Identification of Novel Diarylpyrimidines as Potent HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors by Exploring the Primer Grip Region

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Abstract: HIV-1 reverse transcriptase (RT) plays a crucial role in the viral replication cycle, and RT inhibitors can represent a promising pathway in treating AIDS. To explore the primer grip region of HIV-1 RT, using -CH₂O- as a linker, substituted benzene or pyridine rings were introduced into the left wing of diarylpyrimidines (DAPYs). A total of 17 compounds with new structures were synthesized. It showed that all compounds exhibited anti-HIV-1 (wild-type) activity values ranging from 7.6–199.0 nM. Among them, TF2 (EC₅₀ = 7.6 nM) showed the most potent activity, which was better than that of NVP (EC₅₀ = 122.6 nM). Notably, compared with RPV (CC₅₀ = 3.98 µM), TF2 (CC₅₀ > 279,329.6 nM) showed low cytotoxicity. For HIV-1 mutant strains K103N and E138K, most compounds showed effective activities. Especially for K103N, TF2 (EC₅₀ = 28.1 nM), TF12 (EC₅₀ = 34.7 nM) and TF13 (EC₅₀ = 28.0 nM) showed outstanding activity, being superior to that of NVP (EC₅₀ = 7495.1 nM) and EFV (EC₅₀ = 95.1 nM). Additionally, TF2 also showed the most potent activity against E138K (EC₅₀ = 44.0 nM) and Y181C mutant strains (EC₅₀ = 139.3 nM). In addition, all the compounds showed strong enzyme inhibition (IC₅₀ = 0.036–0.483 µM), which demonstrated that their target was HIV-1 RT. Moreover, molecular dynamics simulation studies were implemented to predict the binding mode of TF2 in the binding pocket of wild-type and K103N HIV-1 RT.

Keywords: HIV-1; reverse transcriptase; NNRTIs; diarylpyrimidines

1. Introduction

Human immunodeficiency virus type 1 (HIV-1), one of the viruses that pose a serious threat to human health worldwide, is the main pathogen of acquired immunodeficiency syndrome (AIDS) [1–3]. UNAIDS 2022 data indicated that 38.4 million people were living with HIV, 1.5 million people were newly infected with HIV, and 650,000 people have died from AIDS in 2021 [4]. Currently, the clinical treatment for AIDS often uses highly active antiretroviral therapy (HAART), which is a combination of two, three, or more drugs, typically including reverse transcriptase inhibitors (RTIs) [5,6]. Because of the high specificity, potent antiviral properties, and favorable pharmacokinetics, nonnucleoside reverse transcriptase inhibitors (NNRTIs) have proven to be an important part of HAART regimens. The NNRTIs are non-competitive inhibitors that act by allosteric inhibition of DNA
polymerization [7–9]. There have been six NNRTIs approved by the U.S. Food and Drug Administration (FDA) [10,11], including nevirapine (1, NVP), delavirdine (2, DLV), efavirenz (3, EFV), etravirine (4, ETR), rilpivirine (5, RPV) and doravirine (6, DOR). In addition, elsulfavirine (7, ESV) was approved by the Russian Ministry of Health (MoH) in 2017 [12], and ainuovirine (8, ANV) was approved by National Medical Products Administration (NMPA) in 2021 for AIDS treatment (Figure 1) [13].

![Image of chemical structures]

**Figure 1.** US FDA, Russian MoH and NMPA-approved NNRTI chemical structures.

Among them, ETR and RPV belong to diarylpyrimidines NNRTIs, exhibiting effective activity against the resistant mutations caused by the first-generation NNRTIs, such as Y181C, P236L and K103N [10,14–16]. In spite of their higher genetic barrier, several single, double or triple mutants have emerged with resistance towards these drugs, such as K101E, E138K, Y181V, Y181C + V179F and V179F + Y181C + F227C [17–20]. Therefore, it is still urgent to develop novel NNRTIs with an improved anti-resistance profile.

The rapid development of HIV-1 RT structural biology provides new information for the rational design of potential anti-HIV-1 agents. The RT is a heterodimer consisting of two subunits, p66 (560 residues) and p51 (440 residues), The NNRTI-binding pocket (NNIBP) is located between the \( \beta 6 \) (residues 142–146), \( \beta 9 \) (residues 227–229), \( \beta 10 \) (residues 232–234) sheets (floor) and the \( \beta 12 \) (residues 326–333), \( \beta 13 \) (residues 336–344), \( \beta 14 \) (residues 347–355) sheets (roof) [21–24]. Diarylpyrimidines tend to bind to HIV-1 RT in a “U” mode (also named “horseshoe mode”), and they can bind to the different NNRTI-binding pockets caused by various resistant RT mutations through torsional flexibility (wiggling) and repositioning flexibility (jiggling) to retain potency against mutant HIV-1 viruses [25,26].

The studies of X-ray crystallography and molecular modeling revealed that diarylpyrimidines exhibited a three-dimensional pharmacophore model in an NNRTI-binding pocket (Figure 2): an aminobenzonitrile moiety (A-ring, right wing), a central pyrimidine ring (B-ring) and the NH linker which form two hydrogen bonds with the backbone of residue K101, and a 2,4,6-trisubstituted phenoxy moiety (C-ring, left wing), and the left wing is situated in the hydrophobic channel which is shaped by amino acid residues Y181, Y188, W229, F227 and L234 [27–29].
The primer grip is a highly conserved structural motif consisting of the β12-β13 hairpin (F227-H235) (Figure 3); it is responsible for locating the 3′-OH end of the primer strand at the polymerase catalytic site, making it important in the catalytic activity of RT [30,31]. The primer grip contains three amino acid residues in the NNRTI-binding pocket: F227, W229 and L234, and some mutations of F227 and L234 have been observed: F227C/L/I/V, W229 and L234. However, the mutation of the key W229 has never been observed. As for the other amino acid residues in the primer grip region, except for M230 (M230/L/I), no other drug-resistance mutations have been reported [32]. Targeting highly conserved amino acid residues is of great significance for the discovery of new compounds with improved drug resistance profiles [33], which inspired us to target the conserved residue W229 in the NNRTI-binding pocket for rational drug design and further explore the chemical space of the primer grip region.

On the basis of the above analysis, with the privileged aminobenzonitrile moiety (right wing) and central pyrimidine ring (B-ring) unchanged, substituted benzene rings or pyridine rings were introduced to the left wing of diarylpyrimidines by a CH2O linker, expecting the newly introduced aromatic rings could develop stronger π-π interaction with the conserved residue (W229) and explore primer grip region to enhance the drug resistance profile (Figure 4). Here, we described the synthesis of these novel diarylpyrimidines as well as their anti-HIV-1 activity, and then we further discussed the preliminary structure-activity relationship (SAR) in detail and selected representative molecules for molecular dynamics simulation studies.
2. Result and Discussion

2.1. Chemistry

As indicated in Scheme 1, using 9 as the starting material, which we reported earlier [34–36], it was subjected to nucleophilic substitution reaction with 3,5-dimethyl-4-hydroxybenzaldehyde under the alkaline condition of potassium carbonate to obtain intermediate 10. Reduction of 10 with NaBH₄ gave intermediate 11, which was brominated with PBr₃ to give key intermediate 12. Finally, 12 was reacted with different substituted phenols and thiophenols to obtain the target compounds TF1-TF16 via nucleophilic substitution reactions. TF17 was obtained by removing the Boc protecting group of TF16.

