Design, synthesis, and biological evaluation of piperidinyl-substituted [1,2,4]triazolo[1,5-a]pyrimidine derivatives as potential anti-HIV-1 agents with reduced cytotoxicity

Boshi Huang^{1,3}, Dongwei Kang¹, Ye Tian¹, Dirk Daelemans², Erik De Clercq², Christophe Pannecouque², Peng Zhan^{1,*}, Xinyong Liu^{1,*}

¹*Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, 44 West Culture Road, 250012 Ji'nan, Shandong, PR China*

²*Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, K.U.Leuven, Herestraat 49 Postbus 1043 (09.A097), B-3000 Leuven, Belgium.*

*Corresponding authors. Tel: +86 531 88380270; fax: +86 531 88382731; E-mail address: zhanpeng1982@sdu.edu.cn (Zhan P.); xinyongli@sdu.edu.cn (Liu X.Y.)

³ Present address: Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, 800 E Leigh Street, Richmond, VA 23298, United States.

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Abstract

Taking the previously reported compound **BH-7d** as the lead, we designed and synthesized a series of piperidinyl-substituted [1,2,4]triazolo[1,5-a]pyrimidines, and their anti-HIV activities as well as cytotoxicities were evaluated. Several compounds exhibited moderate anti-HIV (IIIB) potency, among which **2b** was the most active one ($EC_{50} = 4.29 \mu M$). Structure-activity relationships derived from the antiretroviral results were analyzed. Additionally, most compounds demonstrated reduced cytotoxicity ($CC_{50} > 200 \mu M$) compared to those of **BH-7d** and etravirine. Molecular docking study further revealed the binding conformation of **2b** in the binding pocket of HIV-1 reverse transcriptase.

Keywords: [1,2,4]Triazolo[1,5-a]pyrimidines; Reverse transcriptase; Anti-HIV-1 potency; Molecular docking; Reduced cytotoxicity.

Introduction

Highly active antiretroviral therapy (HAART) comprising three or more antiretroviral agents has brought a tremendous benefit in treating acquired immunodeficiency syndrome (AIDS) patients with HIV-1 infection.(De Clercq, 2012; Zhan, Pannecouque, De Clercq, & Liu, 2016) Reverse transcriptase (RT) inhibitors (RTIs), which mainly consist of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), are indispensable components of HAART.(Zhan et al., 2013) Among RTIs, NNRTIs have been widely used due to their potent antiviral activity, high selectivity, and low cytotoxicity. However, HIV-1 rapidly develops resistance to the available NNRTI drugs through mutations, which has markedly compromised the application of these anti-HIV-1 agents.(Huang, Liang, et al., 2015) Therefore, there is still an urgent need to develop new generation HIV-1 NNRTIs with improved anti-resistance profiles.

Etravirine (ETR) and rilpivirine (RPV) (Figure 1), bearing a diarylpyrimidine (DAPY) skeleton, have been approved for clinical use by U.S. FDA in 2008 and 2011 respectively.(Huang et al.,

2019; D. Kang et al., 2016) Both drugs possess prominent potency in inhibiting the wild-type (WT) and most drug-resistant HIV-1 strains at the nanomolar level.(Gu, Lu, Liu, Ju, & Zhu, 2018; Huang, Kang, Yang, Zhan, & Liu, 2016; Huang et al., 2018) However, poor water solubility, newly emerging HIV-1 mutants, virologic failure, and adverse events related to the two DAPY analogues have been observed.(D. Kang et al., 2016; Liu et al., 2016) In addition, both of them are endowed with high cytotoxicity (the CC_{50} values are about 2-6 μ M in MT-4 cells),(Huang et al., 2017; Dongwei Kang et al., 2019; Sang et al., 2019; Zhou et al., 2018) which impedes their long-term administration for AIDS patients. Taken together, the development of new DAPYs with efficient antiviral activity, improved solubility, as well as low cytotoxicity is required for providing more options to fill the HAART.

To address these issues, we previously synthesized a series of piperidinyl-substituted DAPY derivatives with a [1,2,4]triazolo[1,5-a]pyrimidine core ring and tested their anti-HIV-1 potency against WT (IIIB) and the most prevalent K103+Y181C double mutant (RES056) strains in MT-4 cells. (Huang, Li, et al., 2015) The most active inhibitor **BH-7d** displayed potent HIV-1 inhibition against WT strain (EC_{50} = 8.1 nM). Moreover, **BH-7d** was much more water-soluble than ETR at pH 7 (for **BH-7d**, 19.8 μ M; for ETR, $<<$ 2.3 μ M), and had a favorable logP value (for **BH-7d**, 1.91; for ETR, $>$ 5).(Huang, Li, et al., 2015; Weuts et al., 2011) Furthermore, from NNRTI/RT co-crystal structures and molecular docking results it was found that the benzyl-substituted piperidinyl motif was located in a region called “tolerant region I” which is composed of amino acid residues Val106, Phe227, Tyr318 and Pro236 and is extended to the protein/solvent interface.(Huang et al., 2019; Huang, Li, et al., 2015) It has been demonstrated that diverse substituents could be well tolerated in this region. (Huang et al., 2019; Dongwei Kang et al., 2020; Xiao et al., 2020)

