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Diketo Hexenoic Acid Derivatives Are Novel Selective Non-Nucleoside Inhibitors of Mammalian Terminal Deoxynucleotidyl Transferases, with Potent Cytotoxic Effect against Leukemic Cells

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ABSTRACT

Mammalian terminal deoxyribonucleotidyl transferase (TDT) catalyzes the non–template-directed polymerization of deoxyribonucleoside triphosphates and has a key role in V(D)J recombination during lymphocyte and repertoire development. More than 90% of leukemic cells in acute lymphocytic leukemia and approximately 30% of leukemic cells in the chronic myelogenous leukemia crisis show elevated TDT activity. This finding is connected to a poor prognosis and response to chemotherapy and reduced survival time. On the other hand, recent data indicated that TDT is not the only terminal deoxyribonucleotidyl transferase in mammalian cells. Its close relative, DNA polymerase λ, can synthesize DNA both in a template-dependent (polymerase) and template-independent (terminal deoxyribonucleotidyl transferase) fashion. DNA polymerase λ might be involved in the nonhomologous end-joining recombinational repair pathway of DNA double-strand breaks. In this work, we report the characterization of the mechanism of action of three diketo hexenoic acid (DKHA) derivatives, which proved to be extremely selective for the terminal deoxyribonucleotidyl transferase activity of DNA polymerase λ and TDT. They seem to be the first non–nucleoside-specific inhibitors of mammalian terminal transferases reported. Moreover, the DKHA analog 6-(1-phenylmethyl-1*H*-indol-3-yl)-2,4-dioxo-5-hexenoic acid (RDS2119) was not toxic toward HeLa cells ($CC_{50} > 100$ μM), whereas it showed significant cytotoxicity against the TDT^+ leukemia cell line MOLT-4 ($CC_{50} = 14.9$ μM), thus having the potential to be further developed as a novel antitumor agent.

Mammalian terminal deoxyribonucleotidyl transferase (TDT) was initially isolated from thymus, but it is also expressed in bone marrow. It catalyzes the non–template-directed polymerization of deoxynucleoside triphosphates in vitro (Krayevsky et al., 2000). TDT is influencing the outcome of V(D)J recombination during lymphocyte and repertoire development (Benedict et al., 2000). It was shown that terminal nucleotide (N-) addition at the Ig locus correlated with the presence of TDT (Desiderio et al., 1984), and the deletion of TDT activity in mice by gene inactivation resulted in the suppression of N-addition in adult B and T cell V(D)J junctions (Gilfillan et al., 1993; Komori et al., 1996). TDT is expressed in B cells before D–J rearrangement and ceases before the expression of Ig light chains. Finally, premature introduction of TDT activity in mice during fetal and neona-

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ABBREVIATIONS: TDT, terminal deoxynucleotidyl transferase; ss, single strand; HIV-1 RT, human immunodeficiency virus-1 reverse transcriptase; DKHA, diketo hexenoic acids; pols, DNA polymerases; BSA, bovine serum albumin; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; aa, amino acids; RDS2119, 6-(1-phenylmethyl-1*H*-indol-3-yl)-2,4-dioxo-5-hexenoic acid; RDS2153, 6-(4-benzoyl-1-phenylmethyl-1*H*-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acid; RDS2184, 6-(1-phenyl-1*H*-indol-3-yl)-2,4-dioxo-5-hexenoic acid.

tal life, when such activity is absent, results in an impaired ability to make protective antibodies (Benedict and Kearney, 1999). It has been observed that more than 90% of leukemic cells in acute lymphocytic leukemia and approximately 30% of leukemic cells in the chronic myelogenous leukemia crisis exhibit elevated TDT activity, and the high TDT activity of such leukemic cells is associated with a poor prognosis and response to chemotherapy and reduced survival time. The nucleoside analog cordycepin (3′-dA), which inhibits the activity of TDT, is cytotoxic against TDT^+ cells in vitro in the presence of the antitumor agent deoxycoformycin but not against TDT^- cells (Foss, 2000). However, little is known about the role of TDT in the development of leukemias.

TDT belongs to the family X of DNA polymerases (pols) (Hubscher et al., 2002). Other members of this family are DNA polymerase β, DNA polymerase λ, and DNA polymerase μ in mammalian cells and DNA polymerase IV in the yeast *Saccharomyces cerevisiae*. DNA polymerase β and DNA polymerase IV in the yeast *S. cerevisiae* do not contain terminal deoxyribonucleotidyl transferase catalytic activity (tdt), whereas DNA polymerase μ under certain conditions showed a template-dependent, sequence-independent polymerase activity (Covo et al., 2004). On the other hand, DNA polymerase λ has been shown to possess a strong bona fide tdt activity (Ramadan et al., 2003) that requires either a single-stranded (ss) DNA or a partially double-stranded DNA with a 3′-ss overhang of at least 10 to 12 nucleotides for optimal activity (Maga et al., 2005). DNA polymerase λ has also been shown to interact with the DNA replication protein proliferating cell nuclear antigen, and this complex can perform translesion DNA synthesis past an abasic site (Maga et al., 2002, 2004). Recent data indicate that DNA polymerase λ, DNA polymerase μ, and TDT, but not DNA polymerase β, possess a polynucleotide synthetase activity capable of polymerizing nucleotides even in the absence of any DNA primer and/or template (Ramadan et al., 2004b).

Transgenic mice inactivated for either DNA polymerase μ and DNA polymerase λ were viable and fertile, but immunoglobulin κ light-chain gene rearrangement was impaired in mice deficient for DNA polymerase μ (Bertocci et al., 2003). Finally, recent biochemical studies suggested that DNA polymerase λ and DNA polymerase μ might be involved in the nonhomologous end-joning recombinational repair pathway of DNA double-strand breaks (Mahajan et al., 2002; Fan and Wu, 2004; Lee et al., 2004; Ma et al., 2004), making the precise identification of the in vivo role(s) of these enzymes still a challenging task.

The availability of specific inhibitors for these enzymes might help the investigation of their cellular functions. Moreover, the correlation between high TDT activity and malignancy of acute lymphocytic leukemia further increases the interest in developing TDT-specific inhibitors. Beside the unspecific chain-terminating dideoxynucleotides, which inhibit family X as well as family A pols, some nucleoside analogs have been described to inhibit TDT activity (Krayevsky et al., 2000), whereas the only non-nucleosidic inhibitors of DNA polymerase λ known so far are phenolic compounds of natural origin (Mizushina et al., 2002, 2003). However, these molecules have been shown to inhibit the DNA polymerase activity of DNA polymerase λ, whereas no reports have been published on their effects on its tdt activity.

In an effort to identify potent and selective inhibitors of the tdt activity of DNA polymerase λ and TDT, we undertook a random screening of synthetic non-nucleoside analogs. We report here the characterization of the mechanism of action of three diketo hexenoic acid (DKHA) derivatives (Fig. 1), which proved to be extremely selective for both the tdt activity of DNA polymerase λ and TDT. To the best of our knowledge, they seem to be the first non–nucleoside-specific inhibitors of mammalian terminal transferases reported so far. One of these compounds showed no toxicity against HeLa cells but was able to selectively suppress cell proliferation of TDT^+ leukemic cells, holding the potential to be further developed as a novel antitumor agent.

Materials and Methods

Chemicals

[^{3}H]dTTP (30 Ci/mmol) and [^{3}H]dATP (73 Ci/mmol) were from Amersham Biosciences Inc. (Piscataway, NJ); unlabeled dNTPs, poly(dA), and oligo(dT)$_{12–18}$ were from Roche Molecular Biochemicals (Indianapolis, IN). The oligonucleotides were from MWG Biotech (Florence, Italy). Whatman (Clilfton, NJ) supplied the GF/C filters. All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Chemistry

The synthesis of diketo hexenoic acid derivatives RDS2119, RDS2153, and RDS2184 will be described in detail elsewhere (R. Di Santo, R. Costi, A. Roux, G. A. Locatelli, E. Crespan, U. Hübscher, I. Shevelev, G. Blanca, G. Villani, S. Spadari, and G. Maga, manuscript in preparation).

Enzymes and Proteins

Recombinant full-length human DNA DNA polymerase λ, the DNA polymerase $\lambda\Delta$132 and $\lambda\Delta$244 truncated proteins, and the DNA polymerase λ Y505A, E330A, V334A, and F338A mutants were generated and purified as described previously (Blanca et al., 2003; Shevelev et al., 2003; Maga et al., 2004). After purification, the proteins were >90% homogenous, as judged by SDS-polyacrylamide gel electrophoresis and Coomassie staining (data not shown). Human DNA polymerase α and HIV-1 RT were purified as described previously (Weiser et al., 1991; Maga et al., 1997). Human DNA polymerase β and calf thymus TDT were from Trevigen (Gaithersburg, MD). *Escherichia coli* DNA polymerase I (KF) was from Roche Molecular Biochemicals.

