Expanding the repertoire of DNA polymerase substrates: template-instructed incorporation of non-nucleoside triphosphate analogues by DNA polymerases $\beta$ and $\lambda$

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ABSTRACT

We have recently shown that neither the base nor the sugar moieties of a nucleotide is an essential feature for its incorporation by DNA polymerases (pols) $\lambda$ and $\beta$. Here we present the identification of novel non-nucleoside triphosphate (NNTP) derivatives belonging to three classes: (i) non-substrate-specific inhibitors of DNA pol $\lambda$; (ii) substrate inhibitors which could preferentially be incorporated by either DNA pol $\lambda$ wild type or its Y505A mutant and (iii) the substrate inhibitor N-(Biphenylcarbonyl)-4-oxobutyl triphosphate which could be incorporated exclusively by DNA pol $\beta$ in a $\text{Mg}^{2+}$-dependent manner, and preferentially pairs with A on the template. This compound represents the first example of a substrate lacking both nucleobase and ribose residue, showing distinct base-pairing properties with normal bases. Therefore, this NNTP analog can be considered as the prototype of an entirely novel class of DNA pol substrates.

INTRODUCTION

DNA polymerase (pol) $\lambda$ is a member of the pol family X, together with DNA pol $\beta$, DNA pol $\mu$ and TDT (1). DNA pol $\lambda$ is endowed with both template-dependent and template-independent (i.e. terminal transferase) DNA polymerase activities, as well as dRPlyase activity, suggesting multiple cellular roles for the enzyme (2,3). Indeed, the gene encoding DNA pol $\lambda$ was shown to be expressed at high level in the developing mouse testis (2) indicating a possible function of DNA pol $\lambda$ in DNA synthesis associated with meiosis. Additional results suggest that DNA pol $\lambda$ may be involved in a PCNA-dependent DNA translesion synthesis pathway and in the repair of DSBs (4–8). DNA pol $\lambda$ shares 33% sequence identity with DNA pol $\beta$ (3,9,10). However, the template-dependent polymerase activity of DNA pol $\lambda$ has some distinct features compared with the one of DNA pol $\beta$. In particular, DNA pol $\lambda$ appears to have evolved towards having a preference for $\text{Mn}^{2+}$ as the metal activator (11).

DNA pols 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 shape and size of correct base pairs (12,13). A correct complementarity is required to stabilize the catalytically competent closed conformation (14–16). However, kinetic studies have suggested that there is no rate-limiting step preceding catalysis for DNA pol $\beta$ (17), whereas crystallographic studies have shown that the related enzyme DNA pol $\lambda$ appears to be in a ‘closed’ conformation even in the absence of a bound dNTP (10). Understanding the mechanisms underlying the base selectivity of DNA pols $\beta$ and $\lambda$ will have important implications also in light of their ability to overcome lesions on the template strand such as AP sites and cisplatin adducts. Moreover, a number of evidences suggest that DNA pols $\lambda$ and $\beta$ show altered expression in tumors (18). Abnormal levels of these enzymes might, in turn, lead to genetic instability accelerating the cell progression towards a more severe
tumoral phenotype. Thus, identifying the substrate recognition properties and the selectivity of these DNA pols might lead to the development of specific inhibitors which, in turn, can be developed into novel antitumoral drugs.

Nucleoside analogs have since long been investigated as DNA pols inhibitors, particularly for viral and bacterial enzymes (19). They can be broadly divided in two classes: (i) acyclic compounds having a natural base or a base analog but lacking a sugar and (ii) analogs bearing either non-standard bases or modified sugars or both. The classical example of the first class is the antiviral compound acyclovir, which has been designed to target herpes virus DNA pol, but can also act as a substrate for cellular enzymes (20). Examples of the second class are nucleosides bearing methylated bases, non-polar nucleobase isosteres and modified sugars. All these analogs have been shown to be recognized by viral, bacterial or cellular DNA pols as substrates (21). However, even though modified, all of them possess either a base or a sugar or both and thus can be classified as nucleoside analogs.

As a consequence of the urgent need of novel antivirals, particularly against human immunodeficiency virus infections, novel non-nucleoside inhibitors of DNA pols emerged recently. They have very different chemical structures, but all share two main properties: they do not carry a triphosphate moiety and they are not substrates for the target enzymes, i.e. they are not incorporated into a nascent nucleic acid chain (22,23).

