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Discovery of Novel Pyridazinylthioacetamides As Potent HIV-1 NNRTIs Using A Structure-Based Bioisosterism Approach

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ABSTRACT: In continuation of our endeavors to develop new, potent, selective, and less toxic anti-HIV agents, we describe our structure-based bioisosterism design, synthetic strategy, and structure-activity relationship (SAR) studies that led to the identification of pyridazinylthioacetamides, anovel class of NNRTIs, isosteres of arylazolylthioacetanilide derivatives. Nearly all of the tested compounds inhibited HIV-1 strain IIIB replication at the lower micromolar concentration range (EC_{50}: 0.046-5.46μM). Notably, the most promising compound**8k**exhibited extremely potent inhibitory activity against HIV-1 replication with EC_{50} value of 0.046μM, $CC_{50}$of 99.9μM and the viral selectivity index amounted up to 2149. These values were much better than those of NVP (EC_{50}= 0.09 μM) andDDC (EC_{50}= 1.04 μM). Compound **8k** also exhibited moderate inhibition of enzymatic activity with an IC_{50} value of 4.06 μM, which was at the same order of magnitude as that of NVP (2.74 μM). Docking calculations were also performedto investigate the binding mode of compound **8k** into the non-nucleoside binding site of HIV-1 RT andto rationalize some SARs.

1. Introduction

The reverse transcriptase (RT) is one of the main therapeutic targets for the design of anti-HIV-1 drugs used in thetreatment of acquired immunodeficiency syndrome (AIDS).[1,2]Among RT inhibitors, the non-nucleoside reverse transcriptase inhibitors (NNRTIs), binding to anallosteric pocket located 10 Å away from the DNA polymerase active site, are a major component of the current therapeutic regimens for AIDS, namely as highly active antiretroviral therapy (HAART). Nevertheless, the long-term use of NNRTIs is compounded by thee mergence of drug-resistant viruses and potentially severe side effects. Therefore, to alleviate these problems,

there is a substantial need for the identification of novel NNRTIs with improved activity against clinically relevant resistant mutants, and excellent pharmacokinetic and safety profiles.[3,4]

Among the structurally diverse HIV-1 NNRTIs, substituted arylazolylthioacetanilidescaffolds have been of much interest for the development of novel NNRTIs because oftheir high potency and low toxicity against HIV-1 wild-type and resistant strains.[5-11] Especially,1,2,4-triazole derivatives VRX-480733 [12] and RDEA806 [13], wereselected as candidates for further studies. RDEA806, a promising newdrug candidateundergoing phase IIa clinical trial (by Ardea Biosciences Company), was highly effective against mutant HIV-1 strains and exhibited reduced clinical adverse reactions and serum half-life for once-daily dosing (**Fig. 1**).

Fig. 1. Azolylthioacetanilide-based NNRTIs.

In our previous studies, we reported that a series of new arylazolylthioacetanilide with structurally diverse azoles scaffolds possessed potent anti-HIV activities in a cell-based replicon system.[14-22]Among them, 1,2,3-thiadiazole derivative **ZP7** (**Fig. 1**) exhibited the highestanti-HIV-1 activity (EC_{50}= 36.4 nM), inhibiting HIV-1replication in MT-4 cells with sevenfold and eightfold higher efficiency than nevirapine (NVP)and delavirdine (DLV), respectively.[15]

Encouraged by these promising results, and with the aim tofurther explore thechemical features required for RT inhibition and identification of the new compounds as potential NNRTIs through application of the structure-based bioisosterismapproach, [23]an excellent "follow-on"-basedlead optimization strategy [24] was carriedout by modifying the centralcore structure of the lead compounds to produce the desiredpotency, selectivity, and the required ADME profiles. Structural and structure-activity relationship (SAR) studies of the known arylazolylthioacetanilides illustratedin the literature[14-22] have pointed to the presence ofkey chemical features that correlated with the RT inhibitory ability, i.e., the aryl group linked to the azole core fitted into the important hydrophobic pocket and the carbonyl group of the amide was able to establish a key hydrogen-bond through interaction with the backbone N-H of K103. These functionalities are maintained in a spatial arrangement within the NNRTIs binding pocket bya positioning heterocyclic central core inarylazolylthioacetanilides. There are differences in the electronic and conformational contribution of the five-membered heterocyclic moiety to the binding of the inhibitors to RT.[15]Additional branching moieties, such as the *N*-substitutedphenyl moiety located at the protein/solvent interface near a region of the protein known to be flexible, can be present or extended

to further optimize potency and/or physicochemical properties.

