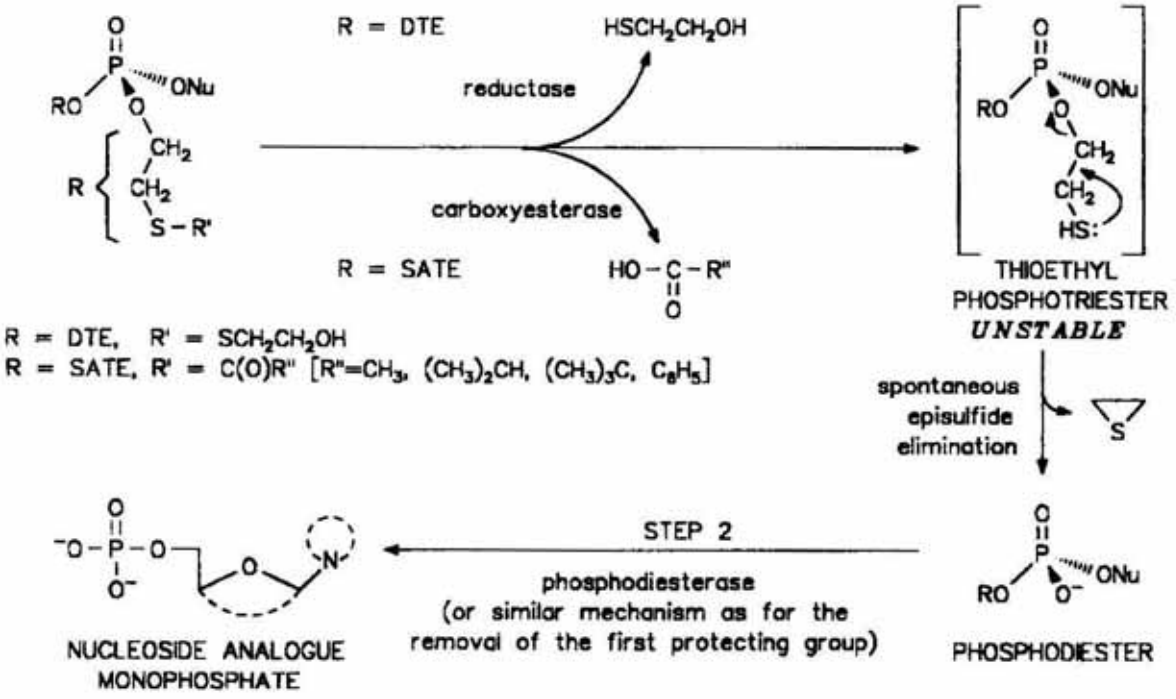


Scheme 2. Transformation pathway of a phosphotriester bearing bio-labile phosphate protecting groups into the corresponding nucleoside monophosphate.

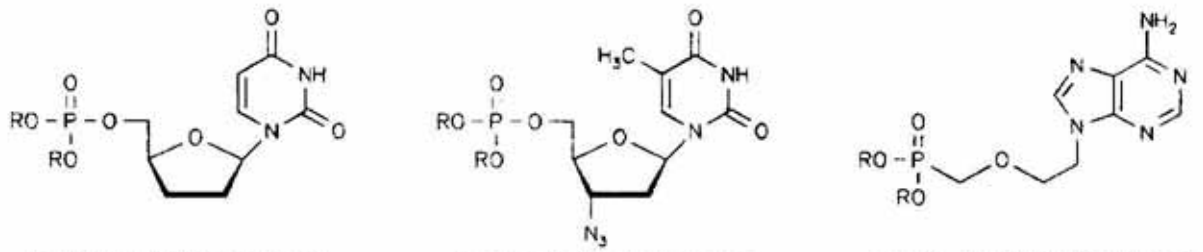


Scheme 3. Transformation pathway of a bis(acyloxymethyl)phosphotriester into the corresponding nucleoside monophosphate.

