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Thiated derivatives of 2′,3′-dideoxy-3′-fluorothymidine: Synthesis, *in vitro* anti-HIV-1 activity and interaction with recombinant drug resistant HIV-1 reverse transcriptase forms

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ABSTRACT

Various thiated analogues of thymine 2′,3′-dideoxy-3′-fluoronucleoside (FLT) and their 5′-monophosphates and 5′-triphosphates were prepared with the use of modified multistep procedures. The thiated analogues of FLT and FLTMP were evaluated against the wild type and drug- and multidrug-resistant strains of HIV-1, using the replicative phenotyping format of the deCIPhR assay, and showed potent inhibition of drug-resistant HIV-1 strains at low cytotoxicity. Additionally, inhibition of recombinant drug resistant forms of reverse transcriptase from single and multiple HIV-1 mutants by the synthesized 5′-triphosphates was investigated. The strongest inhibition was observed for K103N and Δ67 mutants and the most potent anti-HIV-1 activity against drug resistant strains and the lowest cytotoxicity was exerted by S^4FLTMP and FLTMP which may be regarded as potential anti-HIV/AIDS agents.

1. Introduction

2′,3′-Dideoxy-3′-fluorothymidine (FLT, alovudine®) belongs to the most potent agents inhibiting the replication of the human immunodeficiency virus type 1 (HIV-1) (Balzarini et al., 1988). Some aspects of the mechanism of biological action of FLT have been studied including its phosphorylation in a cell-free system and in various cell lines, incorporation into DNA in a cell-free system and *in vitro* termination of DNA synthesis as well as antiproliferative activity towards human cell lines (Langen et al., 1972; Chidgeavadze et al., 1985; Matthes et al., 1988). FLT 5′-triphosphate (FLTTP) is a potent inhibitor of HIV-1 reverse transcriptase (RT) (Cheng et al., 1987; Matthes et al., 1987). In addition, the development of HIV mutants resistant to FLT is slower than of mutants resistant to other RT inhibitors. Various HIV isolates with multidrug resistance-associated mutations showed no evidence of resistance to FLT (Kim et al., 2001). Unfortunately, FLT exerts substantial haematologic toxicity in man. Lipophylic analogues of FLT, 2′,3′-dideoxy-3′-fluoro-2-thiothymidine (S^2FLT) and 2′,3′-dideoxy-3′-fluoro-4-thiothymidine (S^4FLT), as already reported by us (Miazga et al., 2003; Poopeiko et al., 1995), potently inhibit HIV-1 *in vitro* with low cytotoxicity. In the present study the inhibition of HIV-1 drug- and multidrug-resistant strains by newly synthesized thioanalogues of FLT and FLTMP as well as inhibition of HIV-1 RT mutants by thiated analogues of FLTTP were investigated.

2. Materials and methods

2.1. Chemicals

[Methyl-^{3}H] dTTP (45.9 Ci/mmol) was purchased from Moravek Biochemicals Inc., Brea, CA; [α^{32}P] dTTP (5000 Ci/mmol) was obtained from Hartmann Analytic GmbH; dNTPs were from Sigma; DE81 (2.3 cm) circles were from Whatman (Maidstone, UK). Triton X-100 and rotiszint eco plus LSC-universal cocktail were from Roth (Karlsruhe, Germany); HIV PBS DNA template 5′-TTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTG-3′ (50-nt) and Lys 21 primer 5′-CAAGTCCCTGTTCGGGCGCCA-3′ (21-nt) were synthesized in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics of the Polish Academy of Science. All other reagents used in this study were of analytical grade.

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2.2. General methods

Melting points (uncorrected) were measured on a Buchi Melting Point B-540 apparatus, UV spectra were recorded on a Cary 300 instrument, using 10 mm path length cuvettes. Mass spectra were recorded on an AMD-604 spectrometer or a Q-TOF MICROMASS spectrometer. High resolution ^{1}H NMR spectra were recorded on a Varian 500 MHz in D_2O, with DSS as an internal standard or in $CDCl_3$, with tetramethylsilane as an internal standard. ^{31}P NMR spectra were recorded on a Varian 200 MHz in D_2O, with 85% H_3PO_4 as an external standard. Thin-layer chromatography (TLC) was run on Merck silica gel F_{254} glass plates developed with the following solvents (v/v): (A) $CHCl_3$-MeOH, 90:10; (B) $CHCl_3$-MeOH, 95:5.

2.3. Syntheses

2.3.1. 1-(2,3-Dideoxy-3-fluoro-5-O-tosyl-β-D-pentofuranosyl)-thymine (**2**) (Scheme 1)

