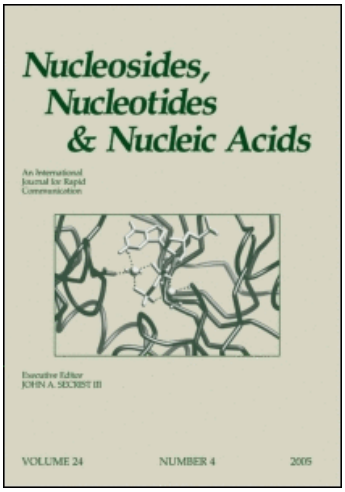

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ABSTRACT

Nucleoside activation by nucleoside diphosphate kinase and inhibition of HIV-1 reverse transcriptase were studied comparatively for a new class of nucleoside analogs with a borano (BH_3^-) or a thio (SH) group on the α-phosphate. Both the α-Rp-borano derivatives of AZT and d4T improved phosphorylation by NDP kinase, inhibition of reverse transcription as well as stability of α-borano monophosphate derivatives in terminated viral DNA chain.

First and second generations of nucleoside analogs like dideoxynucleosides (ddI, ddC), AZT, d4T or 3TC have been extensively used as antiviral drugs targeted

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at the HIV reverse transcriptase. The lack of 3′OH on the ribose moiety prevents the synthesis of the phosphodiester bond between the antiviral drug and a natural 2′-deoxynucleotide (dNTP) by the viral polymerase. DNA chain elongation terminates which accounts for the observed antiviral effect (1). However chemotherapy with these compounds is limited due to poor synthesis of the triphosphate active form in cells and to the appearence of drug-resistant viruses.

It is generally assumed that nucleoside analogs are phosphorylated by the cellular kinases from the salvage pathway that phosphorylate natural nucleotides. The first steps phosphorylating into the mono- and diphosphate derivatives are specific for the nucleobase. The third phosphate addition is catalyzed by nucleoside diphosphate (NDP) kinase with a broad specifity. The X-ray structures of NDP kinases from several organisms have been solved and present a similar fold and a highly conserved active site which binds a single nucleotide in a unique conformation (2). The nucleobase is in hydrophobic interaction with a Phe and a Val residues at the surface of the protein. The ribose moiety located deeper in the active site is stabilized via a hydrogen-bond network involving protein residues (Lys and Asn) and the 3′-OH group. The 3′-OH also H-bonds with the oxygen bridging the β- and γ-phosphates forming an intra-nucleotide H-bond. The phosphate chain is stabilized by Arg residues and one Mg^{2+} ion coordinates an oxygen of each phosphate. The reaction involves the formation of a phosphorylated intermediate on a His residue according to a ping-pong mechanism.

ACTIVATION OF AZT AND d4T BY NDP KINASE

Previous studies have shown that AZT-DP is a poor substrate for human NDP kinase type A and B with a catalytic efficiency of phosphotransfer 10.000 less than the natural counterpart (3). The structure of a point mutant of *Dictyostelium* NDP kinase complexed with AZT-DP has shown that the bulky azido group disturbs the H-bond network surrounding the 3′ group (4). In particular, the ε- amino terminus of Lys16, a catalytic residue involved both in the nucleotide binding and in the phosphotransfer, is displaced by 2.6 Å The internal H-bond appears to be crucial for an optimal nucleotide geometry and an efficient phosphotransfer.

The reaction of NDP kinase with a NTP or a NDP could be studied by monitoring protein fluorescence. The Trp residue located in the vicinity of the catalytic histidine provides a signal that correlates with the phosphorylation level of this His (5). As the ratio of the reaction rates in both directions, *i.e.* the equilibrium constant of the enzyme with nucleosides di- and triphosphate, is 0.2, the reactivity of NDP kinase with a substrate can be studied both in the forward and in the reverse directions. The phosphoryl transfer from NTP to NDP kinase or from the phosphoenzyme to NDP causes monoexponential time courses with either a quenching or an enhancement of the signal depending on the reaction studied. The time courses could be monitored only with antiviral analogs as the reactions with natural nucleotides were too fast to be detected. The constants of these exponential

