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Discovery of Novel Diarylpyrimidine Derivatives as Potent HIV-1 NNRTIs Targeting the "NNRTI Adjacent" Binding Site

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KEYWORDS: HIV-1, AIDS, NNRTIs, drug design, "NNRTI Adjacent" binding site.

ABSTRACT: A novel series of diarylpyrimidine derivatives, which could simultaneously occupy the classical NNRTIs binding pocket (NNIBP) and the newly reported "NNRTI Adjacent" binding site, were designed, synthesized and evaluated for their antiviral activities in MT-4 cell cultures. The results demonstrated that six compounds (**20**, **27** and **31**-**34**) showed excellent activities against wild-type (WT) HIV-1 strain (EC_{50} = 2.4-3.8 nM), which were more potent than that of ETV (EC_{50} = 4.0 nM). Furthermore, **20**, **27**, **33** and **34** showed more potent or equipotent activity against single mutant HIV-1 strains compared to that of ETV. Especially, **20** showed marked antiviral activity, which was 1.5-fold greater against WT and 1.5−3-fold greater against L100I, K103N, Y181C, Y188L, and E138K when compared with ETV. In addition, all compounds showed lower toxicity (CC_{50} = 5.1-149.2 μM) than that of ETV (CC_{50} = 2.2 μM). The HIV-1 RT inhibitory assay was further conducted to confirm their binding target. Preliminary structure-activity relationships (SARs), molecular modeling as well as calculated physicochemical properties of selected compounds were also discussed comprehensively.

Attributing to their potent activity, modest toxicity, high specificity and favorable pharmacokinetic properties, HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs) function as an essential component of highly active antiretroviral therapy (HAART).[1,2] Nevirapine (**1**, NVP), delavirdine (**2**, DLV) and efavirenz (**3**, EFV) are first generation NNRTIs approved by U.S. FDA (Figure **1**), but the rapid emergence of drug-resistant compromised their clinical application, among which K103N and Y181C are the two most prevalent NNRTI resistance-associated mutations selected by NVP and EFV.[3] Although second generation NNRTIs, etravirine (**4**, ETV) and rilpivirine (**5**, RPV), which belongs to the diarylpyrimidine (DAPY) family, showed better anti-resistance profiles than the first generation NNRTIs, they still suffer from bad pharmacokinetics and cross-drug resistance with long-term clinical therapy.[3] Among them, E138K was the most commonly observed resistance mutation in the treatment-emergent of second generation NNRTIs. Therefore, there is an urgent need to exploit novel NNRTI drugs with improved potency against resistance-associated variants.[4]

Based on the recent X-ray crystallographic studies, solvent-accessible regions such as tolerant region I (the Pro236 hairpin loop) and tolerant region II (the entrance channel, namely, the largely open region in front of Leu100, Lys101, Glu138, andVal179) in the NNIBP are revealed as the two broad regions which could accommodate the newly designed NNRTIs.[5,6] Modifications of the DAPY scaffold such as fused heterocycles and/or introducing other groups like piperidine-substituted groups targeting these two tolerant regions have made breakthrough in our lab in recent years (Figure **2**).[7-10] Notably, series of triazine derivatives and piperidine-substituted thiophene[3,2-d]pyrimidine derivatives were the most remarkable, with a prominent antiviral activity against the clinical prevalent mutations. The most potent compounds DCS-a4 (**6**) and K-5a2 (**7**) exhibited EC_{50} values of 7.8 nM and 2 nM against WT HIV-1, respectively.[8,11] Encouragingly, **7** showed improved potency against resistance-associated variants.

Recently, an underexploited sites named as the "NNRTI Adjacent" binding site (PDB code: 4KFB), which formed by Thr139 (p51), Pro140 (p51), Thr165, Leu168, Lys172, and Ile180, was found as an additional opening tunnel-like pocket. It is located at the p66/p51 interface in the palm sub-domain adjacent to Glu138 of p51, separated from the NNIBP by *β*9 strand. Gratifyingly, the highly conserve residues Pro140, Ile180, and Gln182 were related in pivotal interactions, indicating that it is a promising site for the design of novel DAPY derivatives.[12] In this paper, we firstly attempt to design novel diarylpyrimidine derivatives target both NNIBP and the "NNRTI Adjacent" binding site simultaneously. The novel target compounds incorporated the privilege skeleton of ETV and the structure characteristics of K-5a2 (Figure **2**). The linkers with different length and substituents varying in size and electronic feature were primarily designed based on the chemistry space around the original fragment at NNRTI adjacent site. We also proposed that the privileged aromatic/heterocyclic substituents would improve physicochemical properties.[13] Herein, we report the synthesis, biological evaluation and RT inhibition assay of novel diarylpyrimidine derivatives. Furthermore, preliminary SARs, molecular modeling and physicochemical properties study are also discussed.

