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Hydrophobic pocket occupation design of difluoro-biphenyl-diarylpyrimidines as non-nucleoside HIV-1 reverse transcriptase inhibitors: from N-alkylation to methyl hopping on the pyrimidine ring

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Notes

The authors declare that they have no competing interests.

Abstract

Considering the non-ideal metabolic stability of the difluoro-biphenyl-diarylpyrimidine lead compound **4**, a series of novel alkylated difluoro-biphenyl-diarylpyrimidines were structure-based designed and synthesized as non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs). Introducing alkyl or substituted alkyl groups on the linker region (consist of V179, Y181, E138 residues) to block the potential metabolic sensitive sites obtained twenty-two derivatives. Among them, compound **12a** with N-methyl group displayed excellent HIV-1 inhibitory activity ($EC_{50} = 6.16$ nM), moreover, a significant improvement in the selectivity ($SI = 24,069$) compared with existing drugs (**NVP**, $SI > 72$; **ETR**, $SI > 1159$; **EFV**, $SI > 1600$). Finally, the methyl group was hopped to the central pyrimidine ring in order to occupy the small linker region and maintain the water-mediated hydrogen bond observed in the binding of compound **4** with RT. The resulting compound **16y** exhibited an improved HIV-1 inhibitory activity ($EC_{50} = 1.37$ nM) and much lower cytotoxicity with the $SI > 208,333$). In addition, compound **16y** possessed nanomolar activity toward multiple single-mutant virus strains except Y188L. For the metabolic stability, compound **16y** have a better stability in human liver microsomes ($T_{1/2} = 138$ min, $Cl_{int} = 5.0$ μ L/min/mg) than compounds **4** ($T_{1/2} = 44$ min, $Cl_{int} = 16$ μ L/min/mg). No apparent in vivo acute toxicity was observed in **16y**-treated female, and especially pregnant mice. The molecular docking studies predicted the binding modes of **16y** with RT, and the methyl group was displayed as occupying effect in the hydrophobic pocket, explaining the activity differences for **16y** and the precursor **12a**. This series of alkylated compounds with highly potency and safety represent promising lead template for future discovery.

Graphic Abstract

Introduction

Acquired immunodeficiency syndrome (AIDS) is considered as a pandemic caused by human immunodeficiency virus (HIV) infection, and about 37.9 million people in total are living with HIV in 2019 which still indicates a threat to human.^{1, 2} Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are considered as the most successful classes of anti-HIV drug that have been widely used in HIV therapeutic schedule. Diarylpyrimidine (DAPY) derivatives are the most highly potent class of NNRTIs with two FDA-approved drugs (etravirine, ETR, **1**; rilpivirine, RPV, **2**).^{3, 4} With these compounds as the leads, our group first designed the biphenyl-DAPYs with prominent activity against HIV WT and related-mutants (Figure 1).⁵⁻¹¹

Figure 1. Chemical structures of representative biphenyl-DAPYs developed by our group

The biphenyl fragment was predicted to strengthen the π - π interaction targeting W229 consevative region.¹² Compared with the approved drugs, compound **3** possessed great potency to inhibit WT HIV-1 with an EC₅₀ value of 1 nM and single-digit nanomolar activity against various mutant strains. However, its cytotoxicity limited further evaluations probably due to the metabolites of dimethyl biphenyl moiety.⁶ Compound **4** with

replacement of the dimethyl groups by a difluoro moiety maintained the antiviral activity, especially exhibiting much lower cytotoxicity ($SI > 23,8095$). Furthermore, a series of biphenyl-DAPYs compounds (**5-7**) with different CH-R linkers ($R = OH$,¹⁰ CN ⁷ and NOH ¹¹) were also developed in our group by adjusting the flexibility of the molecules to adopt the RT enzyme.¹³ Most of them were demonstrated to show excellent antiviral activities toward WT HIV-1 and the moderate cytotoxicity. Compound **5** carrying a stereogenic center at (CH)-OH linker displayed high anti-HIV activities toward WT ($EC_{50} = 1nM$) and mutant strains. Compared with compound **4** ($SI > 23,8095$), compound **5** displayed a much lower SI value ($SI = 4617$). This non-ideal SI disturbed them for in-depth evaluation.

On the other hand, metabolic stability as an important druggability profile was focused on.¹⁴ Compound **3** showed a half-life of only 14.6 min in human liver microsome, and compound **4** with difluoro group could smoothly improve the metabolic stability which displayed a longer half-life ($t_{1/2} = 31.6$ min).⁸ However, replacement of the NH by a hydroxy group as the linker indicated a poor metabolic stability that cannot be accurately measured.¹⁰ Thus, the linker of biphenyl DAPYs were assumed to be an very important aspect affecting the metabolic, PK and cytotoxicity. In the present study, we still focused on the linker by alkylation design in order to block the potential metabolic sensitive sites and maintain the high antiviral potency.

2. Design Rationale

As illustrated in Figure 2A, compound **4** formed a “U” shape in the RT binding pocket and a classical water-mediated hydrogen-bonding interaction was predicted. This H-bond could be replaced by a C-OH linker in compound **5** (Figure 1) but the importance/necessity of the water-mediated H-bond has not been investigated yet, where it is located in a hydrophobic subpocket (the mesh part in Figure 2). Besides, the NH linker region was a typical metabolic sensitive site.¹⁵ Hence, we first introduced a series of straight-chain, branched alkyl, and substituted alkyl groups in the linker region to investigate the tolerant region and block the potential metabolic sensitive sites (Figure 2A and 2B). Next, the best alkyl group (The methyl was determined by further biological assays) was proposed to be hopped on the central pyrimidine ring to occupy the hydrophobic subpocket and simultaneously remain the water-mediated hydrogen bond (Figure 2C).

Figure 2. Design strategy of alkylated compounds based on the lead compound **4**.

3. Results and discussion

3.1 Chemistry

As depicted in Scheme 1, the target compounds were synthesized by multiple steps of simple substitution reactions. The key intermediate **10** was obtained from the commercial 4-bromo-2,6-difluoroaniline **8** by reacting with 2,4-dichloropyrimidine **9** in the presence of isopropanol/HCl solution.^{6, 8} Then, **10** reacted with various substituted alkyl iodide and immediately reacted with 4-aminobenzonitrile to generate **11**, which were then coupled with (4-cyanophenyl)boronic acid to give the target compounds **12a-t**. For **13a-c** with ester, acetyl and ethanesulfonyl, compound **4** was used as the starting material reacting with the NaH and iodo(methoxy)methane or acetyl chloride or ethanesulfonyl chloride in DMF. The synthetic route of compounds **16y** and **19z** was illustrated in Scheme 2. The intermediate **17** was synthesized from **14a**^{7, 11, 16} and methyl iodide, the Cs₂CO₃ as base in MeCN at 80 °C under N₂ atmosphere. Directly treating **17** with 4-bromo-2,6-difluoroaniline at 80 °C for 3 h in IPA/HCl conditions generated compound **18**. Finally, Compound **18** coupling with (4-cyanophenyl)boronic acid to give the target compound **19z**. Compound **15** was synthesized from **14b** and 4-bromo-2,6-difluoroaniline in IPA/HCl solution and then **15** was converted into the desired target molecules **16** via the Suzuki-Miyaura cross coupling.¹⁷ All of the final structures were

characterized by ^1H NMR and ^{13}C NMR spectroscopy and HRMS. To be mentioned, most of compounds displayed two sets of signals which was combined to one set by rising NMR testing.^{18, 19} This was considered to result from the rotamers of the amido compounds.^{20, 21} In the present study, we further confirmed the rotamer phenomenon taking compound **12a** as the example. Compound **12a** was tested and verified by ^1H NMR and ^{13}C NMR at different temperatures of 30, 50 and 70 °C (see supporting information for spectra). Besides, all target compounds were purified to > 95%, as determined by high-performance liquid chromatography (HPLC) analysis, prior to use in subsequent experiments.

Scheme 1. Synthesis of compounds **12a-t** and **13a-c**.^a

^a Reagents and conditions: (a) IPA, HCl, 60 °C, 4-8 h; (b) substituted alkyl iodide, Cs_2CO_3 , MeCN, 80 °C, 18 h; (c) 4-aminobenzonitrile, IPA, HCl, 80 °C, 4-8 h; (d) (4-cyanophenyl)boronic acid, $\text{Pd}(\text{dppf})\text{Cl}_2$, Cs_2CO_3 , 1,4-dioxane, H_2O , 110 °C, 4-18 h; (e) NaH, iodo(methoxy)methane or acetyl chloride or ethanesulfonyl chloride, DMF, 0-60 °C, 2 h.

Scheme 2. Synthesis of compounds **16y** and **19z**.^b

^b Reagents and conditions: (a) 4-bromo-2,6-difluoroaniline, IPA, HCl, 80 °C, 4-8 h; (b) CH₃I, Cs₂CO₃, MeCN, 80 °C, 4-18 h; (c) (4-cyanophenyl)boronic acid, Pd(dppf)Cl₂, Cs₂CO₃, 1,4-dioxane, H₂O, 110 °C, 4-18 h.

3.2 The in vitro HIV-1 WT inhibitory activities and cytotoxicity of **12a-j** and **19z**

Compared with compound **5** (CC₅₀ = 8.4 μM, SI = 4617),¹⁰ replacement of the chiral hydroxy moiety of biphenyl-DAPYs with a N-alkylation dramatically decreased the cytotoxicity (Table 1, CC₅₀ > 105 μM). Compound **12a** with a N-methyl group remained to possess an EC₅₀ value of 6.2 nM with a CC₅₀ of 148 μM and significantly increased the selectivity index (SI = 24,067). Then, extension of the carbon chain of the N-alkylated substitutions resulted in the generally decreased antiviral activity. For instance, replacement of the ethyl group of **12b** (EC₅₀ = 9.1 nM) by the *n*-propyl diminished the in vitro antiviral activity (**12c**, EC₅₀ = 18.8 nM) in 2-fold. Further extension of the carbon chain to *n*-butyl obtained the compound **12d**, displaying a lower inhibitory activity (EC₅₀ = 40 nM) and SI value (SI = 5241). Compound **12h** with a pentyl group showed the lowest inhibitory activity (EC₅₀ = 241 nM) in this series. In addition, the straight chain substituted compound **12c** (EC₅₀ = 18.8 nM, SI = 11,310) was superior to the branched alkyl substituted compound **12e** (EC₅₀ = 26.6

nM, SI = 6445). Similarly, compound **12d** revealed higher activity and SI value (EC_{50} = 40 nM, SI = 5241) than their branch isomeride compound **12f** (EC_{50} = 70 nM, SI = 3545) and compound **12g** (EC_{50} = 90 nM, SI = 2407). Then, the cycloalkanes were also investigated that was confirmed much lower activity (**12i**, EC_{50} = 120 nM, SI = 1927; **12j**, EC_{50} = 462 nM, SI > 534). Finally, the methyl was migrated onto the other NH linker (**19z**, EC_{50} = 156 nM, SI = 824) leading to significantly reduction of the inhibitory activity in 26-fold compared with its isomer **12a**.

Table 1. Activities and cytotoxicities of compounds **12a-j** and **19z** against HIV-1 (IIIB) strains in MT-4 cells.

12a

12b:R=CH₂CH₃
12c:R=CH₂CH₂CH₃
12d:R=CH₂CH₂CH₂CH₃
12e:R=CH(CH₃)₂
12f:R=CH₂CH(CH₃)₂
12g:R=CH(CH₃)CH₂CH₃
12h:R=CH₂CH(CH₃)CH₂CH₃
12i:R=cyclopentyl
12j:R=cyclopentyl ethyl

19z

Compds	EC ₅₀ (nM)	CC ₅₀ (μM)	SI (III _B)	Compds	EC ₅₀ (nM)	CC ₅₀ (μM)	SI (III _B)
	HIV-1	III _B			HIV-1	III _B	
12a	6±1.9	148±52	24,067	12g	90±34	217±21	2407
12b	9.1±3.0	105±38	11,579	12h	241±82	>255	>1055
12c	19±8.5	212±35	11,310	12i	120±34	232±12	1927
12d	40±11	211±23	5241	12j	462±168	>247	>534
12e	27±8.4	172±47	6445	19z	156±80	129±73	824
12f	70±20	241±11	3545				

3.3 The in vitro HIV-1 WT inhibitory activities and cytotoxicity of 12k-t, 13a-c and 16y

Next, a series of analogues with hydroxyl groups on the carbon chains were synthesized based on chiral hydroxyl compound **5** and alkyl compounds (Figure 2). As a result, introducing hydroxy moieties in the linker

could slightly be favorable to antiviral activities (**12k**, $EC_{50} = 5$ nM; **12l**, $EC_{50} = 7$ nM; **12m**, $EC_{50} = 7$ nM). However, they still showed relative high cytotoxicity. Then, fluoro, chloro, ethanoyl, cyano and allyl groups were introduced into the carbon chains to replace the hydroxyl group, causing the cytotoxicity apparently reduced ($CC_{50} > 200$ μ M). Compound **12s** with a cyano group showed an EC_{50} of 5 nM and high SI ($SI = 42,504$) which was better than methyl (**12a**) and ethyl (**12b**) analogues. Next, the hydroxyl compounds were modified to obtain compound **13a** with etherification exhibiting a high antiviral activity, low cytotoxicity ($CC_{50} = 238$ μ M) and high SI ($SI = 42,800$). In contrast, acetyl, sulfonyl, and allyl groups were favorable and the antiviral activities were kept at the single-digit nanomolar range (**13b**, $EC_{50} = 8$ nM, **12t**, $EC_{50} = 9$ nM, **13c**, $EC_{50} = 47$ nM). Finally, as proposed initially, the methyl group of compound **12a** was migrated to the central pyrimidine ring to occupy the hydrophilic pocket which was formed by residues E138, V179 and Y181 (Fig 2C). Meanwhile, the water-mediated hydrogen-bonding interaction shown in Figure 2A could be maintained in the new compound **16y**. This compound displayed excellent improvement in anti-HIV activities ($EC_{50} = 1$ nM) which was 6-fold than the corresponding compound **12a**, supporting our hypothesis and highlighting the importance of the water-mediated hydrogen-bonding interaction. Especially, this compound exhibited the highest SI value ($SI > 208,333$) in this series showing dramatically decrease in the cytotoxicity compared with the hydroxyl compound **5**. To be metioned here, this compound was also much better than the FDA-approved drugs NVP, ETR, and EFV in antiviral activity and toxicity, representing a promising candidate for further investigation.