The decomposition studies of the AZT pronucleotides bearing bio-labile bis(POM), bis(DTE) and bis(SATE) protections were carried out in several biological media, including human serum, cell culture medium and CEM full cell extracts (Table 2). The more relevant results were those obtained in serum, where the bis(DTE) and the bis(methylSATE) phospho-



Scheme 4. Transformation pathway of a bis(DTE) phosphotriester or a bis(SATE) phosphotriester into the corresponding nucleoside monophosphate.



Bis(POM) pronucleotides, R = $(CH_3)_3CC(O)OCH_2-$
Bis(DTE) pronucleotides, R = $HO(CH_2)_2SS(CH_2)_2-$
Bis(methylSATE) pronucleotides, R = $CH_3C(O)S(CH_2)_2-$

Scheme 5. Structures of the bis(POM), bis(DTE) and bis(methylSATE) pronucleotides of DDU, AZT and PMEA.

triesters were very unstable. On the other hand, the bis(*t*-butylSATE) derivative was the most resistant against hydrolysis in serum with a half-life ($t_{1/2}$ = 7.5 h) more than seven times longer than that of the bis(POM) derivative ($t_{1/2}$ = 1 h).

Thus, the very promising results obtained with the bis(methylSATE) and bis(*t*-butylSATE) phosphotriesters of AZT (cf. Tables 1 and 2) led us to develop further such kind of bis(SATE) pronucleotides, improving their

Table 1
Comparison of the antiviral activities of AZT and its bis(POM), bis(DTE) and bis(SATE) pronucleotides in two CEM cell lines infected with HIV-1-LAI [15].
(SI, selectivity index = CC_{50}/EC_{50})

	CEM-SS			CEM-TK$^-$ (thymidine kinase-deficient)		
	efficiency EC_{50} (μM)	toxicity CC_{50} (μM)	selectivity SI	efficiency EC_{50} (μM)	toxicity CC_{50} (μM)	selectivity SI
AZT	0.006	> 100	> 17300	> 100	> 100	-
Bis(POM)AZTMP	0.077	67	860	0.26	66	254
Bis(DTE)AZTMP	0.023	> 100	> 4300	0.55	> 100	> 180
Bis(methylSATE)AZTMP	0.022	93	4300	0.05	> 100	> 2500
Bis(*i*-propylSATE)AZTMP	0.046	> 10	> 200	0.52	> 10	> 17
Bis(*t*-butylSATE)AZTMP	0.015	> 10	> 670	0.45	> 10	> 22

Table 2
$t_{1/2}$ of the pronucleotides of AZT in three different media [15]