The synthetic protocol for 5-substituted diarylpyrimidine derivatives is depicted in **Scheme 1**.[14-17] Firstly, treatment of commercially available 2,4-dichloro pyrimidine (**8**) with 4-hydroxy-3,5-dimethylbenzonitrile (**9**) afforded intermediate **10**. Then, **12** was obtained by Buchwald-Hartwig reaction of **10** and 4-aminobenzonitrile (**11**). With the presence of *N*-iodosuccinimide (NIS) and CF_3COOH, key intermediate **13** was obtained through electrophilic reaction. Subsequently, **13** reacted with methyl thioglycolate or methyl mercaptopropionate to provide important intermediate **14** or **15** *via* cross-coupling reaction. The oxidative product **16** and **17** was prepared by treating **14** and **15** with *m*-CPBA. Finally, **14** was hydrolyzed with lithium hydroxide to afford **18** and then yield the target compounds **19-34** by amide condensation reaction. Both analytical and spectral data of the newly synthesized compounds were found in full agreement with the proposed structures.

Antiviral activity were evaluated in MT-4 cell cultures infected with WT HIV-1 strain (IIIB), single-mutant strains L100I, K103N, Y181C, Y188L, E138K, and double-mutant strains F227L+V106A and K103N+Y181C (RES056). NVP, EFV, ETV, and azidothymidine (AZT) were selected as reference drugs. The values of EC_{50} (anti-HIV potency), CC_{50} (cytotoxicity) and SI (selectivity index, CC_{50}/EC_{50} ratio) are summarized in **Table 1** and **Table 2**.

All the synthesized compounds were found to be active against WT HIV-1 with an EC_{50} values of 2.4 nM-216.5 nM and SI values between 152-15413, with an exception of **17** (EC_{50} = 1563.9 nM, SI = 9). Notably, 11 compounds (**19**, **20**, **25-27**, **29-34**) displayed superior activity (EC_{50} < 10 nM), among which six compounds (**20**, **27**, **31-34**) exhibited more potent or equipotent antiviral activity (EC_{50} = 2.4−3.8 nM) compared to ETV (EC_{50} = 4.0 nM). The most potent compound **27** (EC_{50} = 2.4 nM) and **33** (EC_{50} = 2.4 nM) exhibited a 1.6-fold greater potency than ETV. Meanwhile, all the compounds showed much lower cytotoxicity (CC_{50} = 5.1-149.2 μM) than that of ETV (CC_{50} = 2.2 μM).

Table 2 showed that compounds with potent activity against WT HIV-1 strain also exhibited potent activity against mutant HIV-1 strains. Among all the target compounds, **20**, **27**, **33**, and **34** were demonstrated with excellent activity against HIV-1 single mutations L100I, K103N, Y181C, Y188L and E138K, being more potent or equipotent compared to ETV. Particularly, **20** was the most potent inhibitor and its antiviral efficacy was 1.5−3-fold greater against these single mutations than ETV. But for the double-mutant strains F227L+V106A and K103N+Y181C, **20** showed less potent activity. It is noteworthy that our newly designed compounds showed an overall and excellent activity for a panel of single mutants and the results are summarized as follows:

1) In case of the K103N mutant strain, all the thirteen tested compounds showed lower EC_{50} values (1.4−44.9 nM) and FR values (0.4-1.9), being more potent than EFV (EC_{50} = 81.0 nM, FR = 16.3). Nine compounds (**19**, **20**, **25**, **27**, **29**, **30** and **32-34**) provided a single-digit nanomolar activity (EC_{50} = 1.4-8.3 nM). In particular, **19**, **20**, **27** and **32-34** displayed prominent inhibitory activity, being 1.3−2.3-fold superior to ETV (EC_{50} = 3.3 nM).