Table 2. Activities and cytotoxicities of compounds **12k-t**, **13a-c** and **16y** against HIV-1 (IIIB) strains in MT-4 cells.

12k-v, 13a-c

12k: $R = CH_2CH_2OH$
12l: $R = CH_2CH_2CH_2OH$
12m: $R = CHCH(OH)CH_3$
12n: $R = CH_2CH(OH)CH_2(OH)$ (-rac)
12o: $R = CH_2CH_2F$
12p: $R = CH_2CHF_2$

12q: $R = CH_2CH_2CH_2F$
12r: $R = CH_2CH_2CH_2Cl$
12s: $R = CH_2CN$
12t: $R = CH_2CH=CH_2$
13a: $R = CH_2OCH_3$
13b: $R = COCH_3$
13c: $R = SO_2CH_2CH_3$

16y

Compds	EC_{50} (nM)	CC_{50} (μ M)	SI (III_B)	Compds	EC_{50} (nM)	CC_{50} (μ M)	SI (III_B)
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HIV-1 III _B				HIV-1 III _B			
12k	5±0.15	5.31±0.45	1058	12t	9±2.1	249±16	28,736
12l	7±0.95	68±7.5	10253	13a	6±0.30	238±18	42,800
12m	7±2.0	9.8±3.3	1465	13b	8±2.4	>268	>34,722
12n	8±3.0	19.5±4.0	2440	13c	47±6.5	>125	>5135
12o	7±0.6	235±4.8	32,449	16y	1±0.32	>285	>208,333
12p	14±7.2	252±3.2	17,740	NVP	209±66	>15	>72
12q	28±7.9	241±14	8688	ETR	4±0.68	>4.60	>1159
12r	40±6.6	>250	>6303	EFV	4±1.5	>6.33	>1600
12s	5±0.76	206±1.7	42,504				

3.4 In vitro Inhibitory Activity of Selected Analogues against HIV-1 Mutants and WT HIV-1 RT Enzyme

Next, representative compounds with good anti-HIV activity, low cytotoxicity and high SI values were selected to investigate their inhibitory activity against a panel of clinically observed drug-resistant mutants (L100I, K103N, Y181C, Y188L, E138K) and WT HIV-1 RT enzyme (Table 3). Most of the compounds had nanomolar anti-HIV mutants.

All the compounds seemed to be particularly active against L100I, Y181C, and E138K mutants, and most of them were more potent than NVP and EFV. All the tested analogues exhibited excellent EC₅₀ values ranging from 8-70 nM against E138K strain, which were better than that of the reference drug NVP (EC₅₀ = 288 nM) and comparable to those of EFV (EC₅₀ = 7.5 nM) and ETR (EC₅₀ = 18 nM). However, most of the test compounds were less active or inactive against Y188L. More importantly, all the test compounds without the hydroxy groups displayed higher SI values (SI > 1964) than reference drugs (NVP, EFV or ETR, SI = 2~1176) excepted for mutant Y188L. For the best compound 16y, it was still the most active and showed the lowest EC₅₀ value of 5 nM, making it 17~1486-fold more potent than NVP or EFV.

Table 3 Inhibitory activity of the selected compounds toward a panel of clinically relevant HIV-1 mutant strains and HIV-1 RT enzyme.

Compds	EC ₅₀ , nM (SI)					IC ₅₀ (nM) ^a	
	L100I	K103N	Y181C	Y188L	E138K		
12a	17±3.2 (8550)	218±8.1 (7141)	415±0.48 (3661)	524±52 (283)	33±26 (4451)	15±0.5	
12b	20.±4.1 (5186)	26±1.1 (3992)	40±4.4 (2665)	767±16 (138)	26±15 (4131)	30±0.3	
12c	95.0±11 (2242)	108±1.4 (1964)	69±8.0 (3091)	1864±597 (114)	34.9±0.15 (6094)	41±1.0	
12k	18.2±2.1 (293)	36.3±5.1 (146)	21.8±6.9 (244)	662±88.7 (8)	7.6±1.1 (701)	15±1.3	
12l	40.9±1.5 (1666)	53.4±4.3 (1274)	34.4±2.1 (1977)	813±29 (84)	21.9±10 (3110)	30±3.4	
12o	84.2±6.8 (2790)	73.5±27 (3193)	52.7±7.1 (4458)	967±204 (243)	28.6±8.6 (8203)	29±11	
12s	47.8±14 (4318)	36.0±1.4 (5744)	43±3.4 (4782)	444±25 (465)	20.7±3.7 (9962)	21±13	
12t	25±3.8 (9916)	46±8.7 (5385)	50±8.5 (4958)	965±86 (258)	42±0.91 (5924)	64±12	
13a	36.5±3.3 (6508)	51.4±1.4 (4627)	49.9±15 (4766)	808±154 (294)	35.0±6.3 (6785)	25±8.0	
13b	64.8±6.4 (>4139)	10.9±2.7 (>24,510)	27.4±5.9 (>9804)	547.7±28 (>490)	9.23±0.30 (>29,070)	40±3.0	
13c	196±85 (>1233)	78±21 (>3086)	114±42 (>2113)	4676±1763 (>52)	70±15 (>3455)	200±78	
16y	16.3±5.3 (>17,483)	5.4±1.8 (>53,191)	11.2±1.9 (>25,510)	-	8.3±1.5 (>34,247)	13±2.5	
NVP	4106±1970	8026±2230	8082±4784	-	288.4±102	580±160	

	(>4)	(>2)	(>2)		(>52)	
EFV	44.7±12.6	91.5±23.6	5.4±1.2	269±31.2	7.5±0.54	10±0
	(>142)	(>69)	(>1176)	(>24)	(>842)	
ETR	10.7±4.6	3.4±0.34	18.9±5.9	29.3±9.3	17.5±7.7	11 ± 9 ^b
	(>428)	(>1356)	(>243)	(>157)	(>263)	

^a IC₅₀: inhibitory concentration of test compound required to inhibit WT HIV-1 RT polymerase activity by 50%.

^b The data were obtained from the same laboratory (Prof. Erik De Clercq, Rega Institute for Medical Research, KU Leuven, Belgium) with the same method

All the new DAPY derivatives exhibited great inhibitory activities against WT HIV-1 RT with the IC₅₀ values ranging from 13 to 41 nM, which were 14~45-fold higher than that of **NVP** (IC₅₀ = 580 nM) and comparable to that of **ETR** (IC₅₀ = 10 nM). The regression analysis of pEC₅₀ and pIC₅₀ with a good correlation (R² = 0.81) indicating that these candidates specifically bind to HIV-1 RT (Figure 3).

Figure 3. Regression analysis pEC₅₀ and pIC₅₀.

3.5 Molecular docking analysis of 12a and 16y with RT enzymes.

Docking analysis was applied to further explain the experimental results on the most promising compound **16y** and the precursor **12a**. Similar to other biphenyl-DAPYs,^{6, 7, 11, 22} these two compounds were predicted to

well fit into the hydrophobic pocket formed by the key amino acid residues Y181, Y188, F227 and W229 (Fig. 4). For the binding modes with WT HIV RT enzyme (Figure 4A and B), as proposed in Figure 2, a water-mediated hydrogen-bonding network ($\text{NH}\cdots\text{O}$ distance = 2.1 Å; $\text{H}\cdots\text{O}=\text{C}$ distance = 1.8 Å) between the amino hydrogen of the linker and the carbonyl group of E138 was observed in compound **16y**. This interaction was lost in compound **12a**, probably contributing to the improvement in the inhibitory effect. As shown in Figures 4C-J, **16y** and **12a** fitted well into the pockets of the L100I, K103N, Y181C, Y188L mutants and several common features ("U" binding conformation, hydrogen bonds, hydrophobic and electrostatic interactions) were still observed as those in the WT RT enzyme. However, the interaction with compound **16y** to the Y188L mutant strain lost the typical π - π stacking interaction. In the Y181C mutant strain remained partly well inhibitory activity where the π - π stacking interaction was also missing. It was indicated that Y188 and Y181 residues had different contribution to the activities. This phenomenon was predicted as consistent with compound **5**.¹⁰ The K138 residue involved in additional water-mediated hydrogen-bonding interactions with the linkage amide of compound **16y** (Figure 4K and 4L) and not observed in compound **12a**, resulting in about 4-fold reduction in the inhibitory activity than that of **16y**. Besides, the methyl group on the pyrimidine of compound **16y** displayed well filling function as the N-methylated **12a**.

Figure 4. Predicted binding modes of **12a** and **16y** with HIV-1 RT and mutants (PDB: 2ZD1). (A) WT with **12a**; (B) WT with **16y**; (C) L100I with **12a**; (D) L100I with **16y**; (E) K103N with **12a**; (F) K103N with **16y**; (G) Y181C with **12a**; (H) Y181C with **16y**; (I) Y188L with **12a**; (J) Y188L with **16y**; (K) E138K with **12a**; (L) E138K with **16y**. Mutated residues are depicted as pink sticks. Hydrogen bonds are depicted as yellow dashed lines.

3.6 Acute toxicity analysis of **16y** in mice and pharmacokinetic (PK) evaluation

Reported claimed plasma concentrations of **RPV** were reduced that was closely related with the drug metabolism during second/third trimesters of pregnancy, with exposed a potential risk of viral breakthroughs.^{13, 23} Hence, the toxicity issue should be taken into consideration in the pregnancy. A single-dose acute toxicity of **16y** was carried out in the healthy and pregnant mouse model. The assay was carried out according to our previous studies.^{8, 24} No death occurred after intragastric (i.g.) administration of **16y** at a dose of 2 g/kg. All mice in the drug administration groups (female and pregnant) grew normally as the mice in the control group. The body weights of these mice gradually increased (Figure 5A and 5B), and no

significant behavioral abnormalities were observed over the period of two weeks. All pregnant mice in the drug administration groups and control groups were spontaneous labor. The offspring mice had the similar number and survival rate and no stillborn foetus, abortion or abnormalities was observed. This indicated that **16y** was well tolerated at a dose of 2 g/kg. The toxicity to the six important organs (brain, heart, liver, spleen, lung, and kidney) was investigated by hematoxylin-eosin (HE) staining. No marked pathological damage was observed as well (Figure 5C)

Figure 5. (A) Body weight of female mice (g)-time (day). (B) Body weight of pregnant mice (g)-time (day). (C) Six organ tissues were collected from the vehicle or treatment groups as soon as the experiment was finished. The histological changes in mice were analyzed by HE staining (magnification: X 200).

Next, the metabolic stability of compound **16y** was assayed (Figure 6). As compared with the lead compound **3** and **4**, the methyl-containing compound **16y** was much more stable in both human and rat liver microsomes. Compound **3** exhibited a high clearance (47.4 $\mu\text{L}/\text{min}/\text{mg}$ proteins) and short half-life (14.6 min) in humans and rats. This situation had partly improved in compound **4** ($\text{Cl}_{\text{int}} = 15.7 \mu\text{L}/\text{min}/\text{mg}$ proteins, $T_{1/2} = 44.3$ min) in rat liver microsomes. However, it was still non-ideal in human liver microsomes ($\text{Cl}_{\text{int}} = 21.9 \mu\text{L}/\text{min}/\text{mg}$ proteins, $T_{1/2} = 31.6$ min). In contrast, **16y** displayed similar stability in mice liver microsomes ($\text{Cl}_{\text{int}} = 30.1 \mu\text{L}/\text{min}/\text{mg}$ proteins, $T_{1/2} = 46.1$ min) and rat liver microsomes ($\text{Cl}_{\text{int}} = 28.4 \mu\text{L}/\text{min}/\text{mg}$ proteins, $T_{1/2} = 48.9$ min). Noticeably, **16y** significantly improved the stability in human liver microsomes ($\text{Cl}_{\text{int}} = 5.0 \mu\text{L}/\text{min}/\text{mg}$

proteins, $T_{1/2} = 138$ min) showing a suitable clearance and a longer half-life. This result highlighted the importance of the position of the methyl group in the biphenyl-DAPY derivatives.

