Letter

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Discovery of Thiophene[3,2-d]pyrimidine Derivatives as Potent HIV-1 NNRTIs Targeting the Tolerant Region I of NNIBP

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KEYWORDS: HIV-1, NNRTIs, thiophene[3,2-d]pyrimidine, tolerant region I, drug design

ABSTRACT: Our previous studies led us to conclude that thiophene[3,2-d]pyrimidine is a promising scaffold for diarylpyrimidine (DAPY)-type anti-HIV agents with potent activity against resistance-associated human immunodeficiency virus (HIV) variants (*J.Med.Chem*. 2016, 59, 7991−8007; *J.Med.Chem*. 2017, 60, 4424−4443). In the present study, we designed and synthesized a series of thiophenepyrimidine derivatives with various substituents in the right wing region of the structure with the aim of developing new interactions with the tolerant region I of the binding pocket of the HIV-1 non-nucleoside reverse transcriptase (NNRTI), and we evaluated their activity against a panel of mutant HIV-1 strains. All the derivatives exhibited moderate to excellent potency against wild-type (WT) HIV-1 in MT-4 cells. Among them, sulfonamide compounds **9b** and **9d** were single-figure-nanomolar inhibitors with EC_{50} values of 9.2 and 7.1 nM, respectively. Indeed, **9a** and **9d** were effective against the whole viral panel except RES056. Notably, both compounds showed potent antiviral activity against K103N (EC_{50} = 0.032 and 0.070 μM) and E138K (EC_{50} = 0.035 and 0.045 μM, respectively). Furthermore, **9a** and **9d** exhibited high affinity for WT HIV-1 RT (IC_{50} = 1.041 and 1.138 μM, respectively) and acted as classical NNRT inhibitors (NNRTIs). These results are expected to be helpful in the design of thiophenepyrimidine-based NNRTIs with more potent activity against HIV strains with RT mutations.

Mutations in the viral reverse transcriptase can reduce the effectiveness of the first-generation FDA-approved HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs), including nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV)[1-3]. On the other hand, second-generation diarylpyrimidine (DAPY) agents, such as etravirine (ETV) and rilpivirine (RPV) (Figure **1**), have enjoyed considerable clinical success. However, drug-resistant mutants with reduced susceptibility to ETV and RPV have emerged rapidly. Among the various mutations, K103N and E138K are the most frequently selected by ETV and RPV, respectively[4, 5]. Therefore, the development of novel NNRTIs with improved efficacy against resistant mutants remains an important research objective in medicinal chemistry[6, 7].

Previous research in our lab showed that the thiophene[3,2-d]pyrimidine can be a useful skeleton for DAPY inhibitors with improved potency against drug-resistant HIV-1 variants[8,9]. In particular, compound **3** (K-5a2) exhibited low-nanomolar anti-HIV activity against HIV-1 WT and a panel of NNRTI-resistant mutant strains. Our previous work indicated that extensive main-chain hydrogen-bonding interactions (double hydrogen-bonding chelation with the amino acid backbone) could contribute to increased activity towards drug-resistant mutants. Therefore, we considered that sulfonamide and amide functional groups would be promising substituents, as they are highly polar and can readily accept or donate a proton, so that they should easily form hydrogen bonds with amino acid residues of the target receptor or receptor-bound water molecules. In addition, the sulfonamide motif could act as a scaffold to orient pharmacophore elements into the proper geometry for binding[9-11].

In the present work, we examined the properties of a series of analogs of **3** synthesized by introducing sulfonamide and amide substituents at the right wing (interacting with tolerant region I) (Figure 2). We retained the left wing (A-ring) and the thiophene[2,3-d]pyrimidine core (B-ring). Thus, we aimed to further explore the tolerant region I of the NNRTIs binding pocket (NNIBP), based on the idea that the newly introduced hydrogen-bonding donors or receptors might form hydrogen bonds with amino acid residues of the NNIBP, resulting in stronger binding and greater potency against resistance-associated variants. The 4-aminopiperidine moiety of the lead **3** was replaced with cyclohexanediamine, and then acylation and sulfonylation with various acyl and sulfonyl chlorides

afforded our target compounds bearing substituents varying in size and polar group substitution. We expected the newly introduced sulfonamide group would increase the binding affinity of the drug molecules with the target proteins[12]. Herein, we report the synthesis, anti-HIV activity and preliminary SAR evaluation of ten thiophene[3,2-d]pyrimidine derivatives.