2) For E138K, all the tested compounds exhibited more potent activity (EC_{50} = 6.0-157 nM) than NVP. The EC_{50} values of ten compounds **19**, **20**, **25**, **27-30** and **32-34** (EC_{50} = 6.0-34.7 nM) were less than 35 nM, showing more potency than AZT (EC_{50} = 38.6 nM). Besides, six compounds (**20**, **27**, **30**, **32-34**) proved to inhibit E138K mutant HIV-1 strain in lower or equal concentration than ETV (EC_{50} = 16.9 nM).

3) As for single-mutant strains L100I, Y181C, and Y188L, it was noted that compounds **20** and **33** had single-digit nanomolar activity (EC_{50} = 6.5 and 7.7 nM, respectively) against L100I being slightly more potent compared to AZT (EC_{50} = 8.3 nM), and compounds **20**, **27**, and **33** exhibited an EC_{50} values ranging from 11.1 to 13.8 nM against Y181C, which were somewhat greater than AZT (EC_{50} = 14.1 nM).

4) Against the double-mutant strains F227L/V106A and Y181C/K103N, the most potent compounds **20**, **27**, **33**, and **34** exhibited a moderate activity (EC_{50} > 100 nM). It was concluded that the inflexible conformation of compounds caused by bulky groups may be account for their moderate activity.

Based on the above results, the preliminary SARs could be concluded in terms of the length of the chain and different terminal substitution group. For the former, detailed comparison of **14** (EC_{50} = 65.9 nM) with **15** (EC_{50} = 60.2 nM), **16** (EC_{50} = 68.8 nM) with **17** (EC_{50} = 1563.9 nM), **19** (EC_{50} = 6.2 nM) with **20** (EC_{50} = 2.6 nM) and **27** (EC_{50} = 2.4 nM) with **31** (EC_{50} = 3.8 nM) for their activity against WT HIV-1 strain suggested that the length of carbon chain couldn't govern the antiviral activity neither proportionally nor inversely. It was anticipated that compounds bearing different length of carbon chain would present distinct conformations and so that had distinguished antiviral potency.

For the latter, when we focused on **14** (EC_{50} = 65.9 nM) and **15** (EC_{50} = 60.2 nM) comparing with their sulfur oxidation products **16** (EC_{50} = 68.8 nM) and **17** (EC_{50} = 1563.9 nM), the result suggested that sulfone group have a negative impact on the antiviral activity. Next, comparison of activity of **34** (methoxy group, 3.8 nM), **19** (morpholinyl, 2.6 nM), and **21** (Boc-protected pyrazinyl, 32.6 nM) with that of **22** (Boc-protected pyrazinyl, 88.5 nM), **23** (Boc-protected 3-aminopyrrolyl, 33.9 nM) and **24** (thiomorpholinyl, 38.3 nM) indicated that polar groups are much preferred than groups with lower polarity distinctly. By comparing the activity between **31-33** and **29-30**, it was suggested that compounds with groups in similar size with short linker had similar activity, and small group with short linker (**31-33**) displayed better activity than those of bulky groups, such as phenol (**29**) and tetrahydrofuran (**30**). It was also found that the terminal *t*-butyloxycarbonyl group was an inferior group that impeded antiviral potency when compared **21**, **22** and **23** to **28**.

In general, these data indicated that modifications at 5-position of pyrimidine ring contribute to improve antiviral activity against WT and a panel of single mutant strains, especially K103N and E138K. The preliminary SARs will support information for the future structure modification. In addition, the compounds did not inhibit HIV-2.

To further validate the binding target, all the newly designed compounds were tested for their ability to inhibit recombinant WT HIV-1 RT enzyme. As shown in Table 3, all the tested compounds showed excellent inhibitory activities toward RT with IC_{50} values ranging from 0.013 to 0.863 μM, being superior to that of NVP (IC_{50} = 2.32 μM). Meanwhile, eight compounds (**14**, **27-29**, and **31-34**) had a better inhibitory activity than EFV (IC_{50} = 0.03 μM). The IC_{50} values of the most potent compounds **20**, **27**, **33**, and **34** (IC_{50} = 0.084, 0.021, 0.023, and 0.026 μM, respectively) were comparable to that of ETV (IC_{50} = 0.011 μM). It was identified that the results of preliminary SARs for IC_{50} were consistent

