

Incorporation of non-nucleoside triphosphate analogues opposite to an abasic site by human DNA polymerases β and λ

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ABSTRACT

A novel class of non-nucleoside triphosphate analogues, bearing hydrophobic groups sterically similar to nucleosides linked to the α -phosphate but lacking the chemical functional groups of nucleic acids, were tested against six different DNA polymerases (polymerases). Human polymerases α , β and λ , and *Saccharomyces cerevisiae* polymerase IV, were inhibited with different potencies by these analogues. On the contrary, *Escherichia coli* polymerase I and HIV-1 reverse transcriptase were not. Polymerase β incorporated these derivatives in a strictly Mn^{++} -dependent manner. On the other hand, polymerase λ could incorporate some alkyltriphosphate derivatives with both Mg^{++} and Mn^{++} , but only opposite to an abasic site on the template strand. The active site mutant polymerase λ Y505A showed an increased ability to incorporate the analogues. These results show for the first time that neither the base nor the sugar moieties of nucleotides are required for incorporation by family X DNA polymerases.

INTRODUCTION

During DNA synthesis, any DNA polymerase is presented with a pool of four structurally similar deoxynucleotide triphosphates (dNTPs) from which it must select the sole

Watson–Crick base-paired (correct) substrate for incorporation into the growing DNA strand (1). The identity of such a correct dNTP changes with each cycle of nucleotide incorporation, as translocation along the DNA strand presents a new ‘templating’ base to the enzyme’s active site. Most polymerases are accurate, with error frequencies for nucleotide incorporation ranging from 10^{-3} to 10^{-6} . However, a Watson–Crick base pair is only 0.2–4 kcal/mol more stable than a mismatched base pair in free solution, accounting for discrimination efficiencies between incorrect and correct base pair of $\sim 10^{-2}$ (2). Indeed, studies with nonpolar analogues of nucleotides have shown that replication can proceed efficiently even in the absence of hydrogen bonds (3–7). This led to the ‘steric-exclusion’ or ‘induced-fit’ model for fidelity of incorporation, whereby neither Watson–Crick hydrogen bonds nor canonical purine and pyrimidine structures are required for enzymatic synthesis of nascent base pairs (8,9). This model predicts that a combination of the active site size (steric exclusion) and flexibility (tightness) drives the selectivity of nucleotide incorporation (10). This model has been substantiated by studies showing efficient incorporation of a large pyrene deoxynucleoside triphosphate in front of an abasic (AP) site by *Escherichia coli* polymerase I (Klenow fragment, KF) and T7 polymerase (11). Furthermore, C4'-alkyl-substituted dTTP analogues were used to show that increasing the size of the sugar ring also leads to steric exclusion (12–15). Polymerases in families A, B, X and RT have binding pockets that tightly accommodate a correct Watson–Crick base pair, supporting the notion that nucleotide selectivity for these enzymes largely depends on geometric selection for the

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The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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shape and size of correct base pairs (16,17). Based on the crystal structures of binary and ternary complexes of polymerases with their substrates, it has been proposed that after the initial encounter between a dNTP and the polymerase-DNA binary complex, a rate-limiting transition from the open to the closed polymerase conformation would carry the dNTP into the active site where it would encounter the templating base. A correct complementarity would stabilize the catalytically competent close conformation (18–20). However, recent data have shown that some family X polymerases are exceptions. For example, kinetic studies have suggested that there is no rate-limiting step preceding catalysis for polymerase β (21), whereas crystallographic studies have shown that the related enzyme polymerase λ appears to be in a ‘closed’ conformation even in the absence of a bound dNTP (22). Understanding the mechanisms underlying the base selectivity of polymerases β and λ will have important implications also in light of their ability to overcome lesions on the template strand, such as AP sites and cisplatin adducts.

Recently, a novel class of non-nucleoside triphosphate analogues has been developed, in which the α -phosphate was esterified to bulky hydrophobic groups sterically similar to nucleosides but lacking the chemical functional groups of

nucleic acids (23,24). These analogues have been shown to be efficient substrates for the template-independent polymerase terminal-deoxynucleotidyl transferase (TdT), another member of the polymerase family X, leading to the suggestion that the base moiety did not participate significantly in dNTP binding to the active site of TdT, whereas the main contribution was made by the triphosphate moiety (23,24). Both polymerase β and polymerase λ share significant sequence and structural similarity with TdT (25). Moreover, polymerase λ possesses a template-independent terminal transferase (tdt) activity, in addition to the normal template-dependent polymerase activity (26). We were interested in investigating the ability of template-dependent polymerases to bind and eventually incorporate such non-nucleoside triphosphate analogues. In fact, no reports to date have shown that template-dependent DNA polymerases can incorporate triphosphate analogues lacking both the sugar and the nucleobase. Such analogues might help to further define the minimal requirements for incorporation by DNA polymerases. To this aim, we synthesized novel alkyltriphosphate analogues, bearing different substituents esterified at the α -phosphate position (Figure 1), and tested them in the presence of polymerases from the A, B, X and RT families, on undamaged and AP sites-containing DNA