Enzymatic Assays

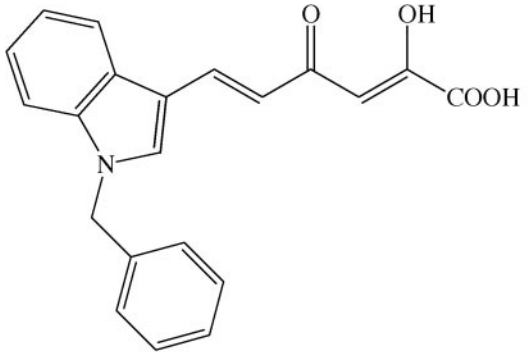
DNA Polymerase Assay. Human DNA polymerase λ and *S. cerevisiae* DNA polymerase IV activity on poly(dA)/oligo(dT)$_{10:1}$ were assayed in a final volume of 25 μl containing 50 mM Tris-HCl, pH 7.0, 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM $MnCl_2$, 0.2 μM poly(dA)/oligo(dT)$_{10:1}$ (3′-OH ends), 50 nM DNA polymerase λ, and 5 μM [^{3}H]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 was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described. DNA polymerase β, DNA polymerase α, *E. coli* DNA polymerase I (KF), and HIV-1 RT activities were assayed as described previously (Blanca et al., 2003).

Terminal Deoxyribonucleotidyl Transferase Assay. DNA polymerase λ and TDT terminal transferase activities 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 $MnCl_2$, 0.2 μM ss 27-mer DNA oligonucleotide, unless otherwise stated. Enzymes and [^{3}H]dNTPs (10 Ci/mmol) were added as indicated in the figure legends. All reactions were incubated at 37°C for 10 min unless otherwise indi-

cated in the figures, and the DNA was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described previously (Hubscher and Kornberg, 1979).

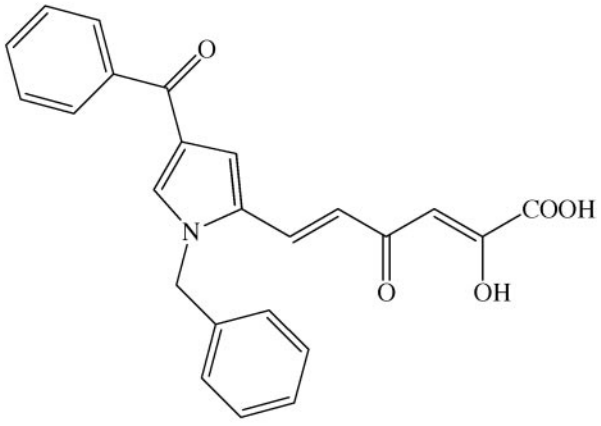
Polynucleotide Synthetase (de Novo DNA Synthesis) Assay. A final volume of 10 μl contained 50 mM Tris-HCl, pH 7.0, 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM $MnCl_2$, 2 μM [α-^{32}P]dCTP (300 Ci/mmol), and 200 nM DNA polymerase λ. Reactions were carried out for 60 min at 37°C and products resolved on a 6 M urea/14% polyacrylamide gel. Radioactive bands were visualized by filmless autoradiographic analysis. The DNA polymerase λ preparation used was tested to be free of any contaminating DNA, as described previously (Ramadan et al., 2004b).

Inhibition Assays. Reactions were performed under the conditions described for the terminal deoxyribonucleotidyl transferase activity assay. Incorporation of radioactive dTTP into the ss 27-mer oligodeoxynucleotide at different concentrations of DNA or dNTP was monitored in the presence of increasing amounts of inhibitor as



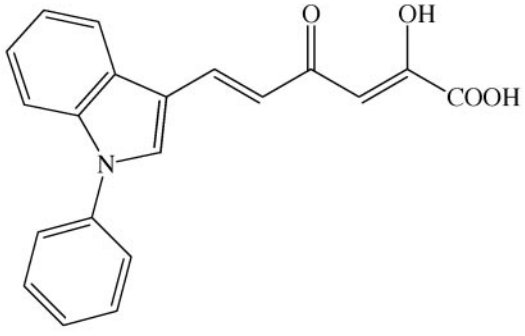
RDS 2119

6-(1-Phenylmethyl-1*H*-indol-3-yl)-2,4-dioxo-5-hexenoic acid



RDS 2153

6-(4-Benzoyl-1-phenylmethyl-1*H*-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acid



RDS 2184

6-(1-Phenyl-1*H*-indol-3-yl)-2,4-dioxo-5-hexenoic acid

Fig. 1. Structures of the three DKHA derivatives used in this study: RDS2119, RDS2153, and RDS2184.

indicated in the figure legends. Dose-response curves were generated by computer fitting of the data to the relationship $E_{(\%)} = E_{max}/[1 + (I/ID_{50})]$, where $E_{(\%)}$ is the fraction of the 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 $E_{(\%)} = 0.5\ E_{max}$.

Steady-State Kinetic Analysis

The mechanism of action of DKHA derivatives was found to be either fully noncompetitive or mixed. A schematic drawing of the different equilibria is depicted in Fig. 2. The steady-state kinetic analysis was simplified by varying one of the substrates (either DNA or dNTP) while the other was kept constant. When the DNA substrate was saturating concentration (50-fold greater than its K_m) and the inhibition analyzed in dependence of varying concentrations of dNTPs, all the input DNA polymerase λ was in the form of the E/DNA binary complex so that only the binary complex and the ternary complex with dNTP could react with the inhibitor (left part of Fig. 2). Likewise, when the dNTP concentration was saturating (5-fold greater than its K_m) and the inhibition analyzed in dependence of varying DNA concentrations, the inhibitor could interact only with the free enzyme or with the ternary complex with DNA and dNTP (right part of Fig. 2).

The general steady-state rate equation used was the one describing a mixed inhibition of a reaction involving only two mechanistic forms of the enzyme (Dixon and Webb, 1979):

$$v = \frac{k_{cat} \times E_0 \times \left[\frac{1 + k'_{cat} \times I}{k_{cat} \times K'_i}\right]}{\left[1 + \frac{K_s}{S} \times \frac{(1 + I \times K_i)}{(1 + I \times K'_i)}\right]} \quad (1)$$

where k_{cat} is the apparent reaction rate in the absence of the inhibitor, k'_{cat} is the reaction rate at infinite inhibitor concentration, E_0 is the total enzyme concentration, I is the inhibitor concentration, S is the substrate concentration and K_i and K'_i are the equilibrium dissociation constants for the different enzyme-substrate complexes. According to Fig. 2, when DNA was held constant and only the dTTP concentration was varied, $K_i = K_i^{bin}$ and referred to the binding of the inhibitor to the binary complex E/DNA, whereas $K'_i = K_i^{ter}$ and referred to the binding of the inhibitor to the ternary E/DNA/dNTP complex. On the other hand, when dTTP was held constant, K_i referred to the binding of the inhibitor to the free enzyme, whereas $K'_i = K_i^{ter}$ described the binding to the ternary E/DNA/dNTP complex. Equation 1 was used to derive the apparent reaction rate $k_{cat(app)}$ and affinity $K_{s(app)}$ for the DNA and dNTP substrates of the reaction at different inhibitor concentrations. Then, K_s, K_i, K'_i and K'_s values were derived by computer fitting of the variation of the $K_{s(app)}$ values as a function of the inhibitor concentrations according to the equations:

$$K_{s(app)} = \frac{I + K_i}{\left(\frac{K_i}{K_s - \frac{I}{K'_s}}\right)} \quad (2)$$

and

$$K_{s(app)} = K_i \times \frac{1 + I}{\left[\frac{\left(1 + \frac{I}{K'_i}\right)}{K_s}\right]} \quad (3)$$

The K'_i and k'_{cat} values were derived by computer fitting of the variation of the $k_{cat(app)}$ values as a function of the inhibitor concentrations according to the equation

$$1/k_{cat(app)} = \frac{K'_i + I}{(K'_i \times k_{cat}) + (I \times k'_{cat})} \quad (4)$$

For the fully noncompetitive case, $k'_{cat} = 0$, $K_s = K'_s$ and $K_i = K'_i$, thus eq. 1 simplifies to

$$v = \frac{\frac{k_{cat} \times E_0}{\left(1 + \frac{I}{K_i}\right)}}{1 + \frac{K_s}{S}} \quad (5)$$