with SARs for EC_{50} that discussed above. Furthermore, the line chart was used to observe the consistency between the value of pEC_{50} (blue line) and pIC_{50} (red line) of target compounds in which the compound is sorted according to their pEC_{50} value in a decreasing order as shown in Figure **3**. The value of pIC_{50} showed a declining trend (purple line) compared with their pEC_{50} and this indicated the relative coherence between inhibitory against WT HIV-1 strain and HIV-1 RT enzyme. However, the EC_{50} value of some derivatives such as **14**, **15**, and **18** were 1.5−5-fold lower than their values of IC_{50} presenting an abnormal deviation, potentially owing to the cellular retention for poor membrane permeability or much higher affinity for the cellular full length enzyme than that of recombinant RT enzyme or metabolism of molecules. Overall these results illustrated that these new compounds displayed high affinity for RT and could be specifically target HIV-1 RT and regarded as typical HIV-1 NNRTIs.

To obtain further insight into the dual-site binding of the newly synthesized compounds to the NNIBP and "NNRTI Adjacent" binding site and rationalize the results of SAR studies, docking experiments were carried out using representative compounds **19**, **20** and **27** into WT HIV-1 RT with bound fragment at NNRTI adjacent site (PDB code: 4KFB) by using the software Surflexe-Dock SYBYL-X 2.0. Docking results were visualized with PyMOL (Figure **4**).

The binding mode of compound **20** resembled RPV as horseshoe conformation in parent scaffold and the morpholine ring stretched into the NNRTI adjacent site as the fragment located (Figure **4a)**. Compound **20** maintained the typical double hydrogen bonds with the backbone of Lys101 as described for many other NNRTIs. Besides, the amide group of the linker formed additional double hydrogen bonds with Ile180 and Glu138. Compared with **20**, the disappearance of hydrogen bond between **19** and Glu138 may account for its reduced activity compared to **20** (Figure **4b)**. A close-up view inward the "NNRTI Adjacent" binding site described the morpholine ring stretched into the pocket and occupied the adjacent site through the linker (Figure **4c**). As shown in Figure **4d**, the detailed docking feature of **27** showed additional hydrogen bonds formed by the NH and C=O of amide with the backbone C=O of Glu138 and NH of Ile180, respectively. Noteworthy, although **20** and **27** had different linker, they both form hydrogen bond with Ile180 and Glu138. It was predicted that different linkers and various terminal groups determine the conformation and then influence the activity. It was also guessed that various conformations as well as additional binding force played an important role in their excellent activity against HIV-1 mutant strains. To sum up, the molecular modeling analysis explained the theoretical binding mode and potent activity of the designed compounds partially, which was consistent with our original design intention and would assist further structural optimization.

Furthermore, the preliminary physicochemical properties of representative compounds **20**, **27**, **33**, and **34** were examined to evaluate their drug-likeness features by utilizing free on-line molinspiration software (http://www.molinspiration.com/). Lipophilic parameter-ligand efficiency (LE) was also calculated.[18,19] The results (Table 4) suggested that parameters like molecular weight (MW), hydrogen bond acceptors (nON), hydrogen bond donors (nOHNH), rotatable bonds (nrotb), miLogp, and ligand efficiency (LE) for all the tested compounds were in coincide with the Lipinski's "rule of five" and in an acceptable level except for slightly deviation for molecular weight and ligand efficiency of compound **20.** Therefore, it is supposed that these four compounds have the desired physicochemical properties. Besides, the topological polar surface area (tPSA) which is characterized the absorption and membrane permeability of molecules, showed the four compounds had a value ranging from 123.73-136.30 $Å^2$ confirming their advantageous for intestinal absorption (< 140 $Å^2$) and inability to penetrate the blood-brain barrier averting the central nervous system toxicity (> 60 $Å^2$).

To the best of our knowledge, we firstly discovered a new series of dual binding site NNRTIs targeting the NNIBP and the "NNRTI Adjacent" binding site simultaneously. Encouragingly, most of the novel derivatives exhibited significant inhibitory activity toward WT (EC_{50} < 10 nM) and a panel of single mutant HIV-1 strains in MT-4 cells. Compound **20** proved to be the most potent inhibitor with EC_{50} values of 2.6 nM (WT), 6.5 nM (L100I), 1.4 nM (K103N), 11.6 nM (Y181C), 16.2 nM (Y188L), and 6.0 nM (E138K); it was more potent than ETV against all single mutant strains, though somewhat weaker against double mutant strains F227L+V106A and RES056. In addition, **20** has much lower cytotoxicity (CC_{50} = 27.2 μM) and higher SI value of 10045. In RT inhibition assay, **20** displayed an IC_{50} value of 0.086 μM, which was in same magnitude with ETV. The current results hold great promise for the identification of potential new drug candidates with high potency and desirable drug-like properties. Further optimizations focusing to improve the HIV-1 mutant strains inhibitory activity, especially for double-mutant strains, are currently in progress and will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