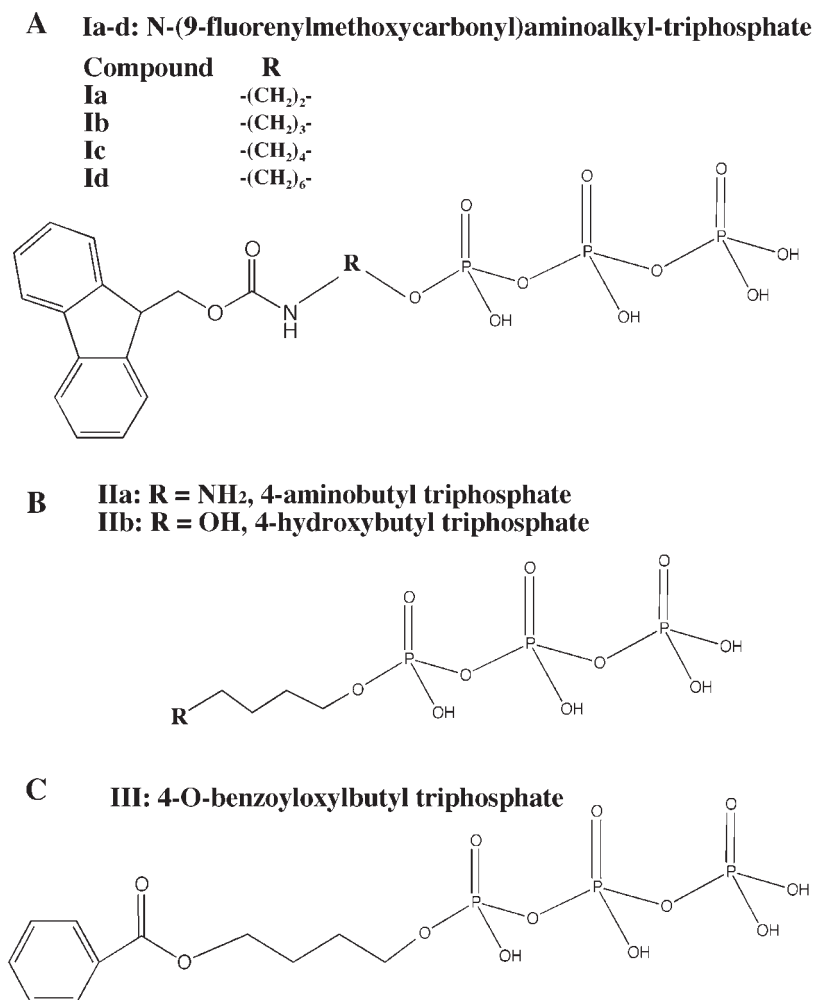


Figure 1. Structures of the compounds used in this study. (A) Ia-d fluorenylmethoxycarbonyl-derivatives. (B) IIa-b butyl-derivatives. (C) III benzoyloxy-derivative.

substrates. Our results showed that neither the base nor the sugar moieties of a nucleotide are an essential feature for its incorporation. Moreover, the incorporation efficiency depends on the particular enzyme, on the structure of the non-nucleoside analogue, on the metal activator and on the nature of the DNA template.

MATERIALS AND METHODS

Chemicals

[³H]dTTP (30 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were from Amersham Biosciences; unlabelled dNTPs, poly(dA) and oligo(dT)_{12–18} were from Roche Molecular Biochemicals. The oligonucleotides were from MWG Biotech (Florence, Italy). Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

Chemistry

Compounds **Ia–c** were synthesized by the reaction of the corresponding amino alcohols with 9-fluorenylmethyl chloroformate and subsequent phosphorylation by the Ludwig procedure without isolating intermediate monophosphates (27,28). Product **Id** was obtained in a similar manner from hexane-1,6-diol (Scheme 1).

Isolation and purification of the synthesized compounds were carried out using a Dowex-50 (NH₄)⁺ column followed by reverse-phase chromatography on LiChroprep RP-18. Compound **III** was synthesized by phosphorylation of 4-hydroxybutyl ester of benzoic acid according to the Ludwig procedure. Compounds **Ia** and **Ib** were obtained by hydrolysis of compounds **Ic** and **III**, respectively.

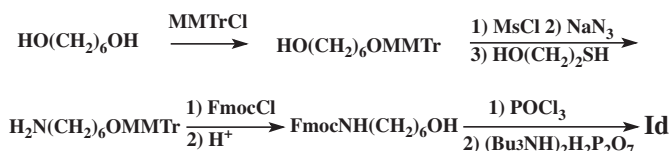
Nucleic acid substrates

The 73mer DNA oligonucleotide, either undamaged or containing the synthetic (tetrahydrofurane) AP site, and the corresponding primers were chemically synthesized and purified on denaturing polyacrylamide gel. The sequence of the d73mer is shown as follows: 5'-GATCGGGAGGGTAGGAA-TATTGAG[X/G]ATGAAGGGTTGAGTTGAGTGGAGAT-AGTGGAGGGTAGTATGGTGGATA-3'.

The sequences complementary to the 17 and 18mer primers are single- and double underlined, respectively. In brackets, the position of the lesion or the corresponding G residue in the undamaged template is indicated in bold letters (X, AP site).

The d66mer and d24mer oligonucleotides were purchased from Roche Molecular Biochemicals. The sequence of the d66mer is shown as follows: 5'-AGGATGTATGTTTAG-TAGGTACATAACTATCTATTGATACAGACCTAAAAC-AAAAAATTTTCCGAG-3'.

The sequence complementary to the d24mer is underlined.



Scheme 1. Schematic diagram of compound Id chemical synthesis.

Enzymes and proteins

Recombinant full-length human polymerase λ and the polymerase λ Y505A mutant were generated and purified as described previously (29–31). After purification, the proteins were >90% homogenous, as judged by SDS-PAGE and Coomassie staining (data not shown). Human polymerase α and HIV-1 RT were purified as described previously (32,33). Human polymerase β and calf thymus TdT were from Trevigen. *E. coli* polymerase I (KF) was from Roche Molecular Biochemicals.

Enzymatic assays

DNA polymerase assay. Human polymerase λ and *Saccharomyces cerevisiae* polymerase IV activity on poly(dA)/oligo(dT)_{10:1} were assayed in a final volume of 25 μ l containing 50 mM Tris-HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM MnCl₂, 0.2 μ M poly(dA)/oligo(dT)_{10:1} (3'-OH ends), 50 nM polymerase λ and 5 μ M [³H]dTTP (5 Ci/mmol), unless otherwise indicated in the figure legends. All reactions were incubated for 15 min at 37°C unless otherwise stated and the DNA precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described previously. Polymerase β , polymerase α , *E. coli* polymerase I (KF) and HIV-1 RT activities were assayed as described previously (29).

Terminal deoxyribonucleotidyl transferase assay. Polymerase λ and TdT terminal transferase activities were assayed in a final volume of 25 μ l containing 50 mM Tris-HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM MnCl₂ and 0.2 μ M of single-stranded 30mer DNA oligonucleotide, unless otherwise stated. Enzymes and [³H]dNTPs (10 Ci/mmol) were added as indicated in the figure legends. All reactions were incubated at 37°C for 10 min., unless otherwise indicated in the figures and the DNA precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described previously (34).

When 5'-³²P-labelled substrates were used, reactions were performed as described above in the presence of 0.2 μ M (3'-OH end) of the labelled substrate and unlabelled dNTPs as indicated in the figure legends. All reactions were incubated for 15 min at 37°C unless otherwise stated and resolved on 14% polyacrylamide-7 M urea. Reactions products were visualized and quantified by storage phosphor analysis with a Molecular Dynamics PhosphorImager using ImageQuant version 5.2 software.