curves (k_{obs}), which represent the rates of the reaction, were found to vary with the concentration of the nucleotides. In case of dideoxynucleotides (ddN), the pseudo-first order rate constant follows a saturation progress versus the concentration of ddNTP. This was analyzed according to a two-step mechanism where fast association of the enzyme with ddNTP is followed by a rate-limiting phosphorylation step (6). Dideoxynucleotides are poor substrates for NDP kinase with a maximum rate of phosphotransfer 1–3 s^{-1}, *i.e.* 1.000 times less than for natural nucleotides. For antiviral analogs available in low amounts like d4T or AZT, k_{obs} varies linearly with the nucleotide concentration. The slope of k_{obs} variation as a function of the nucleotide concentration represents the catalytic efficiency of phosphotransfer. When tested with human NDPK-A, d4T-DP was found the best antiviral drug with a catalytic efficiency of phosphorylation of 2600 $M^{-1}s^{-1}$, whereas phosphorylation of AZT-DP was 10 times less efficient (7).

Is the better reactivity of d4T due to a better binding of the nucleotide to the active site or to a higher rate of phosphotransfer? To address this question, we have engineered an inactive mutant enzyme with both the catalytic histidine changed to a Gly, and the Phe stacking the nucleobase changed to a Trp residue providing a fluorescent signal. The intrinsic fluorescence of the double mutant is 50% enhanced upon binding of NTPs. Fluorescence titrations showed that whereas AZT-TP binds with a dissociation constant (K_D) of 30 μM, d4T-TP binds to the active site with an affinity similar to dTTP (1.5 μM). The reactivity of d4T triphosphate with NDP kinase is therefore due to a better binding of the nucleotide analog to the active site (7). The structure of the binary complex of the inactive mutant with d4T-TP was solved. d4T-TP adopts a conformation similar to a natural nucleotide (Fig. 1A). The sp2 C3′ donates its hydrogen to the oxygen bridging the β- and γ-phosphates and no shift was observed for Lys16 contrary to AZT-DP. The distance and geometry of the contact are consistent with a CH. . .O bond as previously observed in small molecule crystals, proteins and DNA. This particular H-bond, much weaker than the bond with the 3′OH of a natural substrate allows a better affinity of d4T (8). Moreover the planarity of the glycone cycle resulting in a unique conformation of the nucleotide may participate in the formation of the transition state as observed with DNA polymerases (7).

ACTIVATION OF α-BORANO NUCLEOTIDE ANALOGS BY NDP KINASE AND INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY

The close examination of structures of NDP kinase complexed with d4T-TP and of DNA polymerases, such as reverse transcriptase and T7 DNA polymerases, shows that the NDP moieties adopt a similar conformation (Fig. 1B). In particular, in the three proteins, the same oxygen of the α-phosphate ligates a Mg^{2+} ion while the other α-phosphate oxygen interacts neither with the protein, with the nucleotide itself, nor with a metallic ion. The oxygen was therefore substituted