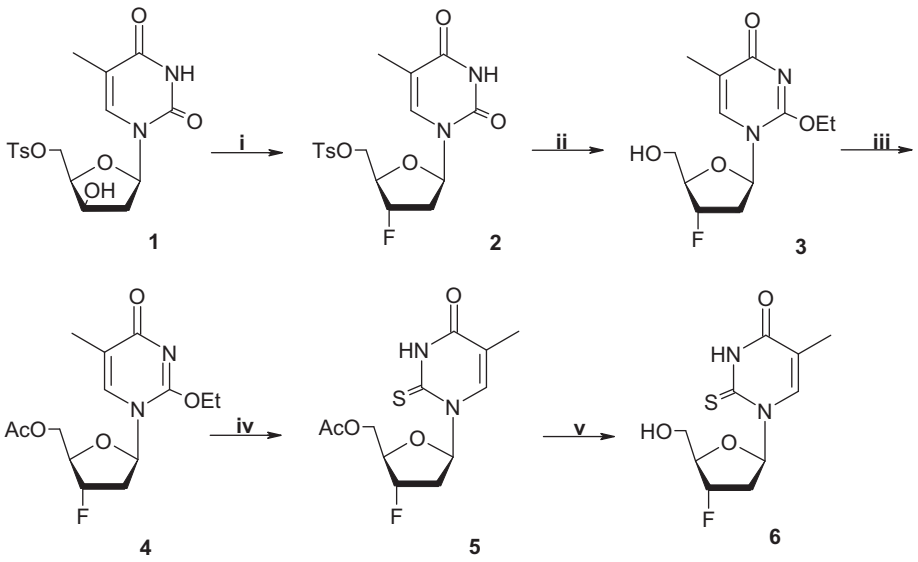
3.6 g (9.08 mmol) of 1-(2-deoxy-5-O-tosyl-β-D-*threo*-pentofuranosyl)-thymine (**1**) was suspended in 30 mL of CH_2Cl_2 and 2 mL of DAST ((diethylamino)sulphur trifluoride) was added at 0 °C. The mixture was stirred for 1 h at 0 °C, diluted with 100 mL of $CHCl_3$ and 50 mL of saturated $NaHCO_3$ was added. The organic layer was separated and extracted with $CHCl_3$. The combined organic layers were washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residual oil was purified by column chromatography on silica gel using $CHCl_3$-MeOH (0–3%) as eluent. Fractions containing **2** were combined, evaporated and crystallized from EtOH to yield 1.95 g (54%) of white crystals: mp. 148–149 °C dec.; UV λ_{max} (pH 0) 265 nm (ε 10.5×10^3), 222 nm (ε 17.6×10^3); λ_{max} (pH 1) 265 nm (ε 10.8×10^3), 223.5 nm (ε 19×10^3); λ_{max} (pH 7) 270 nm (ε 10.4×10^3), 224 nm (ε 18.2×10^3); λ_{max} (pH 12) 273 nm (ε 8.3×10^3), 224.5 nm (ε 22×10^3); TLC (silica gel) R_f (B) 0.63; ^{1}H NMR δ [ppm] ($CDCl_3$) 8.04 (1H, br s, NH), 7.79 (2H, d, Ph), 7.41–7.39 (3H, m, H6, Ph), 6.43 (1H, dd, H1′), 5.21 (1H, dd, H3′), 4.39 (1H, d, H4′), 4.29 (1H, dd, H5′), 4.23 (1H, dd, H5″), 2.63–2.58 (1H, m, H2″), 2.48 (3H, s, $\underline{CH_3}$Ph), 2.24–2.11 (1H, m, H2′), 1.96 (3H, s, CH_3); MS *m/z* 421.0867 $(M+Na)^+$.

2.3.2. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-2-ethoxythymine (**3**)

1.6 g (4 mmol) of **2** was suspended in 90 mL of absolute EtOH and 2.6 mL (17.5 mmol) of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) was added. The mixture was refluxed for 3 h, cooled and treated with 20 mL of Dowex 50 WX8 (pyridine form) in 30 mL of water. The reaction mixture was stirred for 5 min, filtered and evaporated in vacuo. The residual oil was dissolved in 50 mL of $CHCl_3$, washed with water, dried with Na_2SO_4 and concentrated in vacuo. The crude product was purified on a silica gel column using $CHCl_3$-MeOH (0–5%) as eluent to yield **3**: 950 mg (88%); mp. 144–145 °C; UV λ_{max} (pH 0) 264 nm (ε 8.3×10^3); λ_{max} (pH 1) 258.5 nm (ε 9.4×10^3); λ_{max} (pH 2) 256.5 nm (ε 10.8×10^3); λ_{max} (pH 7) 255.5 nm (ε 10.5×10^3); λ_{max} (pH 12) 256 nm (ε 10.1×10^3); TLC (silica gel) R_f (A) 0.47; ^{1}H NMR δ [ppm] (D_2O) 7.48 (1H, d, H6), 6.44 (1H, dd, H1′) 5.37 (1H, dd, H3′), 4.59–4.52 (3H, m, H4′, $\underline{CH_2}CH_3$), 3.85 (2H, d, H5′, H5″), 2.84–2.75 (1H, m, H2″), 2.50–2.36 (1H, m, H2′), 1.97 (3H, s, CH_3), 1.43 (3H, t, $CH_2\underline{CH_3}$); ESI-MS *m/z* 273 $(M+H)^+$.

2.3.3. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-2-thiothymine (**6**)

900 mg (3.3 mmol) of 3, 70 mL of acetic anhydride and 7 mg of DMAP (4-(dimethylamino)pyridine) were stirred for 2 h at room temperature. The mixture was evaporated to dryness with toluene and EtOH. The residual oil was dissolved in 75 mL of $CHCl_3$ and washed twice with water. The organic layer was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to oil of *1*-(2,3-dideoxy-3-fluoro-β-D-pentofuranosyl)-2-ethoxythymine (**4**) which was directly used in the next reaction step. Hydrogen sulphide gas was bubbled into a stirred solution of **4** and 4.15 mL (33.06 mmol) of tetramethylguanidine in 20 mL of dry pyridine at 0 °C for 1 h. The stirred reactants were allowed to warm up to room temperature. After 12 h the mixture was diluted with 75 mL of $CHCl_3$ and argon was bubbled through the solution (1 h). Then, 70 mL of 1 N HCl were added, the organic layer was separated, washed twice with water, dried over Na_2SO_4 and evaporated in vacuo. The product was purified by silica gel column chromatography ($CHCl_3$) and crystallized from EtOH to yield 630 mg (65%) of 1-(5-O-acetyl-2,3-dideoxy-3-fluoro-β-D-pentofuranosyl)-2-thiothymine (**5**). 600 mg (1.98 mmol) of **5** was dissolved in 20 mL of MeOH saturated with ammonia. The mixture was stir-