Inhibition assays

Reactions were performed under the conditions described for the terminal deoxyribonucleotidyl transferase activity assay. Incorporation of radioactive dTTP into poly(dA)/oligo(dT) at different concentrations of DNA or dNTP was monitored in the presence of increasing amounts of inhibitor as indicated in the figure legends. Dose-response curves were generated by computer fitting of the data to the Equation 1:

$$E(\%) = \frac{E_{max}}{\left(1 + \frac{I}{ID_{50}}\right)} \quad 1$$

where $E_{(\%)}$ is the fraction of enzyme's activity measured in the presence of the inhibitor, E_{\max} is the activity in the absence of the inhibitor, I is the inhibitor concentration and ID_{50} is the inhibitor concentration at which $E_{(\%)}$ = $0.5E_{\max}$.

Steady-state kinetic analysis

The K_m and k_{cat} values were calculated according to the Briggs-Haldane model. Inhibition data were analysed according to the equation for the fully competitive mechanism of inhibition. K_i values were calculated from the variation of the values of the apparent affinities for the competing substrate (K_{app} values) as a function of the inhibitor concentrations, according to Equation 2:

$$K_{\text{app}} = \frac{K_m}{K_i} \cdot [I] + K_m. \quad 2$$

Initial velocities of the reaction were determined after 10 min of incubation at 37°C, which represent the midpoint of the linear range of the reaction, as determined in separate experiments (data not shown). Each experiment was performed in triplicate, and mean values were used for the analysis. Curve fitting was made using the computer program GraphPad Prism.

RESULTS

Non-nucleoside alkyltriphosphate analogues are able to inhibit different DNA polymerases

Novel substituted alkyltriphosphates analogues were synthesized (Figure 1, compounds I–III) and tested as inhibitors of various polymerases, such as human polymerase α , polymerase β , polymerase λ (both for its activity as a polymerase and as a terminal transferase), polymerase I of *E.coli*, polymerase IV of *S.cerevisiae* (Sc) and HIV-1 reverse transcriptase (RT). The results are summarized in Figure 2. All compounds were unable to inhibit the polymerase activity of *E.coli* polymerase I and HIV-1 RT. Compound IIa did not affect the polymerase activity of any of the enzymes tested. However,

the other compounds were able to repress the polymerase activity of polymerase α , polymerase β , polymerase λ and Sc polymerase IV, albeit with different potencies. It has been shown that polymerase λ is endowed with both a template-dependent (i.e. DNA polymerase) and a template-independent (i.e. terminal transferase) activity (26). Interestingly, the alkyltriphosphate analogues were able to inhibit polymerase λ both as a polymerase and as a terminal transferase.

Non-nucleoside alkyltriphosphate analogues are competitive inhibitors of DNA polymerases with respect to the nucleotide substrate

In order to determine the precise mechanism of inhibition, the active compounds were titrated in a polymerase assay in the presence of increasing DNA or nucleotide substrates concentrations. The inhibitors caused an increase in the apparent affinity for the nucleotide substrate (K_s), whereas the V_{\max} of the reaction was unaffected. By plotting the apparent K_s values (K_{sapp}) obtained in the presence of the inhibitors, as a function of the inhibitor concentrations, the corresponding K_i values were calculated and reported in Table 1. When analysed in the presence of different DNA substrate concentrations, the active compounds caused a reduction in the V_{\max} of the reaction, without affecting the apparent affinity of the enzyme for the substrate (data not shown). Thus, all the compounds proved to be competitive inhibitors with respect to the nucleotide substrate and non-competitive inhibitors with respect to the DNA substrate.

Non-nucleoside alkyltriphosphate analogues are not incorporated into a normal DNA template by DNA polymerase α , DNA polymerase λ and *S.cerevisiae* DNA polymerase IV

Next, we investigated whether the alkyltriphosphate analogues could be incorporated into the growing DNA chain by the different polymerases, or if these compounds were non-substrate inhibitors of the enzymes. To this aim, incorporation was monitored on the 5'-labelled heteropolymeric DNA

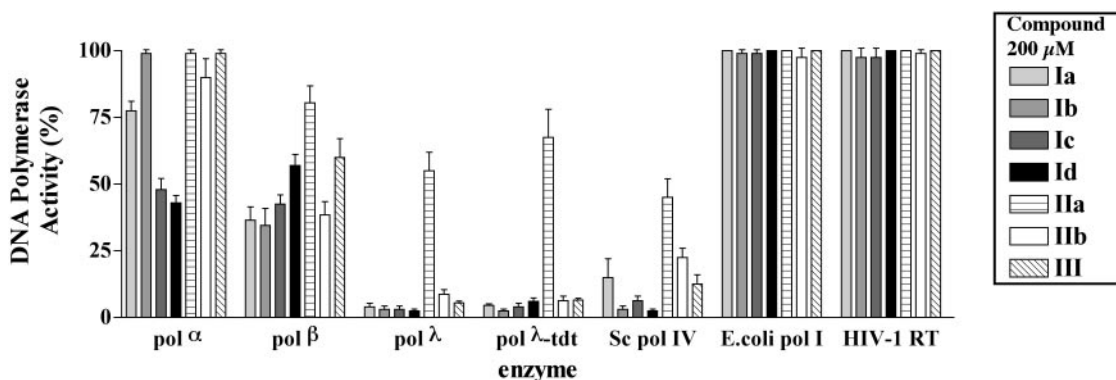


Figure 2. Alkyltriphosphate analogues are inhibitors of the nucleotide incorporation reaction catalysed by various DNA polymerases. The different compounds were tested at 200 μM final concentration in the presence of polymerase α (0.1 U), polymerase β (100 nM), polymerase λ (100 nM), Sc polymerase IV (200 nM), *E.coli* polymerase I (0.1 U) and HIV-1 RT (50 nM), under the conditions described in Materials and Methods. Since the molar concentration of polymerase α and polymerase I was not known, the amount added is expressed in Units. However, in all instances the amounts used for each enzyme resulted in similar values of pmols of dTMP incorporated in the control reactions. One unit of enzyme is equivalent to the incorporation of 1 nmol dTMP/h. Values are the means of three independent experiments; error bars represent SD units.