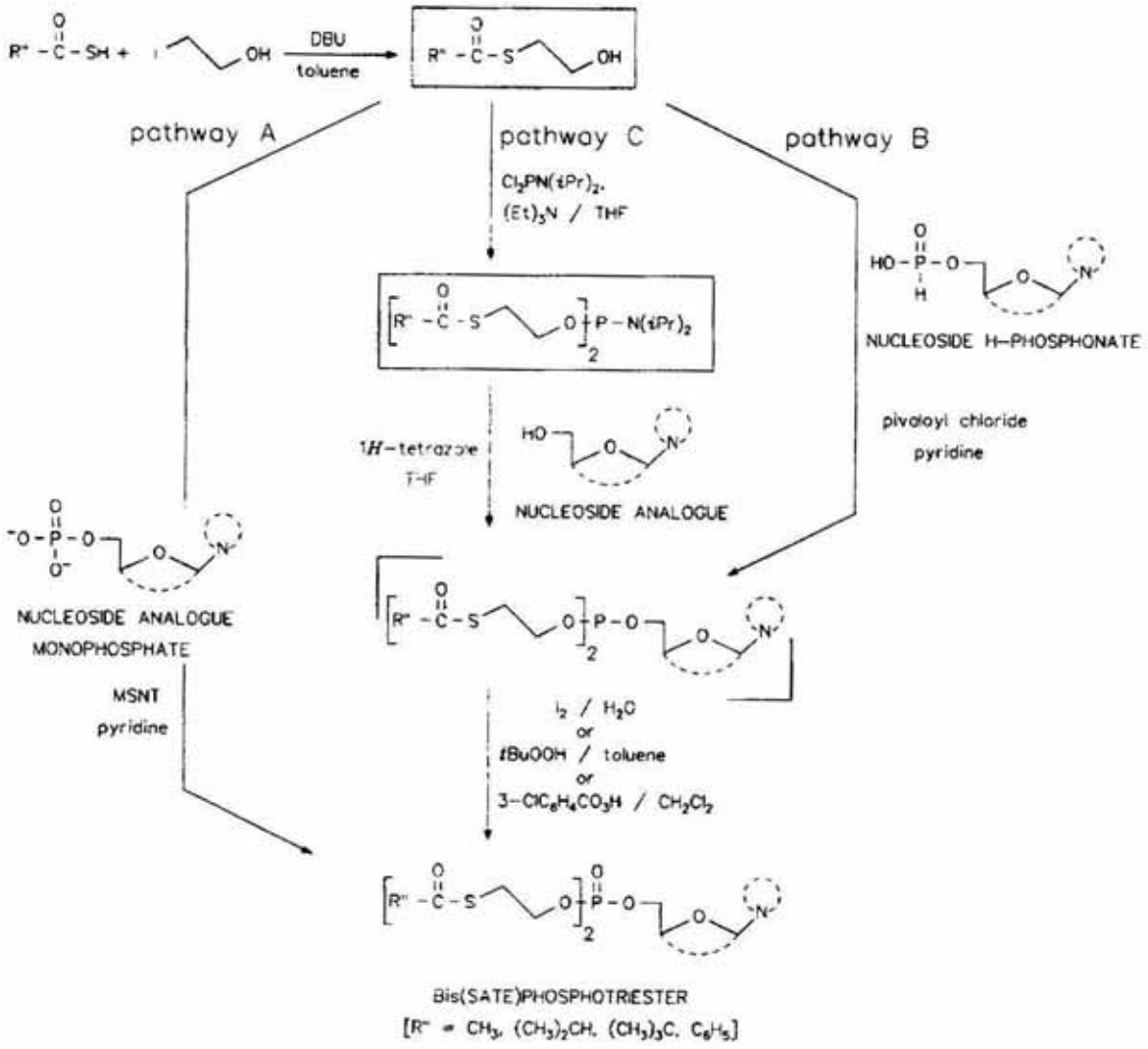
	bis(POM)-AZTMP	bis(DTE)-AZTMP	bis(methylSATE)-AZTMP	bis(*t*-butylSATE)-AZTMP
Serum	1 h	< 5 min	< 5 min	7.5 h
Cell culture medium	3 h	31 h	9 h	99 h
CEM cell full extract	21 min	90 min	8 min	63 min

synthesis and extending this concept to other nucleoside analogue models.

CHEMICAL SYNTHESIS OF bis(SATE) PRONUCLEOTIDES

The chemical synthesis of the bis(SATE) pronucleotides (Scheme 6) involved three different strategies, each of them requiring prior preparation of a *S*-acyl-2-thioethanol or a phosphoramidite reagent which was stable enough to be purified by silica gel flash column chromatography and could be stored in the freezer for several months. The first strategy (pathway A) we used was a P(*V*) approach, starting from an already prepared nucleoside monophosphate which was reacted with a *S*-acyl-2-thioethanol reagent, in pyridine and in the presence of mesitylene sulfonyl nitrotriazole. Note that this strategy was also successful in the case of phosphonate analogues, like PMEA. Alternatively, we also prepared some bis(SATE) pronucleotides following a P(*III*) approach based on hydrogen-phosphonate derivatives (pathway B). In this case, an already prepared H-phosphonate nucleoside was condensed with an *S*-acyl-2-thioethanol reagent in the presence of pivaloyl chloride as catalyst to give a phosphite intermediate which was subsequently oxidized.

However, our preferred approach is now another P(*III*) approach, which does not need any prior derivatization of the nucleoside analogue (pathway C). Thus, the nucleoside is directly condensed with the phosphoramidite reagent



Scheme 6. Chemical synthesis pathways of bis(SATE) phosphotriesters.

in THF and in the presence of tetrazole. Subsequent oxidation of the phosphite intermediate affords the desired bis(SATE) phosphotriester in high yield.