Scheme 1. Synthesis of 3′-fluoro-2-thiothymidine (**6**). (i) DAST, 0 °C, 1 h; (ii) DBU, EtOH, reflux, 3 h; (iii) Ac_2O, DMAP, r.t., 2 h; (iv) H_2S, TMG, Py, 0 °C, 1 h; (v) NH_3/MeOH, r.t., 2 h.

red at room temperature for 2 h, concentrated under reduced pressure and crystallized from EtOH to yield white crystals: 500 mg (97%), mp. 164-165 °C dec.; UV λ_{max} (pH 0) 278.5 nm (ε 18×10^3), 221 nm (ε 15.5×10^3); λ_{max} (pH 1) 277.5 nm (ε 18.2×10^3), 220 nm (ε 16×10^3); λ_{max} (pH 2) 277.5 nm (ε 18.4×10^3), 219 nm (ε 16×10^3); λ_{max} (pH 7) 278 nm (ε 17.8×10^3), 220.5 nm (ε 15.6×10^3); λ_{max} (pH 12) 241.5 nm (ε 24.7×10^3); TLC (silica gel) R_f (A) 0.57; ^{1}H NMR δ [ppm] (D_2O) 7.89 (1H, d, H6), 7.08 (1H, dd, H1′) 5.35 (1H, dd, H3′), 4.45 (1H, m, H4′), 3.87 (2H, d, H5′, H5″), 2.94–2.85 (1H, m, H2″), 2.31–2.18 (1H, m, H2′), 1.95 (3H, s, CH_3); ESI-MS *m/z* 261 $(M+H)^+$.

2.3.4. General procedure for the synthesis of 4-thiated nucleosides (Scheme 2)

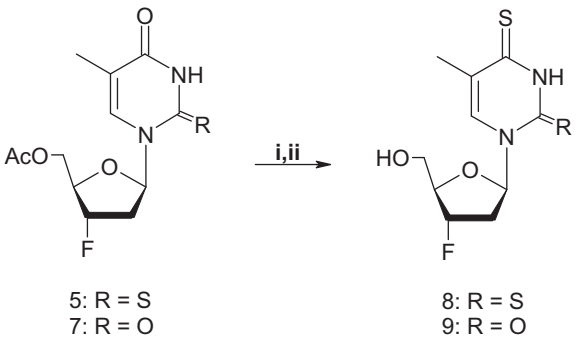
To the solution of 0.5 mmol of acetylated nucleoside analogue (**5**), (**7**) (Huang et al., 1991) in 5 mL of anhydrous 1,4-dioxane 300 mg (0.75 mmol) of Lawesson Reagent was added. The mixture was refluxed for 3 h. The mixture was cooled, 1 mL of H_2O was added and the total was evaporated to dryness. The residue was dissolved in $CHCl_3$, extracted with $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The resulting oil was dissolved in 10 mL of MeOH saturated with ammonia and the mixture was stirred at room temperature for 2 h. The mixture was than evaporated in vacuo and purified on a silica gel column using $CHCl_3$ as eluent.

2.3.5. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-2,4-dithiothymine (**8**)

Crystallized from Et_2O to yield yellow crystals: 125 mg (90%), mp. 155–157 °C dec.; UV λ_{max} (pH 0) 285 nm (ε 20.6×10^3); λ_{max} (pH 1) 284 nm (ε 20.3×10^3); λ_{max} (pH 7) 279 nm (ε 18.7×10^3); λ_{max} (pH 12) 319 nm (ε 22.8×10^3), 273 nm (ε 17×10^3), 222.5 nm (ε 6.8×10^3); TLC (silica gel) R_f (A) 0.75; ^{1}H NMR δ [ppm] (DMSO) 13.88 (1H, br s, NH), 8.02 (1H, d, H6), 6.84 (1H, dd, H1′) 5.32 (1H, dd, H3′), 4.32 (1H, m, H4′), 3.70 (2H, t, H5′, H5″), 2.77–2.62 (1H, m, H2″), 2.31–2.14 (1H, m, H2′), 2.01 (3H, s, CH_3); ESI-MS *m/z* 277 $(M+H)^+$.

2.3.6. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-4-thiothymine (**9**)

Crystallized from Et_2O to yield yellow crystals: 98 mg (75%), mp. 131–132 °C; UV λ_{max} (pH 0) 278.5 nm (ε 18×10^3), 221 nm (ε 15.5×10^3); λ_{max} (pH 1) 277.5 nm (ε 18.2×10^3), 220 nm (ε 16×10^3); λ_{max} (pH 2) 277.5 nm (ε 18.4×10^3), 219 nm (ε 16×10^3); λ_{max} (pH 7) 278 nm (ε 17.8×10^3), 220.5 nm (ε 15.6×10^3); λ_{max} (pH 12) 241.5 nm (ε 24.7×10^3); TLC (silica gel) R_f (A) 0.69; ^{1}H NMR δ [ppm] (D_2O) 7.76 (1H, s, H6), 6.32 (1H, dd, H1′) 5.41–5.29 (1H, dd, H3′, $J_{3',F}$ = 53.3 Hz), 4.45–4.38 (1H, m, H4′, $J_{4',F}$ = 27 Hz), 3.82 (2H, d, H5′, H5″, $J_{4',5'}$ = 4.5 Hz), 2.75–2.67 (1H, m, H2″, $J_{1',2''}$ = 5.5 Hz), 2.46–2.33 (1H, m, H2′, $J_{1',2'}$ = 8.5 Hz), 2.09 (3H, s, CH_3); ESI-MS *m/z* 261 $(M+H)^+$.