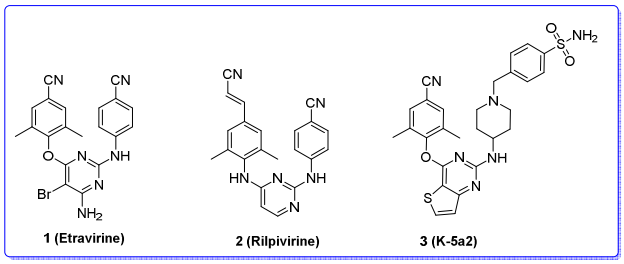

Figure 1. Chemical structures of second-generation NNRTI drugs and thiophene[3,2-d]pyrimidine lead **3** (K-5a2).

The synthetic protocols for the newly designed derivatives are outlined in Scheme 1[8]. Commercially available 2,4-dichlorothiophene[3,2-d]pyrimidine (**4**) was selected as the starting material. Successive nucleophilic substitution reactions with 4-hydroxy-3,5-dimethylbenzonitrile gave the known intermediate **5**. Subsequent reaction with tert-butyl (4-aminocyclohexyl)carbamate afforded **6**, which was directly deprotected without further purification to provide the key intermediate **7**. Treatment of **7** with substituted acyl chloride or sulfonyl chloride in the presence of triethylamine yielded the final products **8a-e** and **9a-e**. All the novel thiophene[3,2-d]pyrimidine derivatives were fully characterized by means of proton nuclear magnetic resonance (^{1}H NMR), carbon nuclear magnetic resonance (^{13}C NMR), and high resolution mass spectrometry (HRMS) measurements.

The antiviral potency of the synthesized compounds was evaluated in MT-4 cell cultures infected with WT HIV-1 strain (IIIB) or with a panel of NNRTI-resistant single- and double-mutant strains, including L100I, K103N, Y181C, Y188L, E138K, F227L+V106A and K103N+Y181C (RES056). Etravirine (ETV) and azidothymidine (AZT) were selected as control drugs. The results, expressed as EC_{50} (anti-HIV potency), CC_{50} (cytotoxicity) and SI (selectivity index, CC_{50}/EC_{50} ratio), are summarized in Tables 1 and 2.

Eight compounds (**8a-c**, **9a-e**) exhibited high potency against WT HIV-1 strain with nanomolar EC_{50} values ranging from 0.0071 to 0.086 μM, which were comparable to that of the reference drug AZT (EC_{50} = 0.011 μM). Among them, **9b** and **9d** turned out to be the most potent NNRTIs with EC_{50} values of 0.0092 and 0.0071 μM, being slightly more potent than AZT, though their activity was about 3-fold lower than that of ETV (EC_{50} = 0.0028 μM). Importantly, compound **9d** exhibited the lowest cytotoxicity (CC_{50} = 9.287 μM) and the highest selectivity index (SI = 1308), being superior to ETV (CC_{50} = 2.18 μM, SI = 776). As expected, the target compounds did not exhibit activity against the HIV-2 strain.

Preliminary SAR analysis indicated that the type of functional group (X) in the cyclohexanediamine significantly influenced anti-HIV activity. The compounds of the 9 sub-series (X = SO_2) showed better activity than those of 8 sub-series (X = CO), i.e., **9b** (EC_{50} = 0.0092 μM) > **8b** (EC_{50} = 0.026 μM), **9c** (EC_{50} = 0.025 μM) > **8c** (EC_{50} = 0.030 μM), **9d** (EC_{50} = 0.0071 μM) > **8d** (EC_{50} = 0.138 μM), **9e** (EC_{50} = 0.086 μM) > **8e** (EC_{50} = 0.196 μM). These results suggested that the tetrahedral structure of the sulfonamide is better than the planar structure of the amide for hydrogen bonding with amino acid residues of the NNIBP. In the 9 sub-series, the order of anti-HIV-1 activity was CN > F > Br > CF_3, whereas in the 8 sub-series, the order was slightly different: F > Br > CN > CF_3.