![Scheme 1](image)

**Scheme 1.** Reagents and conditions: (i) 4-hydroxy-3,5-dimethylbenzaldehyde, K₂CO₃, DMF, 100 °C, 10 h; (ii) NaBH₄, MeOH, 0 °C, 0.5 h, r.t., 4 h; (iii) PBr₃, CH₂Cl₂, 0 °C, 2 h, r.t., 2 h; a (iv) KOH, KI, MeCN, 80 °C, 4 h; (v) CF₃COOH, CH₂Cl₂, r.t., 3 h.

2.2. Biological Evaluation

These 17 newly synthesized compounds were tested in MT4 cells for their antiviral activity using the MTT method [37]. The five marketed drugs approved by the U.S. Food and Drug Administration: Zidovudine (AZT), NVP, EFV, ETR and RPV, were used as positive drugs. EC₅₀, CC₅₀ and SI were used to express the biological evaluation results, which were summarized in Tables 1–4.
As shown in Table 2, compounds TF3, TF4, TF5, TF6, TF7, TF8, TF9, TF10, TF11, TF12, TF13, TF14, TF15, TF16, and TF17 were further evaluated for their activity against drug-resistant strains of NNRTIs in MT-4 cells (L100I, K103N, Y181C, Y188L, E138K, F227L/V106A and RES056).

Furthermore, we found that the substitution of different positions on the introduced substituent benzene rings had a certain effect on cytotoxicity. Compared with compounds containing unsubstituted benzene ring, the order of potency was as follows: OCH3 > OCH2CH3 > NH2 > NO2 > N3 > CN. For the compounds containing CN substituted benzene rings, the activity of compound TF13 (CC50 = 199.0 nM) with an uninfected MT-4 cell viability of 100.1% was significantly higher than that of compound TF15 (CC50 = 253.2 nM). For the compounds containing NO2 substituted benzene rings, the activity of compound TF16 (CC50 = 453.1 nM) with an uninfected MT-4 cell viability of 100.1% was significantly higher than that of compound TF17 (CC50 = 529.3 nM). For the compounds containing CN substituted benzene rings, the activity of compound TF13 (CC50 = 199.0 nM) with an uninfected MT-4 cell viability of 100.1% was significantly higher than that of compound TF15 (CC50 = 253.2 nM).

Table 1. The anti-HIV-1(IIIB) activity and cytotoxicity of TF1-17.

| Compounds | R       | EC50<sup>a</sup>  | CC50<sup>b</sup>  | SI<sup>c</sup>  |
|-----------|---------|-------------------|-------------------|---------------|
|           |         | IIIB (nM)         | ROD (nM)          |               |
| TF1       |         | 17.1 ± 2.3        | >295,865.0        | >17,326.7     |
| TF2       |         | 7.6 ± 0.6         | >279,329.6        | >36,610.9     |
| TF3       |         | 24.5 ± 4.1        | 5522.3 ± 296.6    | 225.2         |
| TF4       |         | 7.8 ± 1.0         | 5293.0 ± 355.6    | 674.4         |
| TF5       |         | 21.7 ± 3.6        | 253,718.1 ± 5720.2| 11,681.3      |
| TF6       |         | 35.3 ± 4.1        | 6896.1 ± 249.1    | 195.6         |
| TF7       |         | 25.5 ± 8.4        | 8152.2 ± 547.6    | 319.6         |
| TF8       |         | 42.8 ± 5.6        | 2917.9 ± 271.5    | 68.1          |
| TF9       |         | 30.5 ± 4.0        | 3458.6 ± 375.4    | 113.3         |
| TF10      |         | 32.2 ± 3.3        | 4804.6 ± 341.7    | 149.1         |
| TF11      |         | 199.0 ± 13.0      | >14,827.6 ± 919.6 | 74.5          |
| TF12      |         | 7.8 ± 0.8         | 2462.8 ± 197.9    | 316.9         |
| TF13      |         | 19.4 ± 3.4        | 4772.8 ± 1561.8   | 246.4         |
| TF14      |         | 23.6 ± 3.8        | >24,438.7 ± 6675.8| 1034.6        |
| TF15      |         | 23.1 ± 3.1        | >215,001.1 ± 20,337.8| 9303.7       |
| TF16      |         | 45.3 ± 14.1       | 6196.6 ± 220.4    | 136.7         |
| TF17      |         | 32.5 ± 1.7        | >5489.9 ± 328.0   | 168.8         |
Table 1. Cont.

| Compounds | R | EC50 \(b\) (nM) | CC50 \(b\) (nM) | SI \(c\) (IIIB) |
|------------|---|-----------------|-----------------|-----------------|
| AZT        | — | 27.3 ± 3.5      | 23.3 ± 2.1      | >7483.6         |
| NVP        | — | 122.6 ± 7.3     | —               | >15,020.7       |
| EFV        | — | 2.4 ± 0.3       | —               | >6335.5         |
| ETR        | — | 2.9 ± 0.4       | —               | >4594.6         |
| RPV \(d\)  | — | 1.00 ± 0.27     | —               | 3.98 (μM)       |

\(a\) EC50: concentration of compounds protecting 50% of MT-4 cells from lesions caused by HIV-1 virus infection. 
\(b\) CC50: concentration of compounds causing 50% of HIV-uninfected MT-4 cells. 
\(c\) SI: selectivity index, CC50/EC50. 
\(d\) Used for comparison [5]. Standard deviation values were obtained from five parallel experiments.

Table 2. The activity of anti-HIV-1 mutant strains of TF1–17.