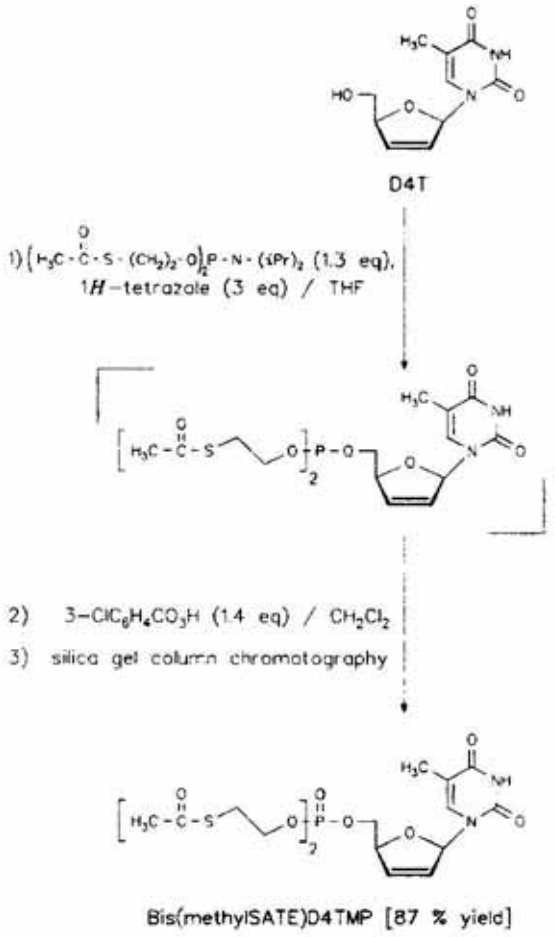
Bis(methylSATE) PRONUCLEOTIDE OF D4T

The bis(methylSATE) pronucleotide approach was implemented in the case of 2',3'-didehydro-3'-deoxythymidine (D4T), an antiviral drug which was licensed last year to treat advanced HIV-infected patients intolerant to or failing on other approved treatments [21]. The mode of action of D4T is similar to that of most nucleoside drugs (Scheme 1), namely successive phosphorylations inside the cell yield the triphosphate derivative. However, in contrast to AZT, the first phosphorylation step of D4T by the cellular thymidine kinase is rate-limiting. Thus, by direct intracellular delivery of D4T monophosphate we hoped to increase the efficiency of this compound in all cell lines.

For the synthesis of the bis(methylSATE) phosphotriester of D4T, we used the phosphoramidite approach which allowed us to obtain the desired pronucleotide in 87% yield after purification by silica gel column chromatography (Scheme 7).

We then evaluated the anti-HIV activity of the bis(methylSATE) phosphotriester of D4T, in comparison with its nucleoside parent, in three cell lines, namely human peripheral blood mononuclear (PBM) cells, CEM-SS cells and CEM thymidine kinase deficient cells [22] (Table 3).

It is noteworthy that in PBM and CEM cells, the efficiency of the bis(methylSATE) phosphotriester was about one order of magnitude higher than that of D4T. Moreover, in CEM thymidine kinase deficient cells, where D4T showed very little activity, the phosphotriester was also very efficient, with an EC_{50} of 0.011 μM and a selectivity index higher than 5000.



Scheme 7. Chemical synthesis of the bis(methylSATE) pronucleotide of D4T.