We aimed to develop non-nucleoside molecules which, on the other hand, could be incorporated by DNA pols. We started from a very simple skeleton, namely an ethyl- or butyl-chain, carrying on one end a triphosphate moiety and on the other end bulky hydrophobic groups sterically similar to nucleosides but lacking the chemical functional groups of nucleobases. Our studies identified non-nucleoside triphosphate analogues (NNTPs) carrying the benzoyl-oxy-butyl moiety (Figure 1), as selective substrates for DNA pol β and λ (24). That was the first demonstration that neither the base nor the sugar moieties of a nucleotide is an essential feature for its incorporation by DNA pols λ and β. However, the incorporation of NNTPs by DNA pol λ was restricted either to DNA strands lacking a templating base, as in the case of abasic (AP) sites, or to the mutant enzyme Y505A, with a larger nucleotide binding pocket. On the other hand, incorporation of NNTPs by DNA pol β was strictly dependent on Mn²⁺ as the metal activator. Thus, starting from these results, we synthesized novel derivatives, with the aim of (i) improving selectivity of the NNTPs towards either DNA pol λ or DNA pol β and (ii) further exploring the molecular determinants linking the incorporation efficiency to the DNA template and the enzyme active site structure. In this study, we used three different classes of NNTPs, whose structure is shown in Figure 1: compounds of the series I have a butyl-triphosphate chain carrying nucleobase-like moieties, but lacking a sugar. Compounds of the series II are close analogs of our lead benzoyl-oxy-butyl triphosphate. Compounds of the series III represent 2,4-dinitrophenyl amino-butyl and -ethyl derivatives carrying various substituents. The aim of the study was to understand how their different chemical structures might modulate their properties as DNA pols substrates.

**MATERIALS AND METHODS**

**Chemicals**

\[{1}\textsuperscript{H}-\textsuperscript{32}P]ATP (3000 Ci/mmol) and \[{3}\textsuperscript{H}]dTTP (40 Ci/mmol) were from GE Healthcare Biosciences, and unlabeled dNTPs, poly(dA)₂₀₀ and oligo(dT)₁₆ were from Roche Molecular Biochemicals. All other reagents were of analytical grade and were purchased from Merck or Fluka.

**Chemistry**

(9-Adenylmethylcarbonyl)-4-aminobutyl triphosphate (Ia) was obtained by phosphorylation of (9-adenylmethylcarbonyl)-4-aminobutanol (25,26). Yield 24%. \[^{1}\text{H}]\text{NMR (D}_2\text{O): 8.20 and 8.10 (2H, 2s, H2 and H8), 4.99 (2H, s, CH}_2\text{Ade), 3.95 (2H, m, CH}_2\text{O), 3.25 (2H, m, CH}_2\text{N), 1.65–1.55 (4H, m, CH}_2\text{CH), 31}^\text{P}\text{NMR (D}_2\text{O): }-5.79 \text{ (1P, } d, J \text{ 17.2, P}_a\text{), }-10.05 \text{ (1P, } d, J \text{ 19.1, P}_b\text{), }-21.68 \text{ (1P, dd, P}_b\text{). UV (H}_2\text{O, pH 6): }A_{\text{max}} \text{ 259 nm ( } \varepsilon \text{ = 15 000). Mass (m/e): 503.0 (M}^+ - 1).
Compounds I b and I I a, b were synthesized according to earlier described procedure (27–29).

2-Thiomethyl-6-phenyl-4-(4′-hydroxybutyl)-1,2,4-triazole (5,1-C(1,2,4)triazine-7-one triphosphate (II b) was obtained according to earlier described procedure (27) starting from 2-thiomethyl-6-phenyl-4-(4′-hydroxybutyl)-1,2,4-triazole(5,1-H) (1,2,4) triazine-7-one. UV (H2O, pH 6): \( \lambda_{\text{max}} 250 \text{ nm} \). Yield 23%. \( ^1 \)H NMR (D2O): 7.89, 7.51 (5H, m, CH2), 4.42 (2H, m, CH2O), 3.99 (2H, m, CH2N), 2.68 (3H, s, SCH2), 2.04, 1.74 (4H, m, (CH2)2). \( ^{31} \)P NMR (D2O): -9.28 (1P, m, P), -10.26 (1P, m, P), -22.10 (1P, m, P).

\( \text{N-(Benzzyloxycarbonyl)-4-aminobutyl triphosphate (IIa)} \)

was obtained by phosphorylation of \( \text{N-(benzyloxycarbonyl)-4-aminobutanol} \) according to (27). Yield 31%. \( ^1 \)H NMR (D2O): 7.40 (5H, m, C6H5), 5.08 (2H, s, CH2 (Cbz)), 3.54 (2H, t, CH2O), 3.45 (2H, m, CH2N), 1.77 (4H, m, (CH2)2). \( ^{31} \)P NMR (D2O): 20.3 Hz, P, 19.3, P, 17.500, 17 500). 1HN M R(D2O): 9.19 (1H, d, 10.12 (1P, d, 19.3, P), -10.36 (1P, d, 18.9, P3), 22.68 (1P, dd, P0).

