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Partial selective inhibition of HIV-1 reverse transcriptase and human DNA polymerases γ and β by thiated 3′-fluorothymidine analogue 5′-triphosphates

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ABSTRACT

3′-Deoxy-3′-fluorothymidine (FLT, alovudine®) belongs to the most potent agents inhibiting HIV-1 replication. Its 5′-triphosphate (FLTTP) is a potent inhibitor of HIV-1 reverse transcriptase (HIV RT). Unfortunately, FLT exerts substantial hematologic toxicity both *in vitro* and *in vivo*. It was suggested that this toxicity may be related to inhibition of human DNA polymerases, especially mitochondrial DNA polymerase γ, by nucleoside analogue 5′-triphosphates leading to termination of DNA synthesis and mitochondrial dysfunction. To decrease the toxicity of FLT, its thiated analogues, 4-SFLT and 2-SFLT, were previously synthesized and shown to be potent inhibitors of HIV-1 with low *in vitro* cytotoxicity. To explain this phenomenon in the present study the synthesis of 5′-triphosphates of thiated FLT analogues was undertaken and their interaction with recombinant HIV-1 RT and human DNA polymerases γ (pol γ) and β (pol β) was investigated. It was shown that 3′-deoxy-3′-fluoro-4-thiothymidine 5′-triphosphate (4-SFLTTP) and 3′-deoxy-3′-fluoro-2-thiothymidine 5′-triphosphate (2-SFLTTP) were, similarly to FLTTP, potent competitive inhibitors of HIV-1 RT, with K_i^{app} values of 0.091 and 0.022 μM respectively. It is of interest that 2-SFLTTP, a compound in an unusual *syn* conformation around the glycosidic bond was an uncompetitive inhibitor of human mitochondrial DNA pol γ with K_i^{app} of 0.174 μM, while 4-SFLTTP in *anti* conformation inhibited this enzyme similarly to FLTTP, i.e., non-competitively, with K_i^{app} of 0.055 μM. Both 4-SFLTTP and 2-SFLTTP were competitive inhibitors of human DNA pol β, with K_i^{app} values of 16.84 and 4.04 μM, respectively. The results point to partially selective inhibition of HIV RT by thiated 3′-fluorothymidine 5′-triphosphate analogues. Of special interest is that 2-SFLTTP, showing *syn* conformation, is a less potent inhibitor of human mitochondrial pol γ than 4-SFLTTP and FLTTP, both in the *anti* conformation, and has a higher inhibitory activity against HIV-1 RT than 4-SFLTTP. Moreover, the parent nucleoside 2-SFLT possessing the *syn* conformation shows a more potent anti-HIV-1 activity and a better selectivity index than its 4-thio isomer in the *anti* conformation (Matthes et al., 1989; Poopeiko et al., 1995), 2-SFLT is a potent and selective anti-HIV-1 agent with the selectivity index 4-fold higher than that of FLT.

Findings regarding the mechanisms of antiviral and cytotoxic activities of FLT and its thioanalogues are discussed.

1. Introduction

Multidrug, highly active antiretroviral therapy (HAART) employed in the treatment of human immunodeficiency virus (HIV) infection uses as its main components nucleoside inhibitors of HIV reverse transcriptase (NRTIs). It was suggested that long term therapy with the NRTIs may be associated with mitochondrial DNA polymerase γ (pol γ) inhibition and subsequent depletion of mitochondrial DNA (Kaguni, 2004; Kakuda, 2000). However, the DNA pol γ hypothesis by itself fails to explain the entire array of metabolic deficiencies associated with NRTI-induced disorders. Direct effects of NRTI's on various mitochondrial targets must also be taken into account (Lund and Wallace, 2004).

3′-Deoxy-3′-fluorothymidine (FLT, alovudine®) belongs to the most potent agents inhibiting HIV-1 replication. In addition, it is highly active against HIV-1 multidrug resistant (MDR) strains. Its 5′-triphosphate (FLTTP) is a potent inhibitor of HIV-1 reverse transcriptase (HIV RT, RNA-directed DNA polymerase, EC 2.7.7.49) (Cheng et al., 1987). Unfortunately, FLT displays high cytotoxicity against host cells which limits its clinical applicability (Balzarini et al., 1988). It was suggested that this toxicity may be related to inhibition of human DNA polymerases (EC 2.7.7.7), especially mito-

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chondrial DNA polymerase γ, by FLT metabolite—5′-triphosphate, leading to termination of DNA synthesis and mitochondrial dysfunction (Kaguni, 2004). To decrease the cellular toxicity of FLT, its thiated analogues 4-SFLT and 2-SFLT were previously prepared and shown to be potent *in vitro* inhibitors of HIV-1 with low toxicity (Matthes et al., 1989; Poopeiko et al., 1995). To establish whether inhibition of pol γ exerted by thiated FLTTP analogues is correlated with development of *in vitro* cytotoxicity in cell cultures, we decided to synthesise 2-SFLTTP, 4-SFLTTP as well as FLTTP and to investigate their interactions with recombinant HIV-1 RT and human polymerases γ and β.

2. Materials and methods

2.1. Chemicals

[Methyl-^{3}H] dTTP (45.9 Ci/mmol) was purchased from Moravek Biochemicals Inc., Brea, CA; nucleotides and activated calf thymus DNA were from Sigma; AZTTP was purchased from Calbiochem; poly(rA)·p(dT)$_{12–18}$ and HIV reverse transcriptase were from GE Healthcare (Amersham Biosciences); DE81 (2.3 cm) circles were from Whatman. Triton X-100 and rotiszint eco plus LSC-universal cocktail were from Roth. Recombinant human DNA polymerases γ and β were provided by EURx Ltd. (Gdańsk, Poland). All other reagents used in this study were of analytical grade.