Compounds	Species	Liver microsome stability		
		k(1/min)	T _{1/2} (min)	Cl _{int} (μL/min/mg protein)
3	rat	0.045	15.4	45.0
	human	0.047	14.6	47.4
4	rat	0.0157	44.3	15.7
	human	0.0219	31.6	21.9
16y	mice	0.0150	46.1	30.1
	rat	0.0142	48.9	28.4
	human	0.0050	138	5.0

Figure 6. The liver microsomal stability of the selected compounds. (A) compound **16y** in mice liver microsome; (B) compound **16y** in rat liver microsome; (C) compound **16y** in human liver microsome.

Finally, the PK profile of **16y** was determined after single i.v. and p.o. administration in Wistar rats. After a single 1 mg/kg i.v. dose of **16y**, the mean clearance rate (CL) and half-lives was 15.8 ml/min/kg and 1.18 h

(Table 4). Absorption of **16y** was assessed after being dosed at 5.0 mg/kg; it reached maximum concentration ($T_{\max}$) at 0.36 h with a maximum concentration ($C_{\max}$) of 38 ng/ml. And the $t_{1/2}$ of **16y** was up to 2.13 h. However, the oral bioavailability (F) was 2.39% as the highly hydrophobic property of this compound that needs to be salified.

Table 4. Pharmacokinetic Profile of **16y**^a

Parameter	5.0 mg/kg (p.o.)	1.0 mg/kg (i.v.)
AUC _{all} (ng*h/ml)	115±34	1082±232
AUC _{inf} (ng*h/ml)	130±42	1087±234
MRT _{inf} (h)	3.50±1.13	0.86±0.10
$t_{1/2}$ (h)	2.13±0.39	1.18±0.06
$T_{\max}$ (h)	0.67±0.29	-
$C_{\max}$ (ng/ml)	38.4±7.3	-
F (%)	2.39±0.78	-

^aPK parameters (mean ± SD, n = 3)

4. Conclusion

In summary, a novel series of compounds with a biphenyl-DAPY scaffold as HIV-1 RT inhibitors have been disclosed through an alkylation design and the tolerance degree in the pocket which is consisted by residuals V179, E138, and Y181 has also been explored. The alkylated compounds in this series have no observable cytotoxicity up to 285 μ M exception of intruding the hydroxy groups in the molecules. Alkylation hopping in the pyrimidine ring discovered the methyl compound **16y** with very potent anti-HIV-1 inhibitory activity at both enzyme and cell levels. The methyl group was predicted to well occupy the hydrophobic cavity, and importantly, maintain the critical water-mediated hydrogen-bonding interaction. This compound showed no apparent toxicity at a dose of 2 g/kg in normal mice and especially pregnant mice. The in vitro metabolic stability and in vivo PK profile was investigated and provided further directions for future development of the DAPY-based drug discovery.

Experimental section

1. Chemistry

Chemical reagents and solvents, purchased from commercial sources, were of analytical grade and used without further purification. All air-sensitive reactions were under a nitrogen atmosphere. All the reactions were monitored by TLC on the pre-coated silica gel G plates at 254 nm under a UV lamp using ethyl acetate/*n*-hexane as the eluent. Column chromatography was performed on glass column packed with silica gel (200-300 mesh) using ethyl acetate/*n*-hexane as the eluent. Melting points were measured on a SGW X-1 microscopic melting point apparatus. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AV400 MHz spectrometer in DMSO-*d*₆. Chemical shifts were reported in δ (ppm) units relative to the internal standard tetramethylsilane (TMS). Mass spectra and HRMS were obtained on a Waters Quattro Micromass instrument and Brukersolari X-70 FT-MS instrument, respectively, using electrospray ionization (ESI) techniques. The purities of the target compounds were $\geq 95\%$, measured by HPLC, performed on a Waters 2695 system with UV detector and Synergi 4 μ m Hydro-RP 80 Å column (250×4.6 mm), eluting with a mixture of water (A) and methanol or acetonitrile (B) ($V_A:V_B = 50:50$ to $10:90$) over 30 min with a flow rate of 1.0 mL/min and detection at 254 nm.

1.1 General procedure to prepare **10**

Commercially available 2,4-dichloropyrimidine **8** (7.35 g, 50.0 mmol, 1.00 eq), 4-bromo-2,6-difluoroaniline (10.0 g, 50.0 mmol, 1.00 eq) and hydrochloric acid (12 M, 7 mL, cat.) in isopropanol (70 mL) were heated at 40 °C under nitrogen atmosphere. Stirring was continued for an additional 24 h until complete consumption of starting material as judged by TLC (PE/EA = 7/1). Then, an additional 400 mL of saturated NaHCO₃ (aq.) was added and the mixture was further stirred for 30 min. The mixture was extracted with ethyl acetate (40 mL * 3) and washed with brine, dried over anhydrous Na₂SO₄, filtered and condensed. The residue was then purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ethe to **10** as white solid in 43% yield. ¹H NMR (400 MHz, DMSO -*d*₆) δ : 10.01(s, 1H, NH), 8.19 (d, *J* = 4 Hz, 1H, pyrimidine-H), 7.60 (d, *J* = 8 Hz, 2H, ArH), 6.79 (d, *J* = 8 Hz, 1H, pyrimidine-H). MS (ESI) *m/z* C₁₀H₅BrClF₂N₃: calcd 320.52, found 321.37 [M+1]⁺.

1.2 : General procedure to prepare **11a~t**

A reaction mixture of the crude *N*-(4-bromo-2,6-difluorophenyl)-2-chloropyrimidin-4-amine **9** (1.0 mmol, 1.0

eq), methyl iodide (1.25 mmol, 1.25 eq), Cs₂CO₃ (0.650 g, 2 mmol, 2.0 eq) in MeCN (1.5 mL) were heated at 20 °C under nitrogen atmosphere. Stirring was continued for an additional 12 h until complete consumption of starting material as judged by TLC (PE/EA = 7/1). The resulting precipitates were filtered and the filtrate was condensed to give *N*-(4-bromo-2,6-difluorophenyl)-2-chloro-*N*-methylpyrimidin-4-amine, which were used immediately.

All the intermediates were synthesized following the general procedure, starting from **10** and Cs₂CO₃ with different alkyl iodides. To form 4-((4-((4-bromo-2,6-difluorophenyl)(alkyl)amino)pyrimidin-2-yl)amino)benzonitrile **11a~t**, the crude *N*-(4-bromo-2,6-difluorophenyl)-2-chloro-*N*-alkylpyrimidin-4-amine (1.0 mmol, 1.00 eq), 4-aminobenzonitrile (0.9 mmol, 0.90 eq), hydrochloric acid (12 M, 5~6 drops, cat.) in isopropanol (3.0 mL) were heated at 80 °C for 4 h under nitrogen atmosphere. Then, an additional 5 mL of saturated NaHCO₃ (aq.) was added and the mixture was further stirred for 10 min. The mixture was extracted with ethyl acetate (10 mL * 3) and washed with brine, dried over anhydrous Na₂SO₄, filtered and condensed. The residue was then purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **11a~t** as white to yellow solids in 15~75% yield in two steps.

1.3 General procedure to prepare **12a~t**

A mixture of (4-cyanophenyl)boronic acid (0.5 mmol, 1.0 eq.), Pd(dppf)Cl₂ (0.025 mmol, 0.05 eq.) Cs₂CO₃ (1.0 mmol, 2.0 eq.) and **11a~t** (0.5 mmol, 1.0 equiv.) in an aqueous solution of dioxane (7 mL, dioxane: H₂O, v: v = 10: 1) was heated to 110 °C under N₂ atmosphere for 3 h. The reaction was monitored by TLC until its completion. The reaction mixture was then poured into brine (20 mL) and extracted with EtOAc (15 mL * 3). The organic layer was separated and dried with anhydrous Na₂SO₄. The mixture was then concentrated under the reduced pressure. The crude residue was purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **12a~t** as white solid.

1.4 Procedure to prepare **13a**

4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**4**, 0.212 g, 0.5 mmol, 1.0 eq.)⁸ and NaH (60%, 30 mg, 0.75 mmol, 1.5 eq.) was added in an 15 mL tube sealing in 0 °C. Then, DMF (2 mL) was added and stirred in 0 °C for 30 min. iodo(methoxy)methane (109 mg, 0.75 mmol, 1.5 eq.) was dropped into the bottom at 0 °C. After addition, the reaction was warmed into 80 °C and stirred for another 1 h. The reaction was monitored by TLC until its completion. The reaction mixture was then

1 poured into water (5 mL) and extracted with EtOAc (15 mL* 3). The organic layer was separated and dried
2 with anhydrous Na₂SO₄. The mixture was then concentrated under the reduced pressure. The crude residue
3 was purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **13a** as white solid.
4

5 *1.5 Procedure to prepare 13b*

6
7 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**4**, 0.212 g,
8 0.5 mmol, 1.0 eq.)⁸ and NaH (60%, 30 mg, 0.75 mmol, 1.5 eq.) was added in an 15mL tube sealing in 0 °C.
9
10 Then, DMF (2 mL) was added and stirred at 0 °C for 30 min. acetyl chloride (58 mg, 0.75 mmol, 1.5 eq.) was
11
12 dropped into the bottom at 0 °C. After addition, the reaction was warmed into 60 °C and stirred for another 1
13
14 h. The reaction was monitored by TLC until its completion. The reaction mixture was then poured into water
15
16 (5 mL) and extracted with EtOAc (15 mL* 3). The organic layer was separated and dried with anhydrous
17
18 Na₂SO₄. The mixture was then concentrated under the reduced pressure. The crude residue was purified via
19
20 flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **13b** as white solid.
21
22

23 *1.6 General procedure to prepare 13c*

24
25 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**4**, 0.212 g,
26 0.5 mmol, 1.0 eq.)⁸ and NaH (60%, 30 mg, 0.75 mmol, 1.5 eq.) was added in an 15mL tube sealing in 0 °C.
27
28 Then, DMF (2 mL) was added and stirred at 0 °C for 30 min. ethanesulfonyl chloride (96 mg, 0.75 mmol, 1.5
29
30 eq.) was dropped into the bottom at 0 °C. After addition, the reaction was warmed into 60 °C and stirred for
31
32 another 1 h. The reaction was monitored by TLC until its completion. The reaction mixture was then poured
33
34 into water (5 mL) and extracted with EtOAc (15 mL* 3). The organic layer was separated and dried with
35
36 anhydrous Na₂SO₄. The mixture was then concentrated under the reduced pressure. The crude residue was
37
38 purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **13c** as white solid.
39
40

41 *1.7 Procedure to prepare 15*

42
43 To form 4-((4-((4-bromo-2,6-difluorophenyl)amino)-5-methylpyrimidin-2-yl)amino)benzonitrile **15**, the 4-
44
45 ((4-chloro-5-methylpyrimidin-2-yl)amino)benzonitrile (2.08 g, 10 mmol, 1.0 eq.)¹⁶ 4-bromo-2,6-
46
47 difluoroaniline (2.06 g, 10 mmol, 1.0 eq.), hydrochloric acid (12 M, 20 drops, cat.) in isopropanol (20.0 mL)
48
49 were heated at 80 °C for 4 h under nitrogen atmosphere. Then, an additional 50 mL of saturated NaHCO₃ (aq.)
50
51 was added and the mixture was further stirred for 10 min. The mixture was extracted with ethyl acetate (10
52
53 mL * 3) and washed with brine, dried over anhydrous Na₂SO₄, filtered and condensed. The residue was then
54
55
56
57
58
59
60

purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ethe to **15** as yellow solid in 70% yield.

1.8 Procedure to prepare **16y**

A mixture of (4-cyanophenyl)boronic acid (0.5 mmol, 1.0 eq.), Pd(dppf)Cl₂ (0.025 mmol, 0.05 eq.) Cs₂CO₃ (1.0 mmol, 2.0 eq.) and **15** (0.5 mmol, 1.0 equiv.) in an aqueous solution of dioxane (7 mL, dioxane: H₂O, v: v = 10: 1) was heated to 110 °C under N₂ atmosphere for 3 h. The reaction was monitored by TLC until its completion. The reaction mixture was then poured into brine (20 mL) and extracted with EtOAc (15 mL * 3). The organic layer was separated and dried with anhydrous Na₂SO₄. The mixture was then concentrated under the reduced pressure. The crude residue was purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ether to **15** as white solid in 85% yield.