5: R = S
7: R = O

8: R = S
9: R = O

Scheme 2. Synthesis of 3′-fluoro-2,4-dithiothymidine (**8**) and 3′-fluoro-4-thiothymidine (**9**). (i) Lawesson Reagent, 1,4-dioxane, reflux, 3 h; (ii) NH_3/MeOH, r.t., 2 h.

2.3.7. General procedure for the synthesis of nucleoside 5′-monophosphates (Scheme 3)

To an ice-cooled solution of dried 1,2,4-triazole (314 mg, 4.4 mmol) in 8 mL of dry 1,4-dioxane was added $POCl_3$ (150 μL, 1.6 mmol) and a solution of triethylamine (0.62 mL, 4.44 mmol) in 2 mL of 1,4-dioxane. After stirring for 1 h at room temperature the reaction mixture was directly filtered into a flask containing 1 mmol of nucleoside. Phosphorylation of nucleoside was completed in 15 min and then 0.5 mL of H_2O was added, and left to stand overnight at room temperature. The solvent was removed *in vacuo* and the product was purified by chromatography on a DEAE-Sephadex A-25 column eluted with a linear gradient of TEAB (triethylammonium bicarbonate buffer) buffer (1 M, pH 7.5) (0–0.4 M). The fractions containing pure monophosphate were pooled and lyophilised to remove excess of buffer to yield nucleoside 5′-phosphate. The product was dissolved in a small amount of water and 5% solution of NaI in acetone was added dropwise; the formed precipitate was filtered off, washed several times with acetone and dried.

2.3.8. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-2-thiothymine 5′-monophosphate disodium salt (**11**)

Yield 276 mg (75%); ^{1}H NMR δ [ppm] (D_2O) 8.03 (1H, psd, H6), 7.13–7.10 (1H, dd, H1′), 5.51–5.39 (1H, dd, H3′, $J_{3',F}$ = 52.5 Hz), 4.61–4.55 (1H, m, 4′, $J_{4',F}$ = 27.5 Hz), 4.13–4.05 (2H, m, H5′, H5″), 2.87–2.79 (1H, m, H2″), 2.36–2.22 (1H, m, H2′), 1.97 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) 1.66; ESI-MS *m/z* 339 $(M-H)^-$.

2.3.9. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-4-thiothymine 5′-monophosphate disodium salt (**12**)

Yield 250 mg (65%); ^{1}H NMR δ [ppm] (D_2O) 7.92 (1H, s, H6), 6.37 (1H, dd, H1′) 5.51–5.36 (1H, dd, H3′, $J_{3',F}$ = 53 Hz), 4.56–4.49 (1H, m, H4′, $J_{4',F}$ = 27 Hz), 4.07–3.96 (2H, d, H5′, H5″), 2.71–2.6 (1H, m, H2″), 2.49–2.3 (1H, m, H2′), 2.1 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) 3.95; ESI-MS *m/z* 339 $(M-H)^-$.

2.3.10. 1-(2,3-dideoxy-3-fluoro-β-D-pentofuranosyl)-2,4-dithiothymine 5′-monophosphate disodium salt (**13**)

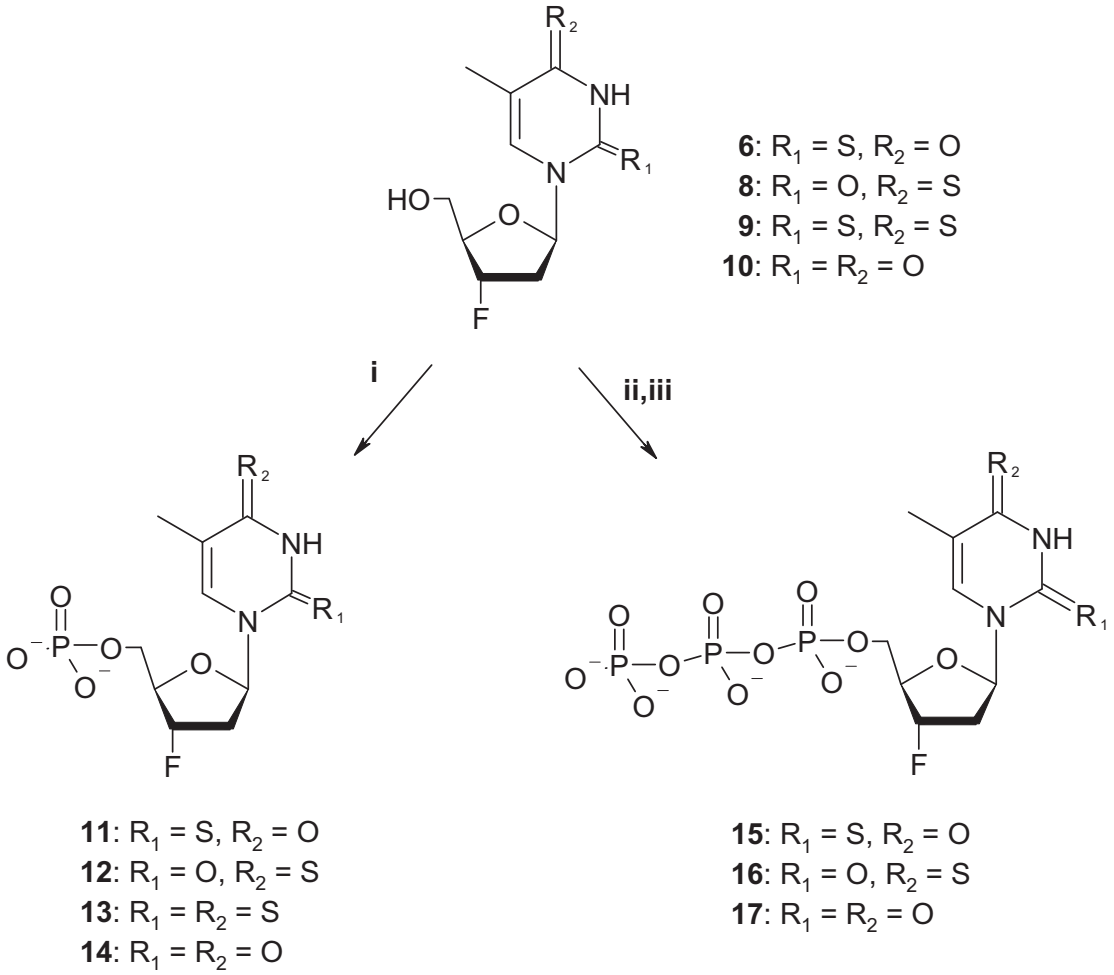
Yield 200 mg (50%); ^{1}H NMR δ [ppm] (D_2O) 8.07 (1H, s, H6), 7.03 (1H, dd, H1′) 5.52–5.38 (1H, dd, H3′, $J_{3',F}$ = 53.1 Hz), 4.62–4.55 (1H, m, H4′, $J_{4',F}$ = 27.1 Hz), 4.08 (2H, m, H5′, H5″), 2.94–2.83 (1H, m, H2″), 2.41–2.24 (1H, m, H2′), 2.15 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) 4.45; ESI-MS *m/z* 356 $(M-H)^-$.