| Compounds | L100I EC50 \(a\) (nM) | K103N | Y181C | Y188L | E138K | F227L + V106A | RES056 |
|-----------|------------------------|-------|-------|-------|-------|---------------|--------|
| TF1       | 221.3 ± 53.5           | 50.7 ± 9.6 | 515.3 ± 60.4 | 1519.8 ± 92.1 | 76.5 ± 9.4 | 1710.6 ± 581.4 | 6853.1 ± 1172.2 |
| TF2       | 117.0 ± 49.5           | 28.1 ± 7.1 | 139.3 ± 34.9 | 1390.3 ± 118.8 | 44.0 ± 2.9 | 1136.6 ± 346.5 | >279,329.6 |
| TF3       | 116.0 ± 5.8            | 48.4 ± 7.5 | 245.0 ± 39.6 | 894.7 ± 90.2 | 87.7 ± 8.5 | 755.4 ± 155.6 | 1543.5 ± 102.5 |
| TF4       | 314.9 ± 30.7           | 75.9 ± 13.9 | 482.3 ± 42.6 | > 5296.1 | 103.1 ± 6.8 | 1279.5 ± 303.3 | >5296.1 |
| TF5       | 126.1 ± 12.6           | 44.5 ± 4.9 | 267.8 ± 28.9 | 963.7 ± 98.1 | 65.3 ± 10.0 | 1406.4 ± 569.6 | >253,718.1 |
| TF6       | 156.0 ± 16.8           | 91.2 ± 14.4 | 386.7 ± 87.4 | 1391.7 ± 83.6 | 149.7 ± 0.7 | 1096.7 ± 281.1 | >6896.1 |
| TF7       | 451.9 ± 70.5           | 164.1 ± 55 | 790.6 ± 62.9 | > 8152.2 | 180.7 ± 10.7 | 1224.1 ± 179.3 | >8152.2 |
| TF8       | 328.9 ± 56.7           | 110.2 ± 4.6 | 667.2 ± 78.0 | 885.5 ± 20.1 | 147.5 ± 4.6 | 853.3 ± 81.4 | 1418.6 ± 9.7 |
| TF9       | 308.1 ± 46.3           | 119.6 ± 18.2 | 499.2 ± 44.6 | 1095.3 ± 95.7 | 147.1 ± 12.0 | 999.8 ± 29.6 | 1586.6 ± 87.5 |
| TF10      | 634.6 ± 28.2           | 158.4 ± 18.6 | 765.6 ± 22.5 | > 4804.6 | 178.8 ± 26.9 | 1487.4 ± 33.9 | >4804.6 |
| TF11      | 9574.0 ± 22.2          | 444.1 ± 68.6 | >14,827.6 | > 14,827.6 | > 3024.0 | > 14,827.6 | >14,827.6 |
| TF12      | 245.6 ± 54.6           | 34.7 ± 8.8 | 384.5 ± 75.5 | > 1560.8 | 46.2 ± 7.1 | 742.4 ± 145.1 | >2462.8 |
| TF13      | 125.3 ± 5.9            | 28.0 ± 2.5 | 265.2 ± 24.6 | 1053.3 ± 66.8 | 57.7 ± 11.6 | 1650.5 ± 19.9 | 1182.9 ± 68.2 |
| TF14      | 185.1 ± 11.6           | 36.7 ± 3.7 | 285.5 ± 52.1 | 773.6 ± 39.2 | 67.4 ± 8.1 | 999.4 ± 125.8 | 1192.8 ± 182.2 |
| TF15      | 105.2 ± 15.2           | 46.3 ± 9.7 | 195.9 ± 25.4 | 1079.6 ± 99.4 | 50.0 ± 4.4 | 1655.9 ± 448.3 | 1801.7 ± 68.5 |
| TF16      | 715.2 ± 183.5          | 131.7 ± 28.4 | 1274.9 ± 153.7 | > 6196.6 | 245.3 ± 30.4 | > 1618.2 | >6196.6 |
| TF17      | 226.5 ± 28.9           | 65.3 ± 7.6 | 552.5 ± 62.5 | 905.1 ± 147.4 | 151.9 ± 10.3 | 747.1 ± 72.2 | > 5,490 |
| AZT       | 10.0 ± 1.2             | 22.8 ± 1.1 | 25.1 ± 3.0 | 19.4 ± 2.9 | 30.1 ± 3.0 | 14.8 ± 1.1 | 25.1 ± 1.8 |
| NVP       | 1856.7 ± 364.6         | 7495.1 ± 866.8 | 10,113.7 ± 850.9 | > 13,781.4 | 149.4 ± 12.0 | > 15,020.7 | >15,020.7 |
| EFV       | 35.0 ± 4.7             | 95.1 ± 9.1 | 5.5 ± 0.5 | 239.6 ± 30.4 | 4.4 ± 0.3 | 200.8 ± 173.0 | 183.8 ± 22.0 |
| ETR       | 5.9 ± 1.0              | 3.0 ± 0.3 | 11.1 ± 1.3 | 18.1 ± 3.2 | 6.3 ± 1.2 | 20.4 ± 7.8 | 29.5 ± 4.8 |
| RPV \(b\) | 1.54 ± 0.00            | 1.31 ± 0.36 | 4.73 ± 0.48 | 79.4 ± 0.77 | 5.75 ± 0.11 | 5.75 ± 0.11 | 10.7 ± 7.96 |

\(a\) EC50: concentration of compounds protecting 50% of MT-4 cells from lesions caused by HIV-1 virus infection. 
\(b\) Used for comparison [5]. Standard deviation values were obtained from five parallel experiments.

According to the results in Table 1, all compounds (EC50 = 7.6 – 199.0 nM) had potent inhibitory activity against WT HIV-1. TF2 (EC50 = 7.6 nM), TF4 (EC50 = 7.8 nM) and TF12 (EC50 = 7.8 nM) exhibited the best anti-HIV-1 activity, being comparable to that of the second-generation drug ETR (EC50 = 3.0 nM) and much superior to those of AZT (EC50 = 27.3 nM) and NVP (EC50 = 122.6 nM). Moreover, at a concentration of 279,329.6 nM, TF2 exhibited no cytotoxicity, which contributes to its higher SI values (SI > 36,610.9) toward HIV-1 IIIB. None of the compounds had inhibitory activity against HIV-2, which demonstrated that these compounds belong to typical HIV-1 inhibitors.
The preliminary SAR was summarized as follows. Inhibitory activity differed depending on the types and positions of substituents on the benzene ring introduced to the left wing by a CH$_2$O linker. For the compounds containing para-substituted benzene ring, the order of potency was as follows: -CN (TF2, EC$_{50} = 7.6$ nM) > -OCH$_3$ (TF5, EC$_{50} = 21.7$ nM) > -NO$_2$ (TF15, EC$_{50} = 23.1$ nM) > -NH$_2$ (TF17, EC$_{50} = 32.5$ nM) > -CH$_2$OH (TF8, EC$_{50} = 42.6$ nM) > -NHBOc (TF16, EC$_{50} = 45.3$ nM). For the compounds containing meta-substituted benzene ring, the order of potency was as follows: -CN (TF3, EC$_{50} = 24.5$ nM) > -CH$_2$OH (TF9, EC$_{50} = 30.5$ nM) > -OCH$_3$ (TF6, EC$_{50} = 35.3$ nM). For the compounds containing ortho-substituted benzene ring, the order of potency was as follows: -CN (TF4, EC$_{50} = 7.8$ nM) > -OCH$_3$ (TF7, EC$_{50} = 25.5$ nM) > -CH$_2$OH (TF10, EC$_{50} = 32.2$ nM). Among them, TF2 and TF4 showed the best antiviral activity. It preliminarily showed that compared to TF1 (-H, EC$_{50} = 17.1$ nM), the introduction of para- and ortho-CN substituted benzene rings were beneficial to the improvement of antiviral potency.

Furthermore, we found that the substitution of different positions on the introduced benzene rings had a certain effect on cytotoxicity. Compared with compounds containing ortho- or meta-substituted benzene rings, TF1 (-H, CC$_{50} > 295,865.0$ nM), TF2 (para-CN, CC$_{50} > 279,329.6$ nM), TF5 (para-OCH$_3$, CC$_{50} = 253,718.1$ nM), and TF15 (para-NO$_2$, CC$_{50} = 215,001.1$ nM) showed low cytotoxicity than that of RPV (CC$_{50} = 3.89$ nM). This suggested that the introduction of the benzene ring and para-substituted benzene ring may help reduce cytotoxicity. However, all three compounds containing hydroxymethyl substituted benzene rings, TF8 (CC$_{50} = 2917.9$ nM), TF9 (CC$_{50} = 3458.6$ nM), TF10 (CC$_{50} = 4804.6$ nM), showed a sharp increase in cytotoxicity compared to TF1, which suggested that the introduction of hydroxymethyl benzene ring may be detrimental to the improvement of the toxicity of compounds.