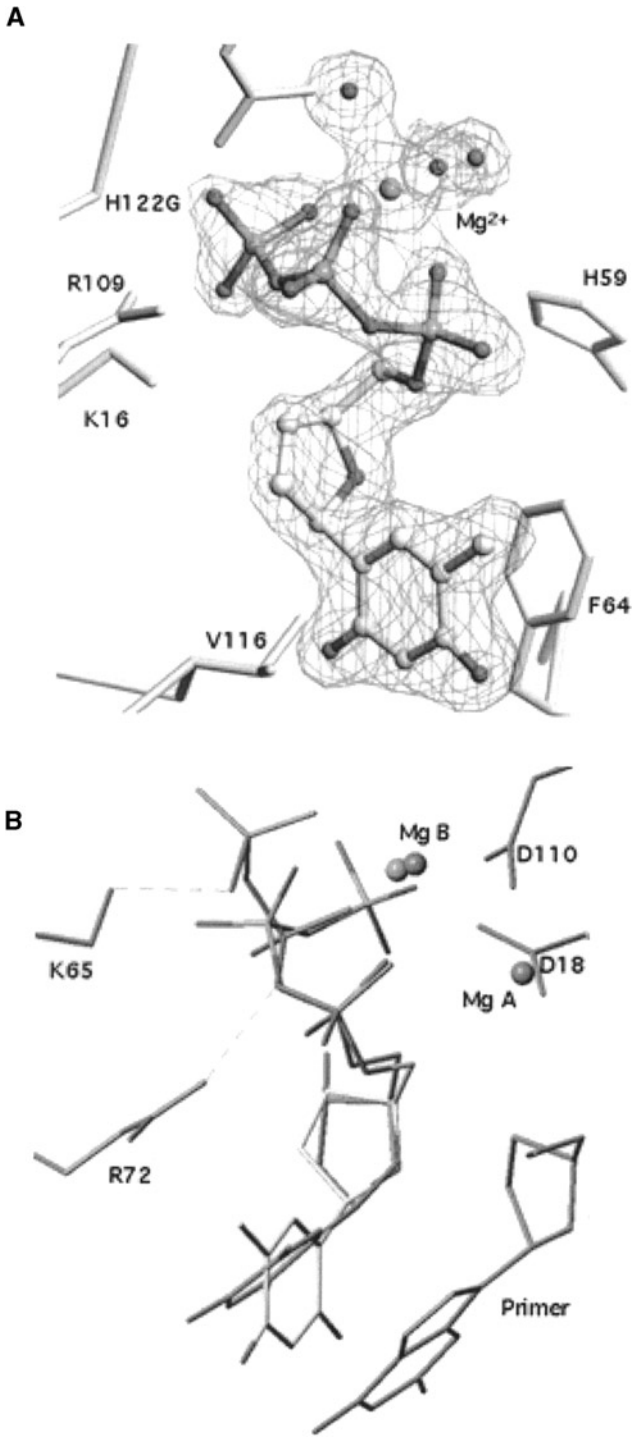

Figure 1. (**A**) d4T-TP bound to the catalytic inactive variant (H122G) of *Dictyostelium* NDP kinase. The 2Fo-Fc electron density map at 1.85 Å resolution is contoured at 1 σ. The Mg^{2+} ion ligates all three phosphates. The thymine base is sandwiched between F64 and V116. **(B)** Conformation of the nucleotide substrate in NDP kinase and HIV reverse transcriptase. d4T-TP from the NDP kinase complex is superimposed onto dTTP in the ternary complex with RT/DNA (PDB file 1RTD) after least-square fitting atom N1 of the base and common atoms in the sugar and the α-phosphate.

and replaced by a borano (BH_3^-) or by a thio group. BH_3 is isosteric and isoelectronic compared to the oxygen atom. **Synthesis**: The syntheses of the triphosphate analogs, namely AZT 5′-O-(1-thiotriphosphate) [AZT-TPαS], AZT 5′-O-(α-P-borano)triphosphate [AZT-TPαB], and d4T 5′-O-(α-P-borano-triphosphate [d4T-TPαB], were performed according to known procedures (9,10). The more handy one-pot synthesis of AZT 5′-O-(α-P-borano)diphosphate [AZT-DPαB] was designed based on a combination of two published methodologies (11,12). The target compounds were characterized by ^{1}H, ^{13}C, and ^{31}P NMR, electrospray mass spectrometry, and HPLC, all data being consistent with their structure and purity. In particular, in the presence of of the borano or the thio group in a nucleoside diphosphate the α-phosphorus atom becomes chiral, leading to two diastereoisomers R_P and S_P. Thus, in each series, the R_P and S_P derivatives were separated by HPLC and isolated as pure isomers, although their absolute configuration could not be ascertained at this stage. The same isomer was found active for both enzymes, when assayed as substrate for NDP kinase activity or as an inhibitor of DNA elongation by HIV-1 reverse transcriptase.

The active isomer was tested as substrate for NDP kinase in fluorescence presteady-state experiments (Fig. 2A). The presence of the α-borano group results in an 10 fold enhancement of the catalytic efficiency of the reaction relative to unsubstituted AZT-DP (Fig. 2B) or dTDP. In the case of thio derivatives, the active isomer also presents enhanced catalytic efficiency. The 2.5-fold increase is slightly less than for the borano derivatives (Fig. 2C). Using the protocol previously described for measuring the binding of α-borano and α-thio active isomers to inactive NDP kinase, we demonstrated that the improved reactivity of α-borano or α-thio derivatives is due to an increase in the affinity (Fig. 3). We crystallized a complex of α-borano TDP with NDP kinase. The structure was solved and revealed the absolute configuration (Rp) of the active α-borano isomer. The α-borano derivative binds like ADP and makes similar interactions. The electron density is weaker at the Rp than the Sp position and is a signature of the presence of boron .As the α-borano group does not interact with either the protein nor the Mg^{2+} ion, metal coordination of the phosphate chain by oxygen atoms likely determines the stereochemistry of recognition of the α-borano group by NDP kinase (8). By analogy, in the case of thio derivatives, the active diastereoisomer for NDP kinase activity is the Sp isomer due to the higher molecular weight of sulfur atom by comparison to oxygen and bore.