1.9 Procedure to prepare **18**:

A reaction mixture of the crude 4-((4-chloropyrimidin-2-yl)amino)benzonitrile **14b** (1.0 mmol, 1.0 eq), methyl iodide (1.25 mmol, 1.25 eq), Cs₂CO₃ (0.650 g, 2 mmol, 2.0 eq) in MeCN (1.5 mL) were heated at 20°C under nitrogen atmosphere. Stirring was continued for an additional 12 h until complete consumption of starting material as judged by TLC (PE/EA = 7/1). The resulting precipitates were filtered and the filtrate was condensed to give *N*-(4-bromo-2,6-difluorophenyl)-2-chloro-*N*-methylpyrimidin-4-amine, which were used immediately. To form 4-((4-((4-bromo-2,6-difluorophenyl)amino)pyrimidin-2-yl)(methyl)amino)benzonitrile **18**, the crude 4-((4-chloropyrimidin-2-yl)(methyl)amino)benzonitrile (1.0 mmol, 1.00 eq), 4-bromo-2,6-difluoroaniline (0.9 mmol, 0.90 eq), hydrochloric acid (12 M, 5~6 drops, cat.) in isopropanol (3.0 mL) were heated at 80 °C for 4 h under nitrogen atmosphere. Then, an additional 5 mL of saturated NaHCO₃ (aq.) was added and the mixture was further stirred for 10 min. The mixture was extracted with ethyl acetate (10 mL * 3) and washed with brine, dried over anhydrous Na₂SO₄, filtered and condensed. The residue was then purified via flash chromatography on silica gel, eluting with EtOAc/petroleum ethe to **18** as white solids in 75% yield in two steps.

1.10 4-((4-((4-bromo-2,6-difluorophenyl)(methyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11a**)

Yield: 45%. pale yellow solid, mp: 171.2–172.0 °C. ¹H NMR (400 MHz, DMSO -d₆) δ: 9.80 (s, 1H, NH), 8.22 (m, 1H, pyrimidine-H), 7.98(m, 1H, ArH), 7.73(d, *J* = 4 Hz, 2H, ArH), 7.46-7.40 (m, 3H, ArH), 6.53 (s, 1H, pyrimidine-H), 3.31 (s, 3H, CH₃). MS (ESI) *m/z* C₁₈H₁₂BrF₂N₅: calcd 415.02, found 416.39 [M+1]⁺.

1.11 4-((4-((4-bromo-2,6-difluorophenyl)(ethyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11b**)

Yield: 50%. white solid, mp: 204.0–204.5 °C. ¹H NMR (400 MHz, CDCl₃) δ: 8.07 (m, 1H, pyrimidine-H), 8.04 (m, 1H, ArH), 7.53–7.29 (m, 5H, ArH), 6.25–5.79 (m, 1H, pyrimidine-H), 3.88 (m, 2H, CH₂), 1.31 (t, *J* = 8 Hz, 3H, CH₃). MS (ESI) *m/z* C₁₉H₁₄BrF₂N₅: calcd 430.25, found 431.47 [M+1]⁺.

1.12 4-((4-((4-bromo-2,6-difluorophenyl)(propyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11c**)

Yield: 42%. white solid, mp: 182.3–183.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.89 (s, 1H, NH), 8.31 (m, 1H, pyrimidine-H), 7.88 (m, 1H, ArH), 7.79 (d, *J* = 8 Hz, 2H, ArH), 7.39 (dd, *J* = 8 Hz, *J* = 56 Hz, 3H, ArH), 6.91 (m, pyrimidine-H), 3.82–3.74 (m, 2H, CH₂), 1.62–1.57 (m, 2H, CH₂), 0.93–0.89 (m, 3H, CH₃). MS (ESI) *m/z* C₂₀H₁₆BrF₂N₅: calcd 444.28, found 446.08 [M+1]⁺.

1.13 4-((4-((4-bromo-2,6-difluorophenyl)(butyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11d**)

Yield: 48%. white solid, mp: 170.6–171.4 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.91 (s, 1H, NH), 8.34 (m, 1H, pyrimidine-H), 7.86 (m, 1H, ArH), 7.83 (dd, *J* = 4 Hz, *J* = 8 Hz, 2H, ArH), 7.41 (dd, *J* = 8 Hz, *J* = 72 Hz, 3H, ArH), 6.93 (d, *J* = 8 Hz, pyrimidine-H), 3.84–3.82 (m, 2H, CH₂), 1.59–1.33 (m, 4H, CH₂CH₂), 0.89–0.88 (m, 3H, CH₃). MS (ESI) *m/z* C₂₁H₁₈BrF₂N₅: calcd 458.31, found 459.31 [M+1]⁺.

1.14 4-((4-((4-bromo-2,6-difluorophenyl)(isopropyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11e**)

Yield: 39%. white solid, mp: 150.0–150.1 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.91 (s, 1H, NH), 8.35 (m, 1H, pyrimidine-H), 7.86–7.83 (m, 3H, ArH), 7.36 (d, *J* = 52 Hz, 3H, ArH), 6.93 (m, 1H, pyrimidine-H), 4.67 (m, 1H, CH), 1.20 (d, *J* = 4 Hz, 6H, CH₃). MS (ESI) *m/z* C₂₀H₁₆BrF₂N₅: calcd 444.28, found 445.51 [M+1]⁺.

1.15 4-((4-((4-bromo-2,6-difluorophenyl)(isobutyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11f**)

Yield: 40%. pale yellow solid, mp: 167.5–168.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.90 (s, 1H, NH), 8.32 (m, 1H, pyrimidine-H), 7.88 (m, 1H, ArH), 7.83 (dd, *J* = 4 Hz, *J* = 8 Hz, 2H, ArH), 7.42 (dd, *J* = 8 Hz, *J* = 72 Hz, 3H, ArH), 6.99 (m, pyrimidine-H), 3.70–3.68 (m, 2H, CH₂), 1.92–1.88 (m, 1H, CH), 0.95 (d, *J* = 8 Hz, 6H, CH₃). MS (ESI) *m/z* C₂₁H₁₈BrF₂N₅: calcd 458.31, found 459.56 [M+1]⁺.

1.16 4-((4-((4-bromo-2,6-difluorophenyl)(sec-butyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11g**)

Yield: 23%. white solid, mp: 141.3–141.6 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.97 (s, 1H, NH), 8.32 (m, 1H, pyrimidine-H), 7.83 (m, 2H, ArH), 7.46–7.21 (m, 4H, ArH), 6.93 (m, 1H, pyrimidine-H), 4.41 (m, 1H, CH), 1.67–1.38 (m, 2H, CH₂), 0.95 (m, 3H, CH₃), 0.91 (m, 3H, CH₃). MS (ESI) *m/z* C₂₁H₁₈BrF₂N₅:

calcd 458.31, found 459.31 [M+1]⁺.

1.17 4-((4-((4-bromo-2,6-difluorophenyl)(2-methylbutyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11h**)

Yield: 31%. white solid, mp: 151.0–151.7 °C. ¹H NMR (400 MHz, DMSO –d₆, CF₃COOH) δ: 10.89 (s, 1H, NH), 8.33 (s, 1H, pyrimidine-H), 7.87 (m, 1H, ArH), 7.83 (dd, *J* = 4 Hz, *J* = 4 Hz, 2H, ArH), 7.41 (dd, *J* = 72 Hz, 3H, ArH), 6.95 (m, pyrimidine-H), 3.81–3.71 (m, 2H, CH₂), 1.52 (d, *J* = 68 Hz, 2H, CH₂), 1.18–1.15 (m, 1H, CH), 0.93–0.84 (m, 6H, CH₃). MS (ESI) *m/z* C₂₂H₂₀BrF₂N₅: calcd 471.08, found 472.85 [M+1]⁺.

1.18 4-((4-((4-bromo-2,6-difluorophenyl)(cyclopentyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11i**)

Yield: 21%. pale yellow solid, mp: 187.8–189.0 °C. ¹H NMR (400 MHz, DMSO –d₆, CF₃COOH) δ: 10.92 (s, 1H, NH), 8.33 (m, 1H, pyrimidine-H), 7.86 (m, 1H, ArH), 7.85 (d, *J* = 8 Hz, 2H, ArH), 7.38 (d, *J* = 104 Hz, 3H, ArH), 6.99 (m, 1H, pyrimidine-H), 4.65 (m, 1H, CH), 2.07–1.26 (m, 8H, CH₂). MS (ESI) *m/z* C₂₂H₁₈BrF₂N₅: calcd 469.07, found 470.64 [M+1]⁺.

1.19 4-((4-((4-bromo-2,6-difluorophenyl)(cyclopentylmethyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11j**)

Yield: 15%. white solid, mp: 190.2–191.3 °C. ¹H NMR (400 MHz, CDCl₃) δ: 8.05 (m, 1H, pyrimidine-H), 7.77 (m, 3H, ArH), 7.30 (m, 3H, ArH), 5.92 (m, 1H, pyrimidine-H), 3.79 (m, 2H, CH₂), 2.28–2.26 (m, 1H, CH), 1.80–1.25 (m, 8H, CH₂). MS (ESI) *m/z* C₂₃H₂₀BrF₂N₅: calcd 484.34, found 485.19 [M+1]⁺.

1.20 4-((4-((4-bromo-2,6-difluorophenyl)(2-hydroxyethyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11k**)

Yield: 42%. pale yellow solid, mp: 178.2–179.3 °C. ¹H NMR (400 MHz, DMSO –d₆, CF₃COOH) δ: 10.91 (s, 1H, NH), 8.31 (d, *J* = 4 Hz, 1H, pyrimidine-H), 8.22 (m, 1H, ArH), 7.83 (d, *J* = 8 Hz, 2H, ArH), 7.74–7.47 (m, 3H, ArH), 6.81–6.08 (m, pyrimidine-H), 3.93–3.92 (m, 2H, CH₂), 3.66–3.63 (m, 2H, CH₂). MS (ESI) *m/z* C₁₉H₁₄BrF₂N₅O: calcd 445.04, found 446.59 [M+1]⁺.

1.21 4-((4-((4-bromo-2,6-difluorophenyl)(3-hydroxypropyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11l**)

Yield: 36%. white solid, mp: 191.4–192.0 °C. ¹H NMR (400 MHz, DMSO –d₆, CF₃COOH) δ: 10.75 (s, 1H, NH), 8.32–8.06 (m, 1H, pyrimidine-H), 7.95 (m, 1H, ArH), 7.80 (d, *J* = 8 Hz, 3H, ArH), 7.40 (dd, *J* = 8 Hz, *J* = 56 Hz, 2H, ArH), 6.85 (m, pyrimidine-H), 3.86 (m, 2H, CH₂), 3.49 (m, 2H, CH₂), 1.77–1.75 (m, 2H, CH₂). MS (ESI) *m/z* C₂₀H₁₆BrF₂N₅O: calcd 460.28, found 462.01 [M+1]⁺.

1.22 4-(((4-((4-bromo-2,6-difluorophenyl)(2-hydroxypropyl)amino)pyrimidin-2-yl)amino)benzonitrile

(11m)

Yield: 30%. white solid, mp:110.3–111.2 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 9.80 (s, 1H, NH), 8.19 (m, 1H, pyrimidine-H), 7.98-7.70 (m, 3H, ArH), 7.42 (m, 3H, ArH), 6.63 (m, 1H, pyrimidine-H), 4.76 (m, 1H, CHOH), 3.89 (m, 2H, CH₂), 3.61-3.55 (m, 3H, CH₃). MS (ESI) *m/z* C₂₀H₁₆BrF₂N₅O: calcd 460.28, found 461.45 [M+1]⁺.

1.23 4-(((4-((4-bromo-2,6-difluorophenyl)(2,3-dihydroxypropyl)amino)pyrimidin-2-yl)amino)benzonitrile

(11n)

Yield: 32%. white solid, mp:158.0–159.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 9.77 (s, 1H, NH), 8.18 (m, 1H, pyrimidine-H), 8.80 (m, 1H, ArH), 7.70 (m, 2H, ArH), 7.40-7.35 (m, 3H, ArH), 6.60 (m, 1H, pyrimidine-H), 4.83 (m, 2H, CH₂), 4.28-4.14 (m, 1H, CHOH), 3.82-3.69 (m, 2H, CH₂OH). MS (ESI) *m/z* C₂₀H₁₆BrF₂N₅O₂: calcd 475.05, found 476.72 [M+1]⁺.

1.24 4-(((4-((4-bromo-2,6-difluorophenyl)(2-fluoroethyl)amino)pyrimidin-2-yl)amino)benzonitrile (11o)

Yield: 42%. white solid, mp: 188.9–190.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.79 (s, 1H, NH), 8.35 (m, 1H, pyrimidine-H), 7.85 (m, 1H, ArH), 7.82 (d, *J* = 8 Hz, 2H, ArH), 7.42 (dd, *J* = 8 Hz, *J* = 56 Hz, 3H, ArH), 6.93 (m, pyrimidine-H), 4.72-4.61 (m, 2H, CH₂), 4.26-4.19 (m, 2H, CH₂). MS (ESI) *m/z* C₁₉H₁₃BrF₃N₅: calcd 448.25, found 450.44 [M+1]⁺.