Our structure-based bioisosterism design of new NNRTIs chemotypes was primarily focused on the identification of additional heterocycle core frame work with synthetic accessibility and drug-like properties, able to replace the central chemical template of arylazolylthioacetanilides, while keeping the above mentioned key interaction features in a spatial orientation appropriate to fit into RT.

Fig. 2. The structure-basedbioisosterism replacement of azoles by pyridazine.

To achieve this goal, a variety of scaffolds easyto synthesize and suitable for appropriate chemical functionalization was scrutinized by athorough bibliographic research. Herein, a series of novel 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-arylacetamide derivatives were synthesized(**Fig.2**), andtheir anti-HIV activities against WT HIV-1 and HIV-2, as well as HIV RT inhibitory potency was evaluated.

2. Results and Discussion

2.1. Chemistry

Scheme 1. Reagents and conditions: (i) NaH, $BrCH_2CH(OEt)_2$, THF; (ii) KOH, EtOH-H_2O; (iii) N_2H_4-AcOH-Water; (iv) Br_2, AcOH; (v) P_2S_5, Pyridine; (vi) $ClCH_2CONHAr$, triethylamine, THF.

Efficient preparations of the novel 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-acetamides (**8**) have been developed, which isoutlined in **Scheme 1**. Using the establishedchemistry, a four-stage synthesis starting from commercially available 2-(naphthalen-1-yl) acetonitrile (**1**) gave the 4-(naphthalen-1-yl)pyridazin-3(2*H*)-one (**6**) in high overall yield, which was the key reaction in this synthetic route.[25] Treatment of **6** with phosphorus pentasulfide in pyridine under reflex afforded 4-(naphthalen-1-yl)pyridazine-3(2*H*)-thione (**7**). The final pyridazinethioacetanilides (**8**) were synthesized by reaction of intermediates **7** with suitable 2-chloro-*N*-aryl-substituted acetamides(or other alkyl halides) in good yields. The 2-chloro-*N*-phenyl acetamides (or other alkyl halides) were synthesized according to the literature.[26] To the best of our knowledge, this is the first example of the synthesis of pyridazinethioacetanilides.The synthesizedcompounds were characterized by MS, IR and/or ^{1}H NMR spectral data together with TLC analysis.

2.2 Biological activities

2.2.1 Anti-HIV activities evaluation

The test of the newly synthesized pyridazinylthioacetamide derivatives for activities against HIV-1 and HIV-2 was performed inMT-4 cell cultures [27]infected with wild-type HIV-1 (strain IIIB) [28] and wild-type HIV-2 (strain ROD)[29]. The methodology of the anti-HIV assay has been previously described.[30,31] Meanwhile, nevirapine (NVP), zidovudine (azidothymidine, AZT), dideoxycytidine (DDC), delavirdine (DLV) and efavirenz(EFV) were used as reference drugs.The cytotoxicity of these compounds was determined in parallel. Comparisons of inhibitory concentration (EC_{50}), cytotoxic concentration (CC_{50}), and SI (selectivity, given by the CC_{50}/EC_{50} ratio) values for different compounds are depicted in **Table 1**.

Table 1. Anti-HIV activity, cytotoxicity and selectivity indices of 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-arylacetamide derivatives (**8a-8p**).