The figures, tables and scheme except figure 2 are concluded in 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; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NNIBP, NNRTIs binding pocket; HAART, highly active antiretroviral therapy; EC_{50},

the concentration causing 50% inhibition of antiviral activity; HIV, human immunodeficiency virus; RT, reverse transcriptase; SAR, structure-activity relationship; SI, selection index; FR, fold resistance; WT, wild-type; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz; ETV, etravirine; RPV, rilpivirine; AZT, azidothymidine; LE, ligand efficiency.

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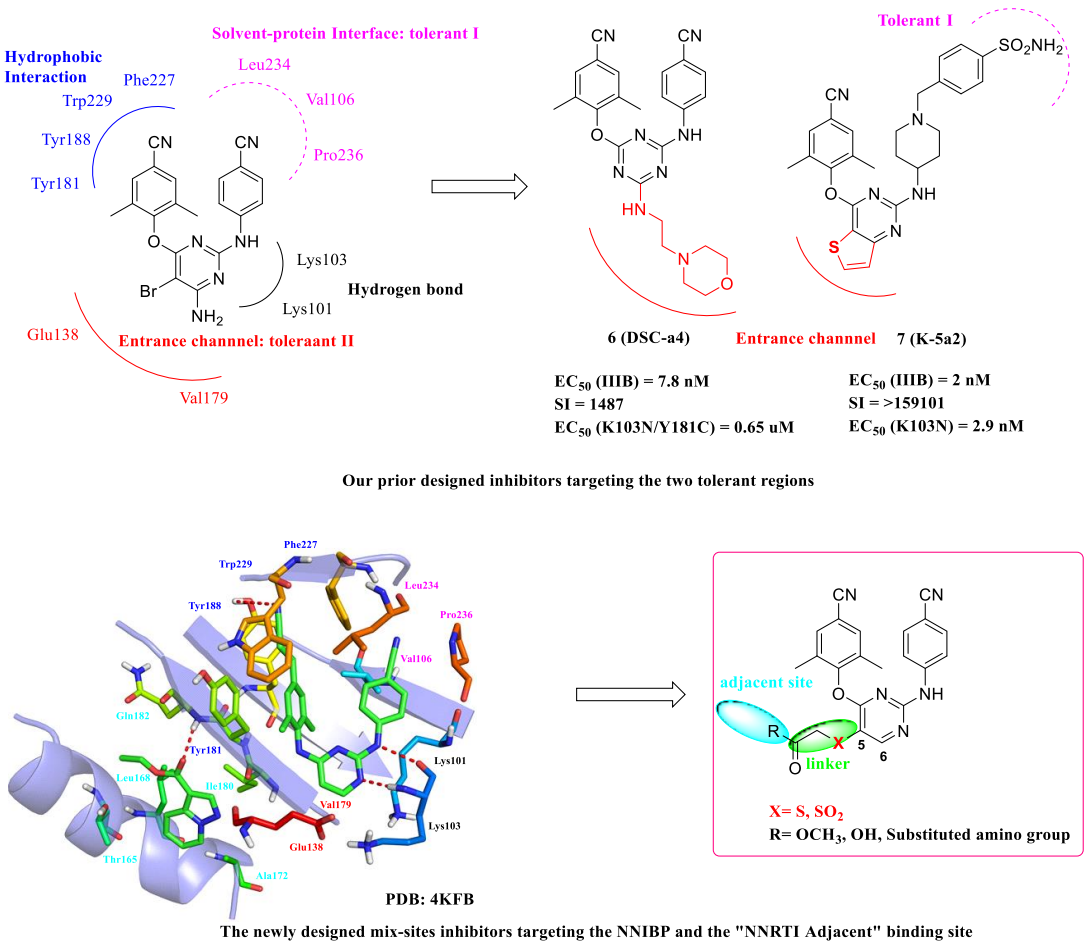

Figure 2. Our prior work and the newly designed compounds targeting the NNIBP and the "NNRTI Adjacent" binding site.

Table of Contents Graphic