1.25 4-(((4-((4-bromo-2,6-difluorophenyl)(2,2-difluoroethyl)amino)pyrimidin-2-yl)amino)benzonitrile

(11p)

Yield: 38%. white solid, mp: 190.4–191.3 °C. ¹H NMR (400 MHz, DMSO *-d*₆) δ: 9.92 (s, 1H, NH), 8.18 (m, 1H, pyrimidine-H), 7.93 (m, 1H, ArH), 7.75 (d, *J*=8 Hz, 2H, ArH), 7.46 (m, 1H, ArH), 7.36 (dd, *J* = 4 Hz, *J* = 4 Hz, 2H, ArH), 6.69 (m, pyrimidine-H), 6.46-6.18 (m, 1H, CH), 4.27-4.20 (m, 2H, CH₂). MS (ESI) *m/z* C₁₉H₁₃BrF₃N₅: calcd 465.02, found 466.48 [M+1]⁺.

1.26 4-(((4-((4-bromo-2,6-difluorophenyl)(3-fluoropropyl)amino)pyrimidin-2-yl)amino)benzonitrile (11q)

Yield: 42%. white solid, mp: 179.3–180.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆) δ: 9.85 (s, 1H, NH), 8.21-7.98 (m, 1H, pyrimidine-H), 7.76 (d, *J* = 8 Hz, 2H, ArH), 7.64-7.40 (m, 2H, ArH), 6.98 (dd, *J* = 12 Hz, *J* = 300 Hz, 2H, ArH), 6.12 (m, pyrimidine-H), 4.53 (dt, *J* = 8 Hz, *J* = 48 Hz, 2H, CH₂), 3.81 (m, 2H, CH₂), 2.00-1.92 (m, 2H, CH₂). MS (ESI) *m/z* C₂₀H₁₅BrF₃N₅: calcd 462.27, found 463.53 [M+1]⁺.

1.27 4-((4-((4-bromo-2,6-difluorophenyl)(3-chloropropyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11r**)

Yield: 42%. white solid, mp:161.0–161.5 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.80 (s, 1H, NH), 8.37 (m, 1H, pyrimidine-H), 7.90 (m, 1H, ArH), 7.84 (dd, *J* = 4 Hz, *J* = 8 Hz, 2H, ArH), 7.50-7.34 (m, 3H, ArH), 6.89 (m, pyrimidine-H), 4.05-3.94 (m, 2H, CH₂), 3.72 (m, 2H, CH₂), 2.10-2.06 (m, 2H, CH₂). MS (ESI) *m/z* C₂₀H₁₅BrClF₂N₅: calcd 477.02, found 478.61 [M+1]⁺.

1.28 4-((4-((4-bromo-2,6-difluorophenyl)(cyanomethyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11s**)

Yield: 48%. white solid, mp: 256.5–257.0 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.39 (s, 1H, NH), 8.43 (m, 1H, pyrimidine-H), 8.22-8.19 (m, 1H, ArH), 8.04 (d, *J* = 8 Hz, 2H, ArH), 7.98-7.74 (m, 3H, ArH), 6.08 (m, pyrimidine-H), 5.06 (m, 2H, CH₂). MS (ESI) *m/z* C₁₉H₁₁BrF₂N₆: calcd 440.02, found 441.51 [M+1]⁺.

1.29 4-((4-((allyl(4-bromo-2,6-difluorophenyl)amino)pyrimidin-2-yl)amino)benzonitrile (**11t**)

Yield: 51%. white solid, mp:155.9–156.3 °C. ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH) δ: 10.79 (s, 1H, NH), 8.34 (m, 1H, pyrimidine-H), 7.90 (m, 1H, ArH), 7.80 (dd, *J* = 4 Hz, *J* = 8 Hz, 2H, ArH), 7.51-7.37 (m, 3H, ArH), 6.81 (m, pyrimidine-H), 5.94-5.84 (m, 1H, CH), 5.41-5.25 (m, 2H, CH₂), 4.49 (m, 2H, CH₂). MS (ESI) *m/z* C₂₀H₁₄BrF₂N₅: calcd 442.27, found 443.25 [M+1]⁺.

1.30 4-((4-((4-bromo-2,6-difluorophenyl)amino)-5-methylpyrimidin-2-yl)amino)benzonitrile (**15**)

Yield: 70%. pale yellow solid, mp:175.4–176.2 °C. ¹H NMR (400 MHz, DMSO *-d*₆) δ: 10.03 (s, 1H, NH), 8.99 (m, 1H, pyrimidine-H), 8.43 (m, 1H, ArH), 8.12-8.02 (m, 4H, ArH), 7.89-7.86 (m, 2H, ArH), 2.94 (s, 3H, CH₃). MS (ESI) *m/z* C₁₈H₁₂BrF₂N₅: calcd 415.02, found 416.67 [M+1]⁺.

1.31 4-((4-((4-bromo-2,6-difluorophenyl)amino)pyrimidin-2-yl)(methyl)amino)benzonitrile (**18**)

Yield: 75%. white solid, mp:150.6-151.5 °C. ¹H NMR (400 MHz, CDCl₃) δ: 8.11 (d, *J* = 8.0 Hz, 1H, pyrimidine-H), 7.61-7.48 (dd, *J* = 8.0 Hz, *J* = 48.0 Hz, 4H, ArH), 7.19 (d, *J* = 8.0 Hz, 2H, ArH), 6.00 (d, *J* = 8.0 Hz, 1H, pyrimidine-H), 3.54 (s, 3H, CH₃). MS (ESI) *m/z* C₁₈H₁₂BrF₂N₅: calcd 415.02, found 416.90 [M+1]⁺.

1.32 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(methyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12a**)

Synthesized following general procedure 1.3. Yield 55%, white solid, mp 267.2-268.4 °C (from MeOH). ¹H NMR (400 MHz, DMSO *-d*₆, 70°C) δ: 9.60 (s, 1H, NH), 8.04 (d, *J* = 8.0 Hz, 2H, ArH), 7.97 (d, *J* = 4.0 Hz,

2H, ArH), 7.77 (d, $J = 8.0$ Hz, 2H, ArH), 7.72-7.44 (m, 2H, ArH), 8.18 (d, $J = 4.0$ Hz, 1H, pyrimidine-H), 6.27 (s, 1H, pyrimidine-H), 3.42 (s, 3H, CH₃). ¹³C NMR (100 MHz, DMSO $-d_6$, 30°C) δ : 162.6, 160.7 (d, $J_{C-F} = 4$ Hz), 159.1, 158.7 (d, $J_{C-F} = 5$ Hz), 158.5, 145.6, 142.0, 139.7, 133.5, 132.9, 128.3, 119.9, 119.0, 118.5, 111.9, 102.3, 96.3, 37.3. ¹³C NMR (100 MHz, DMSO $-d_6$, 70°C) δ : 162.7, 160.8 (d, $J_{C-F} = 4$ Hz), 159.3, 158.8 (d, $J_{C-F} = 5$ Hz), 158.0, 145.6, 142.1, 140.0, 133.4, 133.0, 128.3, 119.8, 118.9, 118.8, 112.0, 111.8 (d, $J_{C-F} = 18$ Hz), 102.5, 96.8, 37.3. HRMS calcd for C₂₅H₁₆F₂N₆ [M+Na]⁺ 438.1405, found 461.1291. HPLC analysis: retention time = 7.51 min; peak area, 99.24% (λ =254 nm).

1.33 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(ethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12b**)

Synthesized following general procedure 1.3. Yield 62%, white solid, mp 242.5-242.7 °C (from MeOH). ¹H NMR (400 MHz, DMSO $-d_6$, CF₃COOH- d) δ : 9.77 (s, 1H, NH), 8.19 (m, 1H, pyrimidine-H), 8.05-7.97 (m, 4H, ArH), 7.83-7.81 (m, 2H, ArH), 7.68-7.23 (m, 3H, ArH), 6.58 (m, 1H, pyrimidine-H), 3.78 (m, 2H, CH₂), 1.19 (t, $J = 4$ Hz, 3H, CH₃). ¹³C NMR (100 MHz, DMSO $-d_6$) δ : 162.1, 161.3 (d, $J_{C-F} = 20$ Hz), 159.3, 158.8 (d, $J_{C-F} = 24$ Hz), 158.4, 157.4, 145.6, 141.9, 139.9, 133.5, 128.3, 120.0, 119.1, 118.4, 111.9, 102.1, 96.1, 44.6, 13.0. HRMS calcd for C₂₆H₁₈F₂N₆ [M+Na]⁺ 452.1561, found 475.1456. HPLC analysis: retention time = 8.03 min; peak area, 97.45% (λ =254 nm).

1.34 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(propyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12c**)

Synthesized following general procedure 1.3. Yield 62%, white solid, mp 228.5-229.1 °C (from MeOH). ¹H NMR (400 MHz, DMSO $-d_6$, CF₃COOH- d) δ : 11.26 (s, 1H, NH), 8.36 (d, $J = 8.0$ Hz, 1H, pyrimidine-H), 8.04 (dd, $J = 8.0$ Hz, $J = 16.0$ Hz, 4H, ArH), 7.88 (s, 4H, ArH), 7.32 (dd, $J = 8.0$ Hz, $J = 16.0$ Hz, 2H, ArH), 7.03 (d, $J = 8.0$ Hz, 1H, pyrimidine-H), 3.88 (m, 2H, CH₂), 1.65 (m, 2H, CH₂), 0.94 (m, 3H, CH₃). ¹³C NMR (100 MHz, DMSO $-d_6$) δ : 163.4, 160.3, 157.8, 152.3, 148.7 (d, $J_{C-F} = 13$ Hz), 141.7 (t, $J_{C-F} = 50$ Hz), 133.5, 133.0, 128.3, 121.2, 120.4, 120.0, 119.0, 117.2, 114.3, 112.1, 111.4, 105.5, 97.0, 52.2, 21.2, 11.0. HRMS calcd for C₂₇H₂₀F₂N₆ [M+Na]⁺ 466.1718, found 489.1610. HPLC analysis: retention time = 8.64 min; peak area, 98.00% (λ =254 nm).

1.35 4'-(butyl(2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12d**)

Synthesized following general procedure 1.3. Yield 45%, pale yellow solid, mp 215.3-216.1 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.2 (s, 1H, *NH*), 8.35 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 8.03 (dd, *J* = 8.0 Hz, *J* = 16.0 Hz, 4H, *ArH*), 7.86 (s, 4H, *ArH*), 7.32 (dd, *J* = 8.0 Hz, *J* = 28.0 Hz, 2H, *ArH*), 7.02 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 3.94-3.89 (m, 2H, *CH*₂), 1.63-1.59 (m, 2H, *CH*₂), 1.41-1.18 (m, 2H, *CH*₂), 0.91-0.84 (m, 3H, *CH*₃). ¹³C NMR (100 MHz, DMSO -*d*₆) δ: 163.4, 160.2, 157.7, 151.8, 147.9, 142.0, 141.7, 133.5, 133.0, 128.3, 121.7, 120.8, 119.9, 118.9, 117.0, 114.2, 111.3, 105.8, 97.0, 50.7, 29.9, 19.5, 14.0. HRMS calcd for C₂₇H₂₀F₂N₆ [M+H]⁺ 480.1874, found 481.1940. HPLC analysis: retention time = 9.30 min; peak area, 100% (λ=254 nm).

1.36 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(isopropyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12e**)

Synthesized following general procedure 1.3. Yield 38%, white solid, mp 248.2-249.3 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.16 (s, 1H, *NH*), 8.37 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 8.06 (dd, *J* = 8.0 Hz, *J* = 12.0 Hz, 4H, *ArH*), 7.88 (s, 2H, *ArH*), 7.24 (dd, *J* = 8.0 Hz, *J* = 32.0 Hz, 2H, *ArH*), 7.02 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 4.74 (m, 1H, *CH*), 1.24 (m, 6H, *CH*₃). ¹³C NMR (100 MHz, DMSO -*d*₆) δ: 163.3, 161.1, 158.6, 152.2, 148.7, 142.3, 141.6, 133.5, 132.9, 128.3, 121.1, 120.2 (d, *J*_{C-F} = 14 Hz), 120.1, 119.0, 117.2, 114.3, 112.2, 111.4, 105.4, 97.2, 51.0, 20.4. HRMS calcd for C₂₇H₂₀F₂N₆ [M+ Na]⁺ 466.1718, found 489.1610. HPLC analysis: retention time = 8.35 min; peak area, 96.88% (λ=254 nm).