In addition, 3-pyridine-containing and substituted 3-pyridine-containing compounds (TF11-TF14) were also synthesized. Compared to TF11 (EC$_{50} = 199.0$ nM) with an unsubstituted benzene ring, the activity of TF11 showed the best antiviral activity. It preliminarily showed that compared to TF11, EC$_{50}$ (TF17) = 2.93 nM, TF16 = 1.97 nM, TF15 = 2.00 nM, TF14 = 1.54 nM, TF13 = 1.47 nM, TF12 = 4.49 nM, TF11 = 2.23 nM, and TF10 = 4.94 nM. The activity of TF17 showed a sharp increase in cytotoxicity compared to TF11, EC$_{50}$ (TF17) = 0.038 ± 0.001 nM, TF16 = 0.057 ± 0.003 nM, TF15 = 0.063 ± 0.001 nM, TF14 = 0.041 ± 0.003 nM, TF13 = 0.059 ± 0.001 nM, TF12 = 0.038 ± 0.004 nM, and TF11 = 0.062 ± 0.000 nM. The activity of TF17 showed a sharp increase in cytotoxicity compared to TF11, EC$_{50}$ (TF17) = 0.038 ± 0.001 nM, TF16 = 0.057 ± 0.003 nM, TF15 = 0.063 ± 0.001 nM, TF14 = 0.041 ± 0.003 nM, TF13 = 0.059 ± 0.001 nM, TF12 = 0.038 ± 0.004 nM, and TF11 = 0.062 ± 0.000 nM. The activity of TF17 showed a sharp increase in cytotoxicity compared to TF11, EC$_{50}$ (TF17) = 0.038 ± 0.001 nM, TF16 = 0.057 ± 0.003 nM, TF15 = 0.063 ± 0.001 nM, TF14 = 0.041 ± 0.003 nM, TF13 = 0.059 ± 0.001 nM, TF12 = 0.038 ± 0.004 nM, and TF11 = 0.062 ± 0.000 nM.
tuted pyridine ring, the activity of TF12–TF14 showed different degrees of increase, among which TF12 (EC\(_{50}\) = 7.8 nM) was the most active compound.

As shown in Table 2, compounds TF1-17 were further evaluated for their activity against drug-resistant strains of NNRTIs in MT-4 cells (L100I, K103N, Y181C, Y188L, E138K, F227L/V106A and RES056).

For single mutant K103N, all compounds (EC\(_{50}\) = 28.0 ~ 444.1 nM) displayed potent inhibitory activity. Notably, TF2 (EC\(_{50}\) = 28.1 nM), TF12 (EC\(_{50}\) = 34.7 nM) and TF13 (EC\(_{50}\) = 28.0 nM) exhibited outstanding activity, which was better than that of NVP (EC\(_{50}\) = 7495.1 nM) and EFV (EC\(_{50}\) = 95.1 nM). As for the effect of the types and positions of substituents on the introduced benzene ring on the activity, compared with TF1 (-H, EC\(_{50}\) = 50.7 nM), the activity of TF2 (para-CN, EC\(_{50}\) = 28.1 nM) was nearly doubled. Moreover, TF3 (meta-CN, EC\(_{50}\) = 48.4 nM), TF5 (para-OCH\(_3\), EC\(_{50}\) = 44.5 nM), and TF15 (para-NO\(_2\), EC\(_{50}\) = 46.3 nM) showed comparable activity with TF1; it preliminarily showed that the para-CN was also the privileged substituent. Regarding the resistance fold (Table 3), compared with EFV (RF = 47.5), the activity of all compounds against the K103N mutant strain was not significantly reduced compared to the wild strain (RF = 1.47 ~ 9.74).

As for single mutant E138K, all compounds showed sub-micromolar inhibitory activity with the exception of TF11. Among them, TF2 (EC\(_{50}\) = 44.0 nM) was the most active compound. TF12 (EC\(_{50}\) = 46.2 nM), TF13 (EC\(_{50}\) = 57.7 nM), TF14 (EC\(_{50}\) = 67.4 nM) and TF15 (EC\(_{50}\) = 50.0 nM) also showed promising activities, which were significantly more potent than NVP (EC\(_{50}\) = 149.4 nM). Similarly, the compounds with para-substituted electron-withdrawing groups on the benzene ring (TF2 (para-CN), TF15 (para-NO\(_2\)) were more active.

In the case of single mutant strains L100I and Y181C, most of the compounds showed sub-micromolar to micromolar inhibitory activity, TF2 (EC\(_{50}\) (L100I) = 117.0 nM, EC\(_{50}\) (Y181C) = 139.3 nM) and TF15 (EC\(_{50}\) (L100I) = 105.2 nM, EC\(_{50}\) (Y181C) = 195.9 nM) had the best activity, which was better than that of NVP (EC\(_{50}\) (L100I) = 1856.7 nM, EC\(_{50}\) (Y181C) = 10113.7 nM). For single mutant Y188L and double mutant F227L + V106A, some compounds were inactive, and most compounds showed sub-micromolar to micromolar inhibitory activity, among which TF14 (EC\(_{50}\) (Y188L) = 773.6 nM) and TF12 (EC\(_{50}\) (F227L + V106A) = 742.4 nM) had the best activity, respectively. For the double mutant RES056, unfortunately, only a few compounds showed micromolar inhibitory activity; TF13 (EC\(_{50}\) = 1182.9 nM) and TF14 (EC\(_{50}\) = 1192.8 nM) had the best inhibitory activity.

To verify the target of synthesized compounds (TF1-TF17), they were further tested for enzyme inhibitory activity by the ELISA method [38], and the results are shown in Table 4. All compounds (IC\(_{50}\) = 0.038 ~ 0.438 \text{ M}) exhibited potent inhibitory activity against WT HIV-1 RT, being superior to that of NVP (IC\(_{50}\) = 0.568 \text{ M}). The three most potent compounds against WT HIV-1 mutant, TF2 (IC\(_{50}\) = 0.055 \text{ M}), TF4 (IC\(_{50}\) = 0.050 \text{ M}) and TF12 (IC\(_{50}\) = 0.038 \text{ M}), displayed higher enzyme inhibitory activity. The weaker inhibitors of the WT HIV-1 strain showed reduced inhibitory activity, such as TF11 and TF16 (IC\(_{50}\) = 0.483 and 0.160 \text{ M}, respectively). It is noteworthy that the antiviral activity of some compounds was inconsistent with their enzyme-inhibitory potency to some extent. This discrepancy may be caused by the variations in the HIV-1 RT-substrate binding affinities and polymerase processivity on different nucleic acid templates [39]. Nonetheless, these novel synthesized compounds functioned as traditional NNRTIs.

2.3. Molecular Dynamics Simulation Studies

In order to predict the binding modes of these novel synthesized diarylpyrimidines in the NNRTI-binding pocket and initially explain the anti-resistance profiles to the K103N strain of TF2, MD simulations were performed in detail using the software Schrödinger [40]. The co-crystal structures of HIV-1 WT RT in complex with RPV (PDB code:2ZD1) [22] and HIV-1 K103N RT in complex with RPV (PDB code:3MEG) [27] were chosen as templates for docking studies.
The binding modes of TF2 to the allosteric pocket in WT and K103N HIV-1 RT were investigated by running a 500 ns MD simulation. Figure 5 shows that the root-mean-square deviation (RMSD) from the initial structure of TF2 was computed throughout the MD simulation for all systems. Figure 5A illustrates the RMSD of ligand TF2; the plot showed that the inhibitor in the WT and K103N mutant strains had deviated from the starting structure. Figure 5B illustrates the RMSD of the complex of RT and ligand TF2, which formed different conformational during the MD time. As a result of the narrow range of RMSD values, these conformational ensembles were structurally similar.

Figure 5. Analysis of MD trajectories for ligand TF2 in complex with HIV-1WT RT (black) and K103N (red) RT. (A) RMSD profiles for the ligand TF2, (B) RMSD profiles for the protein backbone.

Compound TF2 bound to WT and K103N RTs in similar binding modes, the binding modes of TF2 within the NNRTI-binding pocket resembled other diarylpyrimidines as the typical conformation (U shape), as noted in the snapshots collected at the end of the MD trajectories (Figure 6). However, there were some differences in the orientation of some groups and the distance between them and several remarkable features and well-known interactions were delineated here.