As shown in **Table 1**, nearly all of the tested pyridazinylthioacetamide derivatives inhibitedHIV-1 strain IIIB replication in the lower micromolar concentrationrange (EC_{50}: 0.046-5.46μM), exceptfor one compound **8o**, which showed an EC_{50} value >34.51μM. Among them, 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-(2-nitrophenyl)acetamide(**8k**) was the most promising compound. It exhibited extremely potent inhibitory activity against HIV-1 replication with an EC_{50} value of 0.046μM, CC_{50}value of 99.9μM and the viral selectivity amounted up to 2149, which were much better than those of NVP (EC_{50}= 0.09 μM) andDDC (EC_{50}= 1.04 μM). Besides compound **8k**, some other compounds, **8b**, **8f**,**8g**, **8h** and **8j**, were also endowed with the high anti-HIV-1 potencies (EC_{50}= 0.19, 0.20, 0.13, 0.14 and 0.14μM,respectively) and good selectivity indices (SI = 177, 929,370, 1108 and 1438, respectively). Suchresults provide useful information for further development of thisclass of HIV-1 NNRTIs.

A brief investigation of the structure-activity relationships (SARs) revealed that the nature of the *ortho*and the *para* substitution at the phenyl ring of the anilide moiety influenced the anti-HIV activity remarkably. For instance, **Table 1** also revealed the potency order of the *ortho* substitution at the phenyl ring of the anilide moiety:NO_2 (**8k**, EC_{50} = 0.046μM) > halogen atoms {Cl (**8b**, EC_{50} = 0.19μM), Br (**8e**, EC_{50} = 0.21μM), F (**8d**, EC_{50}= 0.26μM)} > Me (**8l**, EC_{50} = 1.09μM) >H (**8a**, EC_{50}= 1.93μM). Introduction of small hydrophobic substituents (methyl group or chlorine atom) at the *para* substitution in the anilide moiety of **8k** and **8b** provided compounds **8j** and **8c**with decreased the anti-HIV activities, respectively. Worthy of note, in the case of compound **8e**, introduction of the methyl, 4-acetyl and methoxy carbonylgroups at the *para* position at the phenyl ring of the anilide moiety led to **8f**, **8g** and **8h** with retained activity. Whereas introduc-

tion of ethoxy carbonyl in **8e** led to **8i** with decreased activity.

Encouraged by these results, in order to find more potent inhibitors and to furtherexplore the salient features controlling the activity, further modificationsat the protein/solvent interface were performed. One *N*-substituted pyridine acetamide derivative **8m** was also synthesized with acceptable anti-HIV-1 profile. Moreover, it is also worth mentioning that the phenylethanone derivative **8n** and 3,4-dihydroisoquinolin-2(1*H*)-ylethanone derivative **8p** also demonstrated reasonable antiviral activity, presumably due to the optimum interactions with the NNRTIs binding pocket (NNIBP). The 3,4-dihydroquinolin-2(1*H*)-ylethanone derivative **8o** was essentially inactive, which could be a result of the unfavorablesteric interactions with the enzyme. We believe that these compounds can be further optimized by more appropriate substitutions in the protein/solvent interface, and work is currently ongoing in this area.

On the whole, we found that the SARfeatures of the pyridazinylthioacetamides were grossly consistent with the previously observed arylazolylthioacetanilide NNRTIs.[14-21] These results confirm the important role of heterocyclic core and the pyridazinylthioacetamide motifas a valuable lead series for the design of the next generation NNRTIs.

None of the pyridazinylthioacetamides derivatives inhibited the replication of HIV-2 ROD in MT-4 cells at a subtoxic concentration. Therefore, based on this fact and the above SAR conclusions, it can be concluded that these compounds were specific for HIV-1 and belonged to typical NNRTIs.

2.2.2 HIV-1 RT inhibition assay

Compound **8k** was evaluated in enzymatic testsfor its ability to inhibit highly purified recombinant HIV-1 RT using poly(rC)-oligo(dG) as template primer[32] to further confirm the drug target of pyridazin-3-ylthioacetamidederivatives. The assay results of the compounds are summarized in **Table 4**. Compound**8k** exhibited moderate inhibition of enzymatic activitywith an IC_{50}value of 4.06 μM, i.e. the same order of magnitude as that of NVP (2.74 μM).

Table 4. Inhibitory activity of compound**8k** against HIV-1 RT.