2.2. Synthesis of thiated nucleoside 5′-triphosphates

The syntheses of 5′-triphosphates of 2-SFLT, 4-SFLT as well as FLT (Fig. 1) were performed with the use of modified Vrang procedure (Vrang et al., 1987) employing direct phosphorylation of unprotected nucleosides.

2.3. Assays of HIV-1 reverse transcriptase and human DNA polymerases γ and β activities

The activities of HIV-1 RT and both human polymerases were assayed by DE81 filter isotopic method (Reardon and Miller, 1990). Unless otherwise indicated, reaction mixtures for RT activity studies contained 50 mM KCl, 50 mM Tris–HCl, pH 7.8, 13 mM sodium phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 0.02% Triton X-100, 0.33 mM DTT, 20 μM [^{3}H]dTTP (128–180 DPM/pmol), 9 μg/ml poly(rA)·p(dT)$_{12–18}$ and 14 ng HIV reverse transcriptase. Reaction mixture for pol γ contained 25 mM HEPES-KOH (pH 8.0), 5 mM Tris–HCl (pH 8.0), 0.25 mM DTT, 0.5 mM $MnCl_2$, 2.5 mM β-ME, 10 μg acetylated BSA, 0.01% Triton X-100, 5 μM [^{3}H]dTTP (4×10^3 DPM/pmol), 0.1 M NaCl, 20 μg/ml poly (rA)·oligo(dT)$_{12–18}$ and 98 ng of polymerase γ. Reaction mixture for pol β contained 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 0.4 mg/ml of BSA, 2.5 mM DTT, 100 mM KCl, 5 mM NaCl, 0.01% Triton X-100, 50 μM dNTP (specific activity of [^{3}H]dTTP was 2×10^2 DPM/pmol), 300 μg/ml activated calf thymus DNA and 18 ng of polymerase β. All the reaction mixtures were initiated with enzyme in a total volume of 20–30 μl and incubated at 37 °C and performed for 30 min. 10–15 μl of a reaction mixture was spotted onto DE81 (2.3 cm) paper circle. In order to remove unincorporated [^{3}H]dTTP, the circles were washed 3× with 5% Na_2HPO_4, 3× with distilled water and once with 95% ethanol. After placing the dry circles in scintillation flasks, 300 μl of the elution mixture (0.2 M KCl, 0.1 M HCl) was added. The amount of [^{3}H]dTMP incorporated into template and adsorbed on the circle was determined in scintillation cocktail using a PerkinElmer® scintillation counter.

2.4. Steady-state kinetic assays

The appropriate concentrations of each inhibitor used for the K_i^{app} determination were calculated using the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [S]/K_m)$ where $[S]$ and K_m were the concentration and affinity of the radioligand, respectively (Cheng and Prusoff, 1973). K_i^{app} values for HIV-1 RT inhibition were determined at minimum two concentrations of each compound tested and four concentrations of dTTP in the range of 1–32 μM and at saturating poly(rA)·p(dT)$_{12–18}$ concentration of 9 μg/ml. K_i^{app} values for human pol γ inhibition were determined at two to three concentrations of each inhibitor tested and four concentrations of dTTP in the range of 1.5–12 μM and at saturating poly(rA)·p(dT)$_{12–18}$ concentration of 20 μg/ml. K_i^{app} values for human pol β inhibition were determined at two concentrations of each inhibitor tested and four concentrations of dTTP in the range of 40–320 μM and saturating concentration of activated calf thymus DNA of 300 μg/ml. V_{max}^{app}, K_m^{app} and K_i^{app} values were calculated using nonlinear regression by fitting of the experimental data to the appropriate equations in GOSA *fit* (Global Optimization by Simulated Annealing) Bio-Log software. To determine the type of inhibition, the experimental data were fitted to the general equation for reversible inhibition in GOSA *fit*, and, additionally, the double-reciprocal plots of dTMP incorporation rate into template–primer and dTTP concentration dependence in the presence of several inhibitor concentrations (Lineweaver–Burk plots) were generated.

2.5. NMR-based conformational analysis

All the spectra of nucleoside 5′-triphosphates and the corresponding nucleosides were recorded for their aqueous solutions at 298 K on 500 MHz Varian Unity Plus spectrometer. The spectra were processed with the aid of MestRec 4.56 using $\pi/4$ shifted sine-bell filter followed by Lorenzian smoothing resulting in 0.5 Hz line broadening and zero-filling up to 32k data points prior to Fourier transformations. The resonance signals of nucleosides were assigned on the basis of the geminal and vicinal scalar couplings extracted from the spectra and verified by comparison of the simulated and experimental spectra. The assignment of those of triphosphates was adopted from the spectra of the corresponding nucleosides. Due to significant broadening of the resonance lines, no furanose ring puckering analysis could have been applied for nucleoside 5′-triphosphates, and only *syn–anti* preferences about the glycosidic bond were followed by the analysis of the two proposed discriminators—e.g., chemical shift of H1′ and the separation of H2′ and H2″ resonances, both of which were previously proven sensitive to relative orientation of ribose and pyrimidine moieties (Poznański et al., 2000).