To further explore the chemical space around the tolerant region I, two series of target compounds were newly designed employing compound **BH-7d** as the lead. Specifically, the left 2,4,6-trimethyl substituted phenoxy pharmacophore, the core ring [1,2,4]triazolo[1,5-a]pyrimidine, and the piperidinyl of **BH-7d** were maintained, meanwhile diverse aromatic substitutes (Ar^1/Ar^2) were introduced to examine their influence on anti-HIV-1 activity. Moreover, the methylene group

was replaced with a sulfonyl group or a dithiocarbamate moiety was inserted (connected with the *N* atom of piperidinyl), anticipating to establish hydrogen bond interactions with surrounding amino acid residues, as these two functional groups could act as favorable hydrogen-bond receptors (Bala et al., 2015; Dongwei Kang et al., 2017) (Figure 2). In the present study, we report the synthesis, anti-HIV-1 evaluation, cytotoxicity evaluation, and molecular docking results of these newly designed piperidinyl-substituted [1,2,4]triazolo[1,5-a]pyrimidine derivatives.

Experimental section

Chemistry

All melting points (Mp) were determined on a micromelting point apparatus and are uncorrected. Mass spectra were performed on a LC Autosampler Device: Standard G1313A instrument by electrospray ionization. ¹H NMR and ¹³C NMR spectra were obtained on a Bruker AV-400 spectrometer (Bruker BioSpin, Fällanden, Switzerland) in the indicated solvent DMSO-*d*₆. Chemical shifts were expressed in δ units (ppm), using TMS as an internal standard, and *J* values were reported in hertz (Hz). TLC was performed on Silica Gel GF254. Spots were visualized by irradiation with UV light (λ 254 nm). Flash column chromatography was carried out on columns packed with silica gel 60 (200–300 mesh). Solvents were of reagent grade and, if needed, were purified and dried by standard methods. The key reagents were purchased from commercial suppliers and with no further purification when used. Rotary evaporators were served in concentration of the reaction solutions under reduced pressure.

General procedure for the preparation of target compounds 2a-f

To a solution of **1** (0.12 g, 0.34 mmol) in 4 mL of methylene dichloride were added substituted benzenesulfonyl chloride (0.34 mmol) and 3 drops of triethylamine. The resulting mixture was stirred at room temperature until completion (monitored by TLC). The excess solvent was removed, and methanol (5 mL) was added. The precipitate was filtered and further dried to offer compounds **2a-f** except **2e**. Column chromatography and recrystallization were implemented to provide pure compound **2e**.

7-(Mesityloxy)-*N*-(1-(phenylsulfonyl)piperidin-4-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-5-amine (**2a**). White solid. Yield: 51%. Mp: 244.2-245.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ : 8.17 (s, 1H, triazole-H), 7.76-7.71 (m, 3H, PhH), 7.67-7.63 (m, 2H, PhH), 7.55 (d, *J* = 7.12 Hz, 1H, NH), 7.06

(s, 2H, PhH), 5.26 (s, 1H, pyrimidine-H), 3.82-3.81 (m, 1H, piperidiny-H), 3.49-3.46 (m, 2H, piperidiny-H), 2.60-2.50 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.07 (s, 6H, CH₃×2), 1.96-1.92 (m, 2H, piperidiny-H), 1.45-1.36 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 160.51, 157.62, 154.48, 153.28, 146.38, 136.71, 136.30, 133.58, 130.50 (Ph-C×2), 129.98 (Ph-C×2), 129.89 (Ph-C×2), 127.82 (Ph-C×2), 79.65, 46.52, 45.03, 30.69, 20.79, 15.73. ESI-MS: *m/z* 493.5 [M+1]⁺, 515.5 [M+23]⁺, C₂₅H₂₈N₆O₃S (492.19).