Table 1. Inhibition constants for alkyltriphosphate analogues towards DNA polymerases

Inhibitor K_i (μM) ^a	Enzyme Polymerase α	Polymerase β		Polymerase λ		Polymerase λ Y505A	Polymerase λ -tdt	ScPol IV
		+1 mM Mn ⁺⁺	+1 mM Mg ⁺⁺	+1 mM Mn ⁺⁺	+1 mM Mg ⁺⁺			
Ia	n.d. ^b	10 (\pm 1)	>400	4.5 (\pm 0.7)	5.6 (\pm 0.9)	0.74 (\pm 0.05)	1.5 (\pm 0.2)	0.45 (\pm 0.02)
Ib	n.d.	63 (\pm 5)	n.d.	2.2 (\pm 0.2)	n.d.	0.07 (\pm 0.01)	0.4 (\pm 0.06)	0.25 (\pm 0.03)
Ic	112 (\pm 15)	71 (\pm 9)	n.d.	2.1 (\pm 0.3)	n.d.	0.09 (\pm 0.01)	0.25 (\pm 0.05)	0.35 (\pm 0.03)
Id	145 (\pm 5)	n.d.	n.d.	2 (\pm 0.2)	n.d.	0.03 (\pm 0.002)	0.5 (\pm 0.06)	0.25 (\pm 0.05)
IIa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IIb	n.d.	39 (\pm 3)	>400	9 (\pm 1)	22 (\pm 1)	0.87 (\pm 0.07)	4.7 (\pm 0.5)	29 (\pm 4)
III	n.d.	n.d.	n.d.	1.5 (\pm 0.2)	2.2 (\pm 0.2)	0.35 (\pm 0.02)	1.4 (\pm 0.1)	2.2 (\pm 0.3)

^aInhibition constant (K_i) values are the means of three independent experiments and were determined as described in Materials and Methods. Numbers in brackets are \pm SD.

^bn.d., not determined.

primer–template d24/d66mer and the reaction products were visualized on a sequencing gel. As shown in Figure 3A (lanes 1–7), none of the compounds was incorporated into the nucleic acid chain by polymerase λ polymerase activity. Similarly, polymerase α and Sc polymerase IV were unable to incorporate the compounds (data not shown). Next, we evaluated whether the inability of the different polymerases to incorporate the alkyltriphosphate analogues was due to the nature of the templating base. The d24/d66mer used in the experiments described above had the base guanosine at +1 position (see the sequence in Figure 3A). In order to evaluate the incorporation of the compounds on substrates having different bases at +1 position, we incubated our substrate d24/d66mer with dCTP at 37°C for 10 min, to obtain the extension of the primer of just one base so as to have adenine as the base at +1 position (Figure 3A, lanes 8–11). Similarly, incubating our template in the presence of both dCTP and dTTP resulted in a primer extended by 2 nt, with cytosine being the base at +1 position (Figure 3A, lanes 12–14), whereas addition of dCTP, dTTP and dGTP resulted in a primer extended by 4 nt, with thymidine as the templating base at +1 position (Figure 3A, lanes 16–19). Primer extension reactions by polymerase λ in the presence of the different combinations of dNTPs were performed for 5 min at 37°C. Then, alkyltriphosphate analogues were added and the reaction was incubated for additional 20 min. As shown Figure 3A, the compounds were not incorporated by polymerase λ , no matter which base was present at the +1 position. Identical results were obtained with polymerase α and Sc polymerase IV (data not shown).

The ability of DNA polymerase λ to incorporate the alkyltriphosphate analogues depends both on the structure of the inhibitor and on the presence of an abasic site in the template strand

The results shown in Figure 3A indicated that alkyltriphosphate analogues were not incorporated into the nucleic acid chain, although they were competitive inhibitors with respect to the nucleotide substrate (Figure 2 and Table 1). One possibility could be that, even though they can bind to the enzyme's nucleotide binding pocket, due to their unusual structures they cannot be properly aligned with the 3'-hydroxyl end of the primer, thus preventing phosphodiester bond formation. However, we hypothesized that, in the presence of particular DNA template/primer structures, the block towards the

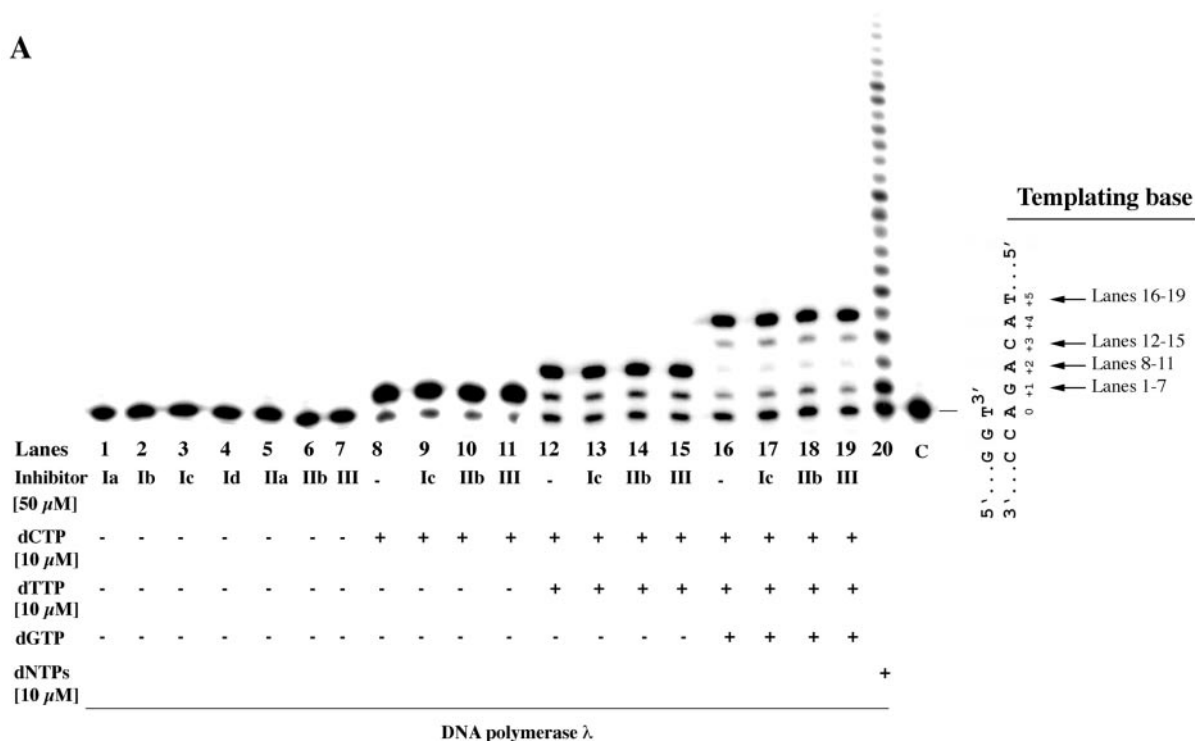
transition to a catalytically competent ternary complex could be removed and the analogues incorporated. It is known that polymerase λ can efficiently perform translesion synthesis in the presence of an AP site on the template strand (35). Owing to the lack of the base moiety, an AP site is considered a non-instructional lesion, and the steric requirements for the alignment and incorporation of an incoming nucleotide in front of an AP site are different from those operating in the presence of a templating base. Thus, we asked whether an AP site would allow incorporation of alkyltriphosphate analogues. To this aim, we employed the heteropolymeric DNA primer–template d18/d73mer as a substrate for our incorporation assay, which bears either a G or a synthetic AP site (tetrahydrofurane moiety) at +1 position. As shown in Figure 3B, polymerase λ (as a polymerase) was unable to incorporate any of the compounds on the intact template, as expected (lanes 5–8), but showed clear incorporation of the compound **III** opposite to an AP site (lane 2). On the other hand, Sc polymerase IV and human polymerase α were unable to incorporate the alkyltriphosphate analogues, either in the absence or in the presence of an AP site (data not shown).

