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Lead optimization at C-2 and N-3 positions of thiazolidin-4-ones as HIV-1 non-nucleoside reverse transcriptase inhibitors

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ABSTRACT

Based on rational drug design approach, a series of novel thiazolidin-4-ones bearing different aryl/heteroaryl moieties at position C-2 and N-3 are synthesized and evaluated as potent inhibitors for human immunodeficiency virus type-1 reverse transcriptase enzyme (HIV-1 RT). An in vitro HIV-1 RT assay showed that the compounds **4**, **5**, **6**, **8**, **12, 13, 14** and **17** have shown high inhibition of reverse transcriptase (75.41, 95.50, 98.07, 91.24, 85.27, 77.59, 84.11 & 76.49% inhibition) enzyme activity. Further, cell based assay showed that compounds **4, 5, 8** & **12** are identified as the best compounds of the series (EC_{50} ranged from 0.09 to 0.8 μg/ml and 0.12 to 1.06 μg/ml) against HIV-1 III_B and HIV-1 ADA5 strains, respectively. Moreover, the compounds which were active against HIV-1 III_B and HIV-1 ADA5 were also found to be active against primary isolates (EC_{50} ranged from 0.10 to 1.55 μg/ml against HIV-1 UG070 and 0.07 to 1.1 μg/ml against HIV-1 VB59), respectively. Structure–activity relationship (SAR) studies demonstrated the importance of the lipophilic bulky substituent pattern on compact heteroaryl ring at N-3, replacement of C4′ at C-2 phenyl by trivalent bioisosteric nitrogen and dihalo groups at C-2 aryl/heteroaryl of thiazolidin-4-ones is crucial for anti-HIV-1 activity. Molecular modeling of compounds **4**, **5**, **8** and **12** in complex with HIV-1 RT demonstrate that there is good correlation of results obtained from SAR studies. Therefore the compounds **4**, **5**, **8** and **12** may be considered as good candidates for further optimization of anti-HIV-1 activity.

1. Introduction

Reverse transcriptase (RT) is the prime target for the development of drugs for HIV/AIDS therapy because it plays an important role in the initial process of HIV replication cycle, and responsible for converting the single-stranded viral RNA genome into the double-stranded DNA form.[1] Compounds that inhibit this enzyme are divided into two classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTI/NtRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a diverse group of compounds that induce allosteric changes in the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, thus rendering the enzyme incapable of converting viral RNA to DNA. However, the long-term usage of NNRTIs in HIV/AIDS patients may eventually lead to the development of virus-drug resistance.[2] Therefore, it is essential to look for novel NNRTIs with potent anti-HIV-1 activity and to address issues related to the drug resistance. In recent years 2,3-diaryl-1, 3-thiazolidin-4-one has emerged as an important chemical scaffold (Fig. 1) having diverse aryl/heteroaryl substituents at the C-2 and N-3 positions of thiazolidin-4-one ring with promising anti-HIV activity.[3–10]

In this context our research group and Pietro Monforte's laboratory have attempted structural modification mainly focused at N-3 position of thiazolidin-4-one ring. Furthermore, a limited structural modifications were carried out at C-2 position in the form of 2′,6′-dihalophenyl group. Our previous explorations of QSAR study on N-3 position of thiazolidine-4-ones as NNRTIs, provided information on the hydrophobic property with less extended or compact structural templates without bulky substitutions on its peripheries for better HIV-1 RT inhibitory activity. Further 3D-QSAR of CoMFA and CoMSIA investigations also suggested the presence of electron rich atom and non-polar group (–CH3) at N-3 substituted heteroaromatic ring system of thiazolidin-4-one would influence the RT inhibitory activity.[11–14] This was corroborated by H. Chen et al., who suggested that the long alkyl chain/bulky substituent at heteroaryl moiety of the N-3 of the thiazolidin-4-one, would have detrimental effect on anti-HIV-RT activity.[15] As far as modifications at C-2 position it is believed that 2′,6′-dihalogen substitution at the

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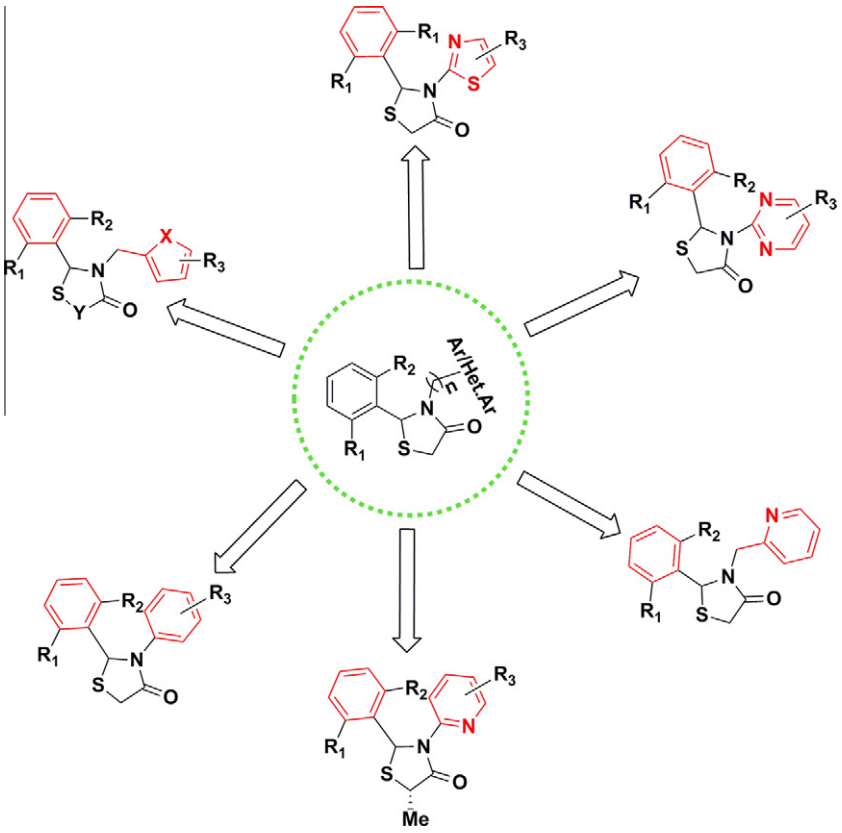


Figure 1. Structural modifications on thiazolidin-4-one derivatives (C-2 & C-3 positions).

phenyl ring of the C-2 facilitates formation of butterfly-like conformation of the molecule which is essential for HIV-RT inhibitory activity. Based on the aforementioned data we have applied bioisosteres replacement strategy by using trivalent replacement of –CH═ with –N═ at C-2 position of phenyl moiety of the thiazolidin-4-one scaffold.[16] Secondly, we decided to introduce compact high electron rich atom containing structural moiety such as isoxazolidin and pyridin-2-yl at N-3 position of the thiazolidin-4-one ring (Fig. 2). In this paper, we report the synthesis, biological evaluation of anti-HIV-1 activity and preliminary structure–activity relationship (SAR) of these new thiazolidin-4-one analogues. Additionally, molecular docking study was performed to investigate binding mode of compounds **4**, **5**, **8** and **12** in the non-nucleoside binding site (NNBS) of HIV-1 RT.