7-(Mesityloxy)-*N*-(1-tosylpiperidin-4-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-5-amine (**2b**). White solid. Yield: 59%. Mp: 232.0-233.8°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.37 (s, 1H, triazole-H), 7.76 (d, *J* = 6.92 Hz, 1H, NH), 7.63 (d, *J* = 8.24 Hz, 2H, PhH), 7.45 (d, *J* = 8.04 Hz, 2H, PhH), 7.07 (s, 2H, PhH), 5.32 (s, 1H, pyrimidine-H), 3.82-3.81 (m, 1H, piperidiny-H), 3.49-3.46 (m, 2H, piperidiny-H), 2.60-2.50 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.07 (s, 6H, CH₃×2), 1.96-1.92 (m, 2H, piperidiny-H), 1.45-1.36 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 160.51, 157.62, 154.48, 153.28, 146.38, 136.71, 136.30, 133.58, 130.50 (Ph-C×2), 129.98 (Ph-C×2), 129.89 (Ph-C×2), 127.82 (Ph-C×2), 79.65, 46.52, 45.03, 30.69, 20.79, 15.73. ESI-MS: *m/z* 507.2184 [M+1]⁺, C₂₆H₃₀N₆O₃S (506.2100).

4-(((4-((7-(Mesityloxy)-[1,2,4]triazolo[1,5-*a*]pyrimidin-5-yl)amino)piperidin-1-yl)sulfonyl)benz onitrile (**2c**). White solid. Yield: 44%. Mp: 275.8-277.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.46 (s, 1H, triazole-H), 8.14 (d, *J* = 8.48 Hz, 2H, PhH), 7.94 (d, *J* = 8.48 Hz, 2H, PhH), 7.87 (d, *J* = 6.92 Hz, 1H, NH), 7.07 (s, 2H, PhH), 5.34 (s, 1H, pyrimidine-H), 3.85-3.80 (m, 1H, piperidiny-H), 3.52-3.49 (m, 2H, piperidiny-H), 2.72-2.67 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.08 (s, 6H, CH₃×2), 1.95-1.91 (m, 2H, piperidiny-H), 1.44-1.37 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 161.47, 154.53, 153.33, 149.73, 146.20, 140.70, 137.00, 134.08 (Ph-C×2), 132.55, 130.57 (Ph-C×2), 129.96 (Ph-C×2), 128.57 (Ph-C×2), 126.88, 118.15, 116.01, 80.84, 46.71, 45.84, 30.48, 20.79, 15.73. ESI-MS: *m/z* 518.1985 [M+1]⁺, C₂₆H₂₇N₇O₃S (517.1896).

N-(1-((4-fluorophenyl)sulfonyl)piperidin-4-yl)-7-(mesityloxy)-[1,2,4]triazolo[1,5-*a*]pyrimidin-5-amine (**2d**). White solid. Yield: 50%. Mp: 219.5-220.4°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.25 (s, 1H, triazole-H), 7.85-7.81 (m, 2H, PhH), 7.63 (d, *J* = 7.08 Hz, 1H, NH), 7.51-7.47 (m, 2H, PhH), 7.07 (s, 2H, PhH), 5.29 (s, 1H, pyrimidine-H), 3.90-3.85 (m, 1H, piperidiny-H), 3.49-3.47

(m, 2H, piperidiny-H), 2.63-2.50 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.08 (s, 6H, CH₃×2), 1.96-1.92 (m, 2H, piperidiny-H), 1.45-1.36 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 165.07 (d, *J* = 250.08 Hz, Ph-C), 160.68, 157.06, 153.65, 153.30, 146.35, 136.77, 132.69 (d, *J* = 2.79 Hz, Ph-C), 130.92 (d, *J* = 9.53 Hz, 2 × Ph-C), 130.52 (Ph-C×2), 129.98 (Ph-C×2), 117.09 (d, *J* = 22.51 Hz, Ph-C×2), 79.86, 46.52, 44.98, 30.62, 20.79, 15.72. ESI-MS: *m/z* 511.1934 [M+1]⁺, C₂₅H₂₇FN₆O₃S (510.1849).

7-(Mesityloxy)-*N*-(1-((4-nitrophenyl)sulfonyl)piperidin-4-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-5-amine (**2e**). White solid. Yield: 36%. Mp: 248.5-249.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.45 (d, *J* = 8.88 Hz, 2H, PhH), 8.19 (s, 1H, triazole-H), 8.03 (d, *J* = 8.92 Hz, 2H, PhH), 7.56 (d, *J* = 7.16 Hz, 1H, NH), 7.06 (s, 2H, PhH), 5.25 (s, 1H, pyrimidine-H), 3.87-3.86 (m, 1H, piperidiny-H), 3.54-3.51 (m, 2H, piperidiny-H), 2.74-2.69 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.09 (s, 6H, CH₃×2), 1.96-1.93 (m, 2H, piperidiny-H), 1.44-1.36 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 160.53, 157.47, 154.29, 153.32, 150.46, 146.36, 142.06, 136.75, 130.51 (Ph-C×2), 129.98 (Ph-C×2), 129.44 (Ph-C×2), 125.17 (Ph-C×2), 79.68, 46.34, 44.96, 30.64, 20.78, 15.72. ESI-MS: *m/z* 538.1887 [M+1]⁺, C₂₅H₂₇N₇O₅S (537.1794).