Likewise, eq. 4 simplifies to

$$1/k_{cat(app)} = \frac{I}{K_i \times k_{cat}} + \frac{1}{k_{cat}} \quad (6)$$

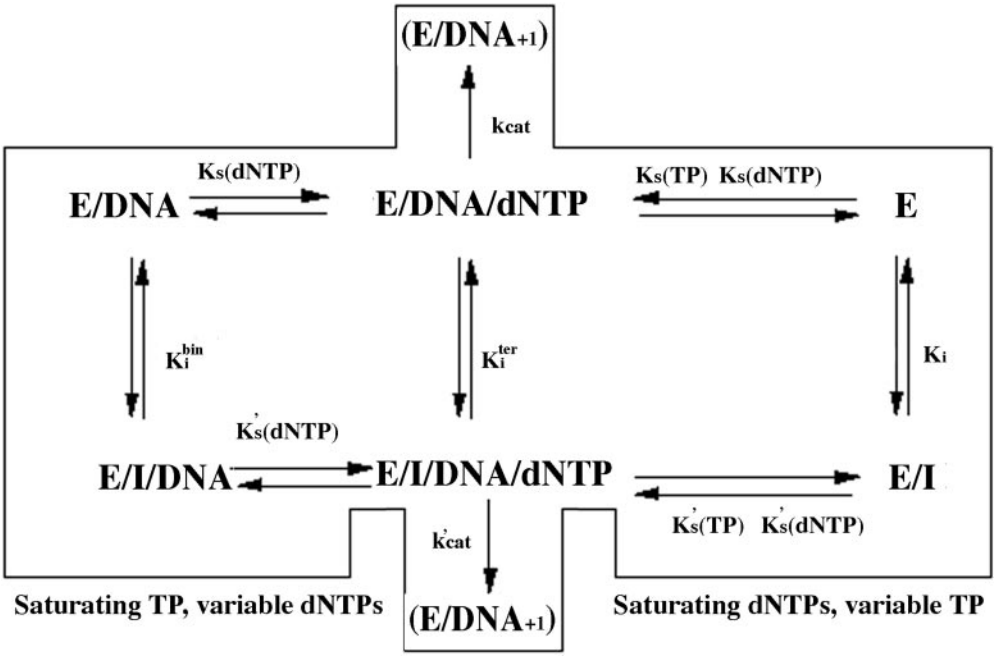


Fig. 2. Simplified kinetic pathway for the DNA elongation reaction catalyzed by the terminal transferase activity of TDT and DNA polymerase λ. Simplified model used for the experimental analysis of the steady-state equilibria. For details, see *Materials and Methods.*

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 done in triplicate, and mean values were used for the analysis. Curve-fitting was made with the computer program Prism (GraphPad Software Inc., San Diego, CA).

Pre–Steady-State Analysis

For the analysis of inhibition under single-turnover conditions, a minimal reaction pathway for the DNA polymerase λ-tdt reaction is depicted in the following scheme:

$$[E] + [DNA] \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} [E:DNA] + [dNTP] \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}}$$

$$[E:DNA:dNTP] \xrightarrow{k_3} [E:DNA_{+1}] \xrightarrow{k_{-1}} [E] + [DNA_{+1}]$$

Time course experiments were performed as described in the figure legends. Samples were taken at 0, 15, 30, 45, 60, 75, 90, 120, 240, 600, and 1800 s. Data were analyzed according to the mixed-exponential equation

$$A \times (1 - e^{-k' \times t}) + k_{ss} \times t \quad (7)$$

where A is the burst amplitude, k' is the apparent first-order rate constant for the single-turnover association of the enzyme to its substrate (burst rate), k_{ss} is the rate-limiting constant for the steady-state reaction, which was assumed to be equal to k_{-1}, and t is time.

Active site concentration was derived from the equation

$$A = [E]_0 \times \left(\frac{k'}{k' + k_3}\right)^2 \quad (8)$$

The catalytic rate k_3 and the affinity of the enzyme for its substrates (K_S) were calculated according to the relationship

$$k' = \frac{k_3 \times [S]}{[S] + K_s} \quad (9)$$

where [S] is the variable substrate concentration.

The actual rates k_1, k_{-1}, k_2, and k_{-2} were calculated according to the relationships

$$k'_{DNA} = \frac{k_{+1} \times k_3}{k_{-1} + k_3}$$

$$k'_{dNTP} = \frac{k_{+2} \times k_3}{k_{-2} + k_3}$$

$$K_{DNA} = \frac{k_{-1}}{k_{+1}}$$

$$K_{dNTP} = \frac{k_{-2}}{k_{+2}}$$

Each experiment was performed in triplicate, and mean values were used for the analysis. Curve-fitting was made with the computer program Prism (GraphPad Software). Error bars (± S.D.) are shown in the plots.

Cell Viability Assays

The TDT$^+$ cell line MOLT-4 (a human early T cell leukemia cell line) and the TDT$^-$ cell line HeLa were used. The TDT status of MOLT-4 cells has been described by McCaffrey and colleagues (McCaffrey et al., 1983; Kodama et al., 2000). Cells were grown in RPMI 1640 (Cambrex Bio Science Baltimore, Inc., Baltimore, MD) supplemented with 10% fetal bovine serum, 50 mg/ml streptomycin, 50 U/ml penicillin, and 2 mM L-glutamine. For cytotoxicity assays, exponentially growing cells were plated at a final concentration of 10^6 cells/ml in six-wells plates (Iwaki Walchem Corp., Holliston, MA) and incubated in the absence or presence of different concentrations of the inhibitors for different times, as indicated in the figure legends. Cell viability was measured by standard dye-exclusion assay with trypan blue.

Western Blot Analysis

Approximately 2×10^7 HeLa or MOLT-4 exponentially growing cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40). Total protein concentration of each extract was determined by Bradford assay. Similar amounts of each extract were loaded onto a 8% SDS-polyacrylamide gel along with different amounts of recombinant DNA polymerase λ and subjected to electrophoresis. After separation, proteins were transferred to a nitrocellulose membrane, which was blocked overnight in 7.5% fat-free milk. The membrane was incubated with anti-DNA polymerase λ polyclonal antibodies. Detection was performed with the enhanced chemiluminescence system. Intensities of the bands were measured by scanning densitometry of the film.

Results

DKHA Derivatives Specifically Target Mammalian Terminal Deoxyribonucleotidyl Transferases. The DKHA derivatives RDS2119, RDS2153, and RDS2184 (Fig. 1 shows the chemical structures) were tested against DNA polymerases belonging to four different families. As shown in Fig. 3, the tdt activity of DNA polymerase λ and TDT were

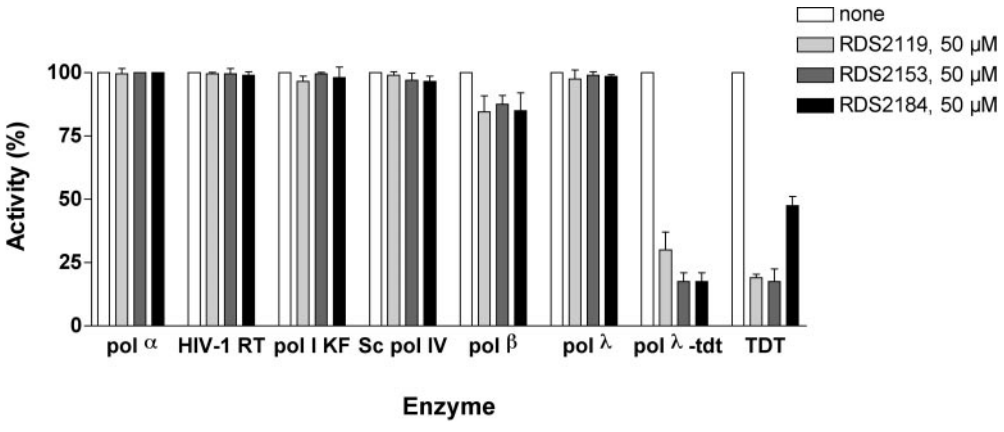


Fig. 3. The inhibitory activity of DKHA derivatives is specific for mammalian terminal transferases. The DNA polymerase and terminal deoxynucletotidytransferase assays were performed as described under *Materials and Methods* in the absence (□) or presence of 50 μM RDS2119 (▨), RDS2153 (▩), or RDS2184 (■). A 0.05-U sample of each of DNA polymerase α, HIV-1 RT, *E. coli* DNA polymerase I (KF), *Sc*DNA polymerase IV, DNA polymerase β, DNA polymerase λ, and TDT were tested. The activities are expressed as the percentage of incorporation compared with the controls without inhibitor. Values represent the means of three independent experiments with error bars (± S.D).

significantly (>50%) inhibited by these compounds, whereas the polymerase activity of human DNA polymerase α (family B), human DNA polymerase λ and *S. cerevisiae* DNA polymerase IV (family X), *E. coli* DNA polymerase I (KF) (family A), and HIV-1 reverse transcriptase (reverse transcriptases family) were unaffected by a drug concentration up to 50 μM. Human DNA polymerase β (family X) was inhibited only by 15%. Thus, the compounds tested seem to be specific for mammalian terminal transferase activities.