2. Results and discussion

2.1. Chemistry

Referring to the reported literature the newly designed compounds were synthesized as shown in Scheme 1.[5] Treatment of substituted hetero aromatic amine with 2′,6′-/3′,5′-dihalo-substituted (hetero/aromatic) aldehyde in the presence of an excess of mercaptoacetic acid in reflux toluene for 24–48 h. After completion

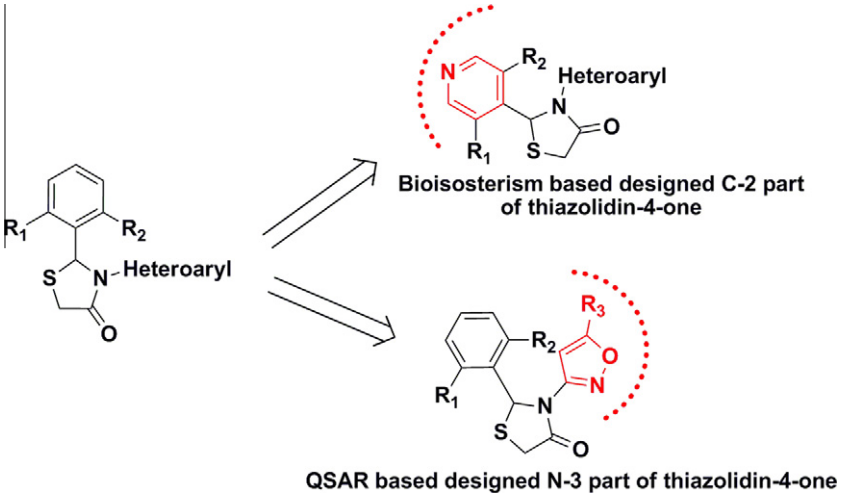
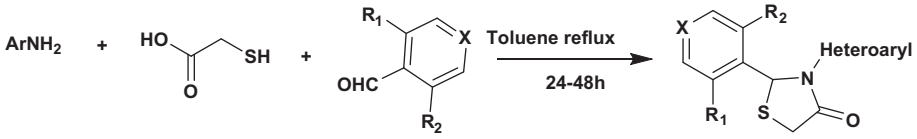


Figure 2. Bioisosterism and QSAR based design of C-2 and N-3 parts of thiazolidin-4-one scaffold.



Compound No	X	R1	R2	Heteroaryl
1	CH	Cl	Cl	5-tert-butylisoxazol-3-yl
2	CH	Cl	F	5-tert-butylisoxazol-3-yl
3	CH	F	F	5-tert-butylisoxazol-3-yl
4	CH	Cl	Cl	5-methylisoxazol-3-yl
5	CH	Cl	F	5-methylisoxazol-3-yl
6	CH	F	F	5-methylisoxazol-3-yl
7	CH	Cl	Cl	5-bromo-4,6-dimethylpyridin-2-yl
8	CH	Cl	F	5-bromo-4,6-dimethylpyridin-2-yl
9	N	Br	Br	5-bromo-4,6-dimethylpyridin-2-yl
10	N	Cl	Cl	5-bromo-4,6-dimethylpyridin-2-yl
11	N	Br	Br	4,6-dimethylpyridin-2-yl
12	N	Cl	Cl	4,6-dimethylpyridin-2-yl
13	N	Br	Br	6-methyl-pyridin-2-yl
14	N	Cl	Cl	6-methyl-pyridin-2-yl
15	N	Br	Br	5-Bromo-6-methyl-pyridin-2-yl
16	N	Cl	Cl	5-Bromo-6-methyl-pyridin-2-yl
17	CH	Cl	F	6-chloro-2-(methylthio)pyrimidin-4-yl

Scheme 1. Synthesis of 2,3-diaryl/heteroaryl-1,3-thiazolidin-4-ones.

of the reaction, desired final products were obtained by conventional work-up procedure followed by flash column chromatography purification on silica-gel (230–400 mesh size) in moderate to excellent yields. All the synthesized compounds were well characterized by IR, ESI-MS, NMR, elemental analyses and HRMS.

2.2. Anti-HIV-1 evaluation

The compounds presented in this study namely 2,3-diaryl/heteroaryl-1,3-thiazolidin-4-ones (**1**–**17**) were evaluated for anti-HIV-1 activity by using enzymatic (RT) and cell based assays. The HIV-1 RT inhibition activity range for these compounds showed from 51–98% inhibition at 100 μg/ml concentrations. The compounds that showed highest inhibitory activity were **4**, **5**, **6**, **8**, **12**, **13**, **14** and **17** (75.41%, 95.50%, 98.07%, 91.24%, 85.27%, 77.59%, 84.11% and 76.49%), respectively, and those with moderate activity were **7** and **11** (60.72% and 62.82%), respectively, whereas the control NNRTI marketed drug nevirapine showed 99.2% inhibition at 100 μg/ml concentration. The enzyme assay results demonstrated that the compound **6** show equally potent to the nevirapine against reverse transcriptase enzyme. Subsequently, the inhibitory activity of HIV-1 viral replication was also assessed by cell based assay. The results are summarized in Table 1 along with standard nevirapine as reference drug. In the cell based assay, the compounds **4**, **5**, **8,** and **12** were the most potent inhibitors of HIV-1 replication against HIV-1 III_B (EC_{50} ranged from 0.09 to 0.8 μg/ml; the selectivity index (SI) ranged from 46.6 to 446.6) and HIV-1 ADA5 (EC_{50} ranged from 0.12 to 1.06 μg/ml; the selectivity index

Table 1
Anti-HIV-1 activity, cytotoxicity and selectivity index in TZM-bl cells and HIV-1 RT kit assay for compounds **1–17**

Compound No.	Anti-HIV-1 activity[a]					
	EC_{50}[b] (μg/ml)		CC_{50}[c] (μg/ml)	SI[d]		% Inhibition (HIV-RT kit assay) 100 μg/ml
	HIV-1 III_B	HIV-1 ADA5	TZM-bl cells	HIV-1 III_B	HIV-1 ADA5	
1	1.49	2.40	48.5	32.6	20	50.40
2	1.95	3.07	40.7	20.8	13.2	51.09
3	2.97	2.58	45.9	15.5	18	71.48
4	0.35	0.65	40.5	115.7	62.3	75.41
5	0.67	1.06	38.5	57.4	36.3	95.50
6	1.01	1.34	62.7	62	46.8	98.07
7	1.04	0.85	2.5	2.4	2.9	60.72
8	0.09	0.12	40.2	446.6	335	91.24
9			NT[e]			62.04
10	1.5	4.8	15.6	10.4	3.2	57.39
11	1.41	1.21	49.8	35.3	41.1	62.84
12	0.8	0.31	37.3	46.6	120.3	85.27
13	2.22	4.11	26.7	12.0	6.5	77.59
14	0.65	1.64	45.0	69.8	27.4	84.11
15	6.09	2.52	26.9	4.4	10.7	75.91
16	6.18	2.17	66.7	10.8	30.7	76.58
17	0.93	1.26	27.0	29	21.4	76.49
Nevirapine	0.05	0.05	76.13	1522.2	1522.2	99.20

[a] Data represent the mean of two and three independent assays for EC_{50} and CC_{50}, respectively.
[b] EC_{50} is the 50% effective concentration required to reduce HIV-1 induced cytopathic effect of HIV-1 III_B and HIV-1 ADA5 in TZM-bl cell line.
[c] The CC_{50} is the 50% cytotoxic concentration for TZM-bl cells.
[d] Selectivity index ratio CC_{50}/EC_{50}.
[e] Not tested.