3. Results and discussion

3.1. Kinetics of incorporation of dTMP

The rates of dTMP incorporation into poly(rA)·p(dT)$_{12–18}$ by HIV-1 RT and human DNA polymerase γ and into activated DNA by human DNA pol β were linear with respect to time and enzyme concentration with V_{max}^{app} values of 370 ± 99, 0.23 ± 0.05 and 64 ± 15 nmol min^{-1} mg^{-1} protein, respectively; the template–primer concentrations used in the assays did not inhibit the enzyme activity (Majumdar et al., 1988). The K_m^{app} value obtained for dTTP for HIV-1 RT (9.05 ± 0.46 μM) was similar to the values determined by others with the use of poly(rA)·p(dT)$_{12–18}$ (Matthes et al., 1989; Reardon and Miller, 1990), whereas K_m^{app} values for the substrate obtained for the studied recombinant human DNA polymerases γ and β (2.61 ± 0.19 μM and 78 ± 14 μM, respec-

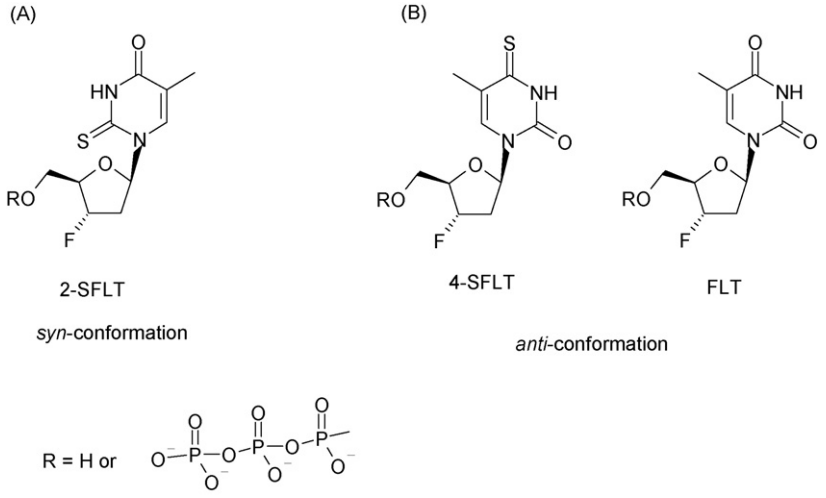

Fig. 1. Structures and conformations around glycosidic bond of 3′-deoxy-3′-fluoro-2-thiothymidine, its 5′-triphosphate (2-SFLT, A), 3′-deoxy-3′-fluoro-4-thiothymidine (4-SFLT) and 3′-deoxy-3′-fluorothymidine (FLT, FLTTP, B).

tively) were significantly higher than those reported for the native polymerases isolated from mammalian cells and assayed with the use of the same template–primers as in the present study (Faraj et al., 2000; Hart et al., 1992; Martin et al., 1994). However, the observed difference between the K_m^{app} values for dTTP for both polymerases studied was similar to that demonstrated by others, and the low K_m^{app} for pol γ was diagnostic for this polymerase (Hart et al., 1992; Martin et al., 1994).

3.2. Effects of 4-SFLTTP and 2-SFLTTP on HIV-1 reverse transcriptase and human DNA polymerases γ and β

FLTTP and AZTTP were used in the present study as controls in HIV-1 RT and human DNA polymerases inhibition assays. The results obtained for inhibition of HIV-1 RT and human polymerase γ and β by the tested compounds are presented in Table 1. Enzyme kinetic studies with HIV-1 RT (Lineweaver–Burk plots) showed that both 4-SFLTTP and 2-SFLTTP were, similarly to the parent compound and to AZTTP, competitive inhibitors of this enzyme with respect to dTMP incorporation into poly(rA)·p(dT)$_{12-18}$. K_i^{app} values for both control inhibitors for HIV-1 RT inhibition with poly(rA)·p(dT)$_{12-18}$ were within the range of values reported in the literature (Cheng et al., 1987; Hart et al., 1992; Matthes et al., 1990; Reardon and Miller, 1990). 4-SFLTTP was about three and four times less inhibitory to HIV-1 RT than FLTTP and AZTTP, respectively (Table 1). In contrast, 2-SFLTTP exhibited exactly the same inhibitory effect toward HIV-1 RT as AZTTP and slightly greater than FLTTP. The obtained difference between the activity of 4-SFLTTP and the parent compound, FLTTP, toward HIV-1 RT was in agreement with the results previously reported (Matthes et al., 1989).

4-SFLTTP and 2-SFLTTP were found to be effective inhibitors of human DNA polymerase γ. Interestingly, 2-SFLTTP acted as an uncompetitive inhibitor of this polymerase with respect to dTMP incorporation into poly(rA)·p(dT)$_{12-18}$ (Fig. 2A), whereas 4-SFLTTP inhibited this enzyme similarly to FLTTP, i.e., non-competitively (Fig. 2B). AZTTP was found to be a mixed inhibitor of human DNA pol γ with respect to dTMP incorporation into poly(rA)·p(dT)$_{12-18}$, with both competitive and uncompetitive mechanisms of inhibition operating with different K_i^{app} values for each of the two types of inhibitor action. The same type of inhibition for AZTTP with similar K_i^{app} values was reported by others, when poly(rA)·p(dT)$_{12-18}$ was used as the template–primer for mitochondrial polymerase (Lewis et al., 1994). The presented data indicate that 4-SFLTTP, similarly to control inhibitors, FLTTP and AZTTP, can bind both the free enzyme and the enzyme–substrate complex, but unlike AZTTP, binds with the same affinity, whereas 2-SFLTTP binds only the enzyme–substrate complex. The obtained results are

Table 1
In vitro anti-HIV-1 activity of thymidine nucleoside analogues and inhibition constants of HIV-1 reverse transcriptase and human DNA polymerases γ and β for the adequate triphosphates.