The DKHA Derivatives Are Noncompetitive Inhibitors of TDT. As shown in Fig. 4A, the three compounds inhibited the activity of TDT in the micromolar range, albeit with different potencies. To elucidate their mechanism of action, the variation of the reaction velocity catalyzed by TDT was measured as a function of the DNA substrate concentration in the absence or in the presence of increasing amounts of the inhibitors. None of the inhibitors affected the apparent affinity [$K_{s(app)}$] of TDT for the DNA template (Fig. 4, B–D). Moreover, the reciprocal of the variation of the apparent rate of the reaction [$k_{cat(app)}$], followed a linear relationship with the inhibitor concentrations (see eq. 6 under *Materials and Methods*). Identical results were obtained by varying the dTTP concentration (data not shown), allowing the conclusion that RDS2119, RDS2153, and RDS2184 were fully noncompetitive inhibitors of mammalian TDT.

The DKHA Derivative RDS2153 Is a Noncompetitive Inhibitor of the DNA Polymerase λ tdt Activity. Next, similar experiments were performed for the tdt activity of DNA polymerase λ. When the inhibition of DNA polymerase λ tdt activity by the DKHA derivative RDS2153 was analyzed as a function of the DNA and dTTP concentrations, the inhibitor did not affect the apparent affinity [$K_{s(app)}$] of DNA polymerase λ for the DNA (Fig. 5A) or for the dTTP substrate (Fig. 5B). Again, the reciprocal of the variation of the appar-

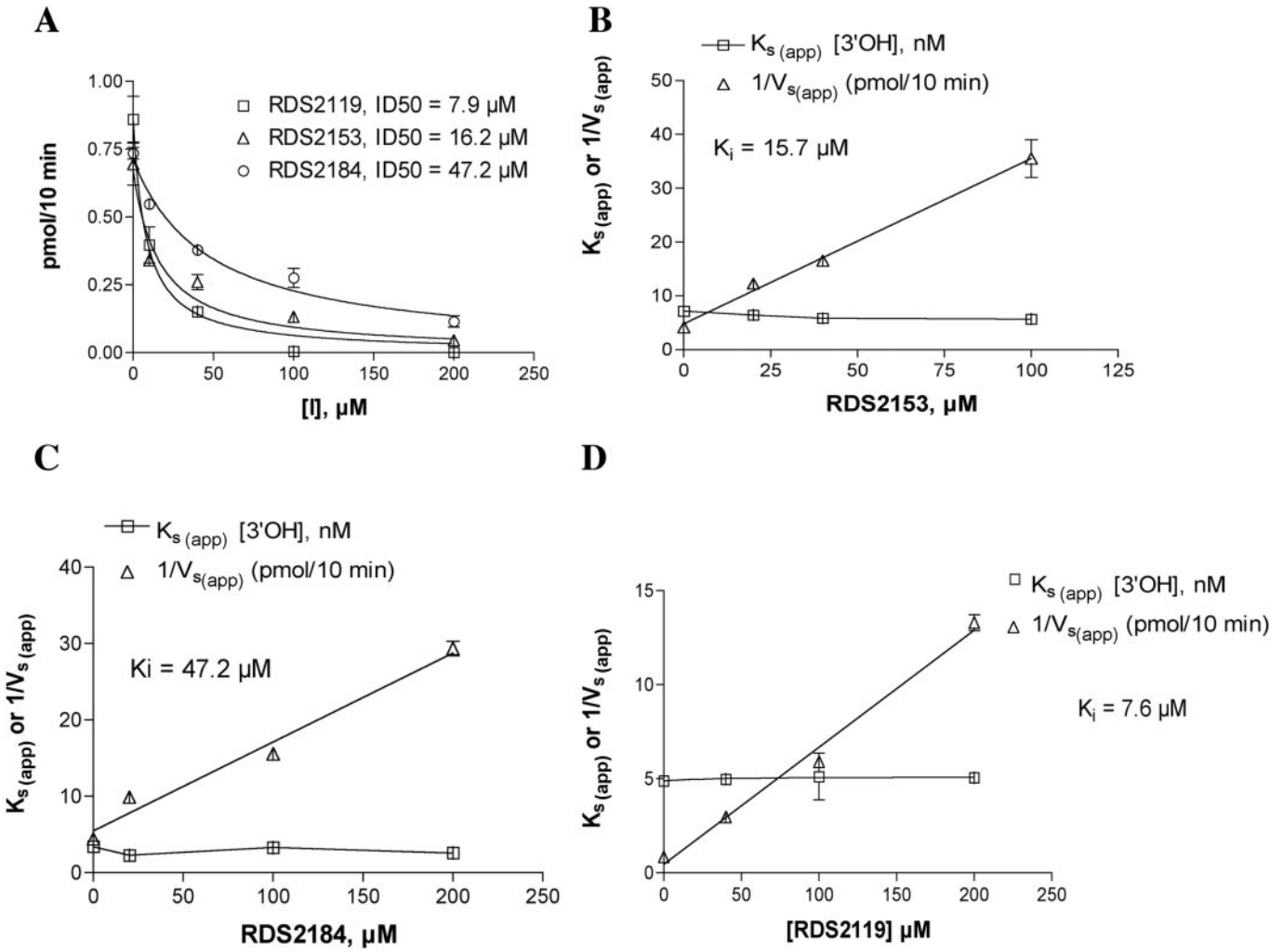


Fig. 4. The DKHA derivatives are noncompetitive inhibitors of terminal transferase. A, dose-response curves were generated as described under *Materials and Methods* in the presence of 0.05 U of TDT and in the absence or presence of 20, 40, 100, and 200 μM RDS2119 (□), RDS2153 (△), or RDS2184 (○). B, the variation of the initial velocity of the reaction catalyzed by 0.05 U of TDT was measured as a function of the DNA substrate concentration in the absence (□) or presence of 20, 40, or 100 μM RDS2153. DNA concentrations used (as 3′-OH ends) were 0.4, 1, 2, 4, and 20 nM. The calculated values for the apparent affinity (K_s, □) for the DNA substrate and apparent maximal velocity (V_s, △) of the reaction were then plotted as a function of the inhibitor concentration. The inhibition constant (K_i) value was derived by linear interpolation of the variation of the V_s values, according to eq. 6 under *Materials and Methods*. C, the variation of the initial velocity of the reaction catalyzed by 0.05 U of TDT was measured as a function of the DNA substrate concentration in the absence or presence of 40, 100, or 200 μM RDS2184. DNA concentrations used (as 3′-OH ends) and the plotting and inhibition constant values calculation were performed as in B. D, the variation of the initial velocity of the reaction catalyzed by 0.05 U of TDT was measured as a function of the DNA substrate concentration in the absence or presence of 40, 100, or 200 μM RDS2119. DNA concentrations used (as 3′-OH ends) and the plotting and inhibition constant values calculation were performed as in B.

ent rate of the reaction [$k_{cat(app)}$] followed a linear relationship with the inhibitor concentrations (see eq. 6 under *Materials and Methods*), indicating a fully noncompetitive mechanism of action.