2-(4-((4-((7-(Mesityloxy)-[1,2,4]triazolo[1,5-*a*]pyrimidin-5-yl)amino)piperidin-1-yl)sulfonyl)phenyl)acetamide (**2f**). White solid. Yield: 50%. Mp: 240.2-241.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 10.38 (s, 1H, CONH₂), 8.17 (s, 1H, triazole-H), 7.82 (d, *J* = 8.84 Hz, 2H, PhH), 7.68 (d, *J* = 8.80 Hz, 2H, PhH), 7.54 (d, *J* = 7.08 Hz, 1H, NH), 7.06 (s, 2H, PhH), 5.26 (s, 1H, pyrimidine-H), 3.82-3.80 (m, 1H, piperidiny-H), 3.45-3.42 (m, 2H, piperidiny-H), 2.59-2.53 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.11 (s, 3H, CH₂, CONH₂), 2.07 (s, 6H, CH₃×2), 1.95-1.93 (m, 2H, piperidiny-H), 1.44-1.35 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 169.55, 160.50, 157.66, 154.54, 153.27, 146.38, 143.86, 136.71, 130.50 (Ph-C×2), 129.99, 129.70 (Ph-C×2), 129.07 (Ph-C×2), 119.15 (Ph-C×2), 79.64, 46.53, 45.00, 30.68, 24.63 (PhCH₂), 20.79, 15.73. ESI-MS: *m/z* 550.2239 [M+1]⁺, C₂₇H₃₁N₇O₄S (549.2158).

General procedure for the preparation of target compounds 4a-d

To a solution of **1** (0.10 g, 0.28 mmol) in methanol (5 mL) were added 30% NaOH solution (0.43 mmol) and carbon disulfide (26 μL, 0.43 mmol). The resulting mixture was stirred at 40°C

for 4h. Benzyl halide or 4-(chloromethyl)pyridine hydrochloride (0.28 mmol or 0.25 mmol) and triethylamine (0.044 g, 0.43 mmol) were added to the mixture, and the reaction mixture was further stirred overnight. The precipitate was filtered and washed with methanol or was recrystallized using methanol to afford compounds **4a-d**.

Benzyl 4-((7-(mesityloxy)-[1,2,4]triazolo[1,5-a]pyrimidin-5-yl)amino)piperidine-1-carbodithioate (**4a**). White solid. Yield: 61%. Mp: 267.8-269.5°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.20 (s, 1H, triazole-H), 7.63 (d, *J* = 7.20 Hz, 1H, NH), 7.40-7.38 (m, 2H, PhH), 7.34-7.24 (m, 3H, PhH), 7.08 (s, 2H, PhH), 5.29 (s, 1H, pyrimidine-H), 4.99-4.96 (m, 1H, piperidiny-H), 4.52 (s, 2H, CH₂), 4.32-4.22 (m, 2H, piperidiny-H), 3.67-3.64 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.09 (s, 6H, CH₃×2), 2.03-2.00 (m, 2H, piperidiny-H), 1.40-1.38 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 194.50(C=S), 160.55, 157.71, 154.61, 153.35, 146.40, 136.73, 130.53 (Ph-C×2), 130.01 (Ph-C×2), 129.66 (Ph-C×2), 128.94 (Ph-C×2), 127.82, 79.68, 50.17, 46.81, 41.51, 31.53, 31.06, 20.80, 15.75. ESI-MS: *m/z* 519.5 [M+1]⁺, 541.5 [M+23]⁺, C₂₇H₃₀N₆OS₂ (518.19).

4-(Methylsulfonyl)benzyl 4-((7-(mesityloxy)-[1,2,4]triazolo[1,5-a]pyrimidin-5-yl)amino)piperidine-1-carbodithioate (**4b**). White solid. Yield: 36%. Mp: 260.5-261.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.20 (s, 1H, triazole-H), 7.86 (d, *J* = 8.36 Hz, 2H, PhH), 7.66-7.62 (m, 3H, NH, PhH), 7.08 (s, 2H, PhH), 5.29 (s, 1H, pyrimidine-H), 4.97-4.94 (m, 1H, piperidiny-H), 4.68 (s, 2H, CH₂), 4.33-4.22 (m, 2H, piperidiny-H), 3.68-3.66 (m, 2H, piperidiny-H), 3.20 (s, 3H, SO₂CH₃), 2.29 (s, 3H, CH₃), 2.09 (s, 6H, CH₃×2), 2.04-2.00 (m, 2H, piperidiny-H), 1.41-1.39 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 193.84 (C=S), 160.55, 157.71, 154.62, 153.36, 146.40, 143.67, 140.08, 136.76, 130.53 (Ph-C×2), 130.42 (Ph-C×2), 130.01 (Ph-C×2), 127.54 (Ph-C×2), 79.69, 50.51, 46.77, 43.99, 31.56, 31.05, 20.80, 15.75. ESI-MS: *m/z* 597.5 [M+1]⁺, 619.5 [M+23]⁺, C₂₈H₃₂N₆O₃S₃ (596.17).