Table 3

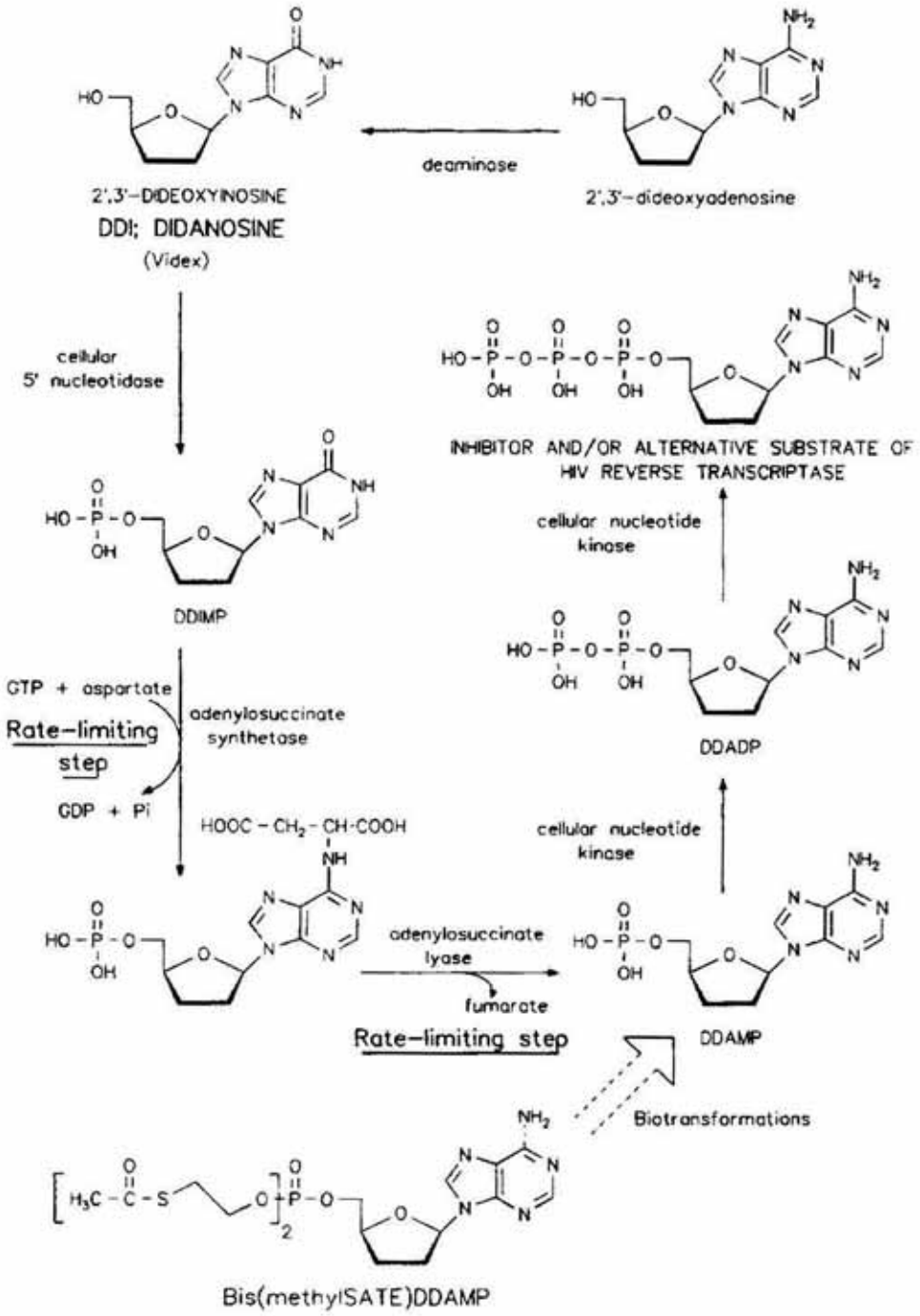
Comparison of the anti-HIV-1 activities of D4T and its bis(methylSATE) pronucleotide in three cell lines [22].

(SI, selectivity index = CC_{50} / EC_{50})

	PBM		CEM-SS		CEM-TK⁻	
	efficiency EC_{50} (μM)	toxicity CC_{50} (μM)	efficiency EC_{50} (μM)	toxicity CC_{50} (μM)	efficiency EC_{50} (μM)	toxicity CC_{50} (μM)
D4T	0.050	42	0.059	> 100	12	> 100
	SI = 840		SI > 1685		SI > 8.3	
Bis(methylSATE)D4TMP	0.007	22	0.006	68	0.011	60
	SI = 3143		SI = 11333		SI = 5456	