In the WT RT protein (Figure 6A), the right benzonitrile moiety was situated in the tolerant region I, where the cyano group could form a water-mediated hydrogen bond with K103. Stable hydrogen-bonding interactions were formed between the 2-aminopyrimidine ring of TF2 and the backbone (both NH and C = O units) of K101. The left 2,4,6-trisubstituted moiety remained stable in a hydrophobic sub-pocket formed by Y181, Y188, and W229. Moreover, the newly introduced p-cyanobenzonitrile group formed π-π stacking interaction with Y188 and hydrogen-bonding interaction with an extra amino acid residue H221. In the K103N RT protein (Figure 6B), compared to the binding mode in WT RT, the overall structure of TF2 was shifted, causing it to move away from K101 and affecting hydrogen-bonding interactions. A hydrogen bond was formed between the pyrimidine-bound NH of TF2 and the carbonyl oxygen of K101, but the N atom on the pyrimidine ring formed water bridge-mediated hydrogen bonds with K101 and E138. Besides, the left 2,4,6-trisubstituted moiety was also positioned in the hydrophobic sub-pocket, and the phenyl group formed π-π stacking interaction with the indole ring of W229. Similarly, the p-cyanophenyl moiety extended out of the hydrophobic channel, but no additional interactions with surrounding amino acids were found.

MMGBSA calculations [41] were used to determine the relative binding strengths of the compound TF2 to the WT and K103N HIV-1 RTs. These estimations are shown in Table 5. Accordingly, the binding affinity (ΔGbind) of TF2 to WT RT was higher than that to K103N RT, supporting the experimental EC50 values. Two important interactions observed in the complex of TF2 and WT were the hydrogen-bonding interaction with an extra residue H221 and a water-mediated hydrogen bond with K103. The H bond (ΔGhbond) contributed more to TF2 binding to WT RT comparing K103N RT, which was consistent with the MD studies.
Figure 6. Final complexes of (A) WT RT-TF2, (B) K103N RT—TF2 obtained from MD simulations. Selected residues are highlighted as pale green lines (carbon atoms in green), the ligand is shown as light pink (carbon atoms) sticks (WT) and light blue (carbon atoms) sticks (K103N), and the primer grip region is colored in pale cyan. (C) Superposition of MD structure of the WT RT-TF2 and K103N RT—TF2.

Table 5. MM/GBSA energy calculations.

| Binding Energies $^a$ (kcal/mol) | TF2 $\rightarrow$ NNIBP (WT) | TF2 $\rightarrow$ NNIBP (K103N) |
|----------------------------------|--------------------------------|---------------------------------|
| $\Delta G_{\text{Hbond}}$        | $-1.1 \pm 0.3$               | $-0.9 \pm 0.3$                  |
| $\Delta G_{\text{bind}}$         | $-113.8 \pm 3.8$             | $-109.0 \pm 4.5$               |

$^a$ $\Delta G_{\text{Hbond}}$ = Hydrogen-bonding correction; $\Delta G_{\text{bind}}$ = total binding free energy.

Overall, a comparison with WT RT revealed that K103N mutation would destabilize the binding mode of TF2 by partially disrupting the protein-ligand hydrophobic and hydrogen bond interactions, which could also contribute to the reduced activity against this strain. Moreover, the newly introduced $p$-cyanophenyl moiety reached the primer grip region, but no interaction with the key amino acid here was observed. It pointed to the outside of the $\beta_{12}$, which may not be an ideal location to interact with amino acids in the primer grip region. This also inspired us to pay attention to the selection of linkers to make it more oriented to explore the primer grip region.

3. Materials and Methods

3.1. Synthesis of Compounds

On a column filled with Silica Gel GF254 for thin-layer chromatography (TLC) and spots were observed using UV radiation at 254 and 365 nm wavelengths. The melting points (mp) of compounds were determined using a micro melting point meter. Flash column chro-
matography was carried out on a column filled with Silica Gel 60 (200–300 mesh). 1H NMR and 13C NMR spectra were acquired on a Bruker AV-400 spectrometer with tetramethylsilane as the internal standard and DMSO-d6 as solvent. The relevant mass spectrometry data were measured by a Standard G1313A Autosampler instrument. Reagents used in this work required no further purification and were bought from commercial sources.

4-((4-(Formyl-2,6-dimethylphenoxy)pyrimidin-2-yl)amino)benzonitrile (10)

9 (3.00 g, 13.04 mmol), 4-hydroxy-3,5-dimethylbenzaldehyde (2.35 g, 15.65 mmol) and K2CO3 (3.60 g, 26.08 mmol) were mixed at 5 mL DMF. The reaction mixture was stirred at 100 °C for 10 h. After the reaction was finished (monitored by TLC), water (40 mL) was added, and the precipitate was filtered to obtain 10, brown solid, yield: 61%. 1H NMR (400 MHz, DMSO-d6) δ 10.13 (s, 1H, -CHO), 10.03 (s, 1H, NH), 8.50 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.80 (s, 2H, Benzene-H), 7.56 (d, J = 8.6 Hz, 2H, Benzene-H), 7.44 (d, J = 8.7 Hz, 2H, Benzene-H), 6.71 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 2.17 (s, 6H, 2×CH3). ESI-MS: m/z calcd for C20H16N4O2 345.13 [M + H]+, found 345.4 [M + H]+.

4-((4-(Hydroxymethyl)-2,6-dimethylphenoxy)pyrimidin-2-yl)amino benzonitrile (11)

10 (1.00 g, 2.91 mmol) was dissolved in 30 mL MeOH, then NaBH4 (0.10 g, 2.91 mmol) was added slowly in portions at 0 °C. The reaction was stirred for 0.5 h at 0 °C and for a further 4 h at room temperature. A saturated brine solution (30 mL) was added, and the mixture was extracted with ethyl acetate (3 × 20 mL) after the reaction solution was concentrated under reduced pressure. Then, the organic layer was dried with anhydrous sodium sulfate. Flash column chromatography was used to separate 11 from the mixture with ethyl acetate and petroleum ether, white solid, yield: 45%. 1H NMR (400 MHz, DMSO-d6) δ 10.11 (s, 1H, NH), 8.44 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.59 (d, J = 8.7 Hz, 2H, Benzene-H), 7.50 (d, J = 8.8 Hz, 2H, Benzene-H), 7.15 (s, 2H, Benzene-H), 6.60 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.25 (t, J = 5.6 Hz, 1H, OH), 4.52 (d, J = 5.6 Hz, 2H, -CH2), 2.07 (s, 6H, 2×CH3). ESI-MS: m/z calcd for C20H18N4O2 347.14 [M + H]+, found 347.3 [M + H]+.

4-(4-(Bromomethyl)-2,6-dimethylphenoxy)pyrimidin-2-yl)amino benzonitrile (12)

11 (1.00g, 2.88 mmol) was dissolved in 20 mL dichloromethane, then PBr3 (0.33 mL, 3.46 mmol) was dissolved in 1.5 mL dichloromethane and added dropwise to the reaction solution at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and for a further 2 h at room temperature until TLC detection of the reaction was complete. A saturated brine solution (30 mL) was added, and the mixture was extracted with ethyl acetate (3 × 20 mL) after the reaction solution was concentrated under reduced pressure. Then, the organic layer was dried with anhydrous sodium sulfate. Flash column chromatography was used to separate 12 from the mixture with ethyl acetate and petroleum ether, white solid, yield: 51%. 1H NMR (400 MHz, DMSO-d6) δ 10.14 (s, 1H, NH), 8.47 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.56 (d, J = 8.6 Hz, 2H, Benzene-H), 7.52 (d, J = 8.2 Hz, 2H, Benzene-H), 7.32 (s, 2H, Benzene-H), 6.66 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 4.79 (d, J = 18.3 Hz, 2H, -CH2), 2.07 (s, 6H, 2×CH3). ESI-MS: m/z calcd for C20H17BrN4O 409.06 [M + H]+, found 409.4 [M + H]+.