3. Molecular modeling

Figure 3.(A) Predicted binding mode and molecular docking of compound **8k** into theallosteric site of HIV-1 RT (PDB code: 3DLG); (B) Superimposition of the docked conformations of **8k** (white) and **ZP7** (purple) in the HIV-1 RT (PDB code: 3DLG); (C) Superimposition of the docked conformations of **8k** (white) and **RDEA806**(red) in the HIV-1 RT (PDB code: 3DLG). The docking resultsareshown by PyMOL. Hydrogen bonds are indicated by dashed lines.

In order to gain a better understanding of how **8k** interacts with RT NNIBP, a hypothetical molecular modelof the complex was constructed using AutodockVina[http://vina.scripps.edu] based on our previously reported methodology (**Fig.3**).The structure of HIV RT (ID: 3DLG) was acquired from the Protein Data Bank (www.rcsb.org). The resulting models showed that **8k** adopted the typical "butterfly" conformation that is characteristic ofmany NNRTIs (Fig. 3).The binding features were similar to those of the previous hypothetical molecularmodels obtained with arylazolylthioacetanilide derivatives (**RDEA806** and **ZP7**). The left hand side naphthalene ring of **8k** laid in a hydrophobic cleft formed by Lue 234, Tyr188, Trp229, and had an apparent π-stackinginteraction with Tyr188. The amide carbonyl of the ligand established a hydrogen bond withthe backbone NH of Lys103. In addition, potential interactions were probably present betweenthe pyridazin linker and residue Lys103. Indeed the conformational and electronic contributionof the heterocyclic linkers have a significant effect on the binding of the inhibitors in the NNIBP, which indicates the importance of central heterocycles for anti-HIV activity. Moreover, the *para* substitution in the anilide moiety (carboxyl in **RDEA806**) extends out of the hydrophobic pocket and points tothe solvent-exposed regions, suggesting that there is a very large space for substitution at this position. In all, the combination of hydrophobic interactions and π-stacking appears to be what governs the binding of the pyridazin-3-ylthioacetamide to RT. Consequently, the design of new arylazinylthioacetanilide derivatives will be further guided by the model of interaction between the lead compound **8k** and the NNIBP of RT.

4. Conclusion

In our on-going efforts to identify structurally diverse compounds with HIV inhibitory activity,we have identified a novel series of 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-arylacetamide by the structure-based bioisosterism approach and also exploredthe SAR in the protein/solvent interface near a flexible region of the RT. Most active compounds showed activity in the lowmicromolecular range with EC_{50} values (varying from 0.046 to 5.46μM).The most activecompound, **8k** showed activity against wild-type HIV-1 in the low micromolar range (EC_{50} = 0.046μM). As inhibitor of the WT RT of HIV-1, compound **8k** showed inhibitory concentrations comparable to the reference drug NVP. Because oftheir excellent potency, these pyridazin-3-ylthioacetamidederivatives may have potentialand should be further pursued as next-generation NNRTIs.Inaddition, molecular modeling studies are being carried out todetermine the effect of structural features on anti-HIV activity. These results have prompted further investigation

into alternative heterocyclic systems which will be disclosed shortly.

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ABBREVIATIONS

SAR,structure-activityrelationship; RT, reverse transcriptase; NNRTIs, non-nucleoside reverse transcriptase inhibitors; HAART, highly active antiretroviral therapy; AIDS, acquired immunodeficiency syndrome; NNIBP, NNRTIs binding pocket.

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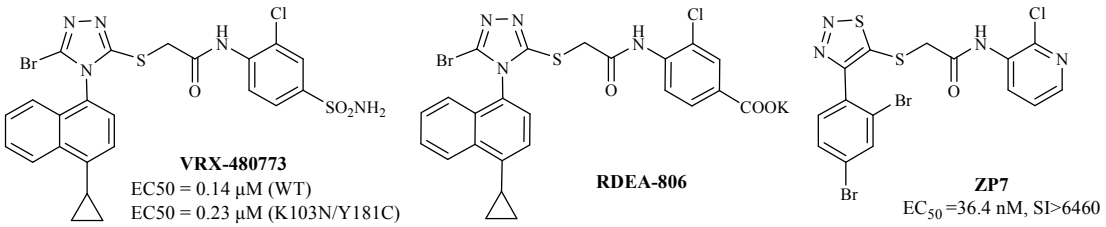


Fig. 1. Azolylthioacetanilide-based NNRTIs.