Nucleoside	Anti-HIV-1 activity EC_{50} [μM][a]	Cytotoxicity CC_{50} [μM][b]	Selectivity index (SI) (CC_{50}/EC_{50})	Enzyme inhibition				
				HIV RT		Human pol γ		Human pol β
				Nucleotide	K_i^{app} ± SD [μM]	Type of inhibition[c]	K_i^{app} ± SD [μM]	K_i^{app} ± SD [μM]
AZT	0.003[e]	4.81[e]	1603	AZTTP	0.022 ± 0.005[d]	Mixed	$K_i^1 = 2.3 \pm 0.09$ $K_i^2 = 9.56 \pm 0.67$	422 ± 38
FLT	0.001[e]	0.197[e]	197	FLTTP	0.031 ± 0.001	Non-competitive	0.054 ± 0.016	2.8 ± 0.39
4-SFLT	1.0[f]	480[f]	480	4-SFLTTP	0.091 ± 0.009	Non-competitive	0.055 ± 0.023	16.84 ± 2.86
2-SFLT	0.269[g]	223[g]	828	2-SFLTTP	0.022 ± 0.004	Uncompetitive	0.174 ± 0.049	4.04 ± 0.12

[a] 50% effective concentration, or concentration required to inhibit HIV-induced cytopathogenicity in MT-4 cells.
[b] 50% cytotoxic concentration, or concentration required to reduce the viability of MT-4 cells.
[c] In all other cases, inhibition was competitive with respect to dTTP.
[d] The results are presented as means ± SD, calculated from three independent experiments.
[e] Balzarini et al. (1989).
[f] Matthes et al. (1989).
[g] Poopeiko et al. (1995).

Table 2
^{1}H resonance assignment of the ribose protons, and the difference, $\Delta\delta$(H2′2″), between H2′ and H2″ resonances, which was found indicative for *syn–anti* conformation.

	H6	H1′	H2′	H2″	H3′	H4′	H5′	H5″	ΔH2′2″
FLT	7.55	6.23	2.26	2.51	5.22	4.25	3.69		0.25
2-SFLT	7.77	6.95	2.12	2.77	5.23	4.33	3.74		0.64
FLTTP	7.66	6.31	2.28	2.50	5.41		4.08	4.16	0.22
2S-FLTTP	7.84	6.98	2.19	2.74	5.44		4.13	4.19	0.55
4S-FLT	7.72	6.29	2.36	2.67	5.31	4.38	3.78		0.32
24diS-FLT	8.02	6.84	2.15	2.70	5.32	4.32	3.70		0.55
4S-FLTTP	7.72	6.27	2.29	2.57	5.41		4.10	4.17	0.28
2,4diS-FLTTP	7.83	6.90	2.17	2.84	5.42		4.20	4.20	0.67
dUrd	7.83	6.27	2.36	2.40	4.45	4.03	3.82	3.74	0.04
2S-dUrd		7.03	2.38	2.70	4.52	4.19	3.97	3.88	0.32

rather intriguing and difficult to explain, however, lack of recognition of studied compounds as thymidine analogues by pol γ might be important reason, that inhibition is not competitive with respect to dTTP. It was demonstrated that AZTTP was non-competitive inhibitor of HIV RT with respect to other nucleotides than dTTP (König et al., 1989). In the case of 2-SFLTTP, it could be possible, that its *syn* conformation contributed to the untypical mechanism of inhibition. Furthermore, the influence of some physico-chemical properties of studied recombinant pol γ (number of free –SH or lack of some posttranslational modifications) or/and additionally template dependence may be also taken into consideration.

It is noteworthy that 2-SFLTTP in contrast to FLTTP and 4-SFLTTP (no selectivity was observed) was several fold more selective toward HIV-1 RT than toward human pol γ, although, it was less selective than AZTTP (Table 1). In comparison to FLTTP and its thionucleotides, AZTTP was found in the present study to be a relatively weak inhibitor of human DNA pol γ, which is consistent with the literature data demonstrating inhibition of this polymerase by AZTTP and other ddNTP (Hart et al., 1992; Martin et al., 1994; Parker et al., 1991). The results obtained for human pol γ inhibition were in agreement with other studies demonstrating that human DNA pol γ is unique among the cellular replicative DNA polymerases in that it is highly sensitive to inhibition by anti-HIV nucleotide analogues (Johnson et al., 2001; Lee et al., 2003; Lim and Copeland, 2001; Martin et al., 1994; Parker et al., 1991). It was demonstrated that mitochondrial polymerase readily incorporated 2′,3′-dideoxy-CTP (ddCTP), 2′,3′-dideoxy-ITP (ddITP) and 2′,3′-didehydro-3′-deoxy-TTP (D4T-TP) with an efficiency similar to the incorporation of normal nucleotides, whereas AZTTP, carbocyclic 2′,3′-didehydro-ddGTP (CBV-TP) and (−)-2′,3′-dideoxy-3′-thiacytidine-TP (3TC-TP) acted as only moderate inhibitors of this enzyme (Bienstock and Copeland, 2004; Eriksson et al., 1995; Hanes and Johnson, 2008; Lim and Copeland, 2001).

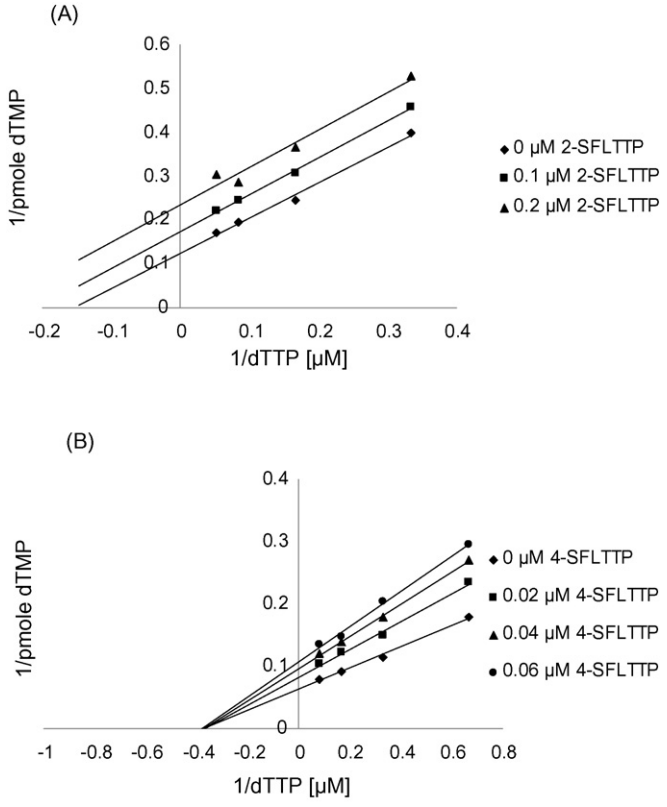

Fig. 2. Lineweaver–Burke plots of human DNA polymerase γ inhibition by 2-SFLTTP (A) and 4-SFLTTP (B).