2.3.11. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-thymine 5′-monophosphate disodium salt (**14**)

Yield 173 mg (47%); ^{1}H NMR δ [ppm] (D_2O) 7.92 (1H, s, H6), 6.53 (1H, dd, H1′) 5.63–5.48 (1H, dd, H3′, $J_{3',F}$ = 52.7 Hz), 4.67–4.6 (1H, m, H4′, $J_{4',F}$ = 27.3 Hz), 4.23–4.14 (2H, m, H5′, H5″), 2.79–2.68 (1H, m, H2″), 2.58–2.41 (1H, m, H2′), 2.03 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) 0.75.

2.3.12. General procedure for the synthesis of nucleoside 5′-triphosphates

To the ice-cold suspension of 0.5 mmol of appropriate nucleoside analogue (**6**, **9**, **10**) in 1 mL of trimethylphosphate, 148 μL (1.6 mmol) of $POCl_3$ were added and the mixture was stirred at 0 °C. After 6 h 1.48 mL of tri-*n*-butylamine and a solution of 1.5 g of bis (tri-*n*-butylammonium) pyrophosphate in 2 mL of DMF were added. The mixture was stirred in an ice-bath for 30 min and TEAB buffer was added, so as to adjust the pH to about 7. The solution was extracted with diethyl ether and the aqueous layer evaporated to dryness. The residue was purified on a DEAE-Sephadex A-25



Scheme 3. Synthesis of nucleoside 5′-monophosphates (**11**–**14**) and 5′-triphosphates (**15–17**). (i) 1,2,4-triazole, $POCl_3$, 1,4-dioxane, H_2O; (ii) $POCl_3$, TMP, 0 °C, 6 h; (iii) bis(tri-*n*-butylammonium) pyrophosphate, *n*-Bu_3N, DMF, 30 min 0 °C, TEAB.

column using linear gradient of TEAB (0.1–0.6 M) as eluent. Fractions containing 5′-triphosphate were collected, lyophilised, and the product was dissolved in a minimum of water and 100 mg of NaI in 10 mL of acetone was added. The precipitated sodium salt was collected by centrifugation, washed with acetone and dried over P_2O_5.

*2.3.13. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-2-thiothymine 5′-triphosphate sodium salt (**15**)*

55 mg (20%). ^{1}H NMR δ [ppm] (D_2O) 8.05 (1H, psd, H6), 7.15–7.13 (1H, dd, H1′), 5.54–5.42 (1H, dd, H3′), 4.63–4.58 (1H, m, 4′), 4.15–4.06 (2H, m, H5′, H5″), 2.90–2.82 (1H, m, H2″), 2.38–2.25 (1H, m, H2′), 1.97 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) −7.11 (γ P), −11.00 (d, α P, J = 19.86), −21.27 (β P); ESI-MS *m/z* 499 $(M-H)^-$.

*2.3.14. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-4-thiothymine 5′-triphosphate sodium salt (**16**)*

49 mg (18%). ^{1}H NMR δ [ppm] (D_2O) 7.85 (1H, d, H6), 6.39 (1H, dd, H1′) 5.58–5.48 (1H, d, H3′), 4.61–4.56 (1H, m, H4′), 4.46–4.18 (2H, m, H5′, H5″), 2.79–2.64 (1H, m, H2″), 2.45–2.36 (1H, m, H2′), 2.12 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) −7.12 (γ P), −10.73 (d, α P), −20.94 (β P); ESI-MS *m/z* 499 $(M-H)^-$.

*2.3.15. 1-(2,3-Dideoxy-3-fluoro-β-D-pentofuranosyl)-thymine 5′-triphosphate sodium salt (**17**)*

21 mg (21%). ^{1}H NMR δ [ppm] (D_2O) 7.80 (1H, d, H6), 6.44–6.41 (1H, dd, H1′), 5.60–5.49 (1H, dd, H3′), 4.57–4.51 (1H, m, H4′), 4.31–4.15 (2H, m, H5′, H5″), 2.65–2.57 (1H, m, H2″), 2.48–2.34 (1H, m, H2′), 1.93 (3H, s, CH_3); ^{31}P NMR δ [ppm] (D_2O) −8.80 (γ P), −11.24 (d, α P), −21.02 (β P); ESI-MS *m/z* 483 $(M-H)^-$.