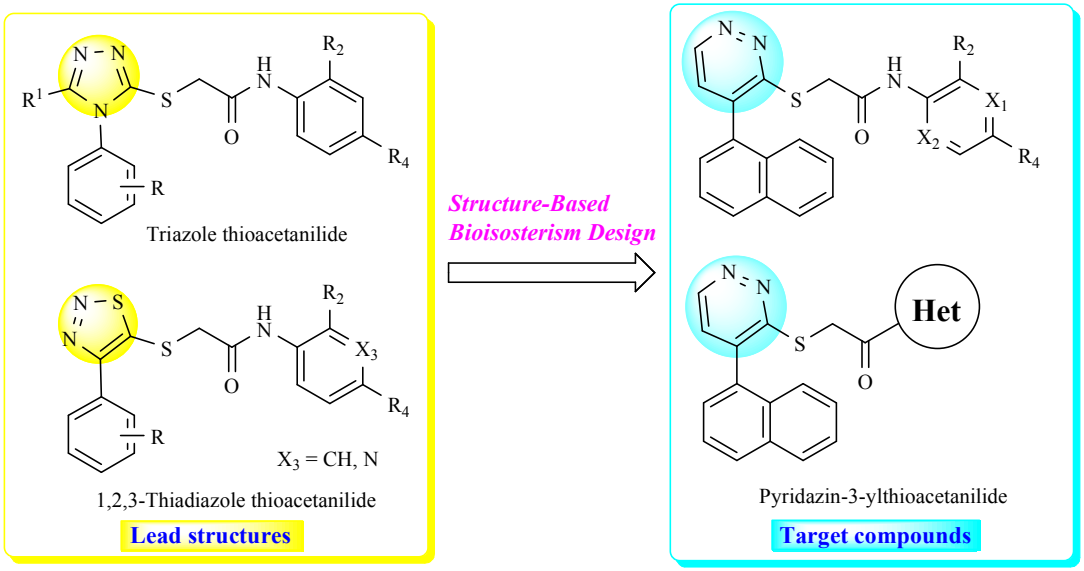
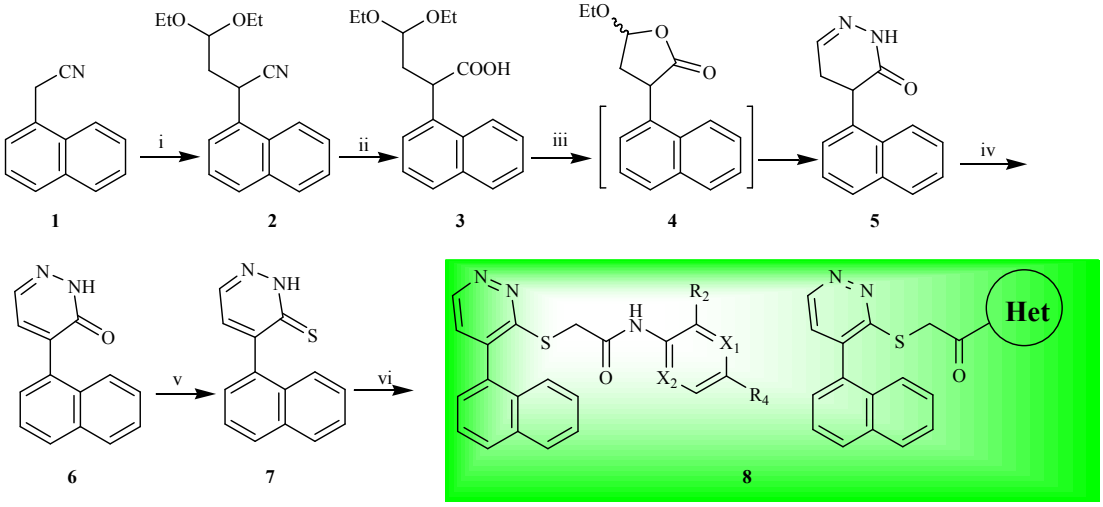
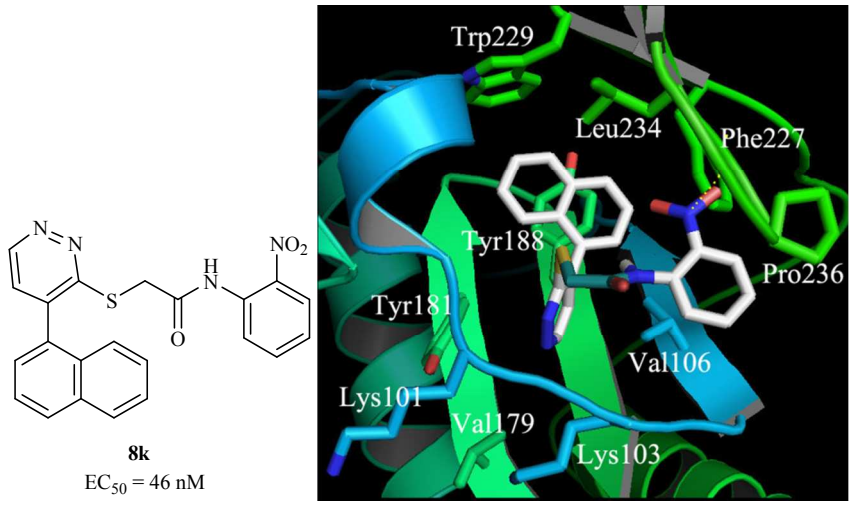