Enzyme kinetic studies with human DNA pol β demonstrated that both 4-SFLTTP and 2-SFLTTP, similarly to AZTTP and FLTTP were competitive inhibitors of that enzyme with respect to dTMP incorporation into activated DNA. The data obtained for inhibition of pol β by 4-SFLTTP and FLTTP were consistent with the previously reported results, demonstrating 4-SFLTTP to be less effective than FLTTP (Matthes et al., 1987, 1989). The reported K_i^{app} values for AZTTP inhibition of dTMP incorporation into endonuclease-activated DNA vary for this polymerase between 0.67 and 810 μM. The reason for this dramatic disparity is not clear. However, AZTTP in the most cases was a less potent inhibitor of polymerase β than the ddNTPs (Copeland et al., 1992; Hart et al., 1992; Martin et al., 1994). In the present study, AZTTP was found to be an over 100-fold less potent inhibitor of human pol β than FLTTP, which is in agreement with the literature data (Martin et al., 1994). The observed general inhibitory effect of the studied compounds was reported for other NRTIs (Kakuda, 2000), with the following hierarchy: HIV-1 RT > human pol γ > human pol β, with the exception of 4-SFLTTP (higher K_i^{app} for HIV-1 RT than for pol γ).

3.3. NMR-based conformational analysis

The different interaction of the 2- and 4-thio FLTTP analogues with polymerase γ is intriguing and may be due to the different, unusual *syn* conformation around the glycosidic bond of the 2-thio FLT analogues (Fig. 1A), in contrast to FLT itself and to its 4-thio analogue which show typical for natural nucleosides conformation *anti* (Fig. 1B). This was more precisely analysed with the aid of NMR spectroscopy. The resonance assignment is summarised in Table 2. On the basis of the data obtained previously for a series of variously substituted α and β 2′-deoxyuridine derivatives (Remin, 2001), we have previously found that the separation of 2′,2″ methylene group resonances is indicative of a *syn–anti* equilibrium. Consequently, the data presented in Fig. 3 clearly indicate that FLT, FLTTP, 4-S-FLT, 4-S-FLTTP and dUrd are in an *anti* conformation,

while in their 2-S substituted derivatives, 2-S-FLT, 2-S-FLTTP, 2,4-S-FLT, 2,4-S-FLTTP and 2-S-dUrd the *syn* conformation predominates (Fig. 3).

3.4. Inhibition of enzymes versus in vitro anti-HIV-1 activity of 4-SFLT and 2-SFLT

The presented results do not show much correlation with the anti-HIV-1 *in vitro* studies on syncytia-inducing laboratory HIV-1 strain in MT-4 cells, which demonstrate that 2-SFLT and 4-SFLT inhibit viral growth with EC_{50} of 0.269 and 1.0 μM (Table 1), respectively, with very low cytotoxicity observed (CC_{50} of 223 and 480 μM, respectively) (Matthes et al., 1989; Poopeiko et al., 1995). 2-SFLT and 4-SFLT were several orders of magnitude weaker inhibitors of HIV replication than FLT (EC_{50} 0.001 μM) (Balzarini et al., 1989), whereas K_i^{app} values obtained for the inhibition of HIV-1 RT by their triphosphates were comparable and 3-fold higher than that for FLTTP, respectively (Table 1). However, it is noteworthy that 4-SFLTTP was a nearly 4-fold weaker inhibitor of HIV-1 RT than 2-SFLTTP, and a similar relation between EC_{50} values for the adequate thionucleosides was observed in *in vitro* anti-HIV-1 activity studies (Matthes et al., 1989; Miazga et al., 2003; Poopeiko et al., 1995).