2.4. Generation of HIV-1 resistant mutants

HIV-1 mutants displaying high resistance to drugs while maintaining sufficient replication capacity were selected (Table 1). Mutations in the reference strain pNL4-3 (GenBank accession number #AF3244939) were inserted using the QuickChange mutagenesis kit (Stratagene) according to Manufacturer's instructions. The presence of mutations in the engineered proviral plasmids was ascertained by sequencing them on ABI 310 prism sequencer.

2.5. Determination of antiviral activity and cytotoxicity

Anti-HIV properties of thiated thymine 2′,3′-dideoxy-3′-fluoronucleosides and their 5′-monophosphates were evaluated against wild-type and multidrug-resistant HIV-1 strains using the replicative phenotyping assay deClPhR as described previously (Louvel et al., 2008; Opravil et al., 2010; Fehr et al., 2011). For determining

Table 1
HIV-1 drug resistant mutants.

Mutant	Number of mutations	Resistance to drug	
M184V	1	Lamivudine,	Emtricitabine
K103N	1	Efavirenz,	Nevirapine
K65R	1	Tenofovir,	Abacavir
M4[a]	4	The class of thymidine analogues	
Δ67[b]	7	All classes of nucleosidic and non-nucleosidic RT	
SQ[c]	28	inhibitors	

[a] D67N, T69D, T215Y, K219Q.
[b] Deletion of D67, substitutions: T69G, K70R, L74I, K103N, T215F, K219Q.
[c] E29Q, M41L, E44D, K49R, K64H, T69D, L74V, V90I, K103N, V108I, F116S, V118I, E169K, M184V, D192N, I195T, G196E, E203K, L210Y, R211S, T215Y, K223E, L228R, M230L, S251N, L283I, R284K, E297K.

the effective concentration of 50% (EC_{50}), test compounds were assayed across a range of concentrations and data were modelled using a curve fitting software (XLfit, idbs) yielding a dose–response curve from which EC_{50} was extrapolated.

In addition, the Alamar blue cell viability assay (Invitrogen) was used to determine the cytotoxic dose of 50% (CD_{50}) for each analogue. Data were modelled with the help of a curve fitting software (XLfit, idbs) yielding a dose–response curve from which CD_{50} was extrapolated.

2.6. Generation of reverse transcriptase mutants

Proviral plasmids containing cDNAs of genomic RNA of the HIV-1 variants resistant to commonly used anti-HIV-1 RT drugs were provided by InPheno. In order to obtain a collection of recombinant RTs, an HIV-1 RT expression vector, generously provided by S.H. Hughes, was applied (Clark et al., 1995). Originally, this vector was used to produce the wild type HIV-1 RT (HIV-1 RT WT) in *Escherichia coli*. In the first stage, fragments of the proviral plasmids encoding the drug-resistant RTs were amplified by PCR. Next, the wild type RT coding sequence present in the expression vector was replaced with the PCR generated products. The structures of the newly created plasmids were determined by sequencing of at least three individual clones for each prepared construct. The expression, isolation and purification of recombinant RTs were carried out according to the protocol previously described for HIV-1 RT WT (Clark et al., 1995). The expression of each recombinant RT: WT, M184V, K103N, K65R, M4, Δ67 and SQ was carried out in the BL21(DE3)pLysS *E. coli* strain. In each case, the total protein fraction was isolated and subjected to a two-step purification: (i) affinity purification on Ni-column, and (ii) ion-exchange column purification. Western blot analysis and MALDI-TOF mass spectrometry analysis were applied to confirm that the expected recombinant HIV-1 RTs were indeed produced in bacteria. The experiments undertaken did not allow to obtain sufficient amounts of homogenous preparations of M184V and K65R mutants. Thus, the collection of five recombinant RTs, WT, K103N, M4, Δ67 and SQ, were used for further studies. The polymerase activity of all obtained HIV-1 RT mutants was confirmed in the primer extension reactions involving either a DNA or RNA template annealed to the 5′ end-labelled DNA primer.

2.7. Assays of HIV-1 reverse transcriptase activity and inhibition studies

2.7.1. Preparation of template-primer

21-nt primer Lys21 and 50-nt HIV PBS DNA were mixed at molar ratio of 3:1 with 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 6 mM $MgCl_2$ and 10 mM DTT, heated at 95 °C for 3 min and then slowly cooled at room temperature. The mixtures were subsequently stored at −20 °C.

2.7.2. Assay of HIV-1 reverse transcriptase activity

The activities of HIV-1 RTs were assayed by DE81 filter isotopic method (Reardon and Miller, 1990) as was described previously (Wińska et al., 2010). Reaction mixtures contained 75 mM KCl, 5 mM NaCl, 50 mM Tris–HCl, pH 8.3, 6 mM $MgCl_2$, 0.01% Triton X-100, 5 mM DTT, 50 μM dATP, dCTP, dGTP, [^{3}H]dTTP (200–600 DPM/pmol), 15 μg/ml HIV PBS DNA and one of the following: 10 ng WT (1 μg/μl), 10 ng Δ67 (1 μg/μl), 30 ng M4 (0.3 μg/μl), 70 ng K103N (0.2 μg/μl) or 132 ng multi-mutant SQ (0.265 μg/μl). All the reactions were initiated with enzyme in a total volume of 20 μl and incubated at 37 °C for 30 min.