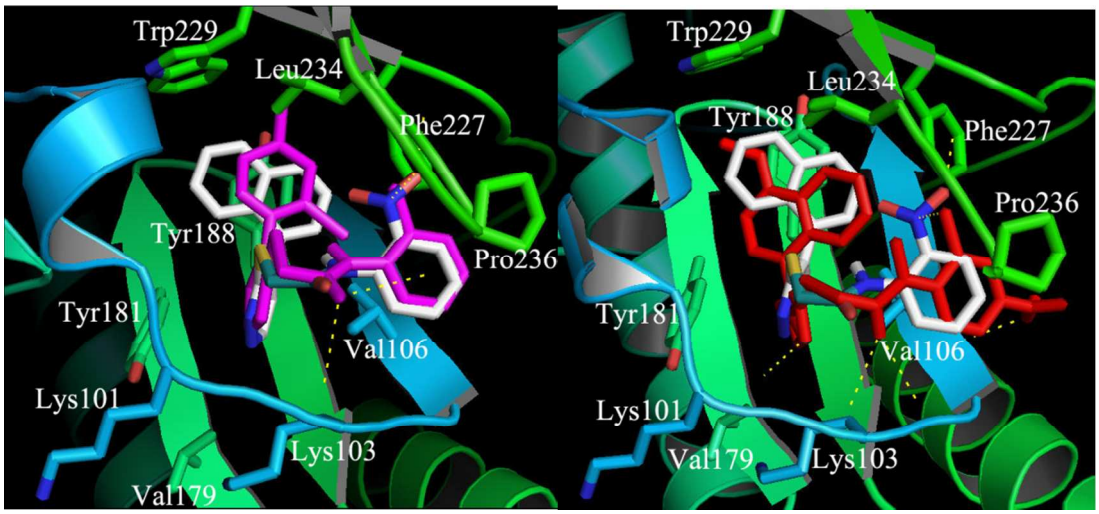
Fig. 2. The structure-based bioisosterismreplacement of azoles by pyridazine.



Scheme 1. Reagents and conditions: (i) NaH, $BrCH_2CH(OEt)_2$, THF; (ii) KOH, EtOH-H_2O; (iii) N_2H_4-AcOH-Water; (iv) Br_2, AcOH; (v) P_2S_5, Pyridine; (vi) $ClCH_2CONHAr$, triethylamine, THF.