3.1.1. General Synthesis Procedure for the Preparation of TF1-TF16

Intermediate 12 (0.25 mmol), different substituted thiophenols (0.30 mmol), KOH (0.30 mmol) and KI (0.30 mmol) were mixed in 10 mL MeOH. Stirred at 80 °C for 4 h, and the reaction was detected by TLC. Dichloromethane (3 × 20 mL) was used to extract the reaction solution after adding saturated sodium chloride solution (30 mL); then, the organic layer was dried with anhydrous sodium sulfate. Using flash column chromatography, the target compounds were separated from ethyl acetate/petroleum ether.

4-((2,6-dimethyl-4-(phenoxymethyl)phenoxy)pyrimidin-2-yl)amino) benzonitrile (TF1)

White solid, yield: 50%, mp: 179–182 °C. 1H NMR (400 MHz, DMSO-d6) δ 10.17 (s, 1H, NH), 8.46 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.57 (d, J = 8.5 Hz, 2H, Benzene-H), 7.49 (d, J = 8.6 Hz, 2H, Benzene-H), 7.33 (d, J = 9.8 Hz, 4H, Benzene-H), 7.09 (d, J = 8.0 Hz, 2H, Benzene-H), 6.97 (t, J = 7.3 Hz, 1H, Benzene-H), 6.65 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.08 (s, 2H, -CH2), 2.09 (s, 6H, 2×CH3). 13C NMR (100 MHz, DMSO-d6) δ 168.97, 160.76, 159.68, 158.95, 149.60, 145.07, 134.90, 133.11, 130.90, 130.01, 129.05, 121.24, 119.90, 118.57,
115.13, 102.90, 99.33, 69.39, 16.55. ESI-MS: m/z calcld for C_{26}H_{22}N_{4}O_{2} 423.17 [M + H]^+; found 423.17 [M + H]^+.

4-((4-(4-cyanophenoxy)methyl)-2,6-dimethylphenoxy)pyrimidin-2-yl)amino) benzonitrile (TF2)

White solid, yield: 62%, mp: 202–205 °C. ^1^H NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H, NH), 8.40 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.76–7.71 (m, 2H, Benzene-H), 7.50 (d, J = 8.6 Hz, 2H, Benzene-H), 7.41 (d, J = 8.6 Hz, 2H, Benzene-H), 7.27 (s, 2H, Benzene-H), 7.19 (d, J = 8.7 Hz, 2H, Benzene-H), 6.58 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.13 (s, 2H, CH_2), 2.02 (s, 6H, 2 × CH_3). 13C NMR (100 MHz, DMSO-d_6) δ 168.93, 160.79, 159.67, 159.08, 149.82, 145.07, 145.10, 133.09, 131.36, 131.04, 129.31, 121.26, 121.00, 119.92, 119.11, 118.59, 118.03, 112.83, 102.90, 99.33, 70.11, 16.54. ESI-MS: m/z calcld for C_{27}H_{21}N_{5}O_{2} 448.17 [M + H]^+; found 448.38 [M + H]^+.

3-((4-(2-(4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzyl)oxy) benzonitrile (TF3)

White solid, yield: 54%, mp: 199–202 °C. ^1^H NMR (400 MHz, DMSO-d_6) δ 10.17 (s, 1H, NH), 8.47 (d, J = 5.7 Hz, 1H, C6-Pyrimidine-H), 7.60–7.43 (m, 9H, Benzene-H), 7.34 (s, 2H, Benzene-H), 6.66 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.17 (s, 2H, CH_2), 2.10 (s, 6H, 2 × CH_3). 13C NMR (100 MHz, DMSO-d_6) δ 168.94, 160.79, 159.67, 159.08, 149.82, 145.07, 134.10, 133.09, 131.36, 131.04, 129.31, 121.26, 121.00, 119.92, 119.11, 118.59, 118.03, 112.83, 102.90, 99.33, 70.11, 16.54. ESI-MS: m/z calcld for C_{27}H_{21}N_{5}O_{2} 446.44 [M + H]^+; found 446.44 [M + H]^+.

2-((4-(2-(4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzyl)oxy) benzonitrile (TF4)

White solid, yield: 61%, mp: 198–201 °C. ^1^H NMR (400 MHz, DMSO-d_6) δ 10.15 (s, 1H, NH), 8.47 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.77 (dd, J = 7.7, 1.7 Hz, 1H, Benzene-H), 7.70 (td, J = 8.0, 7.5, 1.7 Hz, 1H, Benzene-H), 7.61 (d, J = 8.5 Hz, 2H, Benzene-H), 7.48 (d, J = 8.6 Hz, 2H, Benzene-H), 7.43 (d, J = 8.6 Hz, 1H, Benzene-H), 7.35 (s, 2H, Benzene-H), 7.14 (t, J = 7.6 Hz, 1H, Benzene-H), 6.65 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.30 (s, 2H, CH_2), 2.11 (s, 6H, 2 × CH_3). 13C NMR (100 MHz, DMSO-d_6) δ 168.93, 160.72, 160.47, 159.71, 149.73, 145.01, 135.52, 134.30, 133.91, 133.17, 131.07, 128.77, 121.84, 119.86, 118.57, 116.88, 114.01, 103.03, 101.35, 99.42, 70.34, 16.64. ESI-MS: m/z calcld for C_{27}H_{21}N_{5}O_{2} 448.17 [M + H]^+; found 448.30 [M + H]^+.

4-((4-(4-methoxyphenoxy)methyl)-2,6-dimethylphenoxy)pyrimidin-2-yl) amino) benzonitrile (TF5)

White solid, yield: 63%, mp: 188–191 °C. ^1^H NMR (400 MHz, DMSO-d_6) δ 10.16 (s, 1H, NH), 8.46 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.58 (d, J = 8.5 Hz, 2H, Benzene-H), 7.51 (d, J = 8.6 Hz, 2H, Benzene-H), 7.30 (s, 2H, Benzene-H), 7.06–7.00 (m, 2H, Benzene-H), 6.92–6.85 (m, 2H, Benzene-H), 6.65 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.02 (s, 2H, CH_2), 3.71 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3). 13C NMR (100 MHz, DMSO-d_6) δ 168.98, 160.76, 159.68, 153.97, 153.01, 149.52, 145.08, 135.15, 133.12, 130.85, 128.95, 119.91, 118.60, 116.03, 115.10, 102.90, 99.33, 70.00, 55.82, 16.55. ESI-MS: m/z calcld for C_{27}H_{24}N_{4}O_{3} 453.18 [M + H]^+; found 453.20 [M + H]^+.