Compounds **9a**, **9b** and **9d** showed moderate activity towards mutant HIV-1 strain L100I (EC_{50} = 0.562, 0.410 and 0.424 μM, respectively), being far more potent than DLV (EC_{50} = 3.613 μM) or NVP (EC_{50} = 3.251 μM). However, all the compounds in the 8 sub-series lacked activity towards this mutant strain. In the case of the K103N, compounds in the 9 sub-series also exhibited more potent activity than those in the 8 sub-series. In particular, **9a** and **9d** were notably potent (EC_{50} = 0.032 and 0.070 μM). Furthermore, **9d** showed the most potent activity against the single mutants Y181C and Y188L (EC_{50} = 0.428 and 0.675 μM, respectively). Intriguingly, except for **8e**, all the target compounds displayed good activity against E138K with EC_{50} values in the range of 0.035-0.736 μM, although **9a** and **9d** were the most potent (EC_{50} = 0.035 and 0.045 μM, respectively). Moreover, **9a** and **9d** were effective inhibitors of the double-mutant strain F227L+V106A with EC_{50} values of 1.208 and 3.583 μM, respectively. However, both of them were inactive towards RES056 (EC_{50} > 3.727 and 9.280 μM, respectively).

In order to validate the binding target of these newly synthesized thienopyrimidine derivatives, we further tested the representative compounds **9a** and **9d** for the ability to inhibit recombinant WT HIV-1 RT enzyme, versus NVP and ETV as reference drugs (Table **3**). The results demonstrated that **9a** and **9d** exhibit remarkable anti-RT potency (IC_{50} = 1.041 and 1.138 μM, respectively), being superior to NVP but slightly inferior to ETV. Therefore, these novel thienopyrimidine derivatives have high affinity for WT HIV-1 RT and act as classical NNRTIs.

To further understand the binding interactions between the newly designed compounds and the target, we conducted molecular docking studies of the representative compounds **8d** and **9d** with the SurflexeDock SYBYL-X 2.0 software. The X-ray crystal structure of HIV-1 wild-type RT (PDB code: 3M8Q[14]) was used as the input structure for docking calculations. PyMOL was used to visualize the results. The docking protocol is described in the computational section.

As shown in Figure **3** (C), **9d** showed similar binding modes to the lead compound **3**[9]. As with other DAPY NNRTIs, **9d** adopted a horseshoe-like conformation in the NNIBP, whereas the conformation of **8d** showed significant changes. This result suggests that the conformational preference of sulfonamides provides a unique way to control the molecular shape. In contrast to the flat and linear amides, sulfonamides are bent, and this feature has a critical influence on the activity of many NNRTIs, including pyrrolyl aryl sulfones[15] and 1,3-dihydrobenzimidazol-2-one[16]. As illustrated in Figure **3** (B), we can conclude that **9d** displays the following characteristic interactions: (i) the left substituted 4-cyanovinyl-2,6-dimethylphenyl moiety effectively occupies the hydrophobic cavity surrounded by Tyr 181, Tyr 188, Phe 227 and Trp229, and especially is stabilized by π-stacking interactions with Tyr188; (ii) the thiophene ring of **9d** is directed towards the NNIBP entrance channel and there is an electrostatic interaction between the sulphur atom and Val179; (iii) the *N* atom of the central ring and *NH* linker are involved in crucial double hydrogen-bonding with the backbone of Lys101. However, compared to the lead **3**, **9d** lacks the double hydrogen-bonding

(chelate type) interactions with the backbone of Lys104 and Val106, which are important for the activities of the lead towards resistant strains. Fortunately, the newly introduced 4-cyanobenzenesulfonyl group of **9d** extends to the protein-solvent interface, and the amino group of the sulfonamide can form double hydrogen-bonding interactions with oxygen of His235 and Tyr318, which may account for the modest potency towards mutant strains. Compared to **9d**, **8d** shows the double hydrogen bonding interaction with the backbone of Lys101, while the amide functional group of **8d** does not have any other interaction with the NNIBP, providing a rational explanation of the decreased activity of **8d**.

In summary, we have designed and synthesized a novel series of thiophene[3,2-d]pyrimidine HIV-1 NNRTIs targeting the tolerant region I of the NNIBP, and evaluated their activities against HIV-1WT and a panel of mutant strains, as well as HIV-2 (ROD). All the target compounds exhibited moderate to excellent potency against WT HIV-1 with EC_{50} values ranging from 0.0071 to 0.196 μM, and the two most potent compounds **9b** and **9d** proved to be single-figure-nanomolar inhibitors (EC_{50} = 9.2 and 7.1 nM, respectively), being more potent than AZT. These two compounds also showed moderate potency against most of the mutant strains examined. In particular, they exhibited promising activity against K103N (EC_{50} = 0.032 and 0.070 μM) and E138K (EC_{50} = 0.035 and 0.045 μM, respectively). Molecular simulation confirmed that the newly introduced sulfamide group, distinguished from its isostere amide, could develop additional interactions with amino acid residues in tolerant region I of the NNIBP. We believe these results will be helpful in the extending the scope for design of thiophenepyrimidine-based NNRTIs with more potent activity against RT-mutant HIV strains.