1.37 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(isobutyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12f**)

Synthesized following general procedure 1.3. Yield 52%, white solid, mp 229.1-229.8 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.14 (s, 1H, *NH*), 8.35 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 8.04 (dd, *J* = 8.0 Hz, *J* = 20.0 Hz, 4H, *ArH*), 7.89 (s, 4H, *ArH*), 7.33 (dd, *J* = 8.0 Hz, *J* = 20.0 Hz, 2H, *ArH*), 7.07 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 3.76 (d, *J* = 8.0 Hz, 2H, *CH*₂), 1.92 (m, 1H, *CH*), 0.98-0.91 (m, 6H, *CH*₃). ¹³C NMR (100 MHz, DMSO -*d*₆) δ: 163.8, 160.2 (d, *J*_{C-F} = 5 Hz), 157.7 (d, *J*_{C-F} = 5 Hz), 152.4, 148.6, 142.3 (t, *J*_{C-F} = 37 Hz), 133.5, 133.0, 128.3, 120.5 (d, *J*_{C-F} = 14 Hz), 120.0, 119.0, 117.1, 114.3, 112.1, 111.4, 105.5, 97.3, 57.4, 30.5, 30.2, 28.2, 19.8. HRMS calcd for C₂₈H₂₂F₂N₆ [M+H]⁺ 480.1874, found 481.1947. HPLC analysis: retention time = 9.17 min; peak area, 97.75% (λ=254 nm).

1.38 4'-(*sec*-butyl(2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12g**)

Synthesized following general procedure 1.3. Yield 38%, white solid, mp 226.7-227.9 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.18 (s, 1H, NH), 8.36 (d, *J* = 4 Hz, 1H, pyrimidine-*H*), 8.09-7.99 (m, 4H, Ar*H*), 7.93-7.87 (m, 4H, Ar*H*), 7.23 (dd, *J* = 8 Hz, *J* = 16 Hz, 2H, Ar*H*), 7.01 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 4.51-4.46 (m, 1H, CH), 1.75-1.40 (m, 2H, CH₂), 1.00-0.92 (m, 6H, CH₃). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 163.5, 161.2, 158.7, 152.6, 149.0, 142.5, 141.6, 133.5, 132.9, 128.3, 121.1, 119.0, 117.4, 114.5, 112.2, 111.6, 105.4, 97.4, 56.6, 28.0, 17.6, 11.2. HRMS calcd for C₂₈H₂₂F₂N₆ [M+H]⁺ 480.1874, found 481.1933. HPLC analysis: retention time = 8.90 min; peak area, 99.85% (λ=254 nm).

1.39 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2-methylbutyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12h**)

Synthesized following general procedure 1.3. Yield 32%, white solid, mp 224.9-225.8 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.14 (s, 1H, NH), 8.35 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 8.04 (dd, *J* = 8.0 Hz, *J* = 24.0 Hz, 4H, Ar*H*), 7.87 (s, 4H, Ar*H*), 7.31 (d, *J* = 8.0 Hz, *J* = 32.0 Hz, 2H, Ar*H*), 7.21 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 3.88-3.75 (m, 2H, CH₂), 1.67-1.45 (m, 2H, CH₂), 1.28-1.16 (m, 1H, CH), 0.95-0.78 (m, 6H, CH₃). ¹³C NMR (100 MHz, DMSO -*d*₆) δ: 163.8, 160.3 (dd, *J*_{C-F} = 6 Hz, *J*_{C-F} = 3 Hz), 157.8 (dd, *J*_{C-F} = 6 Hz, *J*_{C-F} = 4 Hz), 152.8, 148.2, 141.6 (t, *J*_{C-F} = 12 Hz), 133.5, 128.3, 121.2, 120.4, 120.2, 119.0, 117.3, 114.4, 112.4, 112.1, 111.5, 105.2, 97.2, 56.2, 34.6, 26.4, 16.5, 11.5. HRMS calcd for C₂₇H₂₀F₂N₆ [M+H]⁺ 494.2031, found 495.2097. HPLC analysis: retention time = 9.83 min; peak area, 99.30% (λ=254 nm).

1.40 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(cyclopentyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12i**)

Synthesized following general procedure 1.3. Yield 30%, white solid, mp 265.9-266.9 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 11.17 (s, 1H, NH), 8.37 (m, 1H, pyrimidine-*H*), 8.04 (dd, *J* = 8.0 Hz, *J* = 24.0 Hz, 4H, Ar*H*), 7.89 (d, *J* = 8 Hz, 4H, Ar*H*), 7.24 (d, *J* = 20 Hz, 2H, Ar*H*), 7.03 (m, 1H, pyrimidine-*H*), 5.01-4.68 (m, 1H, CH), 2.10-1.45 (m, 8H, CH₂). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 163.7, 161.0, 158.5, 152.4, 149.0, 141.6, 133.5, 132.9, 128.4, 120.2, 119.0, 117.3, 116.3, 114.4, 112.2, 112.0, 111.6, 105.2, 97.5, 60.2, 30.0, 22.8. HRMS calcd for C₂₉H₂₂F₂N₆ [M+H]⁺ 492.1874, found 493.1947.

HPLC analysis: retention time = 9.22 min; peak area, 99.63% (λ =254 nm).

1.41 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(cyclopentylmethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12j**)

Synthesized following general procedure 1.3. Yield 30%, pale yellow solid, mp 226.5-228.0 °C (from MeOH).

¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.15 (s, 1H, NH), 8.36 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 8.09-7.98 (m, 4H, Ar*H*), 7.92-7.87 (m, 4H, Ar*H*), 7.34 (dd, *J* = 8 Hz, *J* = 16 Hz, 2H, Ar*H*), 7.05 (d, *J* = 4 Hz, 1H, pyrimidine-*H*), 3.87 (d, *J* = 8 Hz, 2H, CH₂), 2.21-2.13 (m, 1H, CH), 1.76-1.17 (m, 8H, CH₂). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 163.4, 160.3 (d, *J*_{C-F} = 5 Hz), 157.8 (d, *J*_{C-F} = 5 Hz), 152.4, 148.8, 141.7 (t, *J*_{C-F} = 36Hz), 133.5, 133.0, 128.3, 121.2, 120.4 (d, *J*_{C-F} = 14 Hz), 120.0, 119.0, 117.2, 114.3, 112.1, 111.4, 105.4, 97.1, 55.0, 38.4, 30.2, 25.1. HRMS calcd for C₃₀H₂₄F₂N₆ [M+H]⁺ 506.2031, found 507.2103.

HPLC analysis: retention time = 10.20 min; peak area, 98.43% (λ =254 nm).

1.42 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2-hydroxyethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12k**)

Synthesized following general procedure 1.3. Yield 56%, white solid, mp 258.0-259.0 °C (from MeOH). ¹H

NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.14 (s, 1H, NH), 8.35 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 8.08-7.98 (m, 4H, Ar*H*), 7.91-7.85 (m, 4H, Ar*H*), 7.34 (dd, *J* = 8 Hz, *J* = 16 Hz, 2H, Ar*H*), 7.03 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 4.03-3.98 (m, 2H, CH₂), 3.70-3.69 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 163.9, 160.2, 157.8, 152.2, 148.2, 142.3, 140.8, 133.5, 133.0, 128.3, 120.6, 119.0, 117.2, 114.3, 112.1, 105.5, 97.6, 59.2, 53.1. HRMS calcd for C₂₆H₁₈F₂N₆O [M+H]⁺ 468.1510, found 469.1583.

HPLC analysis: retention time = 6.28 min; peak area, 97.50% (λ =254 nm).

1.43 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(3-hydroxypropyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12l**)

Synthesized following general procedure 1.3. Yield 49%, white solid, mp 209.4-210.0 °C (from MeOH). ¹H

NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.11 (s, 1H, NH), 8.38 (d, *J*=8 Hz, 1H, pyrimidine-*H*), 8.07-7.85 (m, 8H, Ar*H*), 7.34-7.31 (m, 2H, Ar*H*), 6.95 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 4.06-3.92 (m, 2H, CH₂), 3.54-3.51 (m, 2H, CH₂), 1.83-1.79 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 163.3, 160.3, 158.0, 153.0, 149.7, 142.7, 141.7, 140.9, 133.5, 132.9, 128.3, 120.9, 120.2, 119.0, 117.4, 114.5, 112.1, 105.1, 96.8, 57.8, 48.0, 30.9. HRMS calcd for C₂₇H₂₀F₂N₆O [M+H]⁺ 482.1667, found 483.1739. HPLC

analysis: retention time = 6.56 min; peak area, 99.78% (λ =254 nm).

1.44 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2-hydroxypropyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12m**)

Synthesized following general procedure 1.3. Yield 57%, white solid, mp 248.7-249.2 °C (from MeOH). ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.16 (s, 1H, NH), 8.33(d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 8.19-7.80 (m, 8H, Ar*H*), 7.34-7.29 (m, 2H, Ar*H*), 7.01 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 4.15-3.67 (m, 3H, CH₂-CHOH), 1.17-1.13 (m, 3H, CH₃). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 163.8, 160.2, 157.8, 152.8, 149.0, 142.6, 141.8, 140.6, 133.5, 128.3, 121.0, 120.2, 119.0, 117.5, 114.6, 112.0, 111.8, 111.6, 105.1, 97.8, 65.4, 57.5, 21.4. HRMS calcd for C₂₇H₂₀F₂N₆O [M- H]⁻ 482.1667, found 481.1838. HPLC analysis: retention time = 6.67 min; peak area, 97.45% (λ =254 nm).

1.45 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2,3-dihydroxypropyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12n**)

Synthesized following general procedure 1.3. Yield 63%, white solid, mp 233.2-233.9 °C (from MeOH). ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.16 (s, 1H, NH), 8.35 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 8.07 (d, *J* = 8.0 Hz, 2H, Ar*H*), 7.98 (d, *J* = 12.0 Hz, 2H, Ar*H*), 7.81-7.81(m, 3H, Ar*H*), 7.62-7.45 (m, 1H, Ar*H*), 7.33 (dd, *J* = 8Hz, *J* = 16 Hz, 2H, Ar*H*), 7.00 (d, *J* = 8.0 Hz, 1H, pyrimidine-*H*), 4.48-3.70 (m, 4H, CH₂OH-CH₂OH), 3.48-3.34 (m, 2H, CH₂N). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 164.0, 160.2, 157.7, 152.2, 148.2, 142.3, 141.8, 140.7, 134.6, 133.8, 133.5, 133.0, 128.3, 121.1, 120.5, 119.0, 117.3, 112.0, 105.5, 97.8, 70.6, 63.6, 54.1. HRMS calcd for C₂₇H₂₀F₂N₆O₂ [M- H]⁺ 498.1616, found 499.1689. HPLC analysis: retention time = 5.78 min; peak area, 98.53% (λ =254 nm).

1.46 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2-fluoroethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12o**)

Synthesized following general procedure 1.3. Yield 52%, white solid, mp 232.8-233.4 °C (from MeOH). ¹H NMR (400 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 11.04 (s, 1H, NH), 8.38-8.37 (m, 1H, pyrimidine-*H*), 8.12-7.84 (m, 8H, Ar*H*), 7.42-7.23 (m, 2H, Ar*H*), 7.02-7.00 (m, 1H, pyrimidine-*H*), 4.76 (dd, *J* = 16 Hz, *J* = 32 Hz, 2H, CH₂), 4.30 (d, *J* = 28 Hz, 2H, CH₂). ¹³C NMR (100 MHz, DMSO *-d*₆, CF₃COOH-*d*) δ : 163.8, 160.4, 157.9, 152.9, 149.7, 141.7, 133.5, 128.3, 120.4, 119.9, 119.0, 117.1, 114.2, 112.1, 111.7, 111.3, 105.3, 97.2, 83.1, 81.4. HRMS calcd for C₂₆H₁₇F₃N₆ [M+H]⁺ 470.1467, found 471.1540. HPLC analysis: retention time = 7.20

min; peak area, 98.72% (λ =254 nm).

1.47 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(2,2-difluoroethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12p**)

Synthesized following general procedure 1.3. Yield 53%, white solid, mp 237.5-237.8 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ : 10.76 (s, 1H, NH), 8.40-8.38 (m, 1H, pyrimidine-*H*), 8.05-7.84 (m, 8H, Ar*H*), 7.33 (m, 2H, Ar*H*), 6.99 (m, 1H, pyrimidine-*H*), 6.52-6.05 (m, 1H, CH), 4.44-4.41 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ : 164.0, 163.0, 160.5, 158.0, 156.2, 154.5, 153.4, 152.3, 143.2, 141.7, 141.0, 133.4, 128.3, 120.0, 119.0, 117.1, 114.8, 114.2, 112.1, 111.3, 104.6 (d, *J*_{C-F} = 8 Hz), 97.3, 51.1. HRMS calcd for C₂₆H₁₆F₄N₆ [M+H]⁺ 488.1373, found 489.1445. HPLC analysis: retention time = 7.57 min; peak area, 96.16% (λ =254 nm).