Bis(methylSATE) PRONUCLEOTIDE OF DDA

We also implemented the bis(SATE) pronucleotide approach on 2',3'-dideoxyadenosine (DDA), a well-established anti-HIV nucleoside [23], but which undergoes rapid transformation to 2',3'-dideoxyinosine (DDI) by adenosine deaminase. The mode of action of DDI, currently approved for anti-HIV chemotherapy [24], involves its intracellular monophosphorylation into DDIMP, which is subsequently aminated into DDAMP [25]. DDAMP then is further phosphorylated into its triphosphate DDATP, which is the active form of the drug (Scheme 8). Note that the conversion of DDIMP into DDAMP is the rate-limiting step.



Scheme 8. Mode of action of DDA, DDI and the bis(methylSATE) pronucleotide of DDA.

The synthesis of the mononucleoside bis(methylSATE) phosphotriester of DDA was carried out following the same approach as described above in the case of D4T. However, the yield was slightly lower (about 70% *versus* 87% in the case of D4T) due to some transacetylation reactions on the exocyclic amine function, this side reaction complicating the last step, purification by silica gel column chromatography.

The anti-HIV activity of the bis(methylSATE)DDAMP derivative was compared to those of DDI, DDA and AZT in CEM-SS cells and in macrophages [26, 27] (Table 4). The data obtained underlined the superiority of the bis(methylSATE) phosphotriester of DDA, which was 10000 times more potent than DDI or DDA and even 10 times more efficient than AZT in both cell lines. Note that, although this phosphotriester was a little bit more cytotoxic than the nucleosides DDI, DDA and AZT, its selectivity index remained very high.

Bis(methylSATE) AND bis(*t*-butylSATE) PRONUCLEOTIDES OF ACYCLOVIR

Finally, the bis(SATE) pronucleotide approach was implemented on acyclovir (ACV), a potent drug currently used to treat several affections due to herpes viruses [28]. Regarding the mechanism of action of this compound, ACV is selectively monophosphorylated by herpes virus thymidine kinase before being converted to its triphosphate by cellular nucleotide kinases. The triphosphate interacts with the herpes virus DNA polymerase, giving rise to the anti-herpes-virus activity. However, it is noteworthy that the triphosphate of ACV can also interact with other viral polymerases as the DNA polymerases of cytomegalovirus (CMV) and hepatitis B virus (HBV), or the HIV reverse transcriptase [29]. So, we reasoned that intracellular delivery of ACV monophosphate could also result in inhibition of the replication of viruses other than the herpes viruses. This was the reason why we decided to synthesize and to study the bis(methylSATE) and bis(*t*-butylSATE) phosphotriesters of acyclovir.

In order to increase the solubility of acyclovir in organic solvents and to prevent any side-reaction on its exocyclic amine function, we first prepared in three steps its NH monomethoxytrityl-protected derivative [30] (Scheme 9). Then, this derivative was converted into the fully protected bis(SATE) phosphotriesters using the previously described phosphoramidite approach. Finally, removal of the trityl group under acidic conditions afforded the bis(methylSATE) and bis(*t*-butylSATE) phosphotriesters in high yields (Scheme 9).