(1,2,4) triazine-7-one triphosphate (IIb).

was as described in (33). \( \text{Nucleic acids substrates. All oligonucleotides were purified from polyacrylamide denaturing gels. The sequences are as follows: 18/75merAP/Control: 5′-GATCGGGAGGTTAGA-TATTAG/LX[5]GATGAAAGGTGGATGAG-TGAGATAGTGAGCTG-3′; 18/40merA: 3′-ATAGGTTTATAGGGAACGTAT-GATAGGATGT-5′; 19/40merT: 3′-ATAGGTTTATAGGGAACGTAT-GATAGGATGT-5′; 20/40merG: 3′-ATAGGTTTATAGGGAACGTAT-GATAGGATGT-5′; 19mer: 5′-CCGTCATTACCTGTAGTCT-3′. The sequence underlined corresponds to the primer hybridization position. X indicates the position of the AP site, which corresponds to a G in the control template. The 19mer was used as a primer for TDT. The different primers were 5′-labelled with T4 polynucleotide kinase (New England Biolabs) in the presence of [\( ^32 \)P]nATP. For the preparation of the corresponding primer/template substrates, each labelled primer was mixed to the complementary template oligonucleotide at 1:1 (M/M) ratio in the presence of 25 mM Tris–HCl (pH 8.0) and 50 mM KCl, heated at 80°C for 3 min and then slowly cooled down at room temperature.}

Proteins production and purification

Recombinant histagged human wild-type and Y505A mutant pol \( \lambda \) were expressed and purified as described previously (4.31). After purification, the proteins were >90% homogenous, as judged by SDS–PAGE and Coomassie staining. Human pol \( \beta \) and calf thymus TDT were from Trevigen.
Enzymatic assays

**DNA polymerase assay.** Human pol λ and pol β activities on poly(dA)/oligo(dT)_{10:1} were assayed in a final volume of 25 μl containing 50 mM Tris–HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM MnCl₂, −0.2 μM poly(dA)/oligo(dT)_{10:1} (3'-OH ends), 50 nM pol and 5 μM [³²P]dTTP (5 Ci/mmol), unless otherwise indicated in the figure legends. All reactions were incubated at 37°C for 15 min unless otherwise stated and the DNA precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described in (4).

**Terminal deoxynucleotidyl transferase assay.** Pol λ template-independent (i.e. terminal transferase) activity was assayed in a final volume of 25 μl containing 50 mM Tris–HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, and 30 nM 0.3 pmols of 3'-OH ends of the different 5'-³²P-labelled primer/templates, unless otherwise stated and 30 mM enzyme. Concentrations of NTTPs, dNTPs and either Mg²⁺ or Mn²⁺ were as indicated in the corresponding figure legends. Reactions were incubated at 37°C for 10 min, and then stopped by the addition of standard denaturing gel loading buffer (95% formamide, xylene cyanol and bromophenol blue), heated at 95°C for 3 min and loaded on a 7 M urea/15% polyacrylamide gel. For the elongation reaction by calf thymus TDT, the reaction mixture (10 μl) contained 50 mM cacodylate buffer (pH 7.2), 100 mM 5'-³²P-labelled 19mer primer, 1 mM DTT, 2 mM Co²⁺, 0.25 U of TDT and the tested compounds as shown in the figure legends. After incubation at 37°C for 10 min, the reactions were quenched by the addition of standard denaturing gel loading buffer as described above and the reaction mixtures were loaded onto a 20% denaturing PAGE and subjected to electrophoresis for ~3 h at 2000 V followed by the gel exposure with a Kodak film.

**Inhibition assays**

Reactions were performed under the conditions described for the corresponding enzymatic 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 following equation:

\[ I_{cat}/I_{cat-1} = \frac{V_{max}}{1 + K_m/S} \]

where \( V_{max} \) is the input enzyme concentration.

Since we assumed that \( [E]_0 = [ES] \) at steady-state, \( k_{cat} \) values are upper estimates of the true constants, and thus are referred as apparent \( k_{cat} \) \( [k_{cat,app}] \). Thus, \( k_{cat}K_m \) are also relative values, i.e. upper estimates of the true specificity constant.

Nucleotide incorporation efficiencies were defined as the \( k_{cat}/K_m \) ratio. Under single nucleotide incorporation conditions \( k_{cat} = k_{pol}k_{off}/(k_{pol} + k_{off}) \) and \( K_m = K_{off}(k_{pol} + k_{cat}) \), where \( k_{pol} \) is the true polymerization rate, \( K_{off} \) is the dissociation rate of the enzyme–primer complex and \( K_m \) is the true Michaelis constant for nucleotide binding. Thus, \( k_{cat}/K_m \) values equal to \( k_{pol}/K_e \). Frequency of (Ib) incorporation (\( f \)) was calculated as \( k_{cat}/K_m \)\( [E]_0/k_{cat}/K_m \)\( dNTP \). Selectivity was expressed as \( 1/f \).
Table 1. Inhibition potencies of non-nucleoside triphosphate analogs towards DNA polymerases λ and β