1.48 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(3-fluoropropyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12q**)

Synthesized following general procedure 1.3. Yield 51%, white solid, mp 240.4-241.0 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ : 11.05 (s, 1H, NH), 8.39 (d, *J* = 4 Hz, 1H, pyrimidine-*H*), 8.05-7.80 (m, 8H, Ar*H*), 7.36-7.31 (m, 2H, Ar*H*), 6.95 (m, 1H, pyrimidine-*H*), 4.62-4.50 (m, 2H, CH₂), 4.09-3.98 (m, 2H, CH₂), 2.10-2.00 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO -*d*₆) δ : 163.3, 160.3, 157.9, 153.6, 150.0, 142.6, 141.7, 133.5, 128.3, 120.7 (dd, *J*_{C-F} = 15 Hz, *J*_{C-F} = 56 Hz), 120.0, 119.0, 117.1, 114.3, 112.1, 111.4, 105.2, 96.7, 82.2 (dd, *J*_{C-F} = 51 Hz, *J*_{C-F} = 110 Hz), 47.2 (d, *J*_{C-F} = 35 Hz), 28.8. HRMS calcd for C₂₇H₁₉F₃N₆ [M+H]⁺ 484.1629, found 485.1696. HPLC analysis: retention time = 7.83 min; peak area, 97.66% (λ =254 nm).

1.49 4'-((3-chloropropyl)(2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12r**)

Synthesized following general procedure 1.3. Yield 45%, white solid, mp 217.4-218.6 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ : 11.11 (s, 1H, NH), 8.39 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 8.12-7.79 (m, 8H, Ar*H*), 7.31 (s, 2H, Ar*H*), 6.92 (d, *J* = 8 Hz, 1H, pyrimidine-*H*), 4.09-3.97 (m, 2H, CH₂), 3.75-3.71 (m, 2H, CH₂), 2.13-2.11 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ : 163.1, 160.4 (d, *J*_{C-F} = 5 Hz), 157.9 (d, *J*_{C-F} = 5 Hz), 153.9, 151.1, 143.0, 141.7, 141.0, 133.4, 128.3, 120.3, 118.9, 117.4, 114.5, 112.5, 112.1 (d, *J*_{C-F} = 56 Hz), 104.8, 96.6, 48.1 (d, *J*_{C-F} = 18 Hz), 42.7 (d, *J*_{C-F} = 54 Hz), 30.49 (d, *J*_{C-F}

= 52 Hz). HRMS calcd for $C_{27}H_{19}ClF_2N_6$ $[M+H]^+$ 500.1328, found 501.1401. HPLC analysis: retention time = 8.33 min; peak area, 97.50% ($\lambda=254$ nm).

1.50 4'-((cyanomethyl)(2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12s**)

Synthesized following general procedure 1.3. Yield 51%, white solid, mp 280.1-280.4 °C (from MeOH). 1H NMR (400 MHz, DMSO $-d_6$, $CF_3COOH-d$) δ : 10.63 (s, 1H, NH), 8.45-8.21 (m, 1H, pyrimidine-H), 8.07-7.99 (m, 4H, ArH), 7.91 (d, $J = 8$ Hz, 4H, ArH), 7.83-7.35 (m, 2H, ArH), 6.92-6.11 (m, 1H, pyrimidine-H), 6.11 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO $-d_6$, $CF_3COOH-d$) δ : 162.0, 160.8, 156.9, 144.0, 141.7, 133.5, 128.4, 120.1, 119.9, 119.7, 119.0, 117.0, 116.6, 114.1, 112.2, 111.3, 104.2, 97.9, 65.5, 60.2. HRMS calcd for $C_{26}H_{15}F_2N_7$ $[M+Na]^+$ 463.1357, found 486.1249. HPLC analysis: retention time = 6.22 min; peak area, 99.68% ($\lambda=254$ nm).

1.51 4'-(allyl(2-((4-cyanophenyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**12t**)

Synthesized following general procedure 1.3. Yield 63%, white solid, mp 233.2-233.9 °C (from MeOH). 1H NMR (400 MHz, DMSO $-d_6$, $CF_3COOH-d$) δ : 11.07 (s, 1H, NH), 8.37 (d, $J = 8$ Hz, 1H, pyrimidine-H), 8.06-7.79 (m, 8H, ArH), 7.33 (s, 2H, ArH), 6.86 (d, $J = 8$ Hz, 1H, pyrimidine-H), 6.05-5.89 (m, 1H, $CH=CH_2$), 5.44-5.26 (m, 2H, $CH=CH_2$), 4.59-4.53 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO $-d_6$, $CF_3COOH-d$) δ : 163.2, 160.4, 157.9, 154.6, 153.8, 150.7, 142.9, 141.7, 140.8, 133.4, 133.4, 132.9, 131.8, 128.3, 120.8, 119.5, 119.0, 117.4, 114.5, 112.1, 111.6, 104.8, 97.2, 53.2. HRMS calcd for $C_{27}H_{18}F_2N_6$ $[M+H]^+$ 464.1561, found 465.1634. HPLC analysis: retention time = 8.10 min; peak area, 97.55% ($\lambda=254$ nm).

1.52 4'-((2-((4-cyanophenyl)amino)pyrimidin-4-yl)(methoxymethyl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**13a**)

Synthesized following general procedure 1.4. Yield 72%, white solid, mp 238.6-239.2 °C (from MeOH). 1H NMR (400 MHz, DMSO $-d_6$) δ : 9.92 (s, 1H, NH), 8.25 (m, 1H, pyrimidine-H), 8.04 (dd, $J = 8$ Hz, $J = 2.0$ Hz, 4H, ArH), 7.93-7.22 (m, 6H, ArH), 6.69 (m, 1H, pyrimidine-H), 5.18 (s, 2H, CH_2), 1.20 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO $-d_6$) δ : 174.8, 162.7, 159.1, 158.7, 158.6, 145.4, 141.9, 133.5, 133.0, 130.0, 128.3, 119.9, 119.1, 118.5, 111.9, 111.7, 102.4, 97.4, 55.6, 29.5. HRMS calcd for $C_{26}H_{18}F_2N_6O$ $[M+Na]^+$ 468.1510, found 491.1402. HPLC analysis: retention time = 7.30 min; peak area, 96.70% ($\lambda=254$ nm).

1.53 *N*-(4'-cyano-3,5-difluoro-[1,1'-biphenyl]-4-yl)-*N*-(2-((4-cyanophenyl)amino)pyrimidin-4-yl)acetamide (**13b**)

Synthesized following general procedure 1.5. Yield 64%, white solid, mp 245.9-246.4 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 10.20 (s, 1H, *NH*), 8.12-8.01 (dd, *J* = 8.0 Hz, *J* = 28.0 Hz, 4H, *ArH*), 7.98 (d, *J* = 8.0 Hz, 2H, *ArH*), 7.37-7.29 (dd, *J* = 8.0 Hz, *J* = 16.0 Hz, 4H, *ArH*), 8.56 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 7.60 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 2.21 (s, 3H, CH₃). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 171.2, 162.8, 160.7, 160.4 (d, *J*_{C-F} = 24 Hz), 159.6, 159.0, 158.0 (d, *J*_{C-F} = 20 Hz), 144.7, 141.9 (t, *J*_{C-F} = 40 Hz), 141.6, 133.6, 132.9, 128.4, 119.6, 119.0, 118.5, 112.2, 105.1, 102.9, 25.0. HRMS calcd for C₂₆H₁₆F₂N₆O [M- Na]⁺ 466.1354, found 489.1246. HPLC analysis: retention time = 6.78 min; peak area, 98.44% (λ=254 nm).

1.54 *N*-(4'-cyano-3,5-difluoro-[1,1'-biphenyl]-4-yl)-*N*-(2-((4-cyanophenyl)amino)pyrimidin-4-yl)ethanesulfonamide (**13c**)

Synthesized following general procedure 1.8. Yield 85%, white solid, mp 209.4-210.3 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 10.30 (s, 1H, *NH*), 8.10-8.01 (dd, *J* = 8.0 Hz, *J* = 20 Hz, 4H, *ArH*), 7.94 (s, 1H, *ArH*), 7.92 (s, 1H, *ArH*), 8.45 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 7.71-7.54 (dd, *J* = 8.0 Hz, 4H, *ArH*), 6.34 (d, *J* = 4.0 Hz, 1H, pyrimidine-*H*), 3.95 (m, 2H, CH₂), 1.39 (t, *J* = 4.0 Hz, 3H, CH₃). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 161.5 (d, *J*_{C-F} = 5 Hz), 160.6, 159.7, 159.0 (d, *J*_{C-F} = 4 Hz), 158.8, 144.6, 142.7 (t, *J*_{C-F} = 10 Hz), 141.6, 133.5, 133.2, 128.4, 119.0, 114.0, 112.3, 112.5 (d, *J*_{C-F} = 22 Hz), 103.4, 100.3, 50.0, 7.9. HRMS calcd for C₂₆H₁₆F₂N₆O [M+Na]⁺ 516.1180, found 539.1072. HPLC analysis: retention time = 19.44 min; peak area, 98.24% (λ=254 nm).

1.55 4'-((2-((4-cyanophenyl)amino)-5-methylpyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (**16y**)

Synthesized following general procedure 1.8. Yield 85%, white solid, mp 209.4-210.3 °C (from MeOH). ¹H NMR (400 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 9.58 (s, 1H, *NH*), 8.61 (s, 1H, *NH*), 8.06-7.97 (dd, *J* = 8.0 Hz, *J* = 20.0 Hz, 4H, *ArH*), 7.78 (d, *J* = 8.0 Hz, 2H, *ArH*), 7.63-7.35 (dd, *J* = 12.0 Hz, 4H, *ArH*), 7.99 (s, 1H, pyrimidine-*H*), 2.15 (s, 3H, CH₃). ¹³C NMR (100 MHz, DMSO -*d*₆, CF₃COOH-*d*) δ: 160.8, 160.7-158.2 (dd, *J*_{C-F} = 6 Hz, *J*_{C-F} = 240 Hz), 158.1, 156.5, 146.0, 142.2, 138.3 (t, *J*_{C-F} = 10 Hz), 133.5, 133.0, 128.1, 120.1, 119.1, 118.1, 117.3 (t, *J*_{C-F} = 8 Hz), 111.6, 111.2-111.1 (dd, *J*_{C-F} = 6 Hz, *J*_{C-F} = 13 Hz), 107.2, 101.6, 13.8.

HRMS calcd for $C_{26}H_{16}F_2N_6O$ $[M+H]^+$ 438.1405, found 439.1477. HPLC analysis: retention time = 6.82 min; peak area, 98.23% ($\lambda=254$ nm).

1.56 4'-((2-((4-cyanophenyl)(methyl)amino)pyrimidin-4-yl)amino)-3',5'-difluoro-[1,1'-biphenyl]-4-carbonitrile (19z)

Synthesized following general procedure 1.3. Yield 65%, white solid, mp 214.8-215.4 °C (from MeOH). 1H NMR (400 MHz, DMSO $-d_6$) δ : 9.24 (s, 1H, NH), 7.67 (d, $J = 4.0$ Hz, 2H, ArH), 7.65 (d, $J = 4.0$ Hz, 2H, ArH), 8.00-7.94 (m, 4H, ArH), 7.48-7.46 (m, 2H, ArH), 8.08 (d, $J = 8.0$ Hz, 1H, pyrimidine-H), 6.29 (d, $J = 4.0$ Hz, 1H, pyrimidine-H), 3.41 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO $-d_6$) δ : 161.2 (d, $J_{C-F} = 66$ Hz), 159.9 (d, $J_{C-F} = 6$ Hz), 157.3 (d, $J_{C-F} = 6$ Hz), 156.9, 149.5, 142.0, 137.3 (t, $J_{C-F} = 9$ Hz), 133.4, 132.5, 128.0, 125.5, 119.5 (d, $J_{C-F} = 34$ Hz), 116.4 (t, $J_{C-F} = 16$ Hz), 111.4, 111.1 (d, $J_{C-F} = 7$ Hz), 110.9 (d, $J_{C-F} = 7$ Hz), 105.4, 98.8, 37.2. HRMS calcd for $C_{25}H_{16}F_2N_6$ $[M+H]^+$ 438.1405, found 439.1734. HPLC analysis: retention time = 6.88 min; peak area, 97.75% ($\lambda=254$ nm).