There was no correlation between the obtained K_i^{app} values for human pol γ and pol β inhibition by 5′-triphosphates of FLT and its two thionucleosides (Table 1) and the cytotoxicity observed in *in vitro* tests. 4-SFLT and 2-SFLT exerted very low cytotoxicity in comparison to FLT (CC_{50} of 480, 223 and 0.197 μM, respectively) (Balzarini et al., 1989; Matthes et al., 1989; Poopeiko et al., 1995). The above results are in contrast to the observation that 4-SFLTTP and 2-SFLTTP inhibit pol γ similarly and only 4-fold less than FLTTP, respectively, and, moreover, 1–2 orders of magnitude more strongly than AZTTP (Table 1). On the other hand, AZT exerts much stronger cytotoxicity than thionucleosides with CC_{50} = 4.81 μM (Balzarini et al., 1989). Furthermore, both thionucleoside 5′-triphosphates were found to be much more effective inhibitors of human DNA pol β than AZTTP, but, respectively comparable and 6-fold less effective inhibitors of DNA pol β in the case of 2-SFLTTP and 4-SFLTTP as compared to FLTTP (Table 1). The data on pol γ inhibition are in line with previous results, obtained with other nucleoside analogues, which point to the lack of a universal mechanism of mitochondrial toxicity exerted by NRTIs (Lund and Wallace, 2004; Lund et al., 2007). The poor correlation between inhibition of HIV-1 RT and human DNA polymerases and the effects in cells was observed for many other NRTIs including analogues of thymidine triphosphate, such as ddTTP and d4TTP (Martin et al., 1994). Obviously, a precise correlation between enzyme inhibition and the effects on cells cannot be expected, because of numerous differences in the assays. The templates and primers used in the present study, poly(rA)·p(dT)$_{12-18}$ and activated calf thymus DNA, were artificial and differed from those the enzymes might encounter in HIV-1 infected cells. Different templates and primers will give rise to binary enzyme-template and primer complexes whose interactions with a particular nucleotide might result in significantly different K_i^{app} values (Hart et al., 1992). Many processes of intracellular metabolism of nucleoside analogues, like reduction, oxidation, glycosylation and phosphorylation of nucleosides in cells, can influence the anti-HIV activity and cytotoxicity of NRTIs (Becher et al., 2003; Lund et al., 2007; Steet et al., 2000). It was demonstrated that hematopoietic toxicity of FLT *in vivo* was due in part to its efficient conversion to FLTTP and that FLT induced both damage and apoptosis in human lymphoblastoid cell line (Sundseth et al., 1996). Several physico-chemical effects, like a decrease in the pK_a values for dissociation of N(3)-H or an increase in the hydrophobic properties of the pyrimidine moiety due to the presence of a sulphur atom, can affect the cell membrane interactions and transport of nucleoside analogues into the mitochondria; this would influence the anti-HIV activity and cytotoxicity of 4-SFLT and 2-SFLT (Stahle et al., 1993). Moreover, a decrease in the pK_a values for dissociation of N(3)-H in both 2-SFLT and 4-SFLT as compared to FLT can reduce the phosphorylation efficiency of the thionucleosides by cellular and mitochondrial kinases, thus weakening their cytotoxicity. It was demonstrated that while both HIV-1 RT and human pol γ exhibit 5′-to-3′ DNA polymerase activity, only DNA pol γ has an intrinsic 3′-to-5′ exonuclease proofreading activity, which can reduce the toxicity by removal of nucleoside analogues from DNA termini. Moreover, the rates of the exonuclease excision of different NRTIs by mitochondrial DNA pol γ were found to vary significantly (Graves et al., 1998; Hanes and Johnson, 2008; Lee et al., 2003; Lim and Copeland, 2001; Longley et al., 1998). The presence of a sulphur atom in the pyrimidine moiety may affect the exonuclease efficiency of polymerase γ for both thionucleosides by destabilising binding at the polymerase site (Hanes and Johnson, 2008).

It is worth to notice, that besides examined in this work host polymerases, other cellular polymerases also exposed to triphosphate forms of tested thymidine analogues are potential targets for cytotoxicity. However, Matthes et al. demonstrated poor sensitivity of polymerase α for both 4-SFLTTP and FLTTP with IC_{50} values of 140 μM and above 200 μM, respectively (Matthes et al., 1987, 1989). Additionally, many other inhibition studies showed that polymerase α was less sensitive to other thymidine nucleotide analogues than polymerase γ and mostly similarly or even less sensitive to such compounds than polymerase β (Faraj et al., 2000; Hart et al., 1992; Martin et al., 1994; Matthes et al., 1987). Moreover, it was demonstrated, that both polymerase ε and polymerase δ with IC_{50} values for FLTTP over 100 μM and 200 μM, respectively (von Janta-Lipinski et al., 1998), likewise polymerase α, were mainly less sensitive to thymidine nucleotide analogues than both polymerases γ and β (Focher et al., 1995; Huang et al., 1992; Martin et al., 1994; Yang et al., 2007).

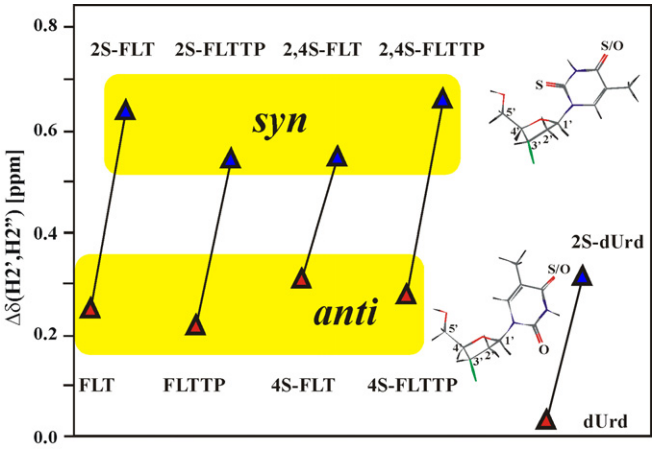

Fig. 3. Conformation of FLT, FLTTP, 4-S-FLT, 4-S-FLTTP and dUrd and their 2-S substituted derivatives, 2-S-FLT, 2-S-FLTTP, 2,4-S-FLT, 2,4-S-FLTTP and 2-S-dUrd.

4. Conclusions

The presented results show that both 4-SFLTTP and 2-SFLTTP are potent, competitive inhibitors of HIV-1 RT. On the other hand, their parent nucleosides, 4-SFLT and 2-SFLT exert reduced, in comparison to FLT, anti-HIV-1 activity as well as cytotoxicity, in spite of the high inhibitory activities of their 5′-triphosphates against human polymerase γ (Table 1). Anti-HIV-1 RT inhibitory activities of 5′-triphosphates of both thiated nucleoside analogues only partially correlated with the antiviral activities of their parent nucleosides.

Direct effects of the nucleoside analogues on various other mitochondrial (and not only mitochondrial) targets causing metabolic dysfunction in cell culture must also be taken into account.