Pyridin-4-ylmethyl 4-((7-(mesityloxy)-[1,2,4]triazolo[1,5-a]pyrimidin-5-yl)amino)piperidine-1-carbodithioate (**4c**). White solid. Yield: 30%. Mp: 239.5-241.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.49 (dd, *J* = 4.40 Hz, *J* = 1.60 Hz, 2H, pyridine-H), 8.20 (s, 1H, triazole-H), 7.64 (d, *J* = 7.20 Hz, 1H, NH), 7.38 (dd, *J* = 4.60 Hz, *J* = 1.36 Hz, 2H, pyridine-H), 7.08 (s, 2H,

PhH), 5.29 (s, 1H, pyrimidine-H), 4.97-4.94 (m, 1H, piperidiny-H), 4.59 (s, 2H, CH₂), 4.35-4.23 (m, 2H, piperidiny-H), 3.71-3.65 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.09 (s, 6H, CH₃×2), 2.03-2.00 (m, 2H, piperidiny-H), 1.44-1.37 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 193.78(C=S), 160.55, 157.71, 154.62, 153.36, 150.06 (Ph-C×2), 146.61, 146.40, 136.75, 130.53 (Ph-C×2), 130.01 (Ph-C×2), 124.52 (Ph-C×2), 79.69, 50.56, 46.77, 31.60, 30.97, 20.80, 15.75. ESI-MS: *m/z* 520.5 [M+1]⁺, 542.5 [M+23]⁺, C₂₆H₂₉N₇OS₂ (519.19).

2-Cyanobenzyl 4-((7-(mesityloxy)-[1,2,4]triazolo[1,5-a]pyrimidin-5-yl)amino) piperidine-1-carbodithioate (**4d**). White solid. Yield: 48%. Mp: 219.5-221.0°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ: 8.20 (s, 1H, triazole-H), 7.84 (d, *J* = 7.20 Hz, 1H, NH), 7.70-7.63 (m, 3H, PhH), 7.51-7.46 (m, 1H, PhH), 7.08 (s, 2H, PhH), 5.29 (s, 1H, pyrimidine-H), 4.96-4.93 (m, 1H, piperidiny-H), 4.70 (s, 2H, CH₂), 4.31-4.22 (m, 2H, piperidiny-H), 3.67 (m, 2H, piperidiny-H), 2.29 (s, 3H, CH₃), 2.09 (s, 6H, CH₃×2), 2.03-2.02 (m, 2H, piperidiny-H), 1.42-1.39 (m, 2H, piperidiny-H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ: 193.21 (C=S), 160.56, 157.71, 154.61, 153.35, 146.40, 140.48, 136.75, 133.84, 133.58, 130.95, 130.52, 130.01, 128.83, 117.82, 112.64, 79.69, 50.30, 46.75, 31.55, 30.93, 20.80, 15.75. ESI-MS: *m/z* 544.1965 [M+1]⁺, 566.1765 [M+23]⁺, C₂₈H₂₉N₇OS₂ (543.1875).

In vitro anti-HIV and cytotoxicity evaluation assays

Evaluation of the antiviral activity and cytotoxicity of the synthesized compounds was performed using the MTT assay as previously described.(Pannecouque, Daelemans, & De Clercq, 2008) Stock solutions (10 × final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells to allow simultaneous evaluation of their effects on mock- and HIV-infected cells at the beginning of each experiment. Serial five-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays by adding 100 μL medium to the 25 μL stock solution and transferring 25 μL of this solution to another well that contained 100 μL medium using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV- and mock-infected cell samples were included for each sample. HIV-1 WT strain (IIIB), HIV-1 K103N+Y181C double mutant strain (RES056) or HIV-2 strain (ROD) stock (50 μL) at 100-300 CCID₅₀ (50% cell culture infectious dose) or culture medium was added

to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compounds on uninfected cells in order to assess its cytotoxicity. Exponentially growing MT-4 cells were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL, and 50 μ L volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT method. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of the test compound that reduced the viability of the mock-infected MT-4 cells by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC₅₀).