ASSOCIATED CONTENT

Supporting Information

Experimental protocols for synthesis and characterization of compounds, *in vitro* anti-HIV assay and modeling study. The Supporting Information is available free of charge on the ACS Publications website.

Supporting Information (file type, PDF)

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Notes

The authors declare no conflict of interest.

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ABBREVIATIONS

AIDS, acquired immunodeficiency syndrome; CC_{50}, 50% cytotoxicity concentration; DAPY, diarylpyrimidine; EC_{50}, the concentration causing 50% inhibition of antiviral activity; HIV, human immunodeficiency virus; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; RT, reverse transcriptase; SAR, structure-activity relationship; SI, selection index; WT, wild-type; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz; ETV, etravirine; RPV, rilpivirine; AZT, azidothymidine.

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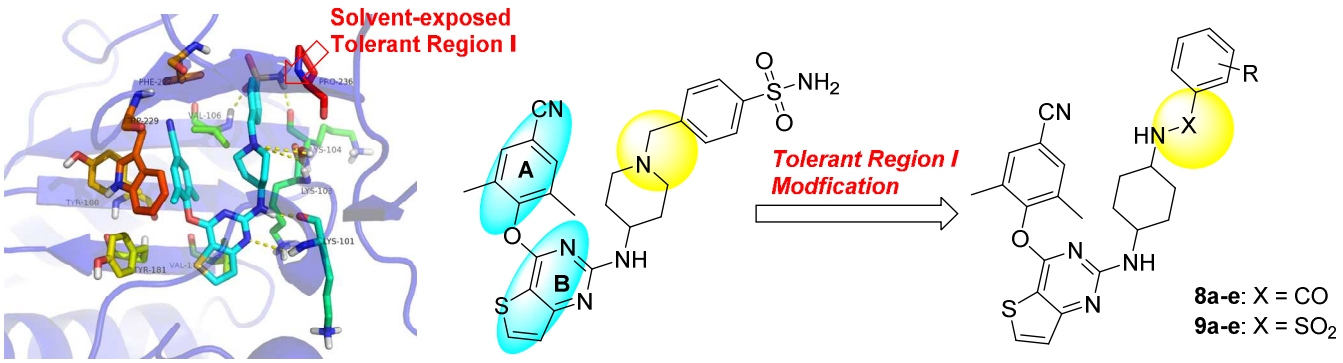
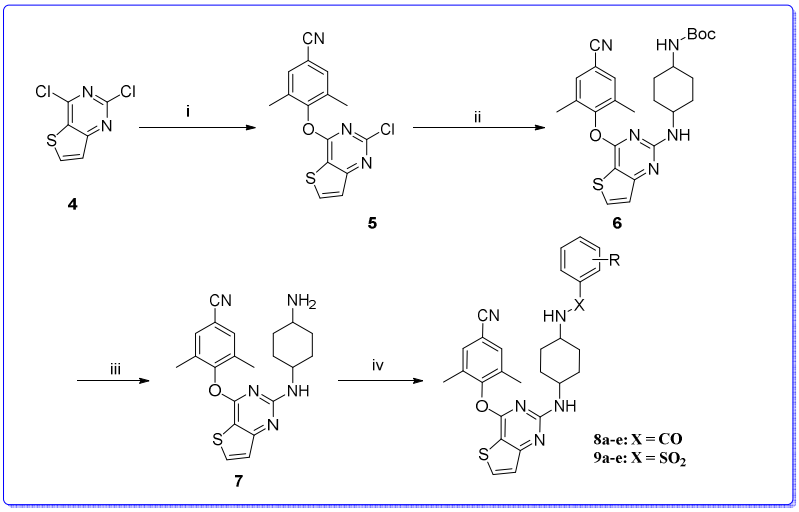


Figure 2. Design of novel NNRTIs utilizing the thiophene[3,2-d]pyrimidine scaffold. The illustration of the co-crystal structure of K-5a2/RT was generated using PyMOL (www.pymol.org).