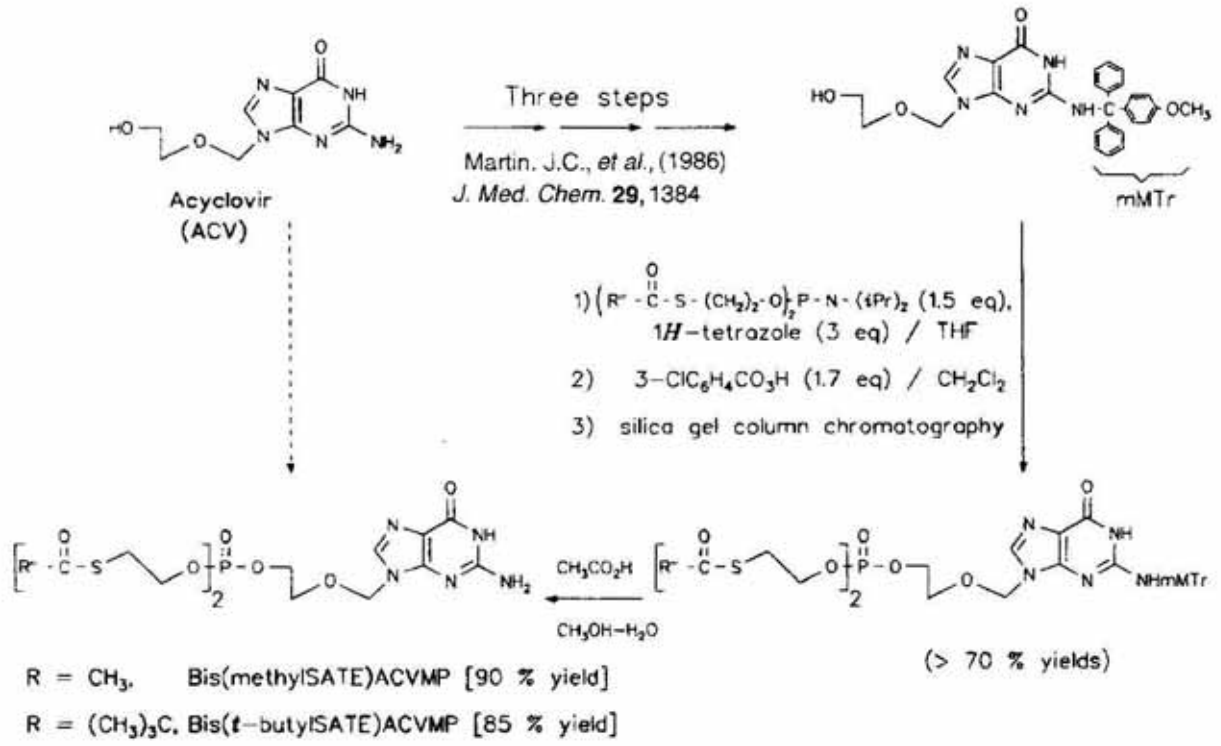
The anti-HBV activities of the bis(methylSATE) and bis(*t*-butylSATE) pronucleotides of acyclovir were evaluated against the HBV transfected human liver HepG2 (2.2.15) cells on day 9. It was found that the two pronucleotides

Table 4

Comparison of the anti-HIV-1 activity of the bis(methylSATE) pronucleotide of DDA with the nucleoside analogues DDI, DDA and AZT in two cell lines [26, 27].

(SI, selectivity index = CC_{50} /EC_{50})

	CEM-SS		Macrophages	
	efficiency (HIV-1 Lai strain) EC_{50} (μM)	cytotoxicity CC_{50} (μM)	efficiency (HIV-1 Bal strain) EC_{50} (μM)	cytotoxicity CC_{50} (μM)
DDI	4.5	> 100	0.64	> 100
	SI > 22		SI > 156	
DDA	0.54	> 100	0.17	> 100
	SI > 185		SI > 588	
Bis(methylSATE)DDAMP	0.00056	24	0.0000010	4.4
	SI = 42860		SI = 440000	
AZT	0.0048	> 100	0.00049	> 100
	SI > 20833		SI > 204081	



Scheme 9. Chemical synthesis of the bis(methylSATE) and bis(t-butylSATE) pronucleotides of acyclovir.

inhibited the replication of this virus as assessed by measurement of HBV replicative intermediate forms and HBV virion levels (Table 5).

The efficiency of the two pronucleotides of ACV was in the same range as that of the reference compound 2',3'-dideoxyguanosine (DDG). However, the pronucleotides were not very cytotoxic in mock-infected HepG2 cells, so their selectivity indexes were more important. Note that in these experiments, the nucleoside parent ACV was fully inactive (Table 5).

Table 5

Comparison of the anti-HBV activity of the bis(methylSATE) and bis(t-butylSATE) pronucleotides of ACV with 2',3'-dideoxyguanosine and acyclovir in human liver HepG2 cells.