Steady-state experiments were conducted using the active diastereoisomer of the α-thio AZT-TP. The presence of the thio group had no effect on either K_m or k_{cat} for wild-type RT(Canard B., Sarfati S., and Richardson C.C., unpublished data). This was not the case for theactive borano derivative for which an improvement in incorporation efficiency could be evidenced (8). Presteady-state experiments with HIV-1 reverse transcriptase and the borano derivative were performed using a primer extension assay and a synthetic DNA-primed DNA template. The time course of the reaction involves a burst phase followed by a linear phase. The analysis of the

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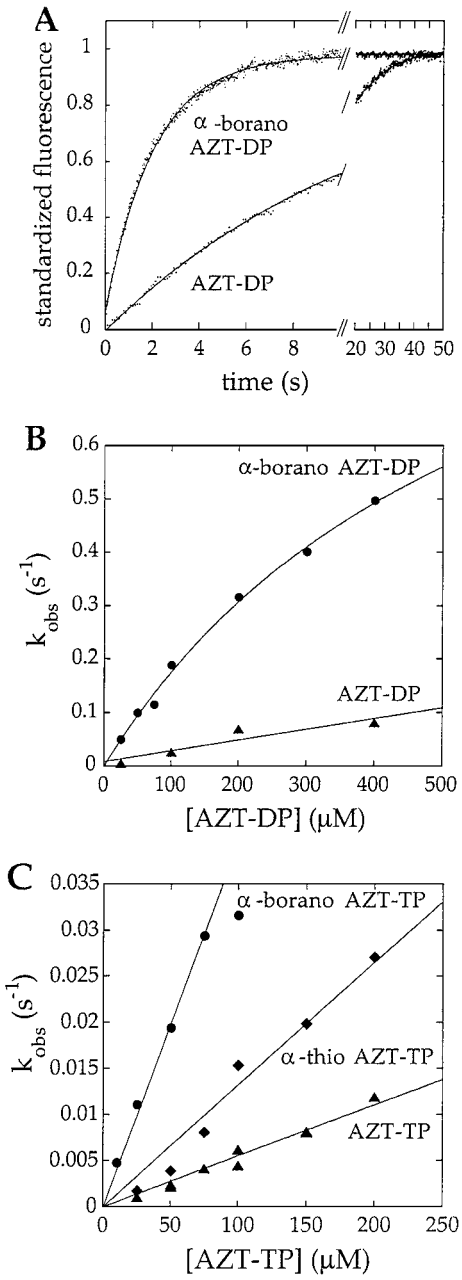

Figure 2. Enhancement of AZT activation by NDPK in the presence of the Rp α-borano or α-thio groups. (**A**) Kinetics of phosphorylation of AZT-DP and α-borano AZT-DP by phosphorylated human NDPK-A. The phosphorylated enzyme (1 μM) is mixed with 400 μM AZT-DP or α-borano AZT-DP. The decrease in fluorescence is monitored with a Hi-tech SF-61DX2 stopped flow device ($\lambda\varepsilon$xc = 304 nm, excitation slit = 2 nm, emission filter <320 nm). The solid lines represent the best fit of each curve to a monoexponential. (**B**) Concentration dependence of the pseudo-first order phosphorylation rate constant k_{obs}: α-borano AZT-DP (●) and AZT-DP (▲). The catalytic efficiency is $k_{max}/K_D = 2000\ M^{-1}s^{-1}$ for α-borano AZT-DP and 200 $M^{-1}s^1$ for AZT-DP. (**C**) Concentration dependence of the pseudo-first order rate constant k_{obs} for NDPK-A phosphorylation by α-borano AZT-TP (●), α-thio AZT-TP (◆) and AZT-TP (▲). The catalytic efficiency is $k_{max}/K_D = 375M^{-1}s^{-1}$ for α-borano AZT-TP, 200 $M^{-1}s^{-1}$ for α-thio AZT-TP and 80 $M^{-1}s^1$ for AZT-TP.