2.7.3. Inhibition studies

IC_{50} values for the studied compounds were determined at 3.5 mM ATP with minimum seven concentrations of each inhibitor tested in the range of 0.01–1000 μM at 10 μM dTTP for Δ67, M4, K103N and WT, and 50 μM dTTP for multi-mutant SQ and calculated using the equation in GOSA *fit* (Global Optimisation by Simulated Annealing) Bio-Log software:

$$\text{Signal} = \min + \frac{(\max - \min)}{1 + 10^{(\log X - \log \text{IC50})}}$$

where IC_{50} is the concentration of unlabelled ligand that inhibits 50% of the binding of a fixed concentration of the radioligand. *X* is the log of the concentration of the unlabelled ligand.

2.8. Steady-state kinetic assays

K_m^{app} values for Δ67, M4, K103N and WT were determined at variable concentrations of [^{3}H]dTTP (200–600 DPM/pmol) in the range of 0.5–192 μM, and for SQ at variable concentrations of [α^{32}P] dTTP (1000 CPM/pmol) in the range of 10–360 μM with 20 μg/ml HIV PBS DNA. V_{max}^{app} and K_m^{app} values were calculated using nonlinear regression by fitting of the experimental data to Michaelis–Menten equation in GOSA *fit* (Global Optimisation by Simulated Annealing) Bio-Log software. K_i^{app} values were calculated with the use of Cheng and Prusoff (1973) equation: $K_i = IC_{50}/(1 + [S]/K_m)$.

3. Results and discussion

3.1. Anti-HIV activity

Anti-HIV properties of thioanalogues of FLT and FLTMP were evaluated against HIV-1 wild type as well as drug and multidrug resistant HIV-1 strains, using the replicative phenotyping format of the deCIPhR assay (Table 2).

Most of the thiated nucleoside analogues are not more active than the parent unthiated compounds. However, S^4FLTMP (**12**) is the exception, as its activity against the HIV-1 WT strain is similar to that of FLTMP (**14**) (and even better in the case of K103N mutant strain) as well as to that of the reference compound FLT (**10**) without increase of cytotoxicity. It should be underlined that S^4FLTMP (**12**) exerts at least equipotent antiviral activity against the multidrug resistant HIV-1 Δ67 (EC_{50} 98 nM) and SQ (EC_{50} 103 nM) strains as the reference compound FLT. Both S^4FLTMP (**12**) and FLTMP (**14**) may be regarded as a potential agent against HIV-1 drug and multidrug resistant HIV-1 strains.

The results obtained for the thiated FLTMP analogues **11**, **12** and **13** point to a more important role of 4-thio- than 2-thio- or 2,4-dithio-substituents in the thymine moiety of FLTMP in enhancing the antiviral activity without increase in cytotoxicity in the deCIPhR assay (Table 2). This may be related to the closer steric anal-

Table 2
Anti-HIV-1 activity of synthesized analogues of FLT in deCIPhR™ assay.

Compound	EC_{50} (μM)[a]							CD_{50} (μM)[b]
	WT	M184V	K103N	K65R	M4	Δ67	SQ	
S^2FLT (**6**)	1.75	1.56	1.12	3.02	0.25	NA[c]	NA[c]	11.7
S^4FLT (**9**)	0.61	0.51	0.27	0.51	0.48	NA[c]	NA[c]	NC[d]
$S^{2,4}$FLT (**8**)	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NC[d]
S^2FLTMP (**11**)	0.22	0.14	0.11	0.5	0.4	NA[c]	NA[c]	NC[d]
S^4FLTMP (**12**)	0.024	0.043	0.011	0.095	0.031	0.098	0.103	NC[d]
$S^{2,4}$FLTMP (**13**)	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NA[c]	NC[d]
FLTMP (**14**)	0.029	0.02	0.03	0.086	0.042	0.139	0.159	NC[d]
FLT (**10**)	0.021	0.027	0.018	0.032	0.016	0.131	0.069	20.2

[a] Effective dose of compound achieving 50% inhibition of HIV-1 replication.
[b] Cytotoxic dose of compound required to reduce the viability of normal uninfected cells by 50%.
[c] Not active at the maximal tested dose of 10 μM.
[d] Not cytotoxic at the maximal tested dose of 10 μM, or extrapolated.

Table 3
Steady-state constants for HIV-1 RT variants: Δ67, M4, K103N, SQ and WT.

HIV-1 RT variant	K_m^{app} for dTTP ± SD[a] (μM)	V_{max}^{app} ± SD[a] pmol/min/μg	K_i^{app} ± SD (μM)			
			AZTTP	FLTTP	S^4FLTTP	S^2FLTTP
WT	8.58 ± 0.90	123.58 ± 33.10	1.26 ± 0.09	0.90 ± 0.08	7.27 ± 0.26	0.85 ± 0.04
Δ67	7.38 ± 0.43	82.66 ± 5.04	0.78 ± 0.14	0.49 ± 0.07	3.76 ± 0.99	0.53 ± 0.08
M4	7.54 ± 0.83	37.27 ± 6.33	1.48 ± 0.23	0.73 ± 0.24	7.28 ± 0.42	1.06 ± 0.16
K103N	2.66 ± 0.35	14.72 ± 2.03	0.44 ± 0.13	0.09 ± 0.03	1.10 ± 0.15	0.14 ± 0.08
SQ	44.08 ± 3.15	7.31 ± 0.83	294.27 ± 0.17	104.77 ± 7.66	116.56 ± 5.21	58.71 ± 10.06

[a] ±SD calculated from a minimum of three independent experiments.

ogy to FLTMP of S^4FLTMP (**12**) (conformation *anti*) than those of S^2FLTMP (**11**) and $S^{2,4}$FLTMP (**13**) (conformations *syn*) congeners (Wińska et al., 2010).