4-((4-(4-(3-methoxyphenoxy)methyl)-2,6-dimethylphenoxy)pyrimidin-2-yl) amino) benzonitrile (TF6)

White solid, yield: 63%, mp: 149–152 °C. ^1^H NMR (400 MHz, DMSO-d_6) δ 10.17 (s, 1H, NH), 8.47 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 7.79 (d, J = 8.5 Hz, 2H, Benzene-H), 7.50 (d, J = 8.6 Hz, 2H, Benzene-H), 7.32 (s, 2H, Benzene-H), 7.22 (t, J = 8.2 Hz, 1H, Benzene-H), 6.71–6.62 (m, 3H, Benzene-H), 6.56 (dd, J = 8.2, 2.3 Hz, 1H, C5-Pyrimidine-H), 5.08 (s, 2H, CH_2), 3.76 (s, 3H, CH_3), 2.10 (s, 6H, 2 × CH_3). 13C NMR (100 MHz, DMSO-d_6) δ 168.97, 161.04, 160.75, 160.23, 159.69, 149.59, 145.07, 134.85, 133.12, 130.89, 130.45, 129.02, 119.90, 118.60, 107.34, 107.06, 102.92, 101.37, 99.35, 69.56, 35.55, 16.55. ESI-MS: m/z calcld for C_{27}H_{24}N_{4}O_{3} 453.18 [M + H]^+; found 453.25 [M + H]^+.
White solid, yield: 58%, mp: 208–211 °C. 3H NMR (400 MHz, DMSO-d6) δ 10.15 (s, 1H, NH), 8.47 (d, J = 5.7 Hz, 1H, C6-Pyrimidine-H), 7.63 (d, J = 8.3 Hz, 2H, Benzene-H), 7.52 (d, J = 8.5 Hz, 2H, Benzene-H), 7.31 (s, 2H, Benzene-H), 7.12 (d, J = 7.4 Hz, 1H, Benzene-H), 7.01 (d, J = 7.5 Hz, 1H, Benzene-H), 6.92 (p, J = 7.4 Hz, 2H, Benzene-H), 6.65 (d, J = 5.7 Hz, 1H, C5-Pyrimidine-H), 5.06 (s, 2H, CH2), 3.78 (s, 3H, CH3), 2.10 (s, 6H, 2×CH3). 13C NMR (100 MHz, DMSO-d6) δ 168.99, 160.65, 159.72, 149.72, 149.50, 148.45, 145.05, 135.02, 132.19, 130.81, 129.05, 121.71, 121.08, 119.89, 118.66, 114.20, 112.62, 102.99, 99.47, 70.27, 55.95, 16.58. ESI-MS: m/z calcd for C27H24N4O3 453.18 [M + H]+, found 453.32 [M + H]+.

4-((4-(4-(4-cyanophenyl)pyrimidin-2-yl)-2,6-dimethylphenoxy)amino)benzonitrile (TF12)

White solid, yield: 41%, mp: 245–248 °C. 1H NMR (400 MHz, DMSO-d6) δ 10.14 (s, 1H, NH), 8.47 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 8.36 (d, J = 4.5 Hz, 1H, Pyridine-H), 7.97...
(d, J = 8.7 Hz, 1H, Pyridine-H), 7.76 (dd, J = 8.8, 4.5 Hz, 1H, Pyridine-H), 7.61 (d, J = 8.5 Hz, 2H, Benzene-H), 7.50 (d, J = 8.5 Hz, 2H, Benzene-H), 7.36 (s, 2H, Benzene-H), 6.65 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.36 (s, 2H, CH₂), 2.11 (s, 6H, 2×CH₃). ¹³C NMR (100 MHz, DMSO-d₆) δ 168.91, 160.75, 159.70, 158.86, 158.53, 158.53, 158.53, 149.81, 149.05, 134.15, 133.50, 133.50, 133.18, 131.15, 129.49, 124.45, 119.22, 116.80, 117.21, 109.75, 102.91, 99.34, 70.61, 16.54. ESI-MS: m/z calcd for C₂₆H₂₆N₃O₂ 422.16 [M + H]^⁺, found 422.61 [M + H]^⁺.

4-(4-(2,6-Dimethyl-4-(4-nitrophenoxy)methyl)phenoxo)pyrimidin-2-ylamino)benzonitrile (TF15)

White solid, yield: 71%, mp: 233–236 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.20 (s, 1H, NH), 8.48 (s, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 8.25 (d, J = 8.8 Hz, 2H, Benzene-H), 7.59 (d, J = 8.6 Hz, 2H, Benzene-H), 7.49 (d, J = 8.5 Hz, 2H, Benzene-H), 7.37 (s, 2H, Benzene-H), 7.31 (d, J = 8.9 Hz, 2H, Benzene-H), 6.67 (s, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.27 (s, 2H, CH₂), 2.11 (s, 6H, 2×CH₃). ¹³C NMR (100 MHz, DMSO-d₆) δ 168.91, 160.80, 159.67, 158.86, 158.53, 158.53, 149.81, 149.05, 134.15, 133.50, 133.18, 131.15, 129.33, 128.26, 128.90, 119.91, 118.59, 115.90, 110.10, 102.89, 99.32, 70.73, 16.54. ESI-MS: m/z calcd for C₂₆H₂₆N₃O₂ 422.16 [M + H]^⁺, found 422.61 [M + H]^⁺.

4-{4-(2,4-Dimethyl-4-(4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzyl)oxy) nicotinonitrile (TF13)

White solid, yield: 52%, mp: 195–198 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.09 (s, 1H, NH), 8.64 (d, J = 2.9 Hz, 1H, Pyridine-H), 8.58 (d, J = 1.5 Hz, 1H, Pyridine-H), 8.40 (d, J = 5.6 Hz, 1H, C6-Pyrimidine-H), 8.05 (t, J = 2.3 Hz, 1H, Pyridine-H), 7.51 (d, J = 8.5 Hz, 2H, Benzene-H), 7.42 (d, J = 8.6 Hz, 2H, Benzene-H), 7.28 (s, 2H, Benzene-H), 6.59 (d, J = 5.6 Hz, 1H, C5-Pyrimidine-H), 5.17 (s, 2H, CH₂), 2.03 (s, 6H, 2×CH₃). ¹³C NMR (100 MHz, DMSO-d₆) δ 168.91, 160.83, 159.67, 154.64, 149.98, 145.05, 145.01, 143.13, 133.54, 133.10, 131.15, 129.49, 124.45, 119.22, 116.80, 117.21, 109.75, 102.91, 99.34, 70.61, 16.54. ESI-MS: m/z calcd for C₂₆H₂₆N₃O₂ 447.16 [M + H]^⁺, found 447.27 [M + H]^⁺.

31.2. Preparation Method of TF17

TF16 (0.2 g, 0.37 mmol) was dissolved in 5 mL dichloromethane. Following the addition of the trifluoroacetic acid (2 mL), the mixture was stirred for 5 hours at room temperature. After the reaction was finished (monitored by TLC), the reaction solution was mixed with water (10 mL) and adjusted pH value to 9–10 with saturated sodium bicarbonate aqueous. Dichloromethane (3 × 20 mL) was used to extract the reaction solution after adding saturated sodium chloride solution (30 mL). Then, the organic layer
was dried with anhydrous sodium sulfate. Flash column chromatography was used to separate TF17 from the mixture with ethyl acetate and petroleum ether.  

4-((4-(4-Aminophenoxy)methyl)-2,6-dimethylphenoxy)pyrimidin-2-yl)amino) benzonitrile (TF17)  

White solid, yield: 65%, mp: 241–244 °C.  

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.17 (s, 1H, NH), 9.35 (s, 2H, NH$_2$), 8.47 (d, $J = 5.7$ Hz, 1H, C6-Pyrimidine-H), 7.56 (d, $J = 8.7$ Hz, 2H, Benzene-H), 7.30 (d, $J = 8.6$ Hz, 2H, Benzene-H), 7.32 (s, 2H, Benzene-H), 7.21 (d, $J = 8.5$ Hz, 2H, Benzene-H), 7.14 (d, $J = 8.5$ Hz, 2H, Benzene-H), 6.66 (d, $J = 5.7$ Hz, 1H, C5-Pyrimidine-H), 5.09 (s, 2H, CH$_2$), 2.09 (s, 6H, 2×CH$_3$).  