The DKHA Derivatives RDS2119 and RDS2184 Are Mixed Inhibitors of DNA Polymerase λ tdt Activity. When the inhibition of DNA polymerase λ tdt activity by the DKHA derivative RDS2119 was analyzed as a function of the DNA and dTTP concentrations, an increase in the $K_{s(app)}$ values for both substrates was noticed. The variation of the $K_{s(app)}$ values with the inhibitor concentrations was not linear (Fig. 5, C and D) but followed eqs. 2 and 3 (see *Materials and Methods*). On the other hand, the reciprocal of the variation of the apparent rate of the reaction [$k_{cat(app)}$] followed a

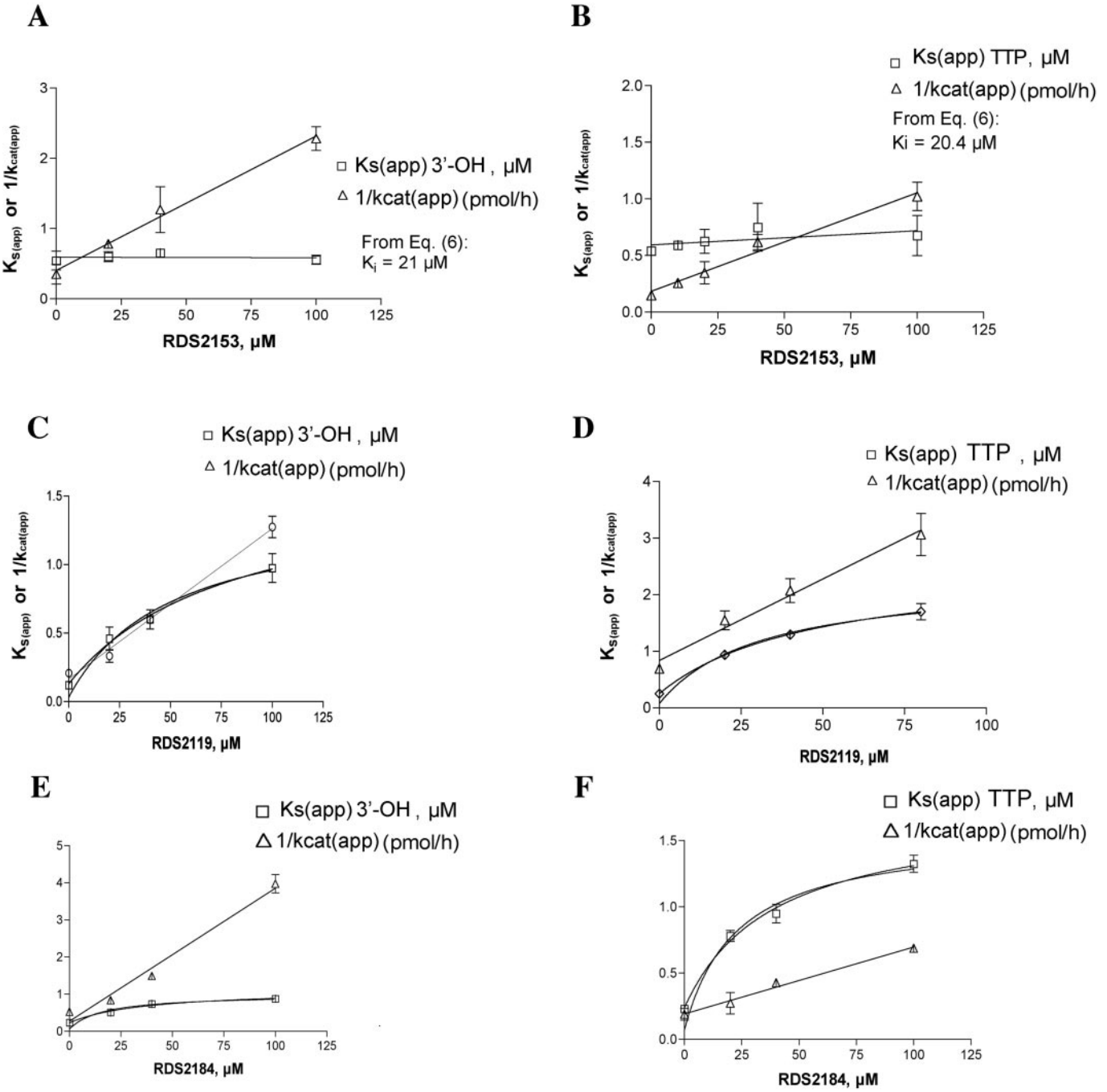


Fig. 5. The DKHA derivative RDS2153 is a noncompetitve inhibitor of DNA polymerase λ terminal transferase activity, whereas RDS2119 and RDS2184 are mixed inhibitors. The variation of the initial velocity of the terminal transferase reaction catalyzed by 50 nM DNA polymerase λ was measured as a function of either the DNA substrate or the TTP substrate concentrations in the absence or presence of different concentrations of RDS2153, RDS2119, and RDS2184. DNA concentrations used (as 3′-OH ends) were 0.1, 0.2, 0.4, 0.8, and 2 μM. TTP concentrations used were 0.4, 0.8, 2, and 10 μM. A, the variation of the apparent affinity (K_s, □) for the DNA substrate and of the apparent reaction rate [$k_{cat(app)}$, △] of the reaction were plotted as a function of RDS2153 concentration. The K_i value was derived by linear interpolation of the variation of the $k_{cat(app)}$ values according to eq. 6 (see *Materials and Methods*). B, as in A but in dependence on the TTP concentrations. C, as in A but in the presence of different RDS2119 concentrations. D, as in C but in dependence of the TTP concentrations. E, as in A but in the presence of different RDS2184 concentrations. F, as in E but in dependence on the TTP concentrations.

linear relationship with the inhibitor concentrations (see eq. 6 under *Materials and Methods*). Thus, RDS2119 caused a concomitant decrease of the apparent affinity of the enzyme for its substrates and a decrease in the rate of the reaction, acting as a mixed inhibitor. Similar results were obtained with the RDS2184 derivative (Fig. 5, E and F). By using the kinetic model depicted in Fig. 2, as explained under *Materials and Methods*, the equilibrium dissociation constants of both derivatives for the different reaction intermediates were derived and calculated (Table 1). Both compounds showed a selective hierarchy of binding, with the free enzyme being the best target, followed by the binary and the ternary complexes, respectively. Because it is known that upon complex formation with their substrates, pols undergo conformational changes, these results suggest that RDS2119 and RDS2184 are sensitive to the structural alterations of their binding site after complex formation of DNA polymerase λ with its substrates.

The Inhibition of DNA Polymerase λ-tdt Nucleotide Addition by the RDS2119 and RDS2184 DKHA Derivatives Is Not Influenced by the Nature of the Substrate and Selectively Affects the Substrate Dissociation Rate. We have shown recently that DNA polymerase λ-tdt activity preferentially uses pyrimidine versus purine nucleotides (Maga et al., 2005). To investigate whether this substrate preference reflected also a different inhibition by RDS2119 and RDS2184 derivatives as a function of the incorporated nucleotide, both DKHA analogs were titrated in tdt reactions in the presence of either dTTP or dATP as substrates. As shown in Fig. 6A, no differences in inhibition were observed, indicating that the compounds tested were not sensitive toward purines versus pyrimidines.

We also have shown previously that DNA polymerase λ-tdt activity was highly distributive, showing a biphasic mode of nucleotide incorporation as a function of time, with a fast (burst) rate of single nucleotide addition followed by a slower (steady-state) rate of subsequent incorporations, which was limited by the dissociation of the enzyme from the DNA substrate (Maga et al., 2005). A typical time-course experiment is shown in Fig. 6B, where it can be seen that the burst rate was increased as a function of the nucleotide substrate concentrations. By performing similar time-course experiments in the absence or presence of fixed amounts of inhibitors, it was possible to precisely determine the variation of different rate constants for the single-turnover reaction of DNA polymerase λ-tdt in response to the inhibitors. As shown in Fig. 6, C and E, neither inhibitor affected the association rate (k_2 according to the reaction pathway in Fig. 2; also see *Materials and Methods*) of the nucleotide to the enzyme but, on the contrary, increased its dissociation rate (k_{-2}). By plotting the variation of the dissociation rate (Δk_{off}) as a function of the inhibitor concentrations (Fig. 6, D and F), it was possible to calculate the dissociation rate at infinite inhibitor concentration (k'_{-2}; see also Fig. 2), as well as the K_i^{ter} values for both inhibitors, which were consistent with the ones calculated with the steady-state approach (Fig. 6). The calculated values are reported in Table 1. These results clearly indicated that the two DKHA derivatives RDS2119 and RDS2184 specifically increased the ground-state nucleotide substrate dissociation rate.

The DKHA Derivatives RDS2119 and RDS2184, but Not RDS2153, Are Partially Sensitive to the Absence of the N-Terminal BRCT Domain of DNA Polymerase λ. DNA polymerase λ has a BRCT domain (aa 36–132) and a proline-rich domain (aa 133–244) at its N terminus (Ramadan et al., 2004a). These domains have been shown to be entirely dispensable for all the catalytic activities of DNA polymerase λ (DNA polymerase, dRP lyase, tdt, and polynucleotide synthetase), which reside in its C-terminal part (aa 244–575). On the other hand, the phenolic compound petasiphenol, an inhibitor of DNA polymerase λ polymerase activity, was shown to bind to a pocket in the BRCT domain (aa 76–104) (Mizushina et al., 2002). Therefore, petasiphenol did not inhibit a truncated form of DNA polymerase λ, lacking the first 132 aa. To test whether the DKHA derivatives also shared the same binding site, their inhibition potencies were determined against two truncated forms of DNA polymerase λ: the Δ132λ mutant, which lacked the first 132 aa, spanning the BRCT domain; and the Δ244λ mutant, which lacked the first 244 aa, comprising both the BRCT and the proline-rich domains. The results are summarized in Table 2. The DKHA derivative RDS2153 inhibited the two truncated enzymes with the same potency as the full-length DNA polymerase λ. On the other hand, the RDS2119 and RDS2184 derivatives showed reduced inhibition toward the mutant enzymes with respect to DNA polymerase λ wild type.