| Inhibitor | DNA pol λ wt $K_i$ (μM)* | DNA pol λ Y505A $K_i$ (μM) | DNA pol β $K_i$ (μM) |
|-----------|--------------------------|-----------------------------|-----------------------|
| Ia        | 2.8 (±0.2) FC versus dTTP<sup>b</sup> | 3.8 (±0.3) FC versus dTTP<sup>b</sup> | >100 n.d. c            |
| Ib        | 2.7 (±0.2) FC versus dTTP<sup>b</sup> | 1.8 (±0.1) FC versus dTTP<sup>b</sup> | 68 (±6) FC versus dTTP<sup>b</sup> |
| Ia        | 1.7 (±0.1) FC versus dTTP<sup>b</sup> | 1.6 (±0.1) FC versus dTTP<sup>b</sup> | 70 (±6) FC versus dTTP<sup>b</sup> |
| Ib        | 1.1 (±0.1) FC versus dTTP<sup>b</sup> | 0.4 (±0.06) FC versus dTTP<sup>b</sup> | 4.8 (±0.2) FC versus dTTP<sup>b</sup> |
| IIa       | 1.2 (±0.1) FC versus dTTP<sup>b</sup> | 3.5 (±0.3) FC versus dTTP<sup>b</sup> | >100 n.d. c            |
| IIIb      | >100 n.d. e | 9.1 (±0.8) FC versus dTTP<sup>b</sup> | >100 n.d. e            |
| IIle      | 3.1 (±0.3) FC versus dTTP<sup>b</sup> | 6.5 (±0.5) FC versus dTTP<sup>b</sup> | >100 n.d. e            |
| IIf       | >100 n.d. e | 14.4 (±0.8) FC versus dTTP<sup>b</sup> | >100 n.d. e            |
| IIIh      | >100 n.d. e | 15.4 (±0.7) FC versus dTTP<sup>b</sup> | >100 n.d. e            |
| IIIIh     | >100 n.d. e | 0.5 (±0.07) FC versus dTTP<sup>b</sup> | >100 n.d. e            |

*Inhibition constants ($K_i$) values represent the mean of three independent experiments. Number in parantheses are ±SD. For details see Materials and Methods.

RESULTS

Inhibition of DNA polymerases β and λ by NNTPs

DNA pols β and λ were challenged with the different NNTPs shown in Figure 1. As summarized in Table 1, several of the tested compounds (Ia, Ib, Ila, IIb, IIIa, IIIc) were able to inhibit pol λ wild type in the low micromolar range. On the contrary, DNA pol β was not inhibited by these compounds, with the exception of compound IIb and, to a much lower extent, compounds Ib and IIa. In all cases, the inhibition was non-competitive with respect to the DNA substrate and purely competitive with the nucleotide substrate (data not shown). Next, the same set of compounds was tested against the DNA pol λ mutant Y505A. As summarized in Table 1, all the tested NNTPs were able to inhibit the mutant enzyme. In particular, compounds IIIb, IIIId, IIIe and IIIIf, which were inactive against the wild type, showed significant inhibitory potencies towards the Y505A mutant. None of these NNTPs showed detectable inhibition against other DNA pols, such as *Escherichia coli* DNA pol I, HIV-1 RT and human DNA pol α, indicating specificity of these compounds for the family X DNA pols λ and β (data not shown). Also, the corresponding unphosphorylated derivatives were tested and none of them proved to be able to inhibit any of the DNA pols used (data not shown).

DNA polymerase λ and its mutant Y505A show different abilities of incorporating NNTPs in the presence of an abasic site on the template strand. The different NNTPs shown in Figure 1 were evaluated for their ability to be incorporated by DNA pol λ wild type and the Y505A mutant on a DNA template either undamaged or bearing an abasic (AP) site at position +1. As shown in Figure 2A, DNA pol λ wild type on the undamaged template was able to incorporate only compound IIb, whereas in the presence of an AP site, also compounds Ia and IIa, together with IIb, could be incorporated. When the mutant Y505A was tested, some striking differences emerged. As shown in Figure 2B, compound IIb was now incorporated opposite to an AP site only. Moreover, compound Ib, which was not a substrate for the wild-type enzyme, was incorporated on both templates, with a higher efficiency on the intact one. Another interesting difference was that compound Ia was now incorporated on both templates by the mutant enzyme. No incorporation could be detected for both enzymes when Mg<sup>2+</sup> replaced Mn<sup>2+</sup>. Thus, it appears that both the presence/absence of tyrosine 505 in the nucleotide binding site, and the nature of the template (either undamaged or carrying an AP site) differentially affected the ability of various NNTPs to act as substrates for DNA pol λ. As apparent by the accumulation of only +1 products, all the compounds acted as chain terminators. This was expected, due to the lack of available 3'-hydroxyl groups for subsequent elongation. In order to investigate in more detail the observed differences in NNTPs incorporation by DNA pol λ and the Y505A mutant, the kinetic parameters ($K_m$, $k_{cat}$, $k_{cat}/K_m$) for the incorporation reaction were determined and the calculated values are summarized in Table 2. As controls, kinetic parameters for the incorporation of dCTP or dATP, depending on the template used, were also determined. Inspection of these data revealed several differences, discussed in detail in the following sections.