Scheme 1. Synthesis of target compounds[a]



[a] Reagents and conditions: (i) 3,5-dimethyl-4-hydroxybenzonitrile, DMF, K_2CO_3, r.t.; (ii) tert-butyl(4-aminocyclohexyl)carbamate, DMF, K_2CO_3, 120□; iii) TFA, DCM, r.t.; (iv) substituted acyl chloride or sulfonyl chloride, DCM, Et_3N, 0□ to r.t.

Table 1. Activity and cytotoxicity against HIV-1 (IIIB) and HIV-2 (ROD) strains in MT-4 cells.

compd	X	R	$EC_{50}(\mu M)^a$		$CC_{50}(\mu M)^b$	SI^c
			IIIB	ROD		IIIB
8a	CO	H	0.013±0.007	>3.930	3.930±0.662	294
8b	CO	4-F	0.026±0.002	>3.182	3.182±1.346	121
8c	CO	4-Br	0.030±0.005	>2.849	2.849±1.225	93
8d	CO	4-CN	0.138±0.018	>2.617	2.617±1.655	19
8e	CO	3-CF_3	0.196±0.086	>3.241	3.241±0.671	17
9a	SO_2	4-$NHCOCH_3$	0.010±0.008	>3.734	3.734±0.157	340
9b	SO_2	4-F	0.0092±0.001	>3.527	3.527±0.372	383
9c	SO_2	4-Br	0.025±0.006	>5.861	5.861±3.624	231
9d	SO_2	4-CN	0.0071±0.0005	>9.287	9.287±6.187	1308
9e	SO_2	3-CF_3	0.086±0.029	>6.683	6.683±3.436	78
ETV	-	-	0.0028±0.0002		2.18±0.029	776
AZT	-	-	0.011±0.005	0.008±0.001	>7.484	>664

[a]EC_{50}: 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]CC_{50}: concentration required to reduce the viability of mock-infected cell cultures by 50%, as determined by the MTT method.

[c]SI: selectivity index, the ratio of CC_{50}/EC_{50}.

Table 2. Anti-HIV-1 activity against mutant strains in MT-4 cells.

Compds	$EC_{50}(\mu M)^a$						
	L100I	K103N	Y181C	Y188L	E138K	F227L+V106A	RES056
8a	>3.922	0.273±0.045	≥1.508	>3.922	0.183±0.039	>3.922	>3.922
8b	>3.183	0.478±0.023	>3.183	>3.183	0.230	>3.183	>3.183
8c	>2.851	0.519±0.023	>2.851	>2.851	0.370±0.086	>2.851	>2.851
8d	>2.623	≥1.436	>2.623	>2.623	0.736±0.017	>2.623	>2.623
8e	>3.237	>3.237	>3.237	>3.237	≥1.327	>3.237	>3.237
9a	0.562±0.487	0.032±0.002	0.513±0.415	0.903±0.248	0.035±0.001	1.208±0.333	>3.727
9b	0.410±0.350	0.103±0.006	0.472±0.323	>3.519	0.076±0.018	>3.519	>3.519
9c	0.841±0.884	0.131±0.003	0.852±0.655	2.250±0.011	0.126±0.014	≥6.136	5.874±0.925
9d	0.424±0.361	0.070±0.025	0.428±0.294	0.675±0.091	0.045±0.001	3.583±0.241	>9.280
9e	≥4.092	0.569±0.31	≥4.341	>6.687	0.642±0.009	>6.687	>6.687
ETV	0.0097±0.003	0.0034±0.0003	0.019±0.007	0.020±0.0034	0.014±0.0025	0.023±0.011	0.026±0.0041
AZT	0.0054±0.0004	0.0078±0.0005	0.0063±0.0009	0.008±0.001	0.017±0.0056	0.0053±0.0011	0.011±0.0029

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

Table 3. Inhibitory activity of the representative compound against WT HIV-1 RT

Compd.	9a	9d	NVP[b]	ETV[b]
IC_{50} (μM)[a]	1.041	1.138	1.39	1.00

[a]IC_{50}: inhibitory concentration of test compound required to inhibit biotin deoxyuridine triphosphate (biotin-dUTP) incorporation into WT HIV-1 RT by 50%.

[b]The data were obtained from the same laboratory with the same method[13].