The mutation Y505A in the active site of DNA polymerase λ allows incorporation of different non-nucleoside alkyltriphosphate analogues in the presence of an abasic site

We have previously shown that the residue Tyr 505 in the polymerase λ catalytic site is important for nucleotide binding (30). Resolution of the 3D structure of the enzyme showed that the Tyr 505 side chain contributes to determine the size and geometry of the nucleotide binding pocket of polymerase λ (22). We, therefore, investigated whether the substitution of Tyr 505 with a shorter Ala side chain had any effect on the binding and incorporation of alkyltriphosphate analogues.

As summarized in Table 1, the compounds **Ia–d**, **IIb** and **III** showed potencies of inhibition 10- to 75-fold higher towards the Y505A mutant than towards the wild-type enzyme. Thus, an increase in the size of the nucleotide binding site of polymerase λ correlated with a tighter binding of the alkyltriphosphate analogues. Starting from these observations, we tested whether polymerase λ Y505A, as a polymerase, could incorporate any of the alkyltriphosphate analogues. As shown in

A



B

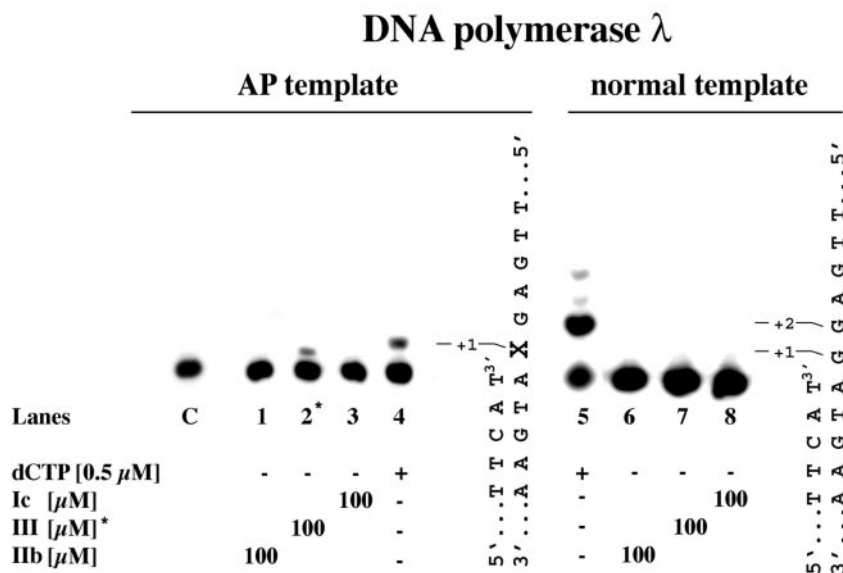


Figure 3. Alkyltriphosphate analogues are incorporated by DNA polymerase λ in the presence of an AP site only. (A) Polymerase λ (100 nM) was incubated in the presence of the 5'-labelled d24/d66mer DNA template and 50 μ M of the various compounds without any preincubation (lanes 1–7) or was preincubated 5 min in the presence of DNA template and 10 μ M dCTP (lanes 8–11), 10 μ M dCTP and dTTP (lanes 12–15), 10 μ M dCTP, dTTP and dGTP (lanes 16–19), before addition of the compounds to be tested. Lane 20, control reaction with all four dNTPs; lane C, labelled template and enzyme only. (B) Polymerase λ (100 nM) was incubated in the presence of the 5'-labelled d18/d73mer DNA template either undamaged (lanes 5–8) or with an AP site at position +1 (lanes 1–4), and in the presence of 100 μ M compound **Ic** (lanes 3 and 8), **III** (lanes 2 and 7), **IIb** (lanes 1 and 6) or 0.5 μ M dCTP (lanes 4 and 5). Lane C, labelled template and enzyme only.

Figure 4A, polymerase λ Y505A was not able to incorporate any of the compounds tested on a normal substrate (lanes 1–8), similarly to the wild-type form of the enzyme. However, after the incubation of the mutant enzyme with an AP substrate, the mutant Y505A was able to incorporate not only compound **III** (lane 11), as the wild-type enzyme, but also the compounds **Ia–c** (lanes 12–14).

DNA polymerase λ as a terminal transferase is not able to incorporate any non-nucleoside alkyltriphosphate analogue

The results obtained with the AP site substrate seemed to indicate that a major barrier for the alkyltriphosphate analogues incorporation by polymerase λ was of steric nature, particularly with respect to the presence of a templating base.

