Molecular docking and anti-viral screening of *N*-substituted benzyl/phenyl-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-c][1,2]benzothiazin-2(4*H*)-yl)acetamides

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Graphical Abstract

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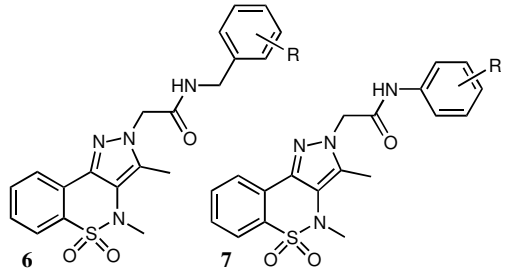
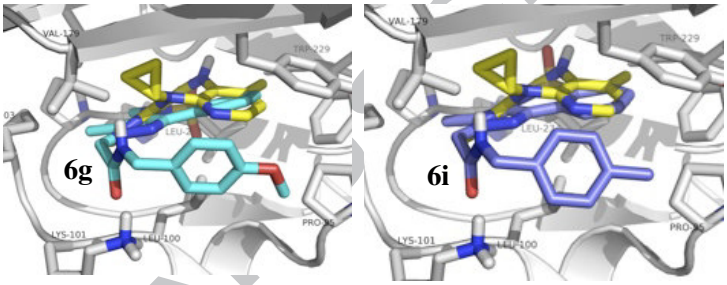


6 9 examples
EC_{50} = 16.7-57.5 µM

7 6 examples
EC_{50} = 6.3-7.6 µM



Benzylamides **6g** and **6i** docked to HIV-1 NNRTI binding site, superimposition with nevirapine (yellow)

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The research work is dedicated to Prof. Dr. Hamid Latif Siddiqui (Late) who was our beloved teacher (MA, SA & SUFR) and our friend and colleague (MD, JMG & RFS).

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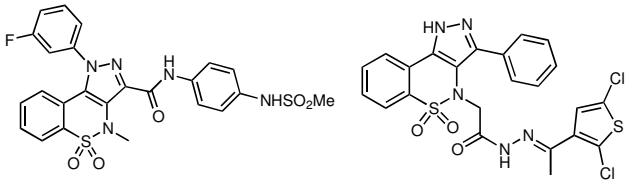
ABSTRACT

Two series of fifteen *N*-substituted benzyl/phenyl-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-*c*][1,2]benzothiazin-2(4*H*)-yl)acetamides were screened for anti-HIV-1 activity and cytotoxicity. The compounds **6a, 6d, 6e, 6g** and **6i** from the series **6a-i** of benzylamides and **7a, 7b, 7c, 7d** and **7e** from the series **7a-f** of anilides were identified as effective anti-HIV-1 agents with EC_{50} values < 20 μM. Among these compounds that displayed anti-HIV-1 activity, **6a, 6e, 6g** and **6i** showed no toxicity in human PBM, CEM and Vero cells, with the exception of **6a** which displayed toxicity in Vero cells. Molecular docking of these compounds provided insight into the molecular mechanism and it was found that **6e, 6g** and **6i** bound deeply in the NNRTI binding pocket of the HIV-1 reverse transcriptase, using RT-bound nevirapine X-ray data and molecular docking for validation, showing the potential of these new structures as inhibitors of this viral enzyme.

Pyrazolobenzothiazines are based on the benzothiazine skeleton, many structurally-varied derivatives of which exhibit a diverse range of biological activities. Various pyrazolobenzothiazine derivatives have been reported to be antipyretic, analgesic and anti-inflammatory,[1,2] antihypertensive,[3] anti-depressant,[4] anti-oxidant and antibacterial agents.[5,6] Recently, compounds based on this ring system have been reported as inhibitors of HCV replication (**a, Figure 1**)[7] and as anti-HIV agents (**b, Figure 1**).[8]

Other derivatives of benzothiazines have been reported to act as anti-inflammatory,[9] antimalarial,[10] antiallergic,[11] anti-thrombotic,[12] antidepressants[13] and antibacterial agents.[14] In our prior work on other 1,2-benzothiazines, we have reported *N'*-arylmethylidene-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-*c*][1,2]benzothiazin-2(4*H*)-yl acetohydrazides,[5] *N*-substitutedbenzyl/phenyl-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-*c*][1,2]benzothiazin-2(4*H*)-yl)acetamides,[15] *N'*-(substituted-2-chloroquinolin-3-yl)methylidene-4-hydroxy-*2H*-1,2-benzothiazine-3-carbohydrazides 1,1-dioxides to be effective antioxidants[6] and pyrazolobenzothiazine-based chalcones as antibacterials.[16]