Compound Ib requires the presence of a templating base and the absence of tyrosine 505 for efficient incorporation by DNA polymerase λ

As shown in Figure 2B, compound Ib could be incorporated on both templates but only by the Y505A mutant. However, as summarized in Table 2, the incorporation efficiency ($k_{cat}/K_m$) for compound Ib was 6.5-fold higher on the intact template than opposite to an abasic site. Together, these results suggest that tyrosine 505 prevents incorporation, very likely through a steric gate mechanism, whereas the presence of a templating base increases incorporation probably helping proper positioning of the analogs in the active site.

Compound IIa requires the absence of a templating base and the absence of tyrosine 505 for efficient incorporation by DNA polymerase λ

As shown in Table 2, compound IIa could be incorporated by DNA pol λ either wild type or the Y505A mutant, opposite to an AP site only. Comparison of the catalytic efficiency of incorporation opposite an AP site by DNA pol λ and the Y505A mutant revealed that the lack of tyrosine 505 resulted in a higher incorporation efficiency (Table 2). Thus, incorporation of compound IIa was affected by the presence of both a templating base and tyrosine 505.
Compound IIb requires the absence of a templating base and the presence of tyrosine 505 for efficient incorporation by DNA polymerase λ.

A different behaviour with respect to compounds Ia and IIa was observed in the case of compound IIb. As shown in Figure 3A and summarized in Table 2, the incorporation efficiency of compound IIb by DNA pol λ wild type was 22-fold higher opposite an abasic site than on the intact template.

**Figure 2.** Incorporation of non-nucleoside triphosphate analogues (NNTPs) by DNA polymerase λ wt and the Y505A mutant. Reactions were carried out as detailed in Materials and Methods. The different migration of the +1 products (indicated by arrows) are due to different electrophoretic mobilities of the incorporated NNTPs. The corresponding incorporation products are indicated with arrows. The different structures resulted in different electrophoretic mobility among the NNTPs. (A) The various NNTPs (10 μM) were tested for incorporation opposite to an abasic site (lanes 1–10) or on an intact template (lanes 11–20) and in the presence of Mn²⁺. Lane 21, reaction was carried out in the presence of 100 μM of all four dNTPs. (B) As in (A) but in the presence of the DNA pol λ Y505A mutant. Lane 16, control reaction in the absence of added nucleotides.

**Table 2.** Kinetic parameters for NNTPs incorporation by DNA polymerases λ and β on intact or abasic site templates

| Compound | DNA pol λ, wild type | DNA pol λ, Y505A | DNA pol β | DNA pol β | DNA pol β | DNA pol β |
|----------|----------------------|------------------|-----------|-----------|-----------|-----------|
|          | AP                   | Control          | AP        | Control   | AP        | Control   |
|          | K<sub>m</sub> (μM)    | k<sub>cat</sub> (min<sup>−1</sup>) | K<sub>m</sub> (μM) | k<sub>cat</sub> (min<sup>−1</sup>) | K<sub>m</sub> (μM) | k<sub>cat</sub> (min<sup>−1</sup>) |
| Ia       | n.a.                 | n.a.             | n.a.      | n.d.      | n.a.      | n.a.      |
| IIa      | n.a.                 | n.a.             | n.a.      | 1.2 × 10<sup>6</sup> | n.a.      | n.a.      |
| IIb      | 0.92 (±0.2)          | 0.046 (±0.002)   | 0.08 (±0.001) | 0.02 (±0.002) | 0.08 (±0.001) | 0.02 (±0.002) |
|          | 1.1 (±0.1)           | 0.048 (±0.002)   | 0.05 (±0.001) | 0.02 (±0.002) | 0.05 (±0.001) | 0.02 (±0.002) |

Values represent the mean of three independent estimations. Numbers in parentheses are ± SD. k<sub>cat</sub>/K<sub>m</sub> are relative values. For details see Materials and Methods.

n.a., not applicable. No incorporation is detected with that particular combination of enzyme/substrate/template.

n.d., not determined.

dCTP was used as control for the undamaged template, bearing a G at position +1, for both DNA polymerases. In the case of the template bearing an AP-site, dCTP, which is preferentially incorporated by DNA pol λ, was used as control.

The different mobility and the corresponding incorporation products strongly support the proposal that the Y505A mutant lacks the ability to use the +1 position.
For comparison, the incorporation of dCTP on both templates by DNA pol λ is shown in Figure 3B. The DNA pol λ mutant Y505A showed an efficiency of incorporation of compound IIb opposite an abasic site, which was 12-fold lower than the corresponding value measured for DNA pol λ wild type (Table 2). No incorporation of this analogue by the Y505A mutant could be detected with the intact template (Figure 3C). Together, these results clearly indicated that incorporation of compound IIb was dependent both on the presence of tyrosine 505 and on the absence of a templating base.