The fact that all three compounds were still able to inhibit the truncated forms of DNA polymerase λ indicated that their binding site is distinct from the one of petasiphenol. However, a difference in the inhibition between the truncated forms and the full-length enzyme could also be seen, but only for the two DKHA derivatives RDS2119 and RDS2184, which both acted as mixed-type inhibitors. This might suggest that the deletion of the N-terminal part of DNA polymerase λ can cause a distortion of the inhibitor binding site, to which those two derivatives are more sensitive than RDS2153.

TABLE 1

Kinetic parameters for pol λ-tdt inhibition by the three DKHA derivatives

Different enzyme-substrate complexes were tested as outlined in Fig. 2 and under *Materials and Methods*, and equilibrium dissociation constants for inhibitor binding to the different enzymatic forms are as described there. k_2 indicates apparent affinity of the enzyme for the nucleotide substrate in the presence of the inhibitor, k_{-2} indicates association and dissociation rates for the nucleotide substrate in the absence of the inhibitor, and k'_{-2} indicates dissociation rate for the nucleotide substrate in the presence of the inhibitor. For details see text. Values are the means of three independent experiments ± S.D.

		Enzymatic Form							
	Type of Inhibition	E (K_I)	E/DNA (K_i^{bin})	E/DNA/dNTP					
				K_i^{ter}	K_{dNTP}	K'_{dNTP}	k_2	k_{-2}	k'_{-2}
		μM	μM		μM		$(M^{-1}s^{-1})\times 10^6$		s^{-1}
RDS2119	Mixed-competitive	1.7 ± 0.2	7 ± 1	19 ± 2	0.3 ± 0.05	2 ± 0.1	1.1 ± 0.1	0.2 ± 0.01	0.99 ± 0.01
RDS2153	Noncompetitive	20 ± 2	20 ± 2	20 ± 2	0.4 ± 0.1	0.3 ± 0.1	0.9 ± 0.1	0.3 ± 0.01	0.2 ± 0.01
RDS2184	Mixed-competitive	2 ± 0.1	7 ± 1	19 ± 2	0.3 ± 0.05	2 ± 0.1	1 ± 0.1	0.2 ± 0.01	0.95 ± 0.01

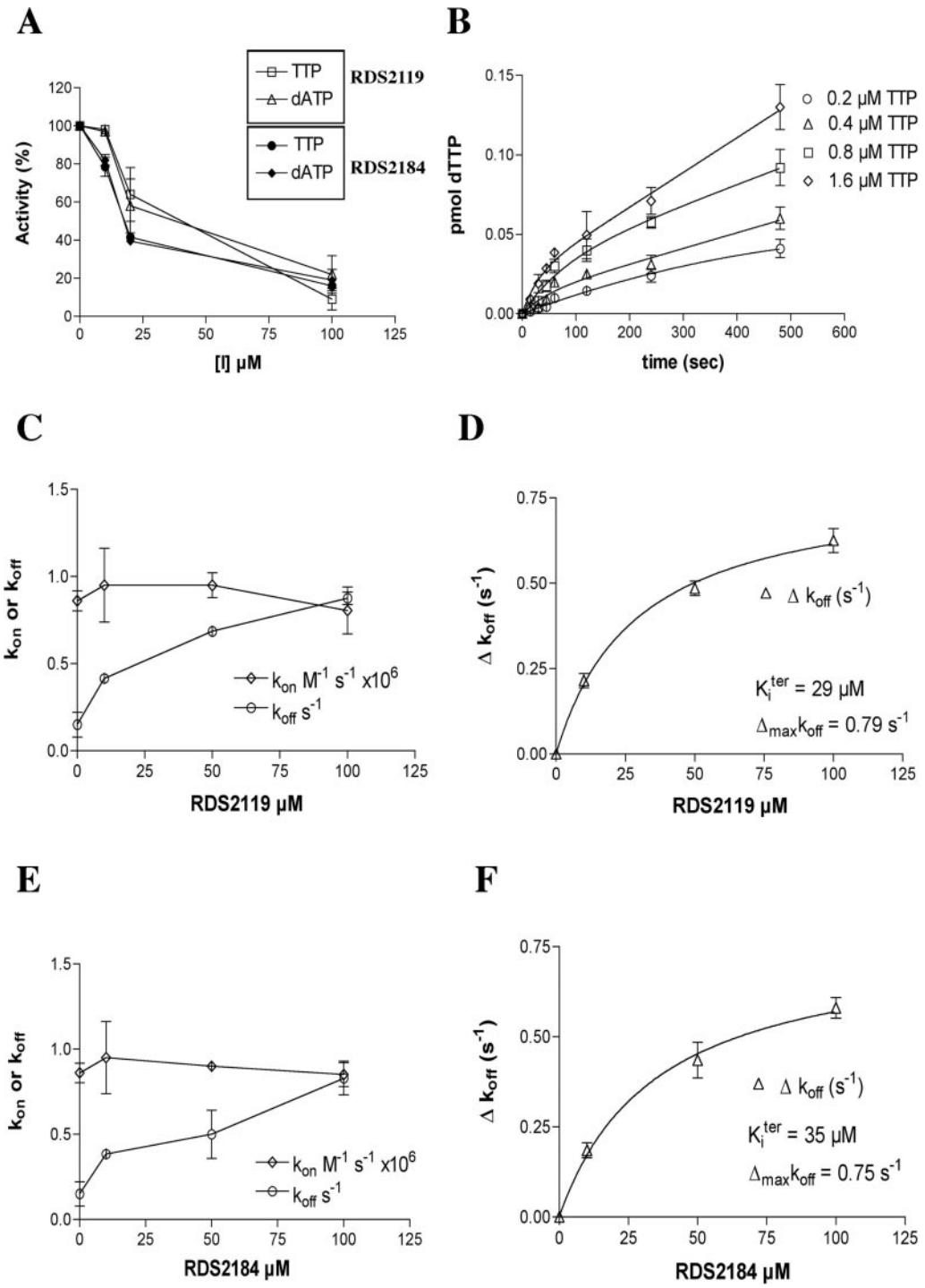


Fig. 6. The inhibition by the DKHA derivatives RDS2119 and RDS2153 of nucleotide incorporation by DNA polymerase λ terminal transferase activity is not influenced by the nature of the substrate and selectively affects the substrate dissociation rate. A, dose-response curves for the inhibition by RDS2119 (open symbols) and by RDS2184 (filled symbols) of TTP and dATP incorporation by 50 nM DNA polymerase λ. Assays were performed under the conditions for terminal transferase activity. □, TTP in the presence of RDS2119; △, dATP in the presence of RDS2119; ●, TTP in the presence of RDS2184; ♦, dATP in the presence of RDS2184. B, time-course experiments for TTP incorporation (terminal transferase activity) by 100 nM DNA polymerase λ in the presence of 0.2 (○), 0.4 (△), 0.8 (■), and 1.6 μM (◇) TTP. Curves were generated as described under *Materials and Methods*. C, variation of the association (k_{on}, ◇) and dissociation (k_{off}, ○) rates for TTP binding to DNA polymerase λ as a function of the RDS2119 concentration. Kinetic constants were calculated as described under *Materials and Methods*. D, the increase in the k_{off} value for TTP dissociation from DNA polymerase λ (Δk_{off}) was calculated from the difference between the k_{off} value in the absence of RDS2119 and the k_{off} values obtained in the presence of increasing amounts of RDS2119 and plotted as a function of the RDS2119 concentration. Data were fitted to the hyperbolic equation $\Delta k_{off} = \Delta_{max}/(1 + K_i^{ter}/[I])$. The value Δ_{max} is the maximum k_{off} increase at infinite inhibitor concentration. The value K_i^{ter} is as defined in Fig. 2. E, variation of the association (k_{on}, ◇) and dissociation (k_{off}, ○) rates for TTP binding to DNA polymerase λ as a function of the RDS2184 concentration. Kinetic constants were calculated as described under *Materials and Methods*. F, the increase in the k_{off} value for TTP dissociation from DNA polymerase λ (Δk_{off}) was calculated from the difference between the k_{off} value in the absence of RDS2184 and the k_{off} values obtained in the presence of increasing amounts of RDS2184 and plotted as a function of the RDS2184 concentration. Data were fitted to the hyperbolic equation $\Delta k_{off} = \Delta_{max}/(1 + K_i^{ter}/[I])$. The value Δ_{max} is the maximum k_{off} increase at infinite inhibitor concentration. The value K_i^{ter} is as defined in Fig. 2.