(SI) ranged from 36.3 to 335), respectively. Some other compounds, **6**, **7**, **11**, **14** and **17**, also showed reasonable anti-HIV-1 potency (EC_{50} = 0.65–1.41 μg/ml and 0.85–1.64 μg/ml) against HIV-1 III_B and HIV-1 ADA5 strains, respectively. Only those compounds having EC_{50} value less than 2.5 μg/ml in HIV-1 III_B and/or HIV-1 ADA5 strains were taken for further anti-HIV-1 screening against the primary HIV-1 isolates of Uganda (HIV-1 UG070) and Indian (HIV-1 VB59). The compounds which were active against HIV-1 III_B and HIV-1 ADA5 were also found to be active against primary isolates (EC_{50} ranged from 0.10 to 1.55 μg/ml; the selectivity index (SI) ranged from 24 to 402 against HIV-1 UG070 strain and 0.07 to 1.1 μg/ml; the selectivity index (SI) ranged from 33.9 to 574 against HIV-1 VB59 strain) as shown in Table 2.

Based on the data shown in Table 1 it is reasonable to infer some important structure–activity information on the role of different aryl/heteroaryl moieties at the C-2 and N-3 of the thiazoldin-4-ones. Analogs having 5-methylisoxazol-3-yl substitution at N-3 position of thiazolidin-4-one ring exhibited enhanced anti-HIV-1 RT activity (compounds **4**–**6**) as compared with the 5-tert-butylisoxazol-3-yl substituted compounds of **1**–**3**. Suggesting that a compact, and electron rich heteroatom containing heterocyclic moiety is crucial for the antiviral activity, and this is in-line with our previous QSAR analysis on thiazolidin-4-ones.[11–14] In the pyridin-2-yl series, a bromine atom at 5-position and a methyl group at 4 and 6-positions is a significant feature for potent anti-HIV-1 agents. The removal of methyl group and bromine atom at 4 and 5 positions of pyridin-2-yl structure invariably causes reduction of antiviral potency in compounds **11, 13, 15** and **16** (EC_{50} = 1.41, 2.22, 6.09 & 6.19 μg/ml against HIV-1 III_B and 1.21, 4.11, 2.52, and 2.17 μg/ml against HIV-1 ADA5 strains, respectively). These substituents on pyridin-2-yl moiety are crucial points to modulate the anti-HIV-1 potency. Additionally we explored the modification at C-2 component of thiazolidinone by using classical trivalent bioisosteric replacement strategy. The replacement of –CH= with –N= at 4-position of phenyl ring at C-2 of the thiazoldin-4-ones displayed moderate to good anti-HIV-1 activity in the range of microgram concentration against HIV-1 III_B (EC_{50} = 0.8–1.5 μg/ml) in compounds **10, 11**, **12** and **14**. The moderate potency of these compounds with respect to compounds **4**, **5** and **8** could be because of variation in polarity, electronic and hydrophobic features at C-2 position. The substitution pattern of two halogen atoms at 2′- and 6′-positions of C-2 aryl/heteroaryl ring is of paramount importance to increase the antiviral activity and it was previously reported,[3–10] these dihalo patterns restrict the rotation of the aryl/heteroaryl ring and allow the thiazolidin-4-ones scaffold to assume the characteristic butterfly-like conformation present in many other known NNRTIs. The above structure–activity relationships (SAR) analysis may be helpful to guide further modification at C-2 & N-3 of positions thiazolidinone analogs.

2.3. Molecular docking analysis

To guide the lead optimization strategy and rationalize the SARs, modeling study was performed to investigate the possible binding conformations of our newly synthesized compounds and their interaction mode with RT, using the AutoDock 4.0.[17] The coordinates of the non-nucleoside binding site were taken from the crystal structure of HIV-1 reverse transcriptase (RT) in complex with TMC278 (Rilpivirine) (pdb code: 2ZD1).[18] Molecules from isoxazol-3-yl (compounds **4** & **5**) and pyridin-2-yl series of **8** and **12** were chosen to be docked into the non-nucleoside inhibitor binding pocket (NNIBP) of HIV-1 RT. As illustrated in Figs. 3 and 4, the 2′,6′-dihalophenyl/3′,5′-dihalopyridyl moiety of compound **4**, **5**, **8** and **12** at C-2 of the thiazolidinone ring interacts through π–π interactions into the hydrophobic binding pocket, surrounded by the aromatic side chains of Tyr181, Tyr188 and Trp229. It appears from the model that Tyr188 is juxtaposed for better interaction with the 2′,6′-dihalophenyl or 3′,5′-dihalopyridyl rings. The carbonyl oxygen atom of the thiazolidin-4-one nucleus and the oxygen atom of isoxazolidin moiety of compounds **4** and **5** form hydrogen bond interactions with the backbone N–H of Lys101 residue. The methyl/bromo substitutions on heteroaryl ring at N-3 of the thiazolidin-4-one ring makes CH–π/hydrophobic interaction with the aromatic portion of Tyr318 residue. The decrease in activity of compounds **13**, **15** and **16** (not shown in the figure) was due to lack of favorable CH–π interaction with Tyr318 by the nonexistence of methyl group at N-3 of heteroaryl ring. The docked binding energy (Table 3), of compounds **5** and **8** displayed strong binding energy values when compared to compounds **4** and **12**. These results indicate the higher stability of the thiazolidin-4-one (**5** and **8**)-RT complex. Finally the docking study proposed some clue for improving thiazolidin-4-ones RT inhibition potency in further drug design attempts by optimization of hydrogen bonding with the main chain backbone of Lys101 and optimization of

Table 2
Results of anti-HIV-1 screening of compounds against primary isolates, X4 tropic HIV-1 UG070 and R5 tropic HIV-1 VB59

Compound No.	Anti-HIV-1 activity[a]				
	EC_{50}[b] (μg/ml)		CC_{50}[c] (μg/ml)	SI[d]	
	HIV-1 UG070	HIV-1 VB59	TZM-bl cells	HIV-1 UG070	HIV-1 VB59
1	1.76	1.8	48.5	27.5	26.9
2	1.6	2.5	40.7	24.4	16.2
4	0.5	0.35	40.5	81	115.7
5	0.75	0.68	38.5	51.3	56.6
6	1.9	1.45	62.7	33	43.2
7	0.29	0.35	2.5	8.6	7.1
8	0.10	0.07	40.2	402	574
11	2.10	1.59	49.8	23.7	31.3
12	1.55	1.1	37.3	24.06	33.9
13	5.55	2.24	26.7	4.8	11.9
14	3.1	1.95	45.0	14.5	23.1
16	8.45	6.5	66.7	7.89	10.3
17	1.8	0.65	27	15	41.5
Nevirapine	0.05	0.05	76.13	1522.2	1522.2

[a] Data represent the mean of two and three independent assays for EC_{50} and CC_{50}, respectively.
[b] EC_{50} is the 50% effective concentration required to reduce HIV-1 induced cytopathic effect of HIV-1 UG070 and HIV-1 VB59 in TZM-bl cell line.
[c] The CC_{50} is the 50% cytotoxic concentration for TZM-bl cells.
[d] Selectivity index ratio CC_{50}/EC_{50}.