The incorporation of compound IIb by DNA polymerase β is modulated by the nature of the activating metal

Nucleotide incorporation by DNA pols is a strictly metal-ion-dependent mechanism (1). In the case of DNA pol λ,
incorporation of the NNTPs was observed only in the presence of its optimal activator, Mn$^{2+}$. As shown in Table 1, among the tested NNTPs, only compound IIb proved to be able to significantly inhibit DNA pol 𝛽 activity. Thus, we tested whether this compound could be incorporated by DNA pol 𝛽. As shown in Figure 4A, in the presence of Mn$^{2+}$, DNA pol 𝛽 incorporated compound IIb both on an intact template and opposite to an AP site. As shown in Figure 4B, DNA pol 𝛽 incorporated dCTP, as expected, on the undamaged template, whereas it exclusively incorporated dATP opposite the AP site. The incorporation efficiency of compound IIb by DNA pol 𝛽 was 2-fold higher in the presence of the undamaged template, with respect to the one carrying an AP site (Figure 3 and Table 2). When Mn$^{2+}$ was replaced by Mg$^{2+}$, this difference became even more striking, so that compound IIb could be exclusively incorporated on the undamaged template.
template by DNA pol β (Figure 4D). Thus, in the presence of Mg\(^{2+}\), the incorporation of compound IIb by DNA pol β became strictly dependent on the presence of a templating base. Replacement of Mn\(^{2+}\) with Mg\(^{2+}\), however, greatly enhanced the preference for incorporation of dCTP versus IIb opposite a template G by DNA pol β, which increased from 23- to 239-fold, as apparent by the comparison of the respective \(k_{\text{cat}}/K_m\) values (Table 2).

DNA polymerases λ and β show different selectivity for incorporation of compound IIb

The selectivity indexes (defined as the reciprocal of the incorporation frequencies, \(1/f\), see Materials and Methods) for incorporation of compound IIb by DNA pol λ and β were determined and their values are shown in Figure 4C. Since the templating base at position +1 was G, and since DNA pol λ preferentially incorporates dCTP opposite an AP site (32) (Figure 3B), selectivity was determined for dCTP incorporation versus IIb in the case of DNA pol λ, both on the undamaged or the AP template. In the case of DNA pol β, dCTP was used as a control for the undamaged template, whereas selectivity for incorporation opposite an AP site was calculated with respect to dATP, which is preferentially incorporated by DNA pol β opposite this particular lesion (32) (Figure 4B). Comparison was possible only for the reaction in the presence of Mn\(^{2+}\), due to the lack of detectable incorporation of this analog by DNA pol λ in the presence of Mg\(^{2+}\). Compound IIb proved to be a poorer substrate than dCTP on the control template for both DNA pol λ and β (24- and 23-fold less efficient, respectively). However, the two enzymes showed a clear difference when incorporating compound IIb opposite an AP site. In fact, whereas DNA pol β still showed a 7.3-fold preference for dATP versus IIb incorporation, DNA pol λ wild type incorporated compound IIb 2.2-fold more frequently than dCTP opposite the lesion. The DNA pol λ Y505A mutant, on the other hand, showed a 5.3-fold preference for dCTP versus IIb incorporation opposite the lesion.

Base-pairing properties of compound IIb as a DNA polymerase β substrate

In the presence of Mg\(^{2+}\), compound IIb strictly required the presence of a templating base for its incorporation by DNA pol β, thus behaving like a bona fide nucleotide substrate, even though biphenyl residue lacks any obvious resemblance to a nucleobase (Figure 4B). Thus, it was interesting to see whether this compound showed any preferential base-pairing capability. To this aim, compound IIb was tested for its ability to be incorporated opposite each of the four possible templating bases A, T, G and C. A 40mer oligonucleotide was hybridized to four different primers of 18 nt, 19 nt, 20 nt and 21 nt, respectively, resulting in each case in a primer/template bearing at +1 position either A, T, G or C as the templating base. A typical experiment is shown in Figure 5A. Kinetic parameters \(K_m\), \(k_{\text{cat(app)}}\), \(k_{\text{cat}}/K_m\) for compound IIb incorporation were determined for each templating base. Incorporation of the correct complementary nucleotide was also monitored in parallel, and the corresponding kinetic parameters determined, allowing the calculation of the relative incorporation frequency for each base pair (\(f\)) defined as \((k_{\text{cat}}/K_m)_{\text{IIb}}/(k_{\text{cat}}/K_m)_{\text{dNTP}}\). The calculated values are listed in Table 3. It can be clearly seen that compound IIb showed a base-pairing hierarchy for incorporation by DNA pol β in the presence of Mg\(^{2+}\), with A being the best templating base, followed by G and T. No incorporation could be detected when C was used as the templating base (Table 3 and Figure 5A). Figure 5B shows a comparison of the selectivity indexes, defined as \(1/f\), for compound IIb incorporation opposite the templating bases A, G and T was calculated as described in Material and Methods. The calculated \(1/f\) values are indicated on top of each bar.