Binding of the DKHA Derivatives Is Not Affected by Mutations in the DNA Primer and Nucleotide Binding Sites of DNA Polymerase λ. We have recently generated and characterized different DNA polymerase λ mutants that carry single amino acidic substitutions in a helix-loop-helix motif (E330A, V334A, and F338A), which we showed was important for primer binding (Maga et al., 2004), and in the enzyme's nucleotide binding site (Y505A) (Shevelev et al., 2003). Because both DKHA derivatives RDS2119 and RDS2184 were found to be partially competitive with the DNA and dNTP substrates, their inhibition potencies were determined also toward the tdt activity of these mutants. The results are summarized in Table 2. The inhibition potencies of all three inhibitors were similar for the different mutants and comparable with the ones obtained for DNA polymerase λ wild type (the biggest differences being a 1.5- to 2-fold decrease in the inhibition potencies). These results clearly indicated that the binding site of the DKHA derivatives neither overlaps with either the DNA primer nor with the nucleotide binding site of DNA polymerase λ. Further site-directed mutagenesis and molecular modeling studies will be necessary to precisely identify the drug binding site.

The DKHA Derivatives also Inhibit the Polynucleotide Synthetase Activity of DNA Polymerase λ. We have shown recently that DNA polymerase λ, DNA polymerase μ, and TDT are able to synthesize short nucleotide oligomers of discrete size (mainly 7- and 14-mer) in the absence of any DNA primer and/or template (Ramadan et al., 2004b). This polynucleotide synthetase activity was shown to be an intrinsic property of these enzymes and not caused by contaminating DNA and/or by an artifact of the reaction conditions. However, because of a lack of specific inhibitors, it was not possible to determine whether the DNA polymerase activity of DNA polymerase λ and DNA polymerase μ played a role in this particular mode of synthesis or whether it had to be ascribed to the tdt activity only. Thus, we tested the DKHA derivatives for their ability to inhibit the polynucleotide synthetase activity of DNA polymerase λ. As shown in Fig. 7, all three compounds reduced the products synthesized by DNA polymerase λ in the presence of dCTP alone in a dose-dependent manner (compare lane 1 with lanes 2–7). These results indicated that the ability of DNA polymerase λ to synthesize DNA de novo is correlated to its tdt activity. Dose-response curves generated over a wider range of inhibitor concentrations (data not shown) allowed the determination of the ID_{50} values for all the inhibitors, which were 260 μM for RDS2119, 170 μM for RDS2153, and 270 μM for RDS2184. These values are much higher than those derived for the inhibition of the tdt activity. This might be caused by a different structure of the binary DNA polymerase λ-dNTP catalytic complex involved in this reaction, compared with the binary DNA polymerase λ-DNA complex responsible for the tdt activity.

TABLE 2
Inhibition potencies of DKHA derivatives against the tdt activity of pol λ wild-type and different mutants
ID_{50} values were calculated as described under *Materials and Methods* and are presented as the mean values of three independent experiments ± S.D.

Enzyme	ID_{50}		
	RDS2119	RDS2153	RDS2184
		μM	
Wild-type	19 ± 2	20 ± 3	15 ± 2
λΔ137	175 ± 10	39 ± 3	71 ± 5
λΔ244	153 ± 10	33 ± 3	55 ± 6
E330A	24 ± 2	24 ± 2	24 ± 2
V334A	32 ± 3	30 ± 3	28 ± 3
F338A	32 ± 2	29 ± 3	27 ± 2
Y505A	32 ± 3	28 ± 2	25 ± 2

The DKHA Derivative RDS2119 Shows High Cytotoxicity against Human TDT$^+$ Leukemic Cells but Not against TDT$^-$ HeLa Cells. TDT has been regarded as an attractive target for human acute lymphocytic leukemia chemotherapy (McCaffrey et al., 1983; Foss, 2000). Because DKHA derivatives represent the first selective inhibitors of mammalian terminal transferases described so far, we decided to test whether these compounds displayed toxicity against TDT$^+$ leukemic cells. We chose RDS2119 as the prototype compound of this class. As a first experiment, we evaluated the toxicity of RDS2119 against human cells. Exponentially growing HeLa cells were incubated for 24 h in the absence or presence of increasing amounts of RDS2119, and then the percentage of viable cells was determined with respect to controls incubated with DMSO. As shown in Fig. 8A, RDS2119 up to a concentration of 100 μM showed no significant toxicity against HeLa cells. Next, exponentially growing MOLT-4 leukemic cells, which overexpress TDT, were incubated for 24 h in the absence or presence of increasing amounts of RDS2119, and then the percentage of viable cells was determined with respect to controls incubated with DMSO. As shown in Fig. 8A, RDS2119 showed high toxicity against MOLT-4 cells, with a CC_{50} value of 14.9 μM. Time-course experiments indicated that RDS2119 showed its maximal toxic effect already after 3 h of exposure of the cells to the drug (Fig. 8B).

HeLa cells do not express TDT, but they express DNA polymerase λ, whose terminal deoxyribonucleotidyl transferase activity is also targeted by RDS2119. To assess the levels of DNA polymerase λ in MOLT-4 versus HeLa cells, Western blot was performed using whole-cell extracts from HeLa and MOLT-4 cells. Increasing amounts of HeLa and MOLT-4 extracts (quantified as total proteins) were loaded on a gel and transferred to a membrane. As shown in Fig. 8C, antibodies specific for DNA polymerase λ detected a polypeptide of the same size in both cell extracts. As an internal standard, known amounts of recombi-

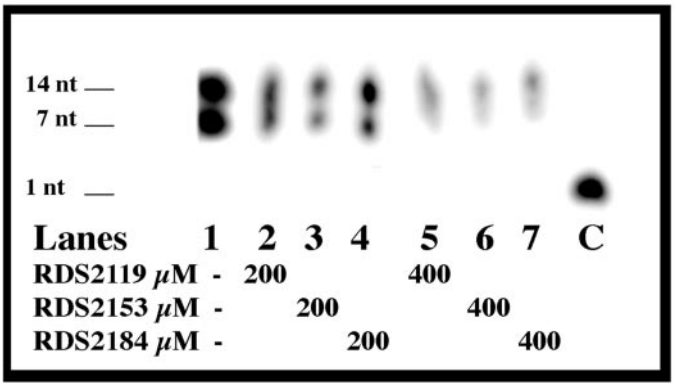


Fig. 7. Effects of the three DKHA derivatives on the polynucleotide synthetase activity of DNA polymerase λ. The polynucleotide synthetase (de novo DNA synthesis; Ramadan et al., 2004) activity of DNA polymerase λ was assayed as described under *Materials and Methods* in the absence (lane 1) or presence of 200 (lane 2) and 400 μM (lane 5) RDS2119; 200 (lane 3) and 400 μM (lane 6) RDS2153; and 200 (lane 4) and 400 μM (lane 7) RDS2184. C, control lane without DNA polymerase λ. The size (in nucleotides) of the products is indicated on the left.

nant human DNA polymerase λ were loaded on the same membrane. They positively reacted with the antibodies, confirming that the polypeptide detected in the cell extracts was indeed DNA polymerase λ. Quantification of the intensities of the bands was performed by scanning densitometry, and the relative intensity normalized to the value measured for 100 ng of recombinant DNA polymerase λ (lane 5) is indicated below each lane. From these measurements, the ratio of DNA polymerase λ over the total protein concentration of each extract was derived. The same membrane was then probed with antitubulin antibodies as an independent control of the relative protein amounts on the membrane (data not shown). As indicated at the top of the figure, HeLa cells and MOLT-4 cells expressed similar amounts of DNA polymerase λ. These results indicate that the selective cytotoxicity of RDS2119 toward MOLT-4 cells was not caused by differences in the levels of DNA polymerase λ-tdt activity and further support the hypothesis that the cytotoxic effect was probably caused by the inhibition of TDT. However, other explanations are still plausible, such as the lack of another protein/enzyme in HeLa cells, which makes them insensitive to DNA polymerase λ-tdt inhibition. Direct knockdown of pol λ expression (e.g., by RNA interference) will be required to directly prove our hypothesis.