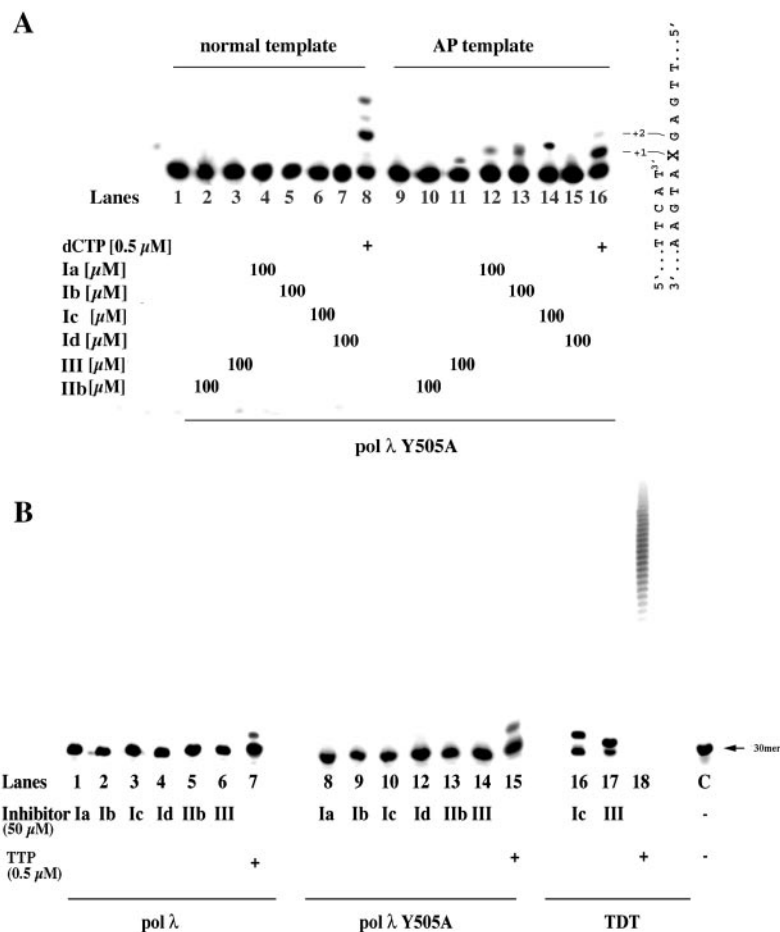


Figure 4. The Y505A mutation increases the ability of incorporation of alkyltriphosphate analogues by DNA polymerase λ as a polymerase but not as a terminal transferase. (A) The polymerase λ mutant Y505A (100 nM) was incubated in the presence of the 5'-labelled d18/d73mer DNA template either undamaged (lanes 1–8) or with an AP site at position +1 (lanes 9–16), and in the presence of 100 μ M compound **Ib** (lanes 2 and 10), **III** (lanes 3 and 11), **Ia** (lanes 4 and 12), **Ib** (lanes 5 and 13), **Ic** (lanes 6 and 14), **Id** (lanes 7 and 15) or 0.5 μ M dCTP (lanes 8 and 16). Lanes 1 and 9, labelled template and enzyme only. (B) An aliquot of 100 nM of polymerase λ wild-type (lanes 1–7), of the polymerase λ mutant Y505A (lanes 8–15) or 0.1 U of calf thymus TdT (lanes 16–18), were incubated in the presence of the 5'-labelled d30mer single-stranded DNA template and 50 μ M of compound **Ia** (lanes 1 and 8), **Ib** (lanes 2 and 9), **Ic** (lanes 3, 10 and 16), **Id** (lanes 4 and 12), **Iib** (lanes 5 and 13), **III** (lanes 6, 14 and 17), or 0.5 μ M dTTP (lanes 7, 15 and 18). Lane C, labelled oligo and enzyme only.

Since polymerase λ is also able to incorporate nucleotides in a template-independent manner, through its tdt activity, we next investigated whether polymerase λ tdt was able to incorporate alkyltriphosphate analogues into a single-stranded DNA chain. As a substrate for the incorporation reaction, we used a single-stranded DNA 30mer oligonucleotide, which proved to be an efficient substrate for dTTP incorporation by polymerase λ tdt (26). As a positive control, we used calf thymus TdT (A. Khandazhinskaya, M. Jasko, L. Alexandrova and M. Kukhanova, manuscript in preparation).

As shown in Figure 4B, although the TdT enzyme was able to incorporate the alkyltriphosphate analogues as expected (lanes 16–18), no incorporation was detected with polymerase λ tdt, either wild-type (lanes 1–7) or the Y505A mutant (lanes 8–15).

DNA polymerase β is able to incorporate different non-nucleoside alkyltriphosphate analogues either in the absence or in the presence of an abasic site

Polymerase λ belongs to the DNA polymerase family X, along with TdT, Sc polIV and polymerase β . The results shown

above already highlighted important differences among polymerase λ and its relatives TdT and Sc polIV, with respect to the incorporation of alkyltriphosphate analogues. As summarized in Table 1, polymerase β activity was also inhibited by compounds **Ia–c** and **Iib**. It was, thus, of interest to investigate the behaviour of polymerase β in the presence of the same compounds. As shown in Figure 5A, polymerase β proved to be able to incorporate compounds **Ia–c** either in the absence (lanes 2–4) or in the presence (lanes 7–9) of an AP site. On the other hand, Compound **Id** was not incorporated. These results indicated a clear difference in the mechanism of nucleotide selection within the polymerase X family, between polymerase β , polymerase λ and Sc polymerase IV.

DNA polymerase β , DNA polymerase λ wild-type and the DNA polymerase Y505A mutant show different specificities for alkyltriphosphate analogues incorporation

The results shown above indicated that both polymerase λ and polymerase λ Y505A were able to incorporate compound **III**, but only in the presence of an AP site on the template strand. In