Antiviral activity of FLTMP (**14**) is similar or several times less (some mutant strains) than that of FLT. On the contrary, S^2- and S^4-thiated nucleoside 5′-monophosphates (**11**, **12**) exert increased antiviral activity against WT and nearly all HIV-1 mutant strains in comparison to their parent nucleos(t)ides (**6**, **9**). The latter *in vitro* effect is rather difficult to explain. Nevertheless it should be added that there would be an advantage to the use in patients of a drug in the form of nucleotide instead of nucleoside as the former is much more water soluble and allows intravenous administration in a small volume. Moreover, nucleoside analogue 5′-monophosphates may act as extracellular prodrugs of nucleoside analogues gradually formed by hydrolytic cleavage of the 5′-monophosphate moiety by membrane-bound enzymes, such as phosphomonoesterase (LePage et al., 1972, 1975). Such a slow conversion of a prodrug to a drug provides sustained blood plasma level of the latter.

3.2. Reverse transcriptase inhibition

3.2.1. Kinetics of dTMP incorporation

The rates of dTMP incorporation into HIV PBS DNA by HIV-1 RTs were linear with respect to time and enzyme concentration with V_{max}^{app} and K_m^{app} values presented in Table 3. Steady state kinetic parameters of HIV-1 SQ RT with K_m^{app} for dTTP of 44.08 ± 3.15 μM and V_{max}^{app} of 7.31 ± 0.83 pmol/min/μg differed strongly from those obtained for other studied HIV-1 RT variants. K_m^{app} value for dTTP for this mutant was about 5-fold higher than for WT, with a V_{max}^{app} that was almost 17-fold lower.

3.2.2. Inhibition of HIV-1 RT variants by nucleotide analogues

3.2.2.1. Primer unblocking studies. The primer unblocking control studies at variable concentration of ATP (2–8 mM) and fixed concentration of AZTTP (10 μM) showed that amongst all the tested RT variants (WT and mutants with TAMs, M4 and Δ67) only M4 demonstrated a very small increase in ATP-phosphorolytic activity in comparison to WT (between 0% and 15%, with the highest difference at 4 mM and the lowest at 8 mM ATP), whereas Δ67 unblocked the AZTMP terminated primer with even lower efficiency than WT (data not shown). Consequently, M4 mutant demonstrated slightly higher IC_{50} for AZTTP (3.44 ± 0.54 μM), than the WT (2.74 ± 0.19 μM). However, with regard to the slightly lower K_m^{app} for dTTP in comparison to WT, the difference in IC_{50} values does not reflect the difference in K_i^{app} values (almost the same values for WT and M4, see Table 3). Similarly to AZTTP, all the compounds tested were ATP-mediated chain-terminated primers excised from DNA by the examined RTs (WT, M4, Δ67 and K103N), with the order of excision: S^4FLTTP > S^2FLTTP > FLTTP (data not shown).

3.2.2.2. Steady-state inhibition results. Amongst the tested compounds the strongest inhibition was observed for K103N and Δ67 RT mutants, with K_i^{app} value for the latter one about 2-fold lower than those for WT and M4 (Table 3).

Amongst the two studied thioanalogues, S^2FLTTP was a better inhibitor of all the examined HIV-1 RT variants than S^4FLTTP, with lower K_i^{app} values as compared to the latter, namely about 7–8-fold lower for WT, Δ67, M4 and K103N and about 2-fold lower for the SQ mutant (Table 3). The activities of S^2FLTTP were comparable (and for SQ RT even almost twice higher) than those obtained for FLTTP.

Anti-HIV-1 inhibitory activities only partially correlate with the steady-state inhibition values, showing FLTTP and S^2FLTTP to be better inhibitors of drug resistant HIV-1-RT variants than S^4FLTTP. Obviously, a precise correlation between enzyme inhibition and the effects on cells cannot be expected, in spite of using a physiological template. Many processes of intracellular metabolism of nucleoside analogues, like reduction, oxidation, glycosylation and especially phosphorylation of nucleosides in cells, can influence

the anti-HIV activity and cytotoxicity of nucleoside reverse transcriptase inhibitors (NRTIs) (Becher et al., 2003; Lund et al., 2007; Steet et al., 2000). Furthermore, although enhanced nucleotide-dependent excision is a major mechanism of resistance to AZT by mutants of HIV-1 and these mutations confer some degree of resistance to most NRTIs (Acosta-Hoyos and Scott, 2010), steady-state inhibition values for AZTTP demonstrated that the drug resistant HIV-1 RT variants with TAMs, with the exception of the SQ mutant, do not show well defined resistance to AZTTP, even in the presence of physiological concentrations of ATP.

4. Conclusions

On the basis of the antiviral profile, inhibition of drug resistant HIV-1 strains and low cytotoxicity, the most effective inhibitors were S^4FLTMP (**12**) and FLTMP (**14**) (anti-HIV-1 K103N activity EC_{50} = 11 nM and 30 nM, respectively, no cytotoxicity up to 10 μM).

The best thiated inhibitor S^4FLTMP (**12**) exerts potent antiviral activity (EC_{50} 103 nM) against the multiresistant SQ mutant, RT of which is 200-fold more resistant to AZTTP than that of the WT enzyme (Table 3). S^4FLTMP (**12**) and FLTMP (**14**) may therefore be regarded as a selective potential agent against HIV-1 drug- and multidrug-resistant strains.

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