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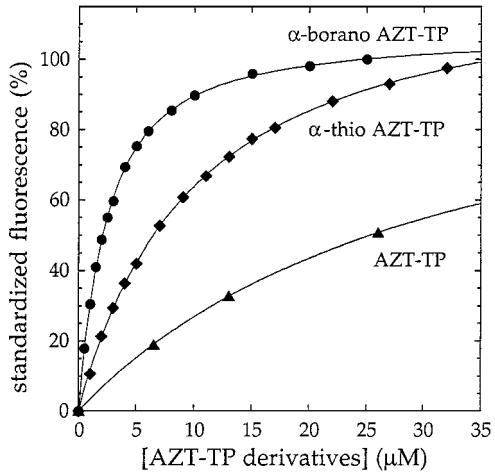

Figure 3. Binding curves of NDP kinase with Rp α-borano or α-thio derivatives. Fluorescence change of the catalytic inactive double mutant (H122G.F64W) of NDP kinase (1 μM) upon binding of triphosphate nucleoside analogs (λexc = 310, λ em = 330 nm). The solid line is the best fit of the data to a quadratic equation curve. It corresponds to a K_D of 2.0 μM for α-borano AZT-TP (●), 10 μM for α-thio AZT-TP (◆) and 30 μM for AZT-TP (▲).

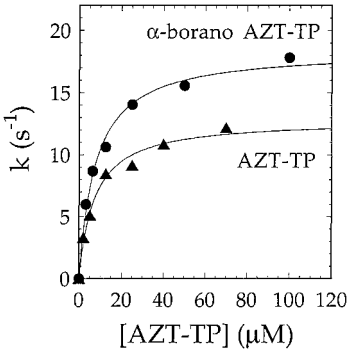

Figure 4. Pre-steady state kinetics of a single α-borano AZT-MP or AZT-MP incorporation into DNA. A 5′ - ^{32}P-labeled 21-primer was annealed to a 31-mer DNA template (100 nM) specifying a single thymidine insertion site immediately adjacent to the 3′-end of the primer, and RT (30 nM active sites) was allowed to bind. The reaction was initiated by the addition of 100 μM of α-borano AZT-TP or AZT-TP, 6 mM $MgCl_2$ in a rapid quench apparatus (Kin Tek, State college, PA), and quenched at various times with 0.3 M EDTA. Products were analysed by denaturing gel electrophoresis and quantitated using photostimulatable plates and a Fuji Imager. Data were fitted to the burst equation: Product = $A(1\text{-}e^{-k}t) + k_{ss}t$, where A is the amplitude, k is the apparent pre-steady state rate, and k_{ss} is the steady-state rate (8). First-order rates were determined as described above for α-borano AZT-TP (●) and AZT-TP (▲) and plotted against nucleotide analog concentration to determine $K_D(\mu M)$ and $k_{pol}(s^{-1})$ using an hyperbolic fit of the data.

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burst phase allows the measurement of the dissociation constant (K_D) for the analogs and their incorporation rate constant k_{pol}. The observed rate constant (k) increases with nucleotide analog concentration as a saturation curve (Fig. 4). The K_D value was measured at half saturation, and k_{pol} was extracted at the plateau. The presence of the Rp α-borano group contributes to a ~50% increase of the catalytic efficiency