Of special interest is the observation that 2-SFLTTP showing a *syn* conformation around the glycosidic bond exerts lower inhibitory activity against pol γ than its 4-thio isomer and FLTTP, both in the *anti* conformation. 2-SFLTTP is also a more potent inhibitor of HIV-1 RT than 4-SFLTTP. In the same way, the parent nucleoside, 2-SFLT also showing a *syn* conformation, exhibits more potent *in vitro* activity against HIV-1, and shows a higher selectivity index than its 4-thio isomer in the *anti* conformation. Moreover, the selectivity index of 2-SFLT is 4-fold higher than that of FLT, which indicates that 2-SFLT is a potent and selective *in vitro* anti-HIV-1 agent.

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References

Balzarini, J., Baba, M., Pauwels, R., Herdewijn, P., De Clerq, E., 1988. Anti-retrovirus activity of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleoside analogues. Biochem. Pharmacol. 37, 2847–2856.

Balzarini, J., Van Aerschot, A., Pauwels, R., Baba, M., Schols, D., Herdewijn, P., De Clercq, E., 1989. Halogeno-3′-fluoro-2′,3′-dideoxyuridines as inhibitors of human immunodeficiency virus (HIV): potent and selective anti-HIV activity of 3′-fluoro-2′,3′-dideoxy-5-chlorouridine. Mol. Pharmacol. 35, 571–577.

Becher, F., Pruvost, A.G., Schlemmer, D.D., Creminon, C.A., Goujard, C.M., Delfraissy, J.F., Benech, H.C., Grassi, J.J., 2003. Significant levels of intracellular stavudine triphosphate are found in HIV-infected zidovudine-treated patients. AIDS 17, 555–561.

Bienstock, R.J., Copeland, W.C., 2004. Molecular insights into NRTI inhibition and mitochondrial toxicity revealed from a structural model of the human mitochondrial DNA polymerase. Mitochondrion 4, 203–213.

Cheng, Y., Dutschman, G.E., Bastow, K.F., Sarngadharan, M.G., Ting, R.Y.C., 1987. Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. J. Biol. Chem. 262, 2187–2189.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Copeland, W.C., Chen, M.S., Wang, T.S.-F., 1992. Human DNA polymerases alpha and beta are able to incorporate anti-HIV deoxynucleotides into DNA. J. Biol. Chem. 267, 21459–21464.

Eriksson, S., Xu, B., Clayton, D.A., 1995. Efficient incorporation of anti-HIV deoxynucleotides by recombinant yeast mitochondrial DNA polymerase. J. Biol. Chem. 270, 18929–18934.

Faraj, A., El Alaoui, A.M., Gosselin, G., Imbach, J.L., Morrow, C., Sommadossi, J.P., 2000. Effects of beta-L-3′-azido-3′-deoxythymidine 5′-triphosphate on host and viral DNA polymerases. Antiviral Res. 47, 97–102.

Focher, F., Maga, G., Bendiscioli, A., Capobianco, M., Colonna, F., Garbesi, A., Spadari, S., 1995. Stereospecificity of human DNA polymerases alpha, beta, gamma, delta and epsilon, HIV-reverse transcriptase, HSV-1 DNA polymerase, calf thymus terminal transferase and *Escherichia coli* DNA polymerase I in recognizing D- and L-thymidine 5′-triphosphate as substrate. Nucleic Acids Res. 23, 2840–2847.

Graves, S.W., Johnson, A.A., Johnson, K.A., 1998. Expression, purification, and initial kinetic characterization of the large subunit of the human mitochondrial DNA polymerase. Biochemistry 37, 6050–6058.

Hanes, J.W., Johnson, K.A., 2008. Exonuclease removal of dideoxycytidine (zalcitabine) by the human mitochondrial DNA polymerase. Antimicrob. Agents Chemother. 52, 253–258.

Hart, G.J., Orr, D.C., Penn, C.R., Figueiredo, H.T., Gray, N.M., Boehme, R.E., Cameron, J.M., 1992. Effects of (−)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrob. Agents Chemother. 36, 1688–1694.

Huang, P., Farquhar, D., Plunkett, W., 1992. Selective action of 2′,3′-didehydro-2′,3′-dideoxythymidine triphosphate on human immunodeficiency virus reverse transcriptase and human DNA polymerases. J. Biol. Chem. 267, 2817–2822.

Johnson, A.A., Ray, A.S., Hanes, J., Suo, Z., Colacino, J.M., Anderson, K.S., Johnson, K.A., 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J. Biol. Chem. 276, 40847–40857.

Kaguni, L.S., 2004. DNA polymerase gamma, the mitochondrial replicase. Annu. Rev. Biochem. 73, 293–320.

Kakuda, N.T., 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin. Ther. 22, 685–708.

König, H., Behr, E., Lower, J., Kurth, R., 1989. Azidothymidine triphosphate is an inhibitor of both human immunodeficiency virus Type 1 reverse transcriptase and DNA polymerase gamma. Antimicrob. Agents Chem. 33, 2109–2114.

Lee, H., Hanes, J., Johnson, K.A., 2003. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry 42, 14711–14719.

Lewis, W., Simpson, J.F., Meyer, R.R., 1994. Cardiac mitochondrial DNA polymerase-gamma is inhibited competitively and noncompetitively by phosphorylated zidovudine. Circ. Res. 74, 344–348.

Lim, S.E., Copeland, W.C., 2001. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase gamma. J. Biol. Chem. 276, 23616–23623.

Longley, M.J., Ropp, P.A., Lim, S.E., Copeland, W.C., 1998. Characterization of the native and recombinant catalytic subunit of human DNA polymerase γ: identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity. Biochemistry 37, 10529–10539.