Molecular docking

The compound **2b** for docking was optimized for 2000-generations until the maximum derivative of energy became 0.005 kcal/(mol*Å), using the Tripos force field. Charges were computed and added according to Gasteiger-Huckel parameters. The published 3D crystal structure of WT RT complexes (PDB code: 3M8Q (Kertesz et al., 2010)) was retrieved from the Protein Data Bank (Berman et al., 2000) and was used for the docking studies by means of surflex-dock module of SYBYL-X 1.1. The protein was prepared by using the Biopolymer application accompanying SYBYL according to SYBYL-X 1.1 manual: The bound ligand was extracted from the complexes, water molecules were removed, hydrogen atoms were automatically added (after which the resulting structures haven't been optimized), and charges and atom types were assigned according to AMBER99. After the protomol was generated, which referred to a computational representation of the intended binding site to which putative ligands are aligned, the optimized compound **2b** was docked into NNIBP, with the relevant parameters set as defaults. The original ligand of the coordinates (3M8Q) was used as reference molecule to calculate the RMSD values. The docking scores related to binding affinities were calculated based on hydrophobic, polar, and repulsive interactions as well as entropic effects and solvation. Top-scoring poses of compounds were shown by the software of PyMOL version 1.5 (<http://www.pymol.org/>). Only the key residues for interactions with the inhibitor were labeled

and shown in sticks. The potential hydrogen bonds were presented by dashed lines in yellow.

Results and Discussion

Chemistry

The synthesis of the target compounds is outlined in Scheme 1. For preparation of both series of compounds **2a-f** and **4a-d**, the previously reported compound **1** was employed as the starting material. (Huang, Li, et al., 2015) Compounds **2a-f** were conveniently obtained by compound **1** reacting with different sulfonyl chlorides. (Dongwei Kang et al., 2017) In addition, compound **1** first reacted with carbon disulfide under a basic condition (30% sodium hydroxide solution with methanol) to afford the intermediate sodium dithiocarbamate **3**. Without purification, intermediate **3** was directly used in the next step to react with different halides in the presence of triethylamine, (Bala et al., 2015) and finally the target compounds **4a-d** were furnished.

Scheme 1. Reagents and conditions: (i) ClSO₂Ar¹, TEA, DCM, r.t., overnight.; (ii) CS₂, 30% NaOH (aq), CH₃OH, 40°C, 4h; (iii) TEA, Ar²CH₂X (X = Cl, Br), r.t., overnight.

Anti-HIV activity and cytotoxicity in MT-4 cells

The anti-HIV-1 activity (expressed as EC₅₀) of all the newly synthesized compounds was biologically evaluated using MTT method in MT-4 cell cultures infected with HIV-1 WT strain (IIIB) or HIV-2 strain (ROD). Some compounds were further tested for their activities against HIV-1 K103N+Y181C double mutant strain (RES056). The cytotoxicity evaluation (expressed as CC₅₀) was conducted in mock-infected MT-4 cells. ETR was used as the control drug. EC₅₀ and CC₅₀ values of the target compounds **2a-f** and **4a-d** are summarized in Table 1.

As shown in Table 1, four compounds (**2a**, **2b**, **2f**, and **4c**) showed moderate activities in inhibiting HIV-1 strain (IIIB) with EC₅₀ values of 7.4 μM, 4.29 μM, 20 μM, and 30 μM, respectively. Among them, **2b** was the most potent and least toxic compound (EC₅₀ = 4.29 μM,

CC₅₀ > 247 μM). None of these new compounds was found to be effective against the K103+Y181C double mutant strain and HIV-2 strain at the highest tested concentration (Table S1).

Detailed structure-activity relationships (SARs) were derived from the antiviral results. For compounds **2a-f**, it was found that electron-donating groups at the 4-position of the phenyl ring might be more favorable for potency than electron-withdrawing groups, of which the order was as follows: electron-donating group [CH₃ (**2b**, EC₅₀ = 4.29 μM)] > H (**2a**, EC₅₀ = 7.4 μM) > electron-withdrawing groups (acetamide, CN, F, and NO₂). Among these compounds with electron-withdrawing groups substituted at the 4-position of the phenyl ring, acetamide with the largest steric size was most favorable for antiretroviral activity. For compounds **4a-d**, only one compound **4c** with a pyridin-4-yl possessed weak anti-HIV-1 activity. Obviously, the pyridin-4-yl group was more profitable than the phenyl group (**4c** vs **4a**), which was in line with our previous study.(Huang, Li, et al., 2015) Comparison between **2a** (EC₅₀ = 7.4 μM) and **4a** (EC₅₀ > 241 μM) both with the terminal phenyl group indicated that the sulfonyl group took advantages for activity over the dithiocarbamate moiety. Additionally, compared to the lead **BH-7d** (EC₅₀ = 0.0081 μM), compound **4b** bearing a SO₂CH₃ group at the 4-position of the phenyl ring did not exhibit antiviral potency (EC₅₀ > 210 μM), implying that the inserted dithiocarbamate moiety might be unfavorable for anti-HIV-1 activity.