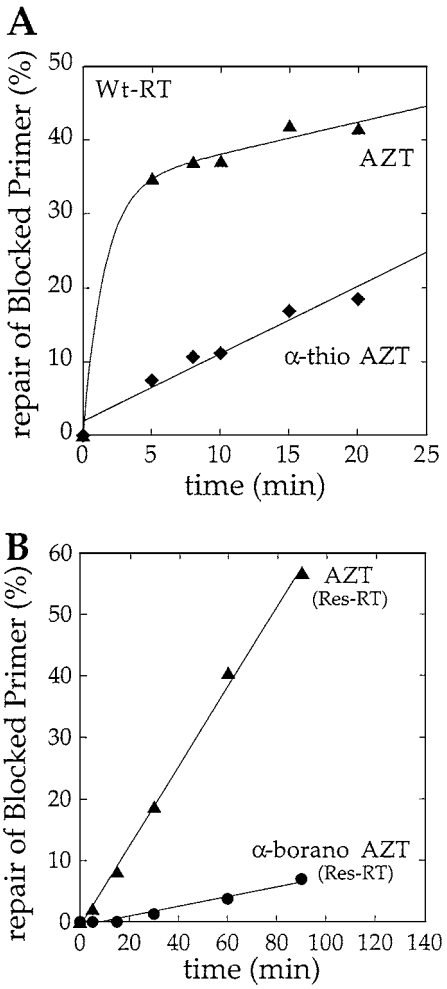

Figure 5. Pyrophosphorolytic repair of incorporated α-thio or α-borano AZT-MP analogs by wild type and "drug-resistant" RT. (**A**) Pyrophosphorolytic repair of AZT-MP- (▲) or α-thio AZT-MP (◆) -terminated DNA primer by wild-type RT. A 5′-^{32}P-labeled 22-primer terminated with either AZT-MP or thio-AZT-MP was annealed to a 31-mer DNA template (100 nM) and incubated with RT (30 nM). The pyrophosphorolytic reaction was started upon addition of 1 mM pyrophosphate (PPi), and products shorter than 22 nucleotides were separated using denaturing 15% polyacrylamide gel electrophoresis. Products were quantitated using photostimulatable plates and a Fuji Imager. The graph shows the quantitation of the appearance of products relative to the starting 22-primer. (**B**) Variant RT D67N/K70R/T215F/K219Q and pyrophosphorolytic repair of α-borano AZT-MP (●) -terminated DNA primer.

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(k_{pol}/K_D) of nucleotide incorporation and is mainly due to an enhanced k_{pol} while the affinity is unchanged (8).

REVERSE TRANSCRIPTASE-CATALYZED PYROPHOSPHOROLYTIC REPAIR OF BLOCKED DNA CHAIN

Drug resistance to nucleoside analogs by HIV has been shown to involve a reverse transcriptase-catalyzed repair mechanism: the incorporated analog is taken off from the 3′end of the viral DNA chain upon nucleophilic attack by either pyrophosphate or ATP (13,14). Pyrophosphorolytic repair of AZT-terminated DNA by wild-type RT was examined using a gel assay. In this assay, a primer is extended by AZT-TP or α-thio AZT-TP, and then subjected to pyrophosphorolysis upon the addition of 1 mM pyrophosphate. The appearance and quantitation of shorter products than the extended primer allows to quantitate the rate of pyrophosphorolytic repair of the terminal analog (8). The results are presented in Figure 5A where It can be seen that the presence of the α-thio decreases the initial rate of pyrophosphorolysis. Although the initial burst of of pyrophosphorolysis is too fast to be measured manually, a lower value of at least 50-fold can be estimated for the decrease in pyrophosphorolysis rate brought by the presence of the thio group. It was thus of interest to test whether substitution at the α-phosphate could be of any use in inhibiting drug-resistant reverse transcriptases. The boranophosphate derivatives were examined using an alternate polymerization assay. Repair of AZT-terminated DNA by two well-known AZT-resistant reverse transcriptases (K65R and D67N/K70R/T215F/K219Q) was assayed. In the presence of pyrophosphate, the K65R excision rate of AZT decreases 5 fold when the α-borano group is present in the phosphodiester bond. Similar results were observed with d4T α-borano. A 2-fold decrease in repair of AZT-terminated DNA was also observed with the quadruple mutant of reverse transcriptase (Fig. 5B). The presence of the α-borano group therefore leads to increased stability towards pyrophosphorolytic repair of blocked DNA chain (8).

CONCLUSION

The better activation by NDP kinase of α-borano derivatives and the increased stability toward repair mechanisms that contributes to drug resistance by HIV reverse transcriptase make α-borano compounds attractive. Our experiments suggest that α-borano nucleotide analogs may be of use *in vivo* against viruses that have become resistant. They could constitute a third generation of drugs in the anti-HIV nucleotide analogs field. As thymidine kinase appears to be the rate limiting step in d4T phosphorylation whereas TMP kinase as well as NDP kinase are the limiting steps in AZT activation, the current aim is to find an α-borano nucleoside phosphate pro-drug that could favor the entry of the borano derivative into cells and that would bypass the rate-limiting steps of intracellular phosphorylation.

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