Lund, K.C., Peterson, L.L., Wallace, K.B., 2007. Absence of a universal mechanism of mitochondrial toxicity by nucleoside analogs. Antimicrob. Agents Chemother. 51, 2531–2539.

Lund, K.C., Wallace, K.B., 2004. Direct, DNA pol-gamma-independent effects of nucleoside reverse transcriptase inhibitors on mitochondrial bioenergetics. Cardiovasc. Toxicol. 4, 217–228.

Majumdar, C., Abhotts, J., Broder, S., Wilson, S.H., 1988. Studies on the mechanism of human immunodeficiency virus reverse transcriptase, steady-state kinetics, processivity, and polynucleotide inhibition. J. Biol. Chem. 263, 15657–15665.

Martin, J.L., Brown, C.E., Matthews-Davis, N., Reardon, J.E., 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. 38, 2743–2749.

Matthes, E., Langen, P., von Janta-Lipinski, M., Will, H., Schröder, H.C., Mery, H., Weiler, B.E., Müller, W.E.G., 1990. Potent inhibition of hepatitis B virus production in vitro by modified pyrimidine nucleosides. Antimicrob. Agents Chemother. 34, 1986–1990.

Matthes, E., Lehmann, C., Scholz, D., von Janta-Lipinski, M., Gaertner, K., Rosenthal, H.A., Langen, P., 1987. Inhibition of HIV-associated reverse transcriptase by sugar-modified derivatives of thymidine 5′-triphosphate in comparison to cellular DNA polymerases alpha and beta. Biochem. Biophys. Res. Commun. 148, 78–85.

Matthes, E., Lehmann, C., von Janta-Lipinski, M., Scholz, D., 1989. Inhibition of HIV-replication by 3′-fluoro-modified nucleosides with low cytotoxicity. Biochem. Biophys. Res. Commun. 165, 488–495.

Miazga, A., Felczak, K., Bretner, M., Siwecka, M.A., Piasek, A., Kulikowski, T., 2003. Thiated analogues of 2′,3′-dideoxy-3′-fluorothymidine and their phosphorylated and phosphonylated derivatives: synthesis, interaction with HIV reverse transcriptase, and in vitro anti-HIV activity. Nucleosides Nucleotides Nucleic Acids 22, 973–976.

Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit Jr., R.W., Germany, J.M., Secrist, J.A., Vince 3rd, R., Shannon, W.M., 1991. Mechanism of inhibition of human immunodeficiency virus type 1 reverse transcriptase and human DNA polymerases alpha, beta, and gamma by the 5′-triphosphates of carbovir, 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyguanosine, and 3′-deoxyguanosine. J. Biol. Chem. 266, 1754–1762.

Poopeiko, N.E., Poznański, J., Drabikowska, A., Balzarini, J., De Clercq, E., Mikhailopulo, I.A., Shugar, D., Kulikowski, T., 1995. Synthesis, solution conformation and biological properties of 2′,3′-dideoxy-3′-fluoro-D-erythro-pentofuranosides of 2-thiouracil and 2-thiothymidine. Nucleosides Nucleotides 14, 435–437.

Poznański, J., Felczak, K., Kulikowski, T., Remin, M., 2000. 1H NMR conformational study of antiherpetic C5-substituted 2′-deoxyuridines: insight into the nature of structure–activity relationships. Biochem. Biophys. Res. Commun. 272, 64–74.

Reardon, J.E., Miller, W.H., 1990. Human immunodeficiency virus reverse transcriptase. Substrate and inhibitor kinetics with thymidine 5′-triphosphate and 3′-azido-3′-deoxythymidine 5′-triphosphate. J. Biol. Chem. 265, 20302–20307.

Remin, M., 2001. A code relating sequence to structure in nucleic acids. Nucleosides Nucleotides Nucleic Acids 20, 841–846.

Stahle, L., Guzenda, E., Ljungdahl-Stahle, E., 1993. Pharmacokinetics and extracellular distribution to blood, brain, and muscle of alovudine (3′-fluorothymidine) and zidovudine in the rat studied by microdialysis. J. Acquir. Immune Defic. Syndr. 6, 435–439.

Steet, R.A., Melancon, P., Kuchta, R.D., 2000. 3′-Azidothymidine potently inhibits the biosynthesis of highly branched N-linked oligosaccharides and poly-N-acetyllactosamine chains in cells. J. Biol. Chem. 275, 26812–26820.

Sundseth, R., Joyner, S.S., Moore, J.T., Dornsife, R.E., Dev, I.K., 1996. The anti-human immunodeficiency virus agent 3′-fluorothymidine induces DNA damage and apoptosis in human lymphoblastoid cells. Antimicrob. Agents Chemother 40, 331–335.

von Janta-Lipinski, M., Costisella, B., Ochs, H., Hübscher, U., Hafkemeyer, P., Matthes, E., 1998. Newly synthesized L-enantiomers of 3′-fluoro-modified beta-2′-deoxyribonucleoside 5′-triphosphates inhibit hepatitis B DNA polymerases but not the five cellular DNA polymerases alpha, beta, gamma, delta, and epsilon nor HIV-1 reverse transcriptase. J. Med. Chem. 41, 2040–2046.

Vrang, L., Bazin, H., Remaud, G., Chattopadhyaya, J., Öberg, B., 1987. Inhibition of the reverse transcriptase from HIV by 3′-azido-3′-deoxythymidine triphosphate and its threo analogue. Antiviral Res. 7, 139–149.

Yang, G., Dutschman, G.E., Wang, C.J., Tanaka, H., Baba, M., Anderson, K.S., Cheng, Y.C., 2007. Highly selective action of triphosphate metabolite of 4′-ethynyl D4T: a novel anti-HIV compound against HIV-1 RT. Antiviral Res. 73, 185–191.