Discussion

In the present study, we have described three novel DKHA derivatives with potent and selective activity against mammalian terminal deoxynucleotidyl transferases activities. These compounds are diketo acids, a family of compounds

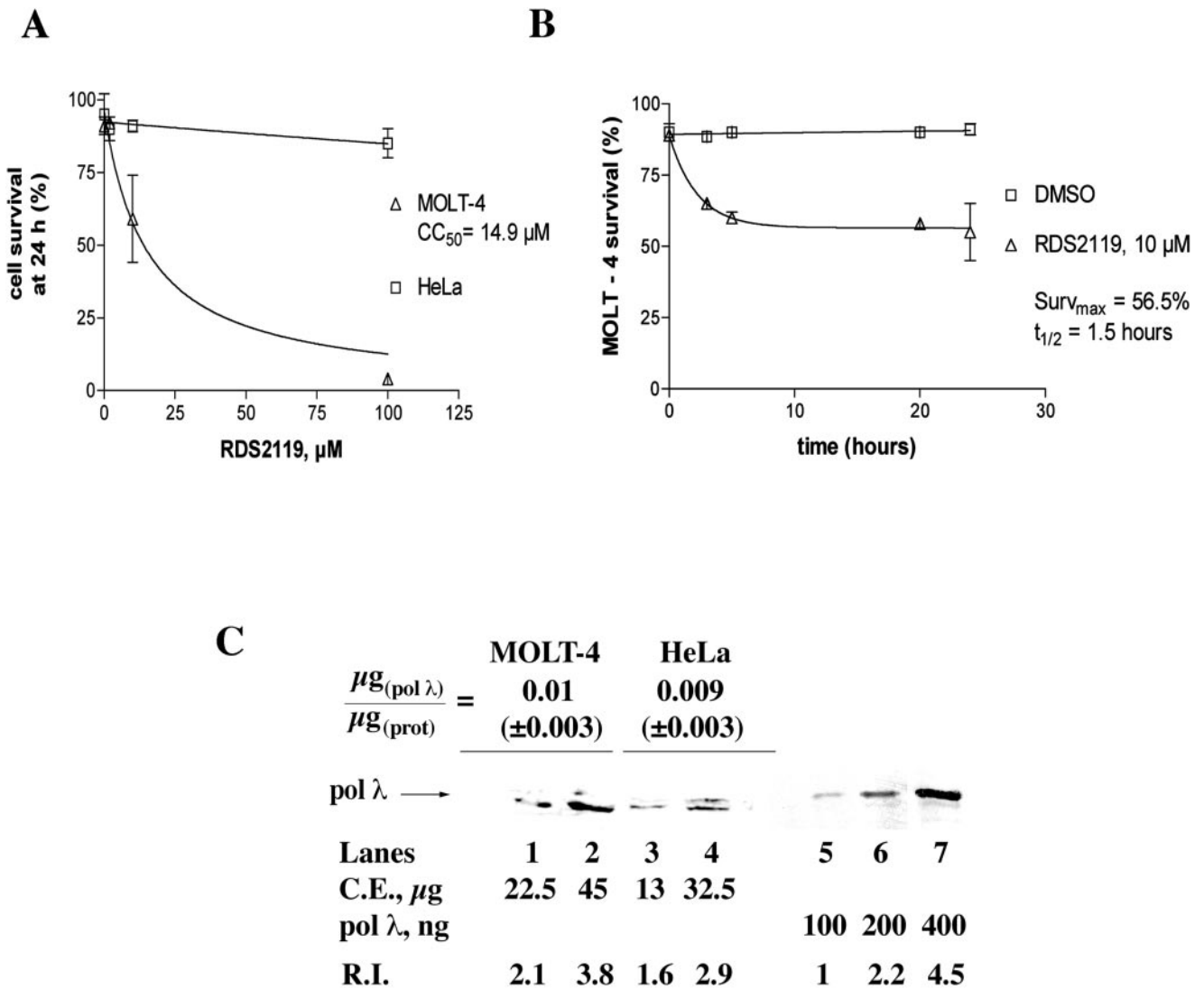


Fig. 8. The DKHA derivatives can specifically inhibit TDT$^+$ leukemic cells proliferation. A, exponentially growing HeLa cells (□) or MOLT-4 cells (TDT$^+$ overproducing cells, △) were exposed for 24 h to increasing amounts of RDS2119 (1, 10, and 100 μM). Cell viability was assessed by the dye-exclusion assay. B, exponentially growing MOLT-4 cells were exposed either to DMSO (□) or to 10 μM RDS2119 (△) for increasing time periods (10, 20, and 30 h). Cell viability was tested at each time point by the dye-exclusion assay. The percentage of viable cells, calculated as the number of living cells at time (t) divided by the number of living cells at time 0, was plotted against time, and the data were fitted to the simple exponential survival (%) = e^{-kt} + $Surv_{max}$(%). C, HeLa or MOLT-4 cells extracts were prepared as described under *Materials and Methods*. The total protein amounts loaded on the gel are indicated (lanes 1–4). Recombinant human DNA polymerase λ was loaded on the same gel (lanes 5–7). After transfer on a nitrocellulose membrane, DNA polymerase λ was revealed by polyclonal antibodies raised against full-length recombinant human DNA polymerase λ. Quantification of the bands was performed as described under *Materials and Methods*. R.I., relative intensities normalized to 100 ng of recombinant DNA polymerase λ (lane 5).

that also includes inhibitors of the RNaseH activity of HIV-1 reverse transcriptase and of the HIV-1 integrase (Shaw-Reid et al., 2003; Costi et al., 2004; Witvrouw et al., 2004; Tramontano et al., 2005). It is interesting that none of the anti–HIV-1 diketo acid derivatives can inhibit the DNA polymerase activity of the viral reverse transcriptase. A similar behavior is shown by the compounds described here, which can selectively inhibit the tdt activity of DNA polymerase λ but not its associated DNA polymerase activity. From a mechanistic point of view, the DKHA derivatives behave as allosteric inhibitors of both DNA polymerase λ-tdt and TDT. They are noncompetitive with either the DNA or the dNTP substrate, but, in the case of RDS2119 and RDS2184, they show a mixed inhibition toward DNA polymerase λ-tdt, reducing both the catalytic rate of the reaction and the apparent affinity of the enzyme for the dNTP substrate. Kinetic analysis showed that this effect was caused by an increase of the nucleotide substrate dissociation rate. Furthermore, the compounds RDS2119 and RDS2184 showed reduced binding affinity toward two truncated DNA polymerase λ mutants lacking the first 137 and 244 amino acids, respectively, whereas the compound RDS2153 was unaffected. The deleted regions corresponded to the BCRT domain and the BRCT plus the proline-rich domains of DNA polymerase λ, respectively (Hubscher et al., 2002). These domains have been shown to be entirely dispensable for the catalytic activities of DNA polymerase λ (Fiala et al., 2004). The fact that their deletion reduced, but did not abrogate, the binding of RDS2119 and RDS2184 suggested that these two inhibitors were sensitive to some structural alteration induced by these deletions. When the relative affinities of RDS2119 and RDS2184 for the different reaction intermediates were determined with wild-type DNA polymerase λ, it was found that both inhibitors bound either the free enzyme or the binary enzyme/DNA complex with higher affinity than the ternary enzyme/DNA/dNTP complex. These results further support the hypothesis that these compounds are very sensitive to rearrangements in the DNA polymerase λ structure.

Besides being valuable tools for investigating the role(s) of mammalian terminal transferases in a cellular context, the DKHA derivatives described here are also potentially interesting from a pharmacological point of view. In fact, the compound RDS2119 proved to be extremely toxic against the TDT^+ leukemic cell line MOLT-4, with a CC_{50} value close to its K_i value for TDT, suggesting that little intracellular degradation occurred but showed almost no toxicity toward HeLa cells. Mammalian TDT seems to be an attractive target for antileukemic chemotherapy, as already suggested by the observation that the nucleoside analog cordycepin (3′-dA), which inhibits TDT, selectively suppresses the proliferation of TDT^+ leukemic cells. However, to exert its activity, cordycepin needs to be administered together with the adenosine deaminase inhibitor deoxycoformycin, which prevents its deamination (Foss, 2000; Kodama et al., 2000). Moreover, cordycepin suffers from a number of drawbacks common to all of the nucleoside analogs. First, it has to be subjected to three independent cellular phosphorylation steps to be converted to its active form cordycepin triphosphate (3′-dATP). Second, it can be readily deaminated by adenosine deaminase. Third, it is subjected to possible degradation by other enzymes, such as nucleosidases and phosphorylases. Fourth, in its active triphosphate form it can inhibit not only TDT but also other cellular enzymes such as RNA polymerase and DNA primase, thus becoming toxic to normal cells. Our DKHA analogs, on the other hand, proved to be very specific for terminal transferases. Moreover, being non-nucleoside analogs, they do not require any intracellular activation step. Thus, DKHAs seem to be the first selective and nontoxic synthetic non-nucleoside TDT inhibitors and hold the potential to be further developed as novel antitumoral agents.

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