$^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 168.95, 160.83, 159.65, 157.18, 149.71, 145.09, 134.59, 133.11, 130.96, 129.20, 127.78, 123.35, 119.95, 118.57, 116.11, 102.85, 99.31, 69.97, 16.54. ESI-MS: $m/z$ calcd for C$_{26}$H$_{23}$N$_5$O$_2$ 438.19 [M + H]$^+$, found 438.57 [M + H]$^+$.  

3.2. In Vitro Anti-HIV Assay  

Evaluation of the anti-HIV activity and cytotoxicity of the target compounds was performed by utilizing the MTT method in MT-4 cells described previously [37]. 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 prepared 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). Each sample includes untreated control HIV- and mock-infected cell samples. WT HIV-1 strain (IIIB), HIV-1 drug-resistant strains including L100I, K103N, Y181C, Y188L, E138K, K103N/Y181C, F227L/V106A or HIV-2 strain (ROD) stock (50 µL) at 100–300 CCID$_{50}$ (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 to evaluate their 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 then transferred 50 µL volumes to the microtiter tray wells. After infection five days, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay. The 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration of the test compound that reduced the viability of the mock-infected MT4 cells by 50%. The 50% effective concentration (EC$_{50}$) was defined as the concentration of the test compound achieving 50% protection from the cytopathic effect of the virus in infected cells.  

3.3. HIV-1 RT Inhibition Assay  

An HIV-1 reverse transcriptase (RT) assay kit produced by Roche was used for the RT inhibition assay. All the reagents for performing the RT reaction came from the kit, and the ELSIA procedures for RT inhibition assay were conducted following the description in the kit protocol [38]. In brief, the reaction mixture containing template/primer complex, viral nucleotides (dNTPs) and HIV-1 reverse transcriptase (RT) enzyme in the incubation buffer with or without inhibitors was incubated for 1 h at 37 °C. Subsequently, the reaction mixture was transferred to a streptavidine-coated microtiter plate (MTP) and incubated for another 1 h at 37 °C to ensure the retranscriptional cDNA chain that consisted biotin labeled dNTPs bound to streptavidine. Then used, a washing buffer was used to remove the unbound dNTPs, and an anti-DIG-POD working solution was added. After incubation for 1 h at 37 °C, the DIG-labeled dNTPs incorporated in cDNA were bound to the anti-DIG-POD antibody. The unbound anti-DIG-PODs were removed, and the peroxidesubstrate (ABST) solution was added to the MTPs. A colored reaction proceeds during the cleavage of the substrate catalyzed by POD. The absorbance of the sample was determined at O.D. 405 nm.
using a microtiter plate ELISA reader. The percentage inhibitory activity of RT inhibitors was calculated by the formula given below:

\[
\% \text{ Inhibition} = \frac{[\text{O.D. value with RT but without inhibitors} - \text{O.D. value with RT and inhibitors}]}{[\text{O.D. value with RT and inhibitors} - \text{O.D. value without RT and inhibitors}]}.
\]

The IC\textsubscript{50} values corresponded to the concentrations of the test compounds required to inhibit the incorporation of biotin-dUTP into RT by 50%.

3.4. MD Simulation Methods

Ligand preparation: The ligand structure TF2 was generated with Chemdraw (version 20.0), and the structure was then transferred to 3D molecules using Chem3D (version 20.0), which was then prepared with the LigPrep module of Schrodinger suites. The ligands were prepared with an OPLS4 force field, and the ionization states were generated via Epik at pH 7.0. A maximum of 32 conformations were generated per ligand, and the best-scoring conformation was used for docking.

Ligand Docking: The co-crystal structures of HIV-1 WT RT in complex with RPV (PDB code:2ZD1) [22], and HIV-1 K103N RT in complex with RPV (PDB code:3MEG) [27] were prepared using the Protein Preparation Wizard module of Schrodinger suites (Release 2022-1). All original hydrogens were removed and re-added. The protein bond order was reassigned with the CCD database, and H\textsubscript{2}O molecules were deleted. The protonation state for the protein complex was set to pH 7.0. Furthermore, the missing side chains were rebuilt using prime. The hydrogen bond order was reassigned with PROPKA, and restrained minimization was carried out with the OPLS4 force field. The generated protein complex structures were then used for docking.

Induced-fit docking (Schrodinger suites, Release 2022-1) was performed to investigate the binding poses of TF2 with WT and K103N RT, respectively. Standard protocol was used, and a maximum of 20 docking poses were generated per ligand. The nonnucleoside reverse transcriptase inhibitor (NNRTI) RPV binding pocket was selected as the binding pocket. The other parameters were set as default. The binding conformations of each analogue with the best IFDs core were selected for analysis and next step MD simulations (Figure S1).

Molecular Dynamic simulations: The input file for MD was the output file from the best docked position of the Schrödinger Induced Fit Docking Panel [40]. A 500 ns molecular dynamic simulation was run using the Desmond module of Schrödinger Suite 2022-1 with an OPLS4 force field. Using the TIP3P water model and Desmond’s system builder module, the protein-ligand complex was solved, and an orthorhombic simulation box with a buffer distance of 10 Å between its edge and the atoms of the complex was created. A sufficient number of counter-ions were added to the system to neutralize it, and 0.15 M NaCl was added to the simulation box to preserve the isotonic condition. Energy minimization was applied to the model up until a gradient threshold of 25 kcal/mol/Å was reached at 300 K and 1 bar of pressure using NPT ensemble class. The trajectory was documented as the MD simulation was running, and using Desmond’s Simulation Event Analysis tool, Protein-Ligand interactions, Protein-Ligand fluctuations, and contacts with different amino acids were used to gauge the stability of the complex.

Free ligand-binding energy calculation (MM-GBSA): Free ligand-binding energy calculation (MM-GBSA) [41]: The MM / GBSA calculation (Schrödinger Release 2022-1) was performed to estimate the binding free energy of ligands when binding the various RTs.

4. Conclusions

In summary, based on the X-ray crystallographic analysis, to explore the primer grip region and target the conserved residues, we have designed and synthesized 17 DAPY derivatives with -CH\textsubscript{2}O- as the linker. According to the biological evaluation results, all compounds (EC\textsubscript{50} = 7.6 nM ~ 199 nM) exhibited potent activity against HIV-1 WT
strain, TF2 (EC50 = 7.6 nM), TF4 (EC50 = 7.8 nM) and TF12 (EC50 = 7.8 nM) showed the most potent activity, which was better than NVP (EC50 = 122.6 nM). Notably, TF2 (CC50 > 279,329.6 nM) showed low cytotoxicity. And for the tested HIV-1 mutant strains, TF2 (EC50 (K103N) = 28.1 nM, EC50 (E138K) = 44.0 nM, EC50 (Y181C) = 139.3 nM) was the most potent compound against HIV-1 K103N, E138K and Y181C mutant strains. Moreover, the WT HIV-1 RT enzyme inhibitory assay indicated that the target of these novel compounds was RT. Furthermore, detailed SARs of compounds and molecular dynamics simulation of TF2 can provide insight into the binding modes of compounds, and the further structural optimization of the compound can be guided accordingly. The molecular dynamics simulation studies of TF2 showed that the newly introduced p-cyanophenyl moiety reached the primer grip region, but no interaction with the key amino acid here was observed. The primer grip region has yet to be explored, and the development of novel NNRTI drugs with improved tolerability and resistance profiles is still required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ph15111438/s1, Figure S1, 1H and 13C NMR spectra of TF1–17.

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