2. In Vitro Anti-HIV Assay.

Evaluation of the antiviral activity of the compounds against HIV in MT-4 cells was performed using the MTT assay as previously described.^{24, 25} Stock solutions (10 x final concentration) of test compounds were added in 25 μ l volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock- and HIV-infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated HIV- and mock-infected cell samples were included as controls. HIV stock (50 μ l) at 100-300 CCID₅₀ (50 % cell culture infectious doses) 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 effects of test compound on uninfected cells in order to assess the cytotoxicity of the test compounds. Exponentially growing MT-4 cells were centrifuged for 5 minutes at 220 g 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 using the MTT assay. The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics) by mitochondrial dehydrogenase activity in metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The

absorbances were read in an eight-channel computer-controlled photometer (Infinite M1000, Tecan), at two wavelengths (540 and 690 nm). All data were calculated using the median absorbance value of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The concentration achieving 50% protection against the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

3. Reverse transcriptase assay.

Recombinant wild type p66/p51 HIV-1 RT was expressed and purified as described.^{26, 27} The RT assay was performed with the EnzCheck Reverse Transcriptase Assay kit (Molecular Probes, Invitrogen), as described by the Manufacturer. The assay is based on the dsDNA quantitation reagent PicoGreen. This reagent shows a pronounced increase in fluorescence signal upon binding to dsDNA or RNA-DNA heteroduplexes. Single-stranded nucleic acids generate only minor fluorescence signal enhancement when a sufficiently high dye:base pair ratio is applied²⁸. This condition is met in the assay. A poly(rA) template of approximately 350 bases long, and an oligo(dT)16 primer, are annealed in a molar ratio of 1:1.2 (60 min. at room temperature). Fifty-two ng of the RNA/DNA is brought into each well of a 96-well plate in a volume of 20 μ l polymerization buffer (60 mM Tris-HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, 100 μ M dTTP, pH 8.1). Five μ l of RT enzyme solution, diluted to a suitable concentration in enzyme dilution buffer (50 mM Tris-HCl, 20% glycerol, 2 mM DTT, pH 7.6), is added. The reactions are incubated at 25°C for 40 minutes and then stopped by the addition of EDTA (15 mM *fc*). Heteroduplexes are then detected by addition of PicoGreen. Signals are read using an excitation wavelength of 490 nm and emission detection at 523 nm using a spectrofluorometer (Safire 2, Tecan). To test the activity of compounds against RT, 1 μ l of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Results are expressed as relative fluorescence i.e. the fluorescence signal of the reaction mix with compound divided by the signal of the same reaction mix without compound.

4. Molecular Modelling

The modeling study was carried out using Schrödinger Maestro 11.4. HIV-1 RT (PDB entry: 2ZD1) is mutated and minimized by Schrödinger BioLuminate. The protein preparation followed a standard protocol.^{5, 14, 29-31} The docking study was performed using Glide. The parameters choose the default without any constraints.

The molecular docking result was generated using PyMol (<http://pymol.sourceforge.net/>).

5. Acute toxicity assay and HE staining of organ tissues

All animal experiments were carried out in adherence with the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011) and were approved by the Fudan University Committee on Animal Care (2020CHEM-JS-005). Compound **16y** was first suspended in 80% PEG400 solution. Pregnant (5-10 days during pregnancy) and female mice (18-22 g) were randomly divided into four groups: pregnant control group (n = 3), female control group (n = 3), pregnant test group (n = 3), and female test group (n = 3). All mice had been fasted for 12 h, and then the mice in test groups were administered intragastrically at a dose of 2 g / kg of **16y** while the mice in control groups were administered with the same volume of vehicle solution on the first day. The death, abnormal behaviors, and body weight of these mice were monitored every day during the subsequent 2 weeks. All the mice were dissected at day 14, and the brain, heart, liver, spleen, lung, and kidney were extracted. These organs were then examined by HE to see if any damage occurred.

ASSOCIATED CONTENT

Supporting Information: [1H and 13C NMR spectra, HPLC and/or HRMS spectra \(DOCX\)](#).

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ABBREVIATIONS

DAPY, diarylpyrimidine; HIV-1, human immunodeficiency virus type one; NNRTI, non-nucleoside reverse transcriptase inhibitor; PK, pharmacokinetics; RT, reverse transcriptase; SI, selectivity index; WT, wild-type; NVP, nevirapine; EFV, efavirenz; ETR, etravirine; RPV, rilpivirine.

REFERENCES

1. https://www.unaids.org/sites/default/files/media_asset/2019-UNAIDS-data_en.pdf. (accessed on Jan, 20, 2021).
2. De Clercq, E. The design of drugs for HIV and HCV. *Nat Rev Drug Discov* **2007**, 6, 1001-1018.
3. Zhan, P.; Chen, X.; Li, D.; Fang, Z.; De Clercq, E.; Liu, X. HIV-1 NNRTIs: structural diversity, pharmacophore similarity, and implications for drug design. *Med Res Rev* **2013**, 33 Suppl 1, E1-72.
4. Namasivayam, V.; Vanangamudi, M.; Kramer, V. G.; Kurup, S.; Zhan, P.; Liu, X.; Kongsted, J.; Byraredy, S. N. The Journey of HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) from Lab to Clinic. *J Med Chem* **2019**, 62, 4851-4883.
5. Jin, K.; Liu, M.; Zhuang, C.; De Clercq, E.; Pannecouque, C.; Meng, G.; Chen, F. Improving the positional adaptability: structure-based design of biphenyl-substituted diaryltriazines as novel non-nucleoside HIV-1 reverse transcriptase inhibitors. *Acta Pharm Sin B* **2020**, 10, 344-357.
6. Jin, K.; Yin, H.; De Clercq, E.; Pannecouque, C.; Meng, G.; Chen, F. Discovery of biphenyl-substituted diarylpyrimidines as non-nucleoside reverse transcriptase inhibitors with high potency against wild-type and mutant HIV-1. *Eur J Med Chem* **2018**, 145, 726-734.
7. Lei, Y.; Han, S.; Yang, Y.; Pannecouque, C.; De Clercq, E.; Zhuang, C.; Chen, F. E. Design of Biphenyl-Substituted Diarylpyrimidines with a Cyanomethyl Linker as HIV-1 NNRTIs via a Molecular Hybridization Strategy. *Molecules* **2020**, 25, 1050.
8. Sang, Y.; Han, S.; Pannecouque, C.; De Clercq, E.; Zhuang, C.; Chen, F. Ligand-Based Design of Nondimethylphenyl-Diarylpyrimidines with Improved Metabolic Stability, Safety, and Oral Pharmacokinetic Profiles. *J Med Chem* **2019**, 62, 11430-11436.
9. Sang, Y.; Han, S.; Pannecouque, C.; De Clercq, E.; Zhuang, C.; Chen, F. Conformational restriction design of thiophene-biphenyl-DAPY HIV-1 non-nucleoside reverse transcriptase inhibitors. *Eur J Med Chem* **2019**, 182, 111603.
10. Chen, X.; Ding, L.; Tao, Y.; Pannecouque, C.; De Clercq, E.; Zhuang, C.; Chen, F. E. Bioisosterism-based design and enantiomeric profiling of chiral hydroxyl-substituted biphenyl-diarylpyrimidine nonnucleoside HIV-1 reverse transcriptase inhibitors. *Eur J Med Chem* **2020**, 202, 112549.
11. Yang, Y.; Pannecouque, C.; Clercq, E.; Zhuang, C.; Chen, F. E. Privileged scaffold inspired design of novel oxime-biphenyl-DAPYs in treatment of HIV-1. *Bioorg Chem* **2020**, 99, 103825.
12. Ding, L.; Zhuang, C.; Chen, F. E. Druggability modification strategies of the diarylpyrimidinetype non-nucleoside reverse transcriptase inhibitors. *Med Res Rev* **2021**, DOI: 10.1002/med.21760.
13. Das, K.; Clark, A. D., Jr.; Lewi, P. J.; Heeres, J.; De Jonge, M. R.; Koymans, L. M.; Vinkers, H. M.; Daeyaert, F.; Ludovici, D. W.; Kukla, M. J.; De Corte, B.; Kavash, R. W.; Ho, C. Y.; Ye, H.; Lichtenstein, M. A.; Andries, K.; Pauwels, R.; De Bethune, M. P.; Boyer, P. L.; Clark, P.; Hughes, S. H.; Janssen, P. A.; Arnold, E. Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* **2004**, 47, 2550-60.
14. Zhuang, C.; Pannecouque, C.; De Clercq, E.; Chen, F. Development of non-nucleoside reverse transcriptase inhibitors (NNRTIs): our past twenty years. *Acta Pharm Sin B* **2020**, 10, 961-978.
15. Apgar, J. M.; Wilkening, R. R.; Greenlee, M. L.; Balkovec, J. M.; Flattery, A. M.; Abruzzo, G. K.; Galgoci, A. M.; Giacobbe, R. A.; Gill, C. J.; Hsu, M. J.; Liberator, P.; Misura, A. S.; Motyl, M.; Nielsen Kahn, J.; Powles, M.; Racine, F.; Dragovic, J.; Habulihaz, B.; Fan, W.; Kirwan, R.; Lee, S.; Liu, H.; Mamai, A.; Nelson, K.; Peel, M. Novel orally active inhibitors of beta-1,3-glucan synthesis derived from enfumafungin. *Bioorg Med Chem Lett* **2015**, 25, 5813-8.
16. Li, T. T.; Pannecouque, C.; De Clercq, E.; Zhuang, C. L.; Chen, F. E. Scaffold Hopping in Discovery of HIV-1 Non-Nucleoside Reverse Transcriptase Inhibitors: From CH(CN)-DABOs to CH(CN)-DAPYs. *Molecules* **2020**, 25, 1581.
17. Fyfe, J. W.; Fazakerley, N. J.; Watson, A. J. Chemoselective Suzuki-Miyaura Cross-Coupling via Kinetic Transmetalation. *Angew Chem Int Ed* **2017**, 56, 1249-1253.

18. Moss, G. P. Basic terminology of stereochemistry (IUPAC Recommendations 1996). *Pure Appl Chem* **2009**, 68, 2193-2222.
19. Hu, D. X.; Grice, P.; Ley, S. V. Rotamers or diastereomers? An overlooked NMR solution. *J Org Chem* **2012**, 77, 5198-202.
20. Heaney, F.; Fenlon, J.; McArdle, P.; Cunningham, D. α -Keto amides as precursors to heterocycles--generation and cycloaddition reactions of piperazin-5-one nitrones. *Org Biomol Chem* **2003**, 1, 1122-32.
21. Lappert, M. F.; Misra, M. C.; Onyszchuk, M.; Rowe, R. S.; Power, P. P.; Slade, M. J. Subvalent group 14 metal compounds XI. Oxidative addition reactions of organic halides or acid anhydrides (including $\text{CH}_4 - \text{nCln}$, PhBr , $\text{BrN}(\text{SiMe}_3)_2$, BuCOCl , or $(\text{CF}_3\text{CO})_2\text{O}$) to some bivalent group 14 metal amides or alkyls. *J Organomet Chem* **1987**, 330, 31-46.
22. Gu, S. X.; Lu, H. H.; Liu, G. Y.; Ju, X. L.; Zhu, Y. Y. Advances in diarylpyrimidines and related analogues as HIV-1 nonnucleoside reverse transcriptase inhibitors. *Eur J Med Chem* **2018**, 158, 371-392.
23. Frange, P.; Tubiana, R.; Sibiude, J.; Canestri, A.; Arvieux, C.; Brunet-Cartier, C.; Cotte, L.; Reynes, J.; Mandelbrot, L.; Warszawski, J.; Le Chenadec, J.; Group, A. E. C. C. S. Rilpivirine in HIV-1-positive women initiating pregnancy: to switch or not to switch? *J Antimicrob Chemother* **2020**, 75, 1324-1331.
24. Pannecouque, C.; Daelemans, D.; De Clercq, E. Tetrazolium-based colorimetric assay for the detection of HIV replication inhibitors: revisited 20 years later. *Nat Protoc* **2008**, 3, 427-34.
25. Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* **1988**, 20, 309-21.
26. Auwerx, J.; North, T. W.; Preston, B. D.; Klarmann, G. J.; De Clercq, E.; Balzarini, J. Chimeric human immunodeficiency virus type 1 and feline immunodeficiency virus reverse transcriptases: role of the subunits in resistance/sensitivity to non-nucleoside reverse transcriptase inhibitors. *Mol Pharmacol* **2002**, 61, 400-6.
27. Ma, H.; Wu, Y.; Zhang, W.; Zhang, H.; Miao, Z.; Zhuang, C. Radiosensitization of human pancreatic cancer by piperlongumine analogues. *Chin Chem Lett* **2020**, DOI:10.1016/j.cclet.2020.08.049.
28. Singer, V. L.; Jones, L. J.; Yue, S. T.; Haugland, R. P. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Anal Biochem* **1997**, 249, 228-38.
29. Wang, H.; Wei, W.; Lan, X.; Liu, N.; Li, Y.; Ma, H.; Sun, T.; Peng, X.; Zhuang, C.; Yu, J. Neuroprotective Effect of Swertiamain on Cerebral Ischemia/Reperfusion Injury by Inducing the Nrf2 Protective Pathway. *ACS Chem Neurosci* **2019**, 10, 2276-2286.
30. Zhuang, C.; Narayanapillai, S.; Zhang, W.; Sham, Y. Y.; Xing, C. Rapid identification of Keap1-Nrf2 small-molecule inhibitors through structure-based virtual screening and hit-based substructure search. *J Med Chem* **2014**, 57, 1121-6.
31. Han, S.; Lei, Y.; Pannecouque, C.; De Clercq, E.; Zhuang, C.; Chen, F. Fragment-based discovery of sulfur-containing diarylbenzopyrimidines as novel nonnucleoside reverse transcriptase inhibitors. *Chin Chem Lett* **2020**, 31, 764-768.