(Selectivity indexes, $SI_a = CC_{50}/EC_{90}$; $SI_b = CC_{50}/EC_{50}$)

	Anti-HBV efficiency				Cytotoxicity	Selectivity index			
	HBV replicative intermediate		HBV virions			HBV replicative intermediate		HBV virions	
	EC_{90} (μM)	EC_{50} (μM)	EC_{90} (μM)	EC_{50} (μM)	CC_{50} (μM)	SI_a	SI_b	SI_a	SI_b
ACV	> 100	> 100	> 100	111	631				5.7
Bis(methylSATE)ACVMP	15	4.3	5.1	0.7	987	66	230	194	1410
Bis(*t*-butylSATE)ACVMP	10	1.1	7.1	0.2	1593	159	1448	224	7965
2',3'-Dideoxyguanosine (reference compound)	18	3.4	11	1.3	219	12	64	20	168

Unpublished data from Dr C. Tseng (National Institutes of Health, Bethesda, U.S.A.)

CONCLUSION

We have shown that in cell culture experiments, the bis(SATE) phosphotriester derivatives of AZT, D4T, DDA and ACV actually lead to the delivery of the corresponding nucleoside monophosphates inside the cells.

Currently, we are thinking about an *in vivo* implementation of the bis(SATE) pronucleotide approach, particularly in the case of: i) nucleoside analogues of which the first anabolic phosphorylation step is rate-limiting (D4T or DDA as anti-HIV agents, ACV as anti-HBV agent) or decreases in the course of the treatment, leading to the emergence of resistance (for instance AZT [31]); ii) nucleoside analogues which are rapidly degraded due to the action of certain enzymes or under acidic conditions. In this regard, we have found that the bis(methylSATE) pronucleotide of DDA is fully resistant to deaminases (in contrast to its nucleoside parent) and is significantly resistant to glycosidic bond breakage in acidic conditions (at pH 2, $t_{1/2}$ = 7.4 h, compared to $t_{1/2}$ = 28 min for DDA and $t_{1/2}$ = 8 min for DDI [27]); iii) nucleoside phosphonates (like PMEA, HPMPC, etc.) which cross with difficulty the cell membranes due to their charges; and, iv) all kinds of nucleoside and nucleotide analogues, in order to modulate both their bioavailability according to their mode of administration (oral, subcutaneous, intramuscular, intraperitoneal, ocular, etc.) and their biodistribution in tissues and organs.

However, the first prerequisite for the *in vivo* implementation of the bis(SATE) pronucleotide approach is that no additional toxicity should be evoked either by the bis(SATE) "pro-moiety", or by its degradation products (i.e. acetic acid, pivaloic acid, episulfide and mercaptoethanol). To answer the question: "does the bis(SATE) "pro-moiety" induce additional toxicity?", we can already provide some answers. For instance, it has been shown that when assessed in a CFU-GM bone marrow progenitor clonogenic assay (a predictive model for drug induced myelotoxicity) the bis(methylSATE) pronucleotide of AZT (IC_{50} = 3.6 μM) was not more toxic than AZT itself (IC_{50} = 1.0 μM). Also, when assessed by PC-12 neurite regeneration and mitochondrial DNA content in NGF-primed PC-12 cells assays (a predictive model for drug induced peripheral neuropathy), the bis(methylSATE) pronucleotide of DDA was less than, or only as, toxic as DDA, DDI and 2',3'-dideoxycytidine (IC_{50} in the range of 1–10 μM) (unpublished data from Prof. J.-P. Sommadossi, University of Alabama at Birmingham, U.S.A.). Furthermore, it has been shown that acetic acid, pivaloic acid, episulfide and mercaptoethanol do not affect the proliferation of CFU-GM, PC-12, CEM-SS, MT-4 and PBM cells at the highest concentrations tested (usually 50 to 200 μM) (unpublished data from Prof. J.-P. Sommadossi, University of Alabama at Birmingham, U.S.A., and from Dr A.-M. Aubertin, Medicine University, Strasbourg, France). Hence, all these data indicate that the SATE protecting groups do not induce additional toxicity. Currently, we are starting to implement the bis(SATE) pronucleotide approach in animal models.

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