Also, it was found that almost all the target compounds (except for **2a** and **2d**) demonstrated very low cytotoxicity, with CC₅₀ values greater than 200 μM. The cytotoxicities of these new compounds were one to two orders of magnitude lower than those of the lead compound **BH-7d** (CC₅₀ = 20 μM) and ETR (CC₅₀ = 2.15 μM). Among compounds **2a-f**, **2a** with the phenyl substituent displayed the highest cytotoxicity (CC₅₀ = 31.7 μM), while a fluorine substitution at the 4-position of the phenyl ring decreased the cytotoxicity slightly (**2d**, CC₅₀ = 33 μM). Interestingly, the shift of sulfonyl moiety from the SO₂CH₃ group of the lead **BH-7d** to the “spacer” portion between the piperidinyl *N* and the terminal phenyl ring of compound **2b** (CC₅₀ > 247 μM) lead to the decreased cytotoxicity profile. The reduced cytotoxicity of the target compounds suggested further modifications could be implemented based on the two skeletons to

yield more potent anti-HIV-1 agents.

Taken together, the present SAR analysis demonstrated that the “spacer” between the piperidinyl *N* and the terminal aromatic ring of these DAPY analogues with a [1,2,4]triazolo[1,5-*a*]pyrimidine core ring should possess the proper length and size, and it seemed that the sulfonyl group was more favorable for anti-HIV-1 potency than the dithiocarbamate methylene moiety. Moreover, from the anti-HIV results it was suggested that compounds bearing electron-donating groups at the 4-position of the phenyl ring demonstrated superior potency than those compounds bearing electron-withdrawing groups among compounds **2a-f**, which furnished further SAR information around the tolerant region I. The work described here offered critical clues for rational design of novel piperidinyl-substituted DAPY-typed HIV-1 NNRTIs in the future.

Molecular docking analysis

To better elucidate the antiviral activity of these new piperidinyl-substituted [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives at the molecular level, a molecular docking study of the most potent compound **2b** was performed. The molecular docking study was accomplished using Surflex-Dock module of Sybyl-X 1.1 software, and the docking results were further analyzed by PyMOL (Figure 3).

As shown in Figure 3, the binding mode of compound **2b** can be described as follows: i) The overall conformation of compound **2b** in the NNRTI binding pocket (NNIBP) was a “U” shape, which was similar to previously reported DAPYs. ii) The 2,4,6-trimethylphenoxy moiety filled a hydrophobic tunnel composed by the aromatic side chains of residues Tyr181, Tyr 188, Phe227, and Trp229, establishing positive hydrophobic interactions. iii) The *NH* linker in **2b** formed a hydrogen bond with the backbone carbonyl of residue Lys101, while 5-*N* of the [1,2,4]triazolo[1,5-*a*]pyrimidine core did not form hydrogen-bonding interaction with residue Lys101. This was somewhat different from that of the lead **BH-7d** (Huang, Li, et al., 2015). iv) The *N*-(1-tosylpiperidin-4-yl) motif was located in tolerant region I, and the terminal methyl phenyl moiety extended to the solvent-exposed region. Furthermore, one oxygen atom of the sulfonyl group formed a hydrogen bond with the side chain of the highly conserved residue

Tyr318, which was not observed in previously reported DAPY-typed compounds. This putative hydrogen bond fulfilled our original drug design, and might compensate for the loss of the hydrogen-bonding interaction between 5-*N* of the [1,2,4]triazolo[1,5-*a*]pyrimidine core and Lys101. Additionally, it was speculated that the terminal electron-donating methyl group might generate more positive interactions with Val106 and Pro236 than the electron-withdrawing groups did in this specific surrounding chemical environment. On the whole, compound **2b** established key interactions with surrounding amino acid residues in the binding pocket, which helped explain its moderate anti-HIV-1 activity.

In silico calculation of physicochemical properties

Some physicochemical properties of the active anti-HIV-1 agents **2a**, **2b**, **2f**, and **4c** as well as the lead compound **BH-7d** and ETR (as controls) using the free online Molinspiration software (<http://www.molinspiration.com/>) were calculated. In addition, the water solubility of compound **2b** was also predicted (free online server, <http://molsoft.com/mprop/>). As depicted in Table 2, the prediction results indicated that compounds **2a**, **2b** and **4c** conformed the Lipinski's rule of five well as **BH-7d** and ETR did. Moreover, compound **2b** possessed moderate water solubility (3.15 µg/mL), which seemed higher than that of ETR, though lower than that of the lead **BH-7d**. We therefore speculated that there would be other factors which might lead to their relatively decreased anti-HIV-1 potencies.