Figure 5. Compound IIb is preferentially incorporated by DNA polymerase β opposite an A on the template strand. (A) Reactions were carried out as detailed in Materials and Methods in the presence of 1 mM Mg\(^{2+}\). Increasing concentrations of compound IIb were titrated in the reaction in the presence of the 18/40merA (lanes 1–5), 19/40merT (lanes 6–10), 20/40merG (lanes 11–15) or 21/40merC (lanes 16–20) templates, respectively. (B) The selectivity index (\(1/f\)) for compound IIb incorporation opposite the templating bases A, G and T was calculated as described in Material and Methods. The calculated \(1/f\) values are indicated on top of each bar.

Family X DNA polymerases and terminal transferases show distinct substrate specificities with respect to NNTPs

Beside DNA pols β, λ, and μ, family X also comprises the TDT enzyme. DNA pol λ is unique among family X members, since it possesses a template-independent DNA synthetic activity very similar to the one of TDT. Thus, it was of interest to compare the substrate specificities of the ttd-like activity of pol λ and the TDT enzyme with respect to the NNTPs. None of the NNTPs tested was incorporated by pol λ-ttd activity on a ssDNA template, not even those which...
Table 3. Kinetic parameters for template-instructed incorporation of compound IIb by DNA pol b with Mg2+.

| Nucleotide substrate | Template base | \(k_{\text{cat(app)}}\) (min^(-1)) | \(k_{\text{cat(app)}}\) (min^(-1)) | \(K_m\) (mM) | \(f\) | \(f_{\text{IIb}}\) | \(f_{\text{IIb}}\) |
|----------------------|--------------|------------------|------------------|----------|----|----------------|------------------|
| IIb                  | dTTP         | 0.035            | 0.003            | 0.003    | 6 x 10^7 | 0.2 | 0.2 |
|                      | dATP         | 0.035            | 0.003            | 0.003    | 6 x 10^7 | 0.2 | 0.2 |
|                      | dCTP         | 0.035            | 0.003            | 0.003    | 6 x 10^7 | 0.2 | 0.2 |
|                      | dGTP         | 0.035            | 0.003            | 0.003    | 6 x 10^7 | 0.2 | 0.2 |

Values represent the mean of three independent estimations. Numbers in parentheses are ±SD. \(k_{\text{cat(app)}}\), apparent \(k_{\text{cat(app)}}\), and \(K_m\) are relative values. For details, see Materials and Methods.

DISCUSSION

Based on their properties, the novel NNTPs described here can be assigned to three different classes: (i) non-substrate-specific inhibitors of DNA pol \(\lambda\) (Figure 1, compounds of the series III); (ii) substrate inhibitors which could be preferentially incorporated by either DNA pol \(\lambda\) wild type or the Y505A mutant (Figure 1, compounds of the series I and II) and (iii) a substrate inhibitor which could be incorporated by DNA pol \(\beta\) exclusively in a strictly template-dependent manner and in the presence of Mg \(^{2+}\) only (Figure 1, compound IIb).

Comparison of the different inhibition potencies of the compounds of the series III against pol \(\lambda\) revealed some interesting features of this class of analogues. In the case of the mutant Y505A, addition of a fluorine substituent at the dinitrophenyl ring of compound IIIa, to generate compound IIIc, decreased the potency of inhibition. Shortening the linker chain from butyl- (compound IIIc) to ethyl- (compound IIIId), also resulted in lower inhibition potency. Replacement of the fluoro-substituent of compound IIIc with an imidazole group (compound IIIIf) generated the most potent inhibitor of this class. Contrary to the fluoro-substituted compounds, in the case of the imidazolyl-derivatives shortening the linker chain from butyl- (compound IIIe) to ethyl- (compound IIIIf), increased the inhibition potency. The same regularity was observed when the compounds were tested as substrates towards TDT, where the potency of IIIIf, bearing an ethyl-linker, was ~100 times higher than that of IIIc bearing a butyl-linker. Thus, both the nature of the substituents and the length of the alkyl chain seemed to be important determinants for the inhibitory activity towards TDT as well as the DNA pol \(\lambda\) Y505A mutant. When the activities of the same compounds were analysed in the case of the wild-type DNA polymerase \(\lambda\), a decrease in inhibitory potency could be observed going from compound IIIa to compound IIIc, similarly to the Y505A mutant. However, the other fluoro- or imidazolyl-substituted compounds (IIIb, IIIId, IIIc and IIIIf) were inactive against the wild-type enzyme, indicating that insertion of a substituent at the dinitrophenyl ring might cause steric hindrance with the side chain of tyrosine 505.

The compounds Ia, Ib, Ila and IIb constitute the second class, i.e. the substrate inhibitors of DNA pol \(\lambda\). All of them were sensitive to steric factors, such as the presence/absence of a templating base and/or of the residue tyrosine 505 in the DNA pol \(\lambda\) active site. The tyrosine 505 residue could be incorporated by its template-dependent activity such as Ib and IIb (data not shown). On the opposite, TDT could efficiently incorporate all NNTPs tested albeit with different efficacy (Figure 6). By comparing the intensities of the bands corresponding to the +1 products, the efficiencies of incorporation for compounds IIIb and IIIe were 50- to 100-fold lower than those of compounds IIIa, IIIc, IIIId and IIIIf, whereas compound Ib was 100-fold less efficient as a substrate than compound Ia. These results suggest different substrate affinities not only among the family X members DNA pol \(\beta\), DNA pol \(\lambda\) and TDT, but also between the template-independent and template-dependent activities of DNA pol \(\lambda\).
has been shown to modulate the ability of DNA pol λ to discriminate between purines and pyrimidines (31). The crystal structure of the complex of DNA pol λ with its substrates, clearly showed that tyrosine 505 acted as a ‘gate’ to limit the access of the incoming nucleotide to its binding pocket (10).