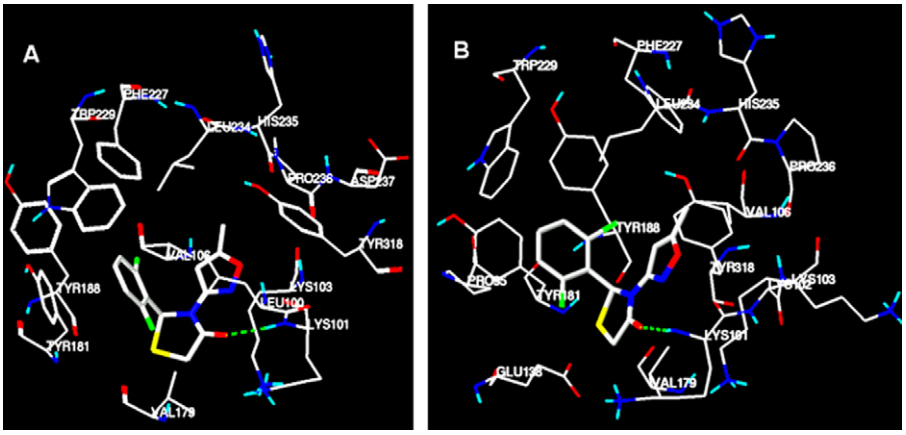


Figure 3. (A & B) Model of 4 (left) and 5 (right) docked into the RT non-nucleoside binding site.

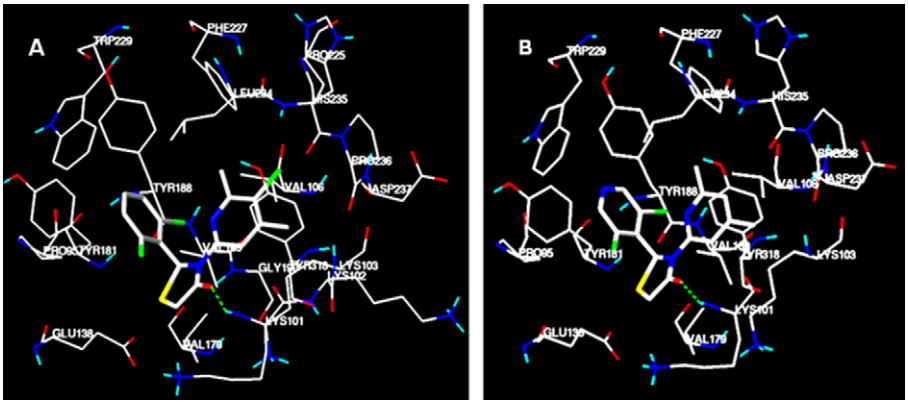


Figure 4. (A & B) Model of 8 (left) and 12 (right) docked into the RT non-nucleoside binding site.

Table 3
The docking score function (kcal/mol) for the thiazolidin-4-one compounds of **4**, **5**, **8** and **12**

Compound No.	$\Delta G^{a}_{Binding}$
4	−8.95
5	−9.75
8	−9.75
12	−8.35

[a] Binding free energy.

hydrophobic contacts with the aromatic side chains of Tyr181, Tyr188 Trp229, and Tyr318.

3. Conclusions

On the basis of our previous QSAR-based drug design strategy and bioisosteric principle, a series of novel thiazolidin-4-ones bearing different aryl/heteroaryl moieties at position C-2 and N-3 of thiazolidin-4-one were synthesized and evaluated as potent inhibitors of human immunodeficiency virus type-1 (HIV-1). In the series of thiazolidin-4-one derivatives, compound **4, 5, 8** and **12** identified as potent inhibitor against all strains HIV-1 III_B, HIV-1 ADA5, primary isolates of HIV-1 VB59 and HIV-1 UG070, respectively. Based upon the preliminary SAR studies of these new 2,3-diaryl/heteroaryl-thiazolidin-4-ones, some structural requirements for high potency against HIV-1 were identified and rationalized by the molecular modeling studies.

4. Experimental protocol

4.1. General

Melting points (mp) were determined on a Complab melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum RX1 (4000–450 cm^{-1}). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a NMR Spectrometer Bruker DRX-300 using $CDCl_3$ as a solvent. The chemical shifts are reported as parts per million (δ ppm) from $(CH_3)_4Si$ (TMS) as an internal standard. HRMS and ESI-MS were performed using a Waters Agilent 6520-Q-TofMS/MS system and JEOL-AccuTOF JMST100LC instruments. Elemental analyses were carried out on CARLO-ERBA EA1108 C, H, N elemental analyzer and values were in the acceptable limits of the calculated values. Column chromatography separations were performed on silica gel (230–400 mesh) as the stationary phase.

4.2. General synthetic procedure for compounds (thiazolidin-4-ones, 1–17)

The appropriate (hetero) aromatic amine (1.0 mmol) and 2′,6′-/3′,5′-dihalo-substituted (hetero/aromatic) aldehyde (1.5 mmol) were stirred in dry toluene under reflux condition followed by addition of mercaptoaceticacid (2.0 mmol). The reaction mixture was refluxed with a Dean-Stark trap for 24–48 h until the complete consumption of (hetero) aromatic amine. The reaction mixture was concentrated to dryness under reduced pressure and the residue

was taken up in ethyl acetate. The organic layer was successively washed with 5% aq citric acid, water, 5% aq sodium hydrogen carbonate, and then finally with brine. The organic layer was dried over sodium sulfate and solvent was removed under reduced pressure to get a crude product that was purified by column chromatography on silica-gel (230–400 mesh) using hexane–ethyl acetate as eluent. The structures of synthesized compounds were characterized by means of TLC, IR, ESI-MS, ^{1}H NMR, ^{13}C NMR, elemental analyses and HRMS.