Conclusions

In summary, based on the structure of our previously reported lead compound **BH-7d**, and careful examination of the structural characteristics of tolerant region I of the NNIBP, we have designed a series of piperidinyl-substituted [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives. The methylene group of **BH-7d** was replaced with a sulfonyl group or a dithiocarbamate moiety was inserted to yield compounds **2a-f** and **4a-d**, respectively. Compound **2b** was found to be the most potent anti-HIV-1 (IIIB) agent. SAR studies revealed that electron-donating groups at the 4-position of the phenyl ring might be more favorable for potency than electron-withdrawing groups for compounds **2a-f**, and the pyridin-4-yl group was most beneficial for activity for compounds **4a-d**. It was also suggested that the sulfonyl group might be a more favorable “spacer”

between the piperidinyll *N* and the terminal aromatic ring of the piperidinyll-substituted [1,2,4]triazolo[1,5-a]pyrimidines. More importantly, most target compounds exhibited very low cytotoxicity. The binding mode of **2b** in NNIBP was investigated by molecular docking study. These valuable information provide important clues for further modifications of this series of piperidinyll-substituted [1,2,4]triazolo[1,5-a]pyrimidine derivatives. More diversified moieties that are prone to establishing hydrogen bond(s) with the surrounding residues in tolerant region I of NNIBP could be introduced onto the terminal phenyl ring of compound **2b** to yield more potent anti-HIV-1 agents.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of the present study are available from the corresponding authors upon reasonable request.

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Table 1. Antiviral activity against HIV-1 IIIB strain and cytotoxicity of target compounds **2a-f** and **4a-d** in MT-4 cells.

Compd	Ar ¹ /Ar ²	EC ₅₀ (μM) ^a	CC ₅₀ (μM) ^b
2a	Phenyl	7.4 ± 1.48	31.7 ± 4.77
2b	4-CH ₃ -Ph	4.29 ± 0.99	>247
2c	4-CN-Ph	≥44.5	>242
2d	4-F-Ph	>33	33 ± 7.17
2e	4-NO ₂ -Ph	>233	>233
2f	4-acetamide-Ph	20 ± 2.62	>228
4a	Phenyl	>241	>241
4b	4-SO ₂ CH ₃ -Ph	>210	>210
4c	Pyridin-4-yl	30 ± 2.73	>241
4d	2-CN-Ph	>230	>230
BH-7d		0.0081 ± 0.0008 ^c	20 ± 9.3 ^c
ETR		0.0040 ± 0.0003	2.15 ± 0.11

^a EC₅₀: concentration of compound required to achieve 50% protection of MT-4 cell cultures against HIV-1-induced cytotoxicity, as determined by the MTT method.

^b CC₅₀: concentration required to reduce the viability of mock-infected cell cultures by 50%, as determined by the MTT method.

^c Data have been reported in Reference (Huang, Li, et al., 2015), and are used here for comparative purposes.

Table 2. Physicochemical properties of **2a**, **2b**, **2f**, **4c**, **BH-7d** and ETR.

Compd	nViol ^a	miLogP ^a	MW ^a	nON ^a	nOHNH ^a	nrotb ^a	Water solubility (μg/mL)
Acceptable range		<5	<500 Da	<10	<5	≤10	
2a	0	4.45	492.61	9	1	6	-
2b	1	4.90	506.63	9	1	6	3.15 ^b
2f	2	3.36	549.66	11	3	8	-
4c	1	4.78	519.70	8	1	8	-
BH-7d	1	1.91 ^c	520.65	9	1	7	10.31 ^c
ETR	1	5.03	435.29	7	3	4	<<1 ^c

^a nViol = number of violations; miLogP = molinspiration predicted LogP; MW= molecular weight; nON = no. of hydrogen bond acceptors; nOHNH = no. of hydrogen bond donors; nroth = no. of rotatable bonds.

^b Using free online server (<http://molsoft.com/mprop/>).

^c See Reference (Huang, Li, et al., 2015).

Figure Legend

Figure 1. Chemical structures of DAPY-type NNRTI drugs ETR and RPV

Figure 2. The lead compound **BH-7d** and newly designed target compounds.

Figure 3. Proposed binding mode of **2b** docked into WT HIV-1 RT (PDB code: 3M8Q). Hydrogen bonds are indicated with dashed lines in yellow, and all the non-polar hydrogen atoms are omitted for clarity.

Etravirine (ETR)

Rilpivirine (RPV)