The presence of a natural base (Ia) or a base analog (Ib) in the place of a phenyl group did not improve the substrate properties of the compounds. For example, incorporation of compound Ib by DNA pol λ wild type occurred only opposite an AP site, but with a lower efficiency than either compound IIb or dCTP (Table 2). Only the DNA pol λ mutant Y505A was able to incorporate the compound Ib on a normal template opposite a G, with an efficiency close to normal dCTP, indicating that the main determinant of selectivity was a steric one.

Small structural modifications in the hydrophobic moiety of the NNTPs can drastically change their substrate properties. For example, incorporation of the benzylxycarbonyl derivative IIa by DNA pol λ was restricted by steric mechanisms, requiring the absence of both the templating base and the ‘gating’ residue tyrosine 505. However, the structurally related biphenylcarbonyl derivative IIb, required the presence of tyrosine 505, suggesting that the pendant hydrophobic group might take stabilizing π-π stacking interactions with the side chain of tyrosine 505.

Whereas several NNTPs were efficiently incorporated by the strictly template-independent DNA pol TDT, paradoxically, none of them was incorporated by the related template-independent activity of DNA pol λ. This observation demonstrates that the presence of a template strand is essential for NNTPs incorporation by DNA pol λ, even if, in some cases, they further require the absence of a templating base (i.e. an abasic site), suggesting that additional interactions with an intact sugar-phosphate backbone on the template strand might be important.

Perhaps, the most surprising finding was the discovery of the non-nucleoside compound IIb, which is incorporated by DNA pol β in a strictly Mg²⁺-dependent and template-dependent manner. Strikingly, DNA pol β showed a clear preference for incorporation of compound IIb opposite an A. The so-called ‘A-rule’ predicts that when a DNA pol encounters a lesion on the template strand, it often incorporates an A opposite the lesion (33). This rule, albeit not absolute, is followed by many DNA pols, and the reason lies in the favourable base-pairing properties of A, which can then be accommodated in front of a variety of modified bases. The results presented here suggest that, in the case of compound IIb, pol β exploits the favourable base-pairing properties of a templating A to accommodate the incoming NNTP in the active site. This observation is in agreement with the hypothesis that A-rich sequences might be more prone to misincorporations by exonuclease-deficient DNA pols, as it has been suggested recently for DNA pol λ (34).

The biphenyl moiety of compound IIb has a length of 6.9 Å and it is 2.7 Å wide. As a comparison, the length of an adenosine nucleoside is 7.4 Å and its wideness is 2.5 Å. Also, the distance between the two phenyl rings in the biphenyl moiety is 1.4 Å, which is almost identical to the length of the glycosidic bond connecting the ribose to the base in a nucleoside, which is 1.5 Å. No structure is available for compound IIb; however, its biphenyl moiety lacks hydrogen (H-) bonding groups and for this reason might be compared to non-polar nucleoside analogs. Toluene, phenyl, styryl, pyrene and benzimidazole moieties have been exploited as non-polar isosteres of natural bases (21). When coupled to a 5’-triphosphate ribose, the resulting nucleoside analogs are incorporated with variable efficiencies by DNA pols. In particular, the non-polar shape analog of thymidine difluorotoluene, could be efficiently processed by bacterial and viral DNA pols, but not by DNA pol β, suggesting that this enzyme is not able to recognize non-H-bonding analogs (35). The results presented here with compound IIb seem to indicate that H-bonding is not an absolute requirement for nucleotide recognition and incorporation by DNA pol β. However, the strong reduction in catalytic efficiency for IIb incorporation opposite normal bases (95- to 1428-fold) might be, at least in part, due to lack of H-bonding capability. Indeed, recent pre-seady-state kinetic analysis have shown that non-polar nucleotide analogs in the nascent base pair caused a reduction of the incorporation efficiency by E.coli DNA pol I (36).

The possibility of directing incorporation of non-nucleoside molecules by DNA pols, not only opposite to DNA lesions, as in the case of DNA pol λ, but also in a template-dependent manner, as for compound IIb and DNA pol β, can be exploited for the development of a novel class of molecular probes that can be used either for investigating the DNA polymerase mechanisms and for potential biotechnological applications. For example, non-nucleoside chain-terminating triphosphates bearing markers groups (such as fluorescent groups) can be successfully applied for DNA labelling during post-PCR target preparation for microarray analysis.
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