4.2.1. 3-(5-*tert*-Butylisoxazol-3-yl)-2-(2,6-dichlorophenyl) thiazolidin-4-one (1)

White solid, yield: 95.5%. mp: 85–87 °C; IR (KBr): ν_{max} C=O 1714 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.36–7.14 (m, 3H, H_3, H_4, & H_5-PhH), 6.84 (s, 1H, H_4, isoxazol-3-yl), 6.83 (s, 1H, CH), 4.13 (dd, 1H, J = 15.9 Hz, 1.83 Hz, CH_2), 3.88 (d, 1H, J = 15.9 Hz, CH_2), 1.31 (s, 9H, *tert*-butyl-H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.34 (C=O), 157.21 (isoxazol-3-yl-C5), 150.21 (isoxazol-3-yl-C3), 135.40, 133.68, 133.07, 133.52, 129.59, 128.95 (Ph-C), 92.07 (isoxazol-3-yl-C4), 58.02 (C2), 34.03 (C5), 28.64 (*tert*-butyl-C); MS (ESI): *m/z* 371.1 (M^+). Anal. calcd for $C_{16}H_{16}Cl_2N_2O_2S$: C, 51.76; H, 4.34; N, 7.55. Found: C, 51.77; H, 4.27; N, 7.51.

4.2.2. 3-(5-*tert*-Butylisoxazol-3-yl)-2-(2-chloro-6-fluorophenyl) thiazolidin-4-one (2)

Light yellow solid, yield: 97.2%. mp: 82–84 °C; IR (KBr): ν_{max} C=O 1712 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.28–6.99 (m, 3H, H_3, H_4, & H_5-PhH), 6.84 (s, 1H, isoxazol-3-yl-H), 6.83 (s, 1H, CH), 4.13 (d, 1H, J = 15.8 Hz, CH_2), 3.78 (d, 1H, J = 15.8 Hz, CH_2), 1.31 (s, 9H, *tert*-butyl-H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 171.08 (C=O), 162.61 (Ph-C), 159.29 (isoxazol-3-yl-C5), 157.28 (isoxazol-yl-C3), 129.88, 129.75, 125.98, 115.57, 115.27 (Ph-C), 91.97 (isoxazol-3-yl-C4), 56.58 (C2), 33.04 (C5), 28.64 (*tert*-butyl-C); MS (ESI): *m/z* 355.1 $(M+H)^+$. HRMS (ESI): $(M+H)^+$, calcd for $C_{16}H_{16}ClFN_2O_2S+H^+$ *m/z* 355.0683, found 355.0646.

4.2.3. 3-(5-*tert*-Butylisoxazol-3-yl)-2-(2,6-difluorophenyl) thiazolidin-4-one (3)

Light yellow solid, yield: 93.4%. mp: 80–82 °C; IR (KBr): ν_{max} C=O 1717 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.28–6.87 (m, 3H, H_3, H_4, & H_5-PhH), 6.82 (s, 1H, isoxazol-3-yl-H), 6.68 (s, 1H, CH), 4.17 (d, 1H, J = 15.9 Hz, CH_2), 3.79 (d, 1H, J = 15.9 Hz, CH_2), 1.31 (s, 9H, *tert*-butyl-H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.84 (C=O), 162.85, 162.91 (Ph-C), 157.16 (isoxazol-3-yl-C5), 151.21 (isoxazol-3-yl-C3), 129.91, 117.19, 112.18, 111.84 (Ph-C), 91.99 (isoxazol-3-yl-C4), 52.23 (C2), 33.37 (C5), 28.62 (*tert*-butyl-C); MS (ESI): *m/z* 339.1 $(M+H)^+$. HRMS (ESI): $(M+H)^+$, calcd for $C_{16}H_{16}F_2N_2O_2S+H^+$ *m/z* 339.0979, found 339.0932.

4.2.4. 2-(2,6-Dichlorophenyl)-3-(5-methylisoxazol-3-yl) thiazolidin-4-one (4)

White solid, yield: 96.5%. mp: 90–92 °C; IR (KBr): ν_{max} C=O 1711 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.35–7.13 (m, 3H, H_3, H_4, & H_5-PhH), 6.84 (s, 1H, isoxazol-3-yl-H), 6.83 (s, 1H, CH), 4.11 (dd, 1H, J = 15.8 Hz, 1.71 Hz, CH_2), 3.88 (d, 1H, J = 15.9 Hz, CH_2), 2.36 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.17 (C=O), 169.88 (isoxazol-3-yl-C5), 157.48 (isoxazol-3-yl-C3), 135.56, 133.53, 133.02, 130.48, 129.61, 128.90 (Ph-C), 95.45 (isoxazol-3-yl-C4), 57.99 (C2), 34.34 (C5), 12.58 (CH_3); MS (ESI): *m/z* 329 (M^+). Anal. calcd for $C_{13}H_{10}Cl_2N_2O_2S$: C, 47.43; H, 3.06; N, 8.51. Found: C, 47.38; H, 3.12; N, 8.59.

4.2.5. 2-(2-Chloro-6-fluorophenyl)-3-(5-methylisoxazol-3-yl) thiazolidin-4-one (5)

Light yellow solid, yield: 95.8%. mp: 95–97 °C; IR (KBr): ν_{max} C=O 1717 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.23–7.17 (m, 3H, H_3, H_4, & H_5-PhH), 6.93 (s, 1H, isoxazol-3-yl-H), 6.85 (s, 1H, CH), 4.11 (d, 1H, J = 15.9 Hz, CH_2), 3.79 (d, 1H, J = 15.9 Hz, CH_2), 2.37 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.02 (C=O), 170.11, (isoxazol-3-yl-C5), 157.58 (isoxazol-3-yl-C3), 134.10, 129.91, 129.77, 125.91, 115.52, 115.23 (Ph-C), 95.27 (isoxazol-3-yl-C4), 56.59 (C2), 33.20 (C5), 12.60 (CH_3); MS (ESI): *m/z* 313.1 $(M+H)^+$. HRMS (ESI): $(M+H)^+$, calcd for $C_{13}H_{10}ClFN_2O_2S+H^+$ *m/z* 313.0214, found 313.0228.

4.2.6. 2-(2,6-Difluorophenyl)-3-(5-methylisoxazol-3-yl) thiazolidin-4-one (6)

White solid, yield: 96.7%. mp: 91–93 °C; IR (KBr): ν_{max} C=O 1717 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 7.25–6.89 (m, 3H, H_3, H_4, & H_5-PhH), 6.86 (s, 1H, isoxazol-3-yl-H), 6.69 (s, 1H, CH), 4.15 (d, 1H, J = 15.7 Hz, CH_2), 3.79 (d, 1H, J = 15.9 Hz, CH_2), 2.36 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.75 (C=O), 170.11 (isoxazol-3-yl-C5), 162.23, 162.13 (Ph-C), 158.91 (isoxazol-3-yl-C3), 129.95, 116.64, 112.14, 111.80 (Ph-C), 95.29 (isoxazol-3-yl-C4), 52.18 (C2), 33.23 (C5), 12.58 (CH_3); MS (ESI): *m/z* 297.1 $(M+H)^+$. HRMS (ESI): $(M+H)^+$, calcd for $C_{13}H_{10}F_2N_2O_2S+H^+$ *m/z* 297.0509, found 297.0605.