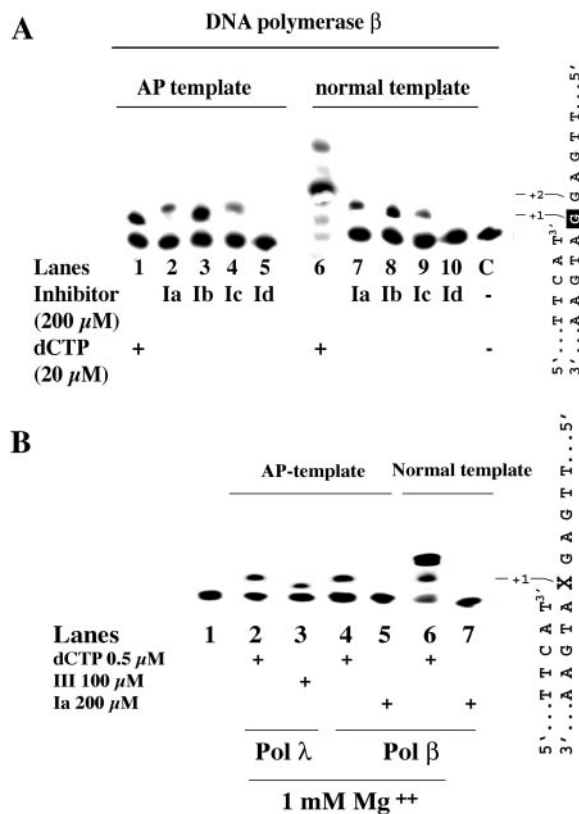


Figure 5. DNA polymerase β , but not DNA polymerase λ , can incorporate alkyltriphosphate analogues in a Mn^{++} -dependent manner exclusively. (A) Polymerase β (100 nM) was incubated in the presence of the 5'-labelled d18/d73mer DNA template either undamaged (lanes 6–10) or with an AP site at position +1 (lanes 1–5), and in the presence of 200 μ M of compound **Ia** (lanes 2 and 7), **Ib** (lanes 3 and 8), **Ic** (lanes 4 and 9), **Id** (lanes 5 and 10) or 20 μ M dCTP (lanes 1 and 6). Lane C, labelled template and enzyme only. (B) An aliquot of 100 nM of polymerase λ or polymerase β were incubated in the presence of the 5'-labelled d18/d73mer DNA template either undamaged (lanes 6 and 7) or with an AP site at position +1 (lanes 1–5), and in the presence of 100 μ M compound **III** (lane 3), 200 μ M compound **Ia** (lanes 5 and 7) or 0.5 μ M dCTP (lanes 2 and 4). Lane 1, labelled template and enzyme only.

addition, polymerase λ Y505A was also able to incorporate compounds **Ia–c** in front of an AP site. Conversely, polymerase β could not utilize compound **III**, but was able to incorporate compounds **Ia–c** both in the absence and in the presence of an AP site. In order to better understand the molecular basis for these different specificities, we determined the kinetic parameters (K_m , k_{cat} and k_{cat}/K_m) for alkyltriphosphate analogue incorporation of polymerase β , polymerase λ and polymerase λ Y505A. The calculated values are reported in Table 2. As can be seen, polymerase λ wild-type mutant incorporated compound **III** with a 380-fold lower efficiency than dCTP in front of the AP site. This large difference was reflected by a higher K_m value of compound **III** with respect to dCTP. The mutation Y505A improved the efficiency of incorporation of compound **III** of \sim 4-fold, with an increase in the apparent affinity (lower K_m value) of compound **III** for the polymerase λ mutant. Polymerase β incorporated compound **Ic** on the undamaged template (i.e. with a G on the template strand) with a 60-fold lower efficiency than dCTP. Again, this low incorporation efficiency was reflected by a

Table 2. Kinetic parameters for incorporation of alkyl triphosphate analogues by polymerase λ and polymerase β on normal and AP-containing templates^a

Substrate	Polymerase λ		Polymerase λ Y505A		Polymerase β	
	AP-site template K_m (μ M)	k_{cat} (min^{-1})	AP-site template K_m (μ M)	k_{cat} (min^{-1})	Undamaged template k_{cat} (min^{-1})	AP-site template K_m (μ M)
dCTP	0.016 (\pm 0.001)	0.005 (\pm 0.001)	0.03 (\pm 0.003)	0.005 (\pm 0.001)	0.14 (\pm 0.05)	2 (\pm 0.2)
III	1.3 (\pm 0.2)	0.001 (\pm 0.0002)	0.5 (\pm 0.07)	0.0008 (\pm 0.0001)	n.a.	n.a.
Ic	n.a.	n.a.	0.03 (\pm 0.002)	0.004 (\pm 0.0005)	14 (\pm 3)	9 (\pm 1)
			k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)
			0.31 (\pm 0.05)	0.17 (\pm 0.02)	0.17 (\pm 0.02)	0.008 (\pm 0.001)
			0.0008 (\pm 0.0001)	0.0016 (\pm 0.0002)	n.a.	n.a.
			n.a.	0.13 (\pm 0.01)	0.02 (\pm 0.008)	0.002 (\pm 0.001)

^a K_m and k_{cat} values were determined as described in Materials and Methods. Values are the means of three independent experiments. Numbers in brackets are \pm SD.

large difference in the apparent binding constant (K_m) between dCTP and compound **Ic**. Interestingly, both polymerase λ Y505A and polymerase β incorporated compound **Ic** in front of an AP site with similar apparent binding affinities (K_m) and catalytic rates (k_{cat}) to dCTP, resulting in nearly the same efficiency. This indicated that the presence of a templating base played a key role in determining the specificity of incorporation of the alkyltriphosphate analogue **Ic** versus a canonical nucleotide.

The binding and incorporation of alkyltriphosphate analogues depends on Mn^{++} as the metal activator for DNA polymerase β but not for DNA polymerase λ

All the experiments discussed above for polymerase β and polymerase λ were performed in the presence of Mn^{++} , which is the optimal metal coactivator for both enzymes *in vitro*. However, it is well known that Mn^{++} can alter the fidelity of DNA synthesis by polymerase β . In addition, kinetic and structural studies have revealed differences in the utilization of Mn^{++} and Mg^{++} between polymerase β and polymerase λ (36,29). In order to investigate whether the observed effects of alkyltriphosphate analogues were dependent on the metal coactivator, inhibition studies were performed in the presence of Mg^{++} for some representative compounds. As reported in Table 1, inhibition of polymerase λ was not significantly affected by replacement of Mn^{++} with Mg^{++} . However, polymerase β , in the presence of Mg^{++} , became completely resistant to inhibition. Accordingly, polymerase λ was still able to incorporate compound **III** in front of an AP site in the presence of Mg^{++} (Figure 5B, lanes 1–3), whereas polymerase β could not incorporate compound **Ia** either in the presence or in the absence of an AP site (Figure 5B, lanes 4–7).