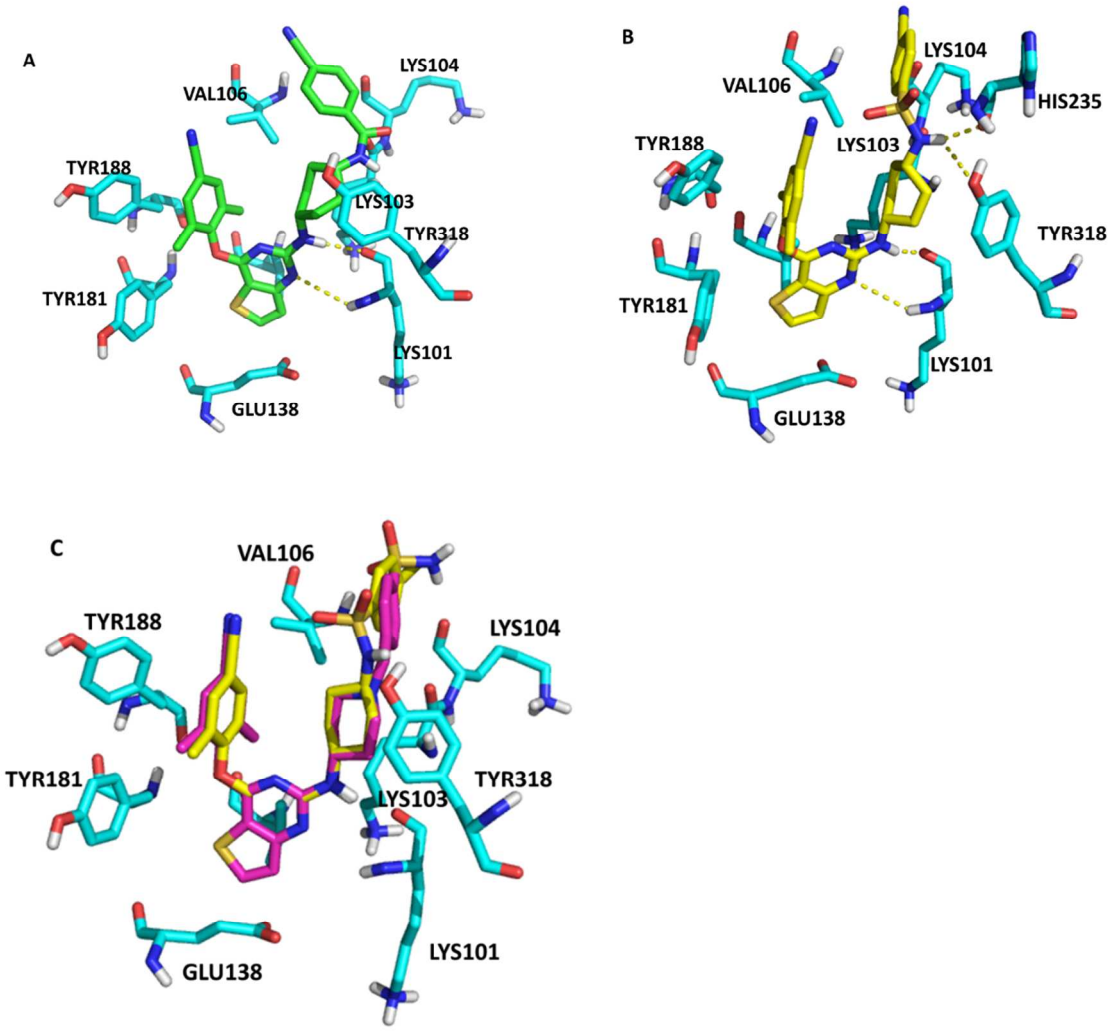


Figure 3. (A) and (B): Predicted binding modes of **8d**, **9d** with the HIV-1 WT RT crystal structure (PDB: 3M8Q). (C) Superimposition of **9d** and **3** with WT RT (yellow, **9d**; magentas, **3**). Hydrogen bonds are indicated with dashed lines (yellow). Nonpolar-hydrogen atoms are not shown for clarity.

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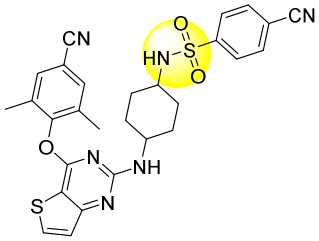

9d