4.2.7. 3-(5-Bromo-4,6-dimethylpyridin-2-yl)-2-(2,6-dichlorophenyl)thiazolidin-4-one (7)

White solid, yield: 84.5%. mp: 112–113 °C; IR (KBr): ν_{max} C=O 1696 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 8.34 (s, 1H, H_3-pyridin-2-yl), 7.99–7.48 (m, 3H, H_3, H_4, & H_5-PhH), 6.90 (s, 1H, CH), 4.59 (dd, 1H, J = 15.7 Hz, 2.13 Hz, CH_2), 4.38 (d, 1H, J = 15.7 Hz, CH_2), 2.82 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.79 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.18 (C=O), 155.15, 149.12, 148.01 (pyridin-2-yl-C6, C4 & C2), 135.58, 134.78, 133.50, 130.48, 129.21, 128.45 (Ph-C), 119.95, 115.20 (pyridin-2-yl-C5 & C3), 59.04 (C2), 35.77 (C5), 24.59, 23.78 (CH_3 at $C_{4,6}$-pyridin-2-yl); MS (ESI): *m/z* 433 $(M+H)^+$. Anal. calcd for $C_{16}H_{13}BrCl_2N_2OS$: C, 44.47; H, 3.03; N, 6.48. Found: C, 44.50; H, 3.09; N, 6.41.

4.2.8. 3-(5-Bromo-4,6-dimethylpyridin-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one (8)

Light yellow solid, yield: 89.5%. mp: 112–114 °C; IR (KBr): ν_{max} C=O 1693 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 8.02 (s, 1H, H_3-pyridin-2-yl), 7.31–7.00 (m, 3H, H_3, H_4, & H_5-PhH), 6.90 (s, 1H, CH), 4.22 (d, 1H, J = 15.8 Hz, CH_2), 3.96 (d, 1H, J = 16.1 Hz, CH_2), 2.45 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.40 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.11 (C=O), 162.03 (Ph-C), 155.18, 149.18, 148.18 (pyridin-2-yl-C6, C4 & C2), 131.29, 131.15, 129.40, 129.26, 125.51 (Ph-C), 115.16, 114.20 (pyridin-2-yl-C5 & C3), 57.57 (C2), 34.98 (C5), 24.72, 23.72 (CH_3 at $C_{4,6}$-pyridin-2-yl); MS (ESI): *m/z* 415.1 (M^+). HRMS (ESI): $(M+H)^+$, calcd for $C_{16}H_{13}BrClFN_2OS+H^+$ *m/z* 414.9683, found 414.9717.

4.2.9. 3-(5-Bromo-4,6-dimethylpyridin-2-yl)-2-(3,5-dibromopyridin-4-yl)thiazolidin-4-one (9)

White solid, yield: 90.3%. mp: 116–118 °C; IR (KBr): ν_{max} C=O 1691 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 8.64, 8.49 (s, 2H, H_2, H_6-pyridin-4-yl), 8.03 (s, 1H, H_3-pyridin-2-yl), 7.40 (s, 1H, CH), 4.20 (dd, 1H, J = 15.8 Hz, 2.0 Hz, CH_2), 3.99 (d, 1H, J = 15.8 Hz, CH_2), 2.42 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.39 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.07 (C=O), 155.09, 152.96 (pyridin-2-yl-C6 & C4), 150.83, 149.52, 147.78 (pyridin-4-yl-C4, C2 & C6), 145.79 (pyridin-2-yl-C2), 123.09 (pyridin-4-yl-C3

& C5) 120.16, 114.49 (pyridin-2-yl-C5 & C3), 62.43 (C2), 35.81 (C5), 24.53, 23.72 (CH_3 at $C_{6,4}$-pyridin-2-yl); MS (ESI): *m/z* 523 $(M+H)^+$. Anal. calcd for $C_{15}H_{12}Br_3N_3OS$: C, 34.51; H, 2.32; N, 8.05. Found: C, 34.57; H, 2.38; N, 8.10.

4.2.10. 3-(5-Bromo-4,6-dimethylpyridin-2-yl)-2-(3,5-dichloropyridin-4-yl)thiazolidin-4-one (10)

White solid, yield: 82.5%. mp: 115–117 °C; IR (KBr): ν_{max} C=O 1691 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.48, 8.33 (s, 2H, H_2, H_6-pyridin-4-yl), 8.05 (s, 1H, H_3-pyridin-2-yl), 7.39 (s, 1H, CH), 4.20 (dd, 1H, *J* = 15.9 Hz, 2.03 Hz, CH_2), 3.98 (d, 1H, *J* = 15.9 Hz, CH_2), 2.42 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.38 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.17 (C=O), 158.09, 152.96 (pyridin-2-yl-C6 & C4), 147.78, 146.34 (pyridin-4-yl-C4, C2 & C6), 144.79 (pyridin-2-yl-C2), 133.09 (pyridin-4-yl-C3 & C5) 118.16, 116.49 (pyridin-2-yl-C5 & C3), 61.24 (C2), 34.62 (C5), 24.65, 23.45 (CH_3 at $C_{6,4}$-pyridin-2-yl); MS (ESI): *m/z* 434 $(M+H)^+$. Anal. calcd for $C_{15}H_{12}BrCl_2N_3OS$: C, 41.59; H, 2.79; N, 9.70. Found: C, 41.61; H, 2.76; N, 9.65.

4.2.11. 2-(3,5-Dibromopyridin-4-yl)-3-(4,6-dimethylpyridin-2-yl)thiazolidin-4-one (11)

Light yellow solid, yield: 80.4%. mp: 114–116 °C; IR (KBr): ν_{max} C=O 1691 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.62, 8.47 (s, 2H, H_2, H_6-pyridin-4-yl), 7.88 (s, 1H, H_3-pyridin-2-yl), 7.46 (s, 1H, H_5-pyridin-2-yl), 6.70 (s, 1H, CH), 4.20 (dd, 1H, *J* = 15.7 Hz, 2.01 Hz, CH_2), 4.00 (d, 1H, *J* = 15.7 Hz, CH_2), 2.32 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.22 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.03 (C=O), 155.88 (pyridin-2-yl-C6), 152.84 (pyridin-4-yl-C4), 150.73 (pyridin-2-yl-C4), 149.64 (pyridin-4-yl-C2 & C6), 146.08 (pyridin-2-yl-C2), 123.34 (pyridin-4-yl-C3 & C5), 121.53 (pyridin-2-yl-C5), 112.94 (pyridin-2-yl-C3), 62.67 (C2), 35.94 (C5), 23.26, 21.31 (CH_3 at $C_{6,4}$-pyridin-2-yl); MS (ESI): *m/z* 444 $(M+H^+)$. Anal. calcd for $C_{15}H_{13}Br_2N_3OS$: C, 40.65; H, 2.96; N, 9.48. Found: C, 40.56; H, 2.90; N, 9.52.