DISCUSSION

We have employed non-nucleoside triphosphate analogues bearing different substituents at the α -phosphate (Figure 1) as means to probe the ability of template-dependent polymerases to bind and incorporate triphosphates lacking both the sugar and the base moieties. This information could be helpful in determining the minimal requirements for incorporation by template-dependent DNA polymerases. The substituents esterified to the α -phosphate in place of the nucleoside moiety ranged from relatively small and hydrophilic groups (as in compounds **Ia** and **Ib**) to bulky hydrophobic molecules (as in compounds **Ia–d** and **III**). As shown in Figure 2 and Table 1, polymerase α (family A) and polymerase β , polymerase λ and Sc polymerase IV (family X) were inhibited by some of the tested compounds, whereas *E. coli* polymerase I (KF) (family A) and HIV-1 RT were completely unaffected. The inhibition was competitive with the nucleotide substrate in all cases, indicating binding of the tested compounds to the enzyme's nucleotide binding pocket. In the case of polymerase λ and Sc polymerase IV, the potency of inhibition increased with the length of the alkylic chain, whereas polymerase β showed an opposite trend (Table 1). Compounds **Ic**, **Ia**, **Ib** and **III** are 1,4-butyldiene-triphosphate derivatives (Figure 1) bearing different substituents. It can be seen that for polymerase λ and Sc polymerase IV, the inhibition potencies

increased with an increase in the size and hydrophobicity of the substituent (compare compound **III** with compound **Ic** in Table 1). Again, polymerase β behaved differently, since compound **III** was totally inactive, whereas compound **Ib** was more active than **Ic** (Table 1). Thus, even though one has to be cautious in drawing general conclusions, our results suggest that binding of alkyltriphosphate analogues to polymerase λ and Sc polIV positively correlated with the length of the alkylic chain and the size and hydrophobicity of the substituent, whereas binding to polymerase β showed an opposite trend. As shown by structural studies, DNA polymerase β undergoes open-to-closed conformational transition when binding a dNTP (37,38), whereas polymerase λ adopts always a 'closed' structure even in the absence of substrates (39). Thus, it is possible to hypothesize that an increased spacing between the fluorenylmethoxy substituent and the triphosphate moiety in compounds **Ia–d** would inhibit polymerase β , by impairing its open-to-closed conformational transition, but not polymerase λ which seems not to be dependent on such transition. The significantly lower potencies of inhibition showed by some compounds towards polymerase α with respect to polymerase λ and polymerase β suggest that family B polymerases are more efficient in discriminating against non-nucleoside triphosphate analogues than family X enzymes.

Kinetic and structural studies have shown that the residue Tyr 505 of polymerase λ plays an important role in dNTP binding (30). In the DNA template–polymerase λ binary complex, Tyr 505 obstructs the nucleotide binding pocket through an hydrogen bond with the templating base (39). The alkyltriphosphate analogues showed potencies of inhibition 10- to 75-fold higher towards the polymerase λ Y505A mutant, where the Tyr side chain has been replaced by a shorter Ala, than towards the wild-type enzyme (Table 1). Interestingly, the largest effect was observed for compounds **Ia–d**, bearing the large fluorenylmethoxy substituent. The better fitting of this bulky substituent in the larger nucleotide binding pocket of the Y505A mutant polymerase λ supports the notion of a steric-check based mechanism for discrimination against this non-nucleoside analogue.

Interestingly, polymerase λ was able to incorporate only compound **III** and exclusively when presented with an AP site on the template strand. Replacement of Tyr 505 with Ala also allowed incorporation of compounds **Ia–c** by the mutant Y505A but always only in front of an AP site, i.e. in the absence of a templating base. As reported in Table 2, incorporation of compound **Ic** in front of the AP site by polymerase λ Y505A occurred with an efficiency identical to the nucleotide substrate dCTP. We have already shown that polymerase λ bypasses an AP site by a template-slippage mechanism, using the base downstream the lesion as the templating one (40). Thus, dCTP incorporation in the presence of the AP site in our experiments was driven by the G at position +2 of the template (Figures 3C and 4A). Conversely, compound **Ic** was likely incorporated in front of the lesion, since we could not detect incorporation of any of the alkyltriphosphate analogues opposite to any of the four bases A, T, G and C (Figure 3A) by polymerase λ . It has to be mentioned here that, in all cases, the incorporated analogues could not be elongated, thus acting as true chain terminators of the DNA synthesis reaction (data not shown).

DNA polymerase β was able to incorporate compounds **Ia–c** independently from the presence of an AP site on the template, but this effect was strictly dependent on Mn^{++} as the metal activator. Structural studies have shown that in the presence of Mn^{++} , the nucleotidyl transfer reaction by polymerase β takes place in a random manner without the need for instructions from the template (36). When polymerase β was tested in the presence of Mg^{++} as the metal activator, it was completely unable to bind and incorporate the alkyltriphosphate analogues (Table 1 and Figure 5). Conversely, in the presence of Mg^{++} , polymerase λ was able to bind and incorporate our compounds in a very similar manner as in the presence of Mn^{++} (Table 1 and Figure 5). The crystal structure of the ternary polymerase λ -DNA-dNTP complex showed only one Mg^{++} ion coordinated with the α , β and γ phosphates of the incoming dNTP (39), contrary to the two metals usually present in polymerase β structures (37,38). Thus, it is possible that either one Mg^{++} or one Mn^{++} might bind to polymerase λ without causing a significant alteration of the active site. However, the differences in size, binding and reactivity between Mn^{++} and Mg^{++} ions are exacerbated in polymerase β , which requires two metal ions for catalysis.

DNA polymerase λ also possesses a template-independent tdt-like activity, which was potentially inhibited by all the compounds tested, with the exception of **IIa** (Table 1). However, the tdt activity of polymerase λ , either wild-type or the Y505A mutant, was completely unable to incorporate any of the tested compounds, contrary to TdT (Figure 4B). Superimposition of the available crystal structures of human polymerase λ and murine TdT revealed a number of differences in the nucleotide binding pocket (22,39,41). Thus, the different nature of the amino acid side chains involved in stabilization of the incoming dNTP might explain the different behaviour of polymerase λ -tdt and TdT with respect to alkyltriphosphate analogues incorporation.

In summary, our results showed that both the nucleobase and the ribose sugar are neither essential for binding nor for incorporation by family X DNA polymerases and can be substituted by hydrophobic molecules of a suitable size. The incorporation efficiencies of the alkyltriphosphate analogues did not correlate with their binding affinities to the different polymerases. For example, polymerase λ , polymerase α and Sc polymerase IV were able to bind but not to incorporate the tested compounds on a normal DNA template, irrespectively from the identity of the templating base. Thus, binding of the non-nucleoside triphosphate analogues to the DNA-polymerase complex can occur with high affinity even though the compound is not incorporated. In addition, incorporation of non-nucleoside alkyltriphosphate analogues of the kind of compounds **Ia–c** by DNA polymerase λ occurs only opposite to AP sites. Thus, non-nucleoside chain-terminating triphosphate analogues specific for AP sites, as the ones identified in this study, warrant further development in light of their potential biotechnological applications.

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