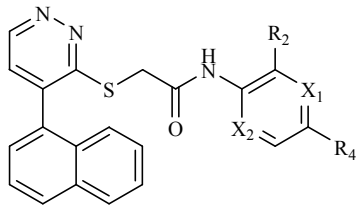
(A)

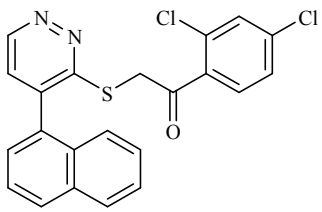


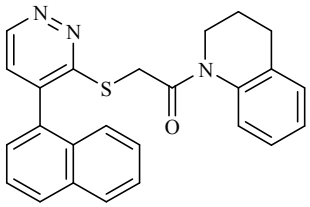
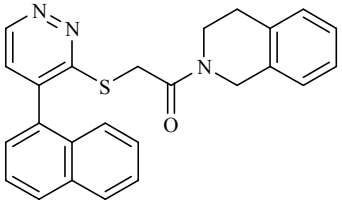
(B) (C)

Figure 3.(A) Predicted binding mode and molecular docking of compound **8k** into theallosteric site of HIV-1 RT (PDB code: 3DLG); (B) Superimposition of the docked conformations of **8k** (white) and **ZP7** (purple) in the HIV-1 RT (PDB code: 3DLG); (C) Superimposition of the docked conformations of **8k** (white) and **RDEA806** (red) in the HIV-1 RT (PDB code: 3DLG). The docking results are shown by PyMOL. Hydrogen bonds are indicated by dashed lines.

Table 1. Anti-HIV activity, cytotoxicity and selectivity indices of 2-(4-(naphthalen-1-yl)pyridazin-3-ylthio)-*N*-arylacetamide derivatives (**8a-8p**).



Code	X_1	X_2	R_2	R_4	EC_{50}(μM)		CC_{50} (μM)		SI	
					HIV-1 III_B	HIV-2 ROD	HIV-1 III_B	HIV-2 ROD	HIV-1 III_B	HIV-2 ROD
8a	CH	CH	H	H	**1.93**	>173.91	203.0	173.91	105	< 1
8b	CH	CH	Cl	H	**0.19**	>34.98	34.25	34.98	177	<1
8c	CH	CH	Cl	Cl	**0.42**	>231.63	>283.87	231.63	>684	<1
8d	CH	CH	F	H	**0.26**	>240.60	204.39	240.60	799	<1
8e	CH	CH	Br	H	**0.21**	>31.31	31.53	31.31	151	<1
8f	CH	CH	Br	Me	**0.20**	>215.34	182.40	215.34	929	<1
8g	CH	CH	Br	COMe	**0.13**	>48.54	46.31	48.54	370	<1
8h	CH	CH	Br	COOMe	**0.14**	>245.87	158.74	>245.87	1106	×1
8i	CH	CH	Br	COOEt	**0.56**	>239.28	237.36	>239.28	420	×1
8j	CH	CH	NO_2	Me	**0.14**	>243.91	204.42	243.91	1438	<1
8k	CH	CH	NO_2	H	**0.046**	>134.71	99.90	134.71	2149	<1
8l	CH	CH	Me	H	**1.09**	>150.46	141.90	150.46	130	<1
8m	N	CH	Cl	H	**0.27**	>164.42	156.06	164.42	573	<1
8n					**5.46**	>55.02	54.55	55.02	10	<1

8o		>34.51	>34.99	34.51	34.99	<1	<1
8p		**1.15**	>36.45	34.02	36.45	29	<1
NVP[e]		0.09		>15.02	>15.02	>168	
AZT[e]		0.021	0.0045	249.5	249.5	12221	56907
DDC[e]		1.04	1.28	>94.69	>94.69	>93	>74
EFV[e]		0.0054		>6.34	>6.34	>1187	
DLV[e]		0.03257		>36.19	>36.19	>1096	

[a]EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cells against HIV-1-induced cytotoxicity, as determined by the MTT method. In bold are the values of active compounds.

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

[c]SI: selectivity index (CC_{50}/EC_{50}). The SI values: x 1 stand for ≥1or<1.

[e]The data were obtained from the Rega Institute for Medical Research, KU Leuven, Belgium.

Table 2. Inhibitory activity of compound **8k** against HIV-1 RT.

Compd.	**8k**	NVP
IC_{50}(μM)[a]	4.06	2.74

[a]50% of the inhibitory concentration of tested compounds required to inhibit biotin deoxyuridine triphosphate (biotin-dUTP) incorporationinto the HIV-1 RT by 50%.