4.2.12. 2-(3,5-Dichloropyridin-4-yl)-3-(4,6-dimethylpyridin-2-yl)thiazolidin-4-one (12)

White solid, yield: 81.2%. mp: 117–119 °C; IR (KBr): ν_{max} C=O 1695 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.47, 8.32 (s, 2H, H_2, H_6-pyridin-4-yl), 7.90 (s, 1H, H_5-pyridin-2-yl), 7.45 (s, 1H, H_3- pyridin-2-yl), 6.71 (s, 1H, CH), 4.21 (dd, 1H, *J* = 15.7 Hz, 2.04 Hz, CH_2), 3.99 (d, 1H, *J* = 15.7 Hz, CH_2), 2.32 (s, 3H, CH_3 at C_6-pyridin-2-yl), 2.21 (s, 3H, CH_3 at C_4-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.88 (C=O), 155.90 (pyridin-2-yl-C6),150.18 (pyridin-2-yl-C4) 149.72, 149.56 (pyridin-4-yl-C2 & C6), 147.67 (pyridin-4-yl-C4), 144.19 (pyridin-2-yl-C2), 131.96, 129.79 (pyridin-4-yl-C3 & C5), 121.49 (pyridin-2-yl-C5), 112.64 (pyridin-2-yl-C3), 58.50 (C2), 35.81 (C5), 23.27, 21.33 (CH_3 at $C_{6,4}$-pyridin-2-yl); MS (ESI): *m/z* 354.2 (M^+). HRMS (ESI): $(M+H^+)$, calcd for $C_{15}H_{13}Cl_2N_3OS+H^+$ *m/z* 354.0235, found 354.0193.

4.2.13. 2-(3,5-Dibromo-pyridin-4-yl)-3-(6-methyl-pyridin-2-yl)-thiazolidin-4-one (13)

Light yellow solid, yield: 78.6%. mp: 110–112 °C; IR (KBr): ν_{max} C=O 1688 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.63, 8.47 (s, 2H, H_2, H_6-pyridin-4-yl), 8.09 (d, 1H, *J* = 8.3 Hz, H_3-pyridin-2-yl), 7.62 (d, 1H, *J* = 7.7 Hz, H_4-pyridin-2-yl), 7.46 (d, 1H, *J* = 2.01 Hz, H_5-pyridin-2-yl), 6.88 (s, 1H, CH), 4.21 (dd, 1H, *J* = 15.7 Hz, 2.1 Hz, CH_2), 4.00 (d, 1H, *J* = 15.8 Hz, CH_2), 2.26 (s, 3H, CH_3 at C_6-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.11 (C=O), 156.37 (pyridin-2-yl-C6), 152.93 (pyridin-4-yl-C4), 150.82 (pyridin-2-yl-C2), 149.58, 145.92 (pyridin-4-yl-C2 & C6), 138.22 (pyridin-2-yl-C4), 123.31 (pyridin-4-yl-C3 & C5), 118.64 (pyridin-2-yl-C5), 112.24 (pyridin-2-yl-C3), 62.57 (C2), 35.87 (C5), 23.47 (CH_3 at C_6-pyridin-2-yl); MS (ESI): *m/z* 429 (M^+). Anal. calcd for $C_{14}H_{11}Br_2N_3OS$: C, 39.18; H, 2.58; N, 9.79. Found: C, 39.11; H, 2.60; N, 9.82.

4.2.14. 2-(3,5-Dichloro-pyridin-4-yl)-3-(6-methyl-pyridin-2-yl)-thiazolidin-4-one (14)

Light yellow solid, yield: 77.4%. mp: 108–110 °C; IR (KBr): ν_{max} C=O 1699 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.47, 8.32 (s, 2H, H_2, H_6-pyridin-4-yl), 8.11 (d, 1H, *J* = 8.3 Hz, H_3-pyridin-2-yl), 7.61 (d, 1H, *J* = 7.7 Hz, H_4-pyridin-2-yl), 7.44 (d, 1H, *J* = 1.9 Hz, H_5-pyridin-2-yl), 6.88 (s, 1H, CH), 4.21 (dd, 1H, *J* = 12.8 Hz, 2.2 Hz, CH_2), 3.99 (d, 1H, *J* = 12.9 Hz, CH_2), 2.25 (s, 3H, CH_3 at C_6-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.93 (C=O), 156.37 (pyridin-2-yl-C6), 149.66 (pyridin-2-yl-C2), 148.52 (pyridin-4-yl-C2 & C6), 147.71 (pyridin-4-yl-C4), 138.27 (pyridin-2-yl-C4), 131.90, 129.70 (pyridin-4-yl-C3 & C5), 120.16 (pyridin-2-yl-C5), 111.92 (pyridin-2-yl-C3), 58.42 (C2), 35.70 (C5), 23.43 (CH_3 at C_6-pyridin-2-yl); MS (ESI): *m/z* 340.1 (M^+). HRMS (ESI): $(M+H^+)$, calcd for $C_{14}H_{11}Cl_2N_3OS^+$ *m/z* 339.0000, found 339.0052

4.2.15. 3-(5-Bromo-6-methyl-pyridin-2-yl)-2-(3,5-dibromo-pyridin-4-yl)-thiazolidin-4-one (15)

Light yellow solid, yield: 75.5%. mp: 118–120 °C; IR (KBr): ν_{max} C=O 1695 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.66, 8.49 (s, 2H, H_2, H_6-pyridin-4-yl), 8.07 (d, 1H, *J* = 8.7 Hz, H_3-pyridin-2-yl), 7.81 (d, 1H, *J* = 8.7 Hz, H_4-pyridin-2-yl), 7.39 (s, 1H, CH), 4.21 (dd, 1H, *J* = 15.9 Hz, 1.9 Hz, CH_2), 4.00 (d, 1H, *J* = 15.9 Hz, CH_2), 2.37 (s, 3H, CH_3 at C_6-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.16 (C=O), 155.09 (pyridin-2-yl-C6), 153.01(pyridin-4-yl-C4), 150.87 (pyridin-2-yl-C2), 148.30, 145.63 (pyridin-4-yl-C2 & C6), 141.63 (pyridin-2-yl-C4), 123.22 (pyridin-4-yl-C3 & C5), 116.97 (pyridin-2-yl-C5), 113.88 (pyridin-2-yl-C3), 62.40 (C2), 35.73 (C5), 23.86 (CH_3 at C_6-pyridin-2-yl); MS (ESI): *m/z* 509.8 $(M+H^+)$. Anal. calcd for $C_{14}H_{10}Br_3N_3OS$: C, 33.10; H, 1.98; N, 8.27. Found: C, 33.19; H, 1.91; N, 8.36.

4.2.16. 3-(5-Bromo-6-methyl-pyridin-2-yl)-2-(3,5-dichloro-pyridin-4-yl)-thiazolidin-4-one (16)

Light yellow solid, yield: 77.8%. mp: 120–122 °C; IR (KBr): ν_{max} C=O 1695 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.50, 8.34 (s, 2H, H_2, H_6-pyridin-4-yl), 8.09 (d, 1H, *J* = 8.7 Hz, H_3-pyridin-2-yl), 7.80 (d, 1H, *J* = 8.7 Hz, H_4-pyridin-2-yl), 7.37 (s, 1H, CH), 4.26 (dd, 1H, *J* = 15.9 Hz, 1.9 Hz, CH_2), 3.98 (d, 1H, *J* = 15.9 Hz, CH_2), 2.36 (s, 3H, CH_3 at C_6-pyridin-2-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.97 (C=O), 155.08 (pyridin-2-yl-C6), 149.68 (pyridin-2-yl-C2), 148.40 (pyridin-4-yl-C2 & C6), 147.77 (pyridin-4-yl-C4), 141.68 (pyridin-2-yl-C4), 131.74 (pyridin-4-yl-C3 & C5), 116.86 (pyridin-2-yl-C5), 113.58 (pyridin-2-yl-C3), 58.24 (C2), 35.55 (C5), 23.82 (CH_3 at C_6-pyridin-2-yl); MS (ESI): *m/z* 419.9 (M^+). Anal. calcd for $C_{14}H_{10}BrCl_2N_3OS$: C, 40.12; H, 2.40; N, 10.03. Found: C, 40.21; H, 2.45; N, 10.16.

4.2.17. 3-(6-Chloro-2-(methylthio)pyrimidin-4-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one (17)

White crystals, yield: 78.8%. mp: 120–122 °C; IR (KBr): ν_{max} C=O 1695 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$); δ 8.12 (s, 1H, H_5-pyrimidin-4-yl), 7.09–7.45 (m, 3H, H_3, H_4, & H_5-PhH), 6.95 (s, 1H, CH), 4.18 (d, 1H, *J* = 16.14 Hz, CH_2), 3.88 (d, 1H, *J* = 16.13 Hz, CH_2), 2.27 (s, 3H, SCH_3 at C2- pyrimidin-4-yl); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.36 (C=O), 170.35 (pyrimidin-4-yl-C2), 163.23, 161.83 (pyrimidin-4-yl-C6 & C4), 161.83, 135.23, 129.62, 125.97, 125.54, 115.52 (Ph-C), 105.98 (pyrimidin-4-yl-C5), 57.65 (C2), 34.32 (C5), 13.78 (SCH_3 at C2- pyrimidin-4-yl); MS (ESI): *m/z* 390 (M^+). HRMS (ESI): $(M+H^+)$, calcd for $C_{14}H_{10}Cl_2FN_3OS_2+H^+$ *m/z* 389.9626, found 389.9677.

4.3. In vitro HIV-RT kit assay[19]

The enzymatic HIV-RT inhibition assay was performed by using an RT assay kit as described in the kit protocol (Roche Kit). Briefly, the reaction mixture containing template/primer complex, 2′-deoxy-nucleotide-5′-triphosphates (dNTP's) and reverse transcriptase (RT) enzyme in the lysis buffer was incubated for 1 h at 37 °C and subsequently, the reaction mix was transferred to streptavidine-coated microtitre plate (MTP). The biotin labeled dNTPs that are incorporated in the template due to activity of RT bind to streptavidine. The unbound dNTPs were washed using wash buffer and anti-digoxigenin-peroxidase (DIG-POD) was added onto MTP. The DIG-labeled dNTPs incorporated in the template was bound to anti-DIG-POD antibody. The unbound anti-DIG-POD was washed and the peroxide substrate (ABST) was added to the MTP. A colored reaction product was produced during the cleavage of the substrate catalyses by a peroxide enzyme. The absorbance of the sample was determined at O.D. 405 nM using microtiter plate ELISA reader. The resulting color intensity is directly proportional to the actual RT activity. The percentage inhibitory activity of RT inhibitors was calculated by comparing to a sample that does not contain an inhibitor. The percentage inhibition was calculated by formula as given:

$$\% \text{ Inhibition} = 100 - \left[\frac{O.D.\ 405\ nm\ with\ inhibitor}{O.D.\ 405\ nm\ without\ inhibitor} \times 100\right]$$

4.4. In vitro anti-HIV assay[20]

4.4.1. Cell line and viral stocks

Cell Line: TZM-bl cells were obtained from NIH AIDS Research and Reference Reagent Programme (ARRRP), NIH, USA. These are genetically engineered HeLa cell clone that expresses CD4, CXCR4, and CCR5 and contains Tat-responsive reporter genes for firefly luciferase (Luc) and Escherichia coli β-galactosidase under regulatory control of an HIV-1 long terminal repeat. The cells were maintained in Dulbecco's modified Eagle's medium DMEM (Gibco, USA) containing 10% heat inactivated fetal bovine serum (Morgate, Australia), HEPES (Gibco, USA), penicillin and streptomycin (Gibco, USA) and gentamicin (Sigma, USA). Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Viral Stocks: The lab adapted strains HIV-1 III_B (X4, subtype B), HIV-1 ADA5 (R5, Subtype B) and the primary isolates HIV-1 UG070 (X4, Subtype D) were obtained from NIH ARRRP, whereas R5 tropic isolate HIV-1 VB59 (Subtype C) is an Indian isolate from the National AIDS Research Institute, Pune. The viruses were grown in PHA-P activated (Sigma, USA) peripheral blood mononuclear cells (PBMC) derived from healthy donors. Virus production was quantified in cell culture supernatants by HIV-p24 antigen detection kit (Vironostika HIV-1 Antigen, Biomerieux, Netherlands). Aliquots of cell-free culture viral supernatants, stored at −70 °C were used as the viral stocks and the 50% tissue culture infectivity dose ($TCID_{50}$) of each isolate was determined in the appropriate cell line by spearman Karber formula.

4.4.2. Cell associated anti-HIV-1 assay

A 96-well plate was seeded with 1×10^4 TZM-bl cells per well and incubated overnight at 37 °C with 5% CO_2. On the next day the medium was replaced with HIV-1 stock and incubated for 2 h at 37 °C. Concentrations of each compound showing more than 50% viability were then added onto the cells. After two days, cells were assayed for luciferase activity using Britelite Plus substrate (Perkin Elmer) by measuring the relative luminescence units (RLU). Nevirapine was used as positive control. Two independent assays using duplicate measurements were carried out for each virus. In this assay, RLU is directly proportional to the number of virus particles and inversely to the percent of inhibition. IC_{50} was determined by Luc software.

4.4.3. Cytotoxicity assay in TZM-bl cell line

A 96-well plate was seeded with 1×10^4 TZM-bl (JC53BL-13) cells per well and incubated overnight at 37 °C with 5% CO_2 atmosphere. On the next day, the medium was replaced with 100 μL of twofold dilution series of each compound and was incubated further. After 48 h, cell viability was determined by MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, USA). Three independent assays using quadruplicate measurements were carried out for each compound. In this assay, the measured absorbance is proportional to the viable cell number and inversely to the degree of cytotoxicity.

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