(**a**, EC_{50} = 3.6 μM) (b, EC_{50} = 3.2 μM)

Figure 1. Structures of antiviral agents belonging to pyrazolobenzothiazine ring system.[7,8]

Human immunodeficiency virus (HIV) is responsible for Acquired Immunodeficiency Syndrome (AIDS) and is treated

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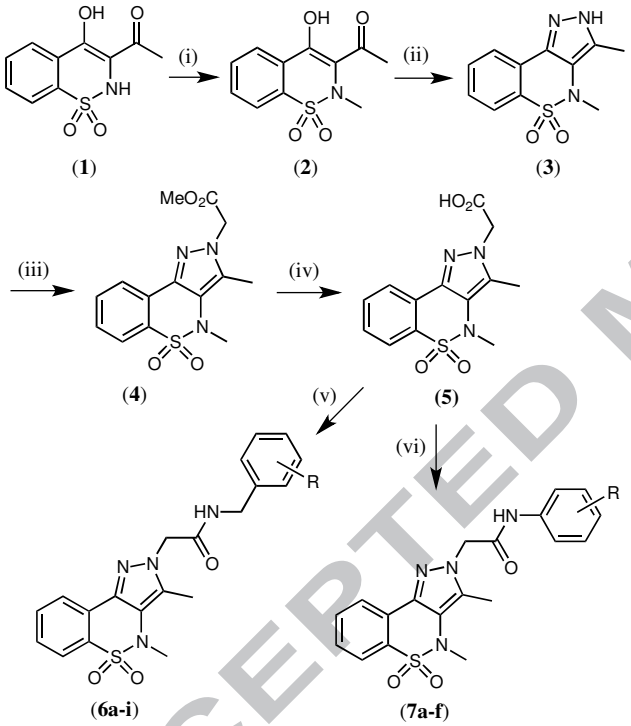
by combination antiretroviral therapy.[17,18,19,20] Such antiretroviral therapy uses a combination of various antiviral drugs and such therapies have now converted AIDS from a routinely fatal, to a chronic disease which can be controlled.[21,22] However, there remains important impetus for the discovery of new anti-HIV leads to address the future risks of resistance to current generations of drugs.[23] Recently, we have evaluated pyrazolobenzothiazine derivatives family having hydrazone moiety in the side chain as anti-HIV agents.[8] Herein we report the further extension of this previous work by exploring anti-HIV-1 potential of the titled carboxamides (**Scheme 1**) providing new SAR information and low-toxicity agents with good anti-HIV activity.

Pyrazolobenzothiazine 5,5-dioxide precursor **1** was prepared from sodium saccharine as a starting material,[5,24] and thence to the target compounds **6a-i** and **7a-f**, as previously reported (**Scheme 1**).[15]

Scheme 1. Synthesis of *N*-substituted benzyl/phenyl-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-*c*][1,2]benzothiazin-2(4*H*)-yl)acetamides. Reagents: (i) $(CH_3)_2SO_4$, $NaOH_{(aq)}$, Acetone (ii) $N_2H_4.H_2O$, EtOH (iii) $BrCH_2COOMe/K_2CO_3$; DMF (iv) NaOH (aq), MeOH (v) Borane-THF complex/Ar-CH_2-NH_2; toluene-THF (1:2) (vi) $SOCl_2$; Ar-NH_2.



a R = 2-Cl **f** R = 3-OMe
b R = 2-F **g** R = 4-OMe
c R = 4-F **h** R = 2-Me
d R = 2-Br **i** R = 4-Me
e R = 2-OMe

a R = H **d** R = 3-Br
b R = 2-F **e** R = 3-OMe
c R = 2-Br **f** R = 2,4-$(OMe)_2$

All the fifteen compounds belonging to both series i.e. (**6a-i & 7a-f)** were tested for their anti-HIV-1 activity and ten compounds exhibited moderate activity (**Table 1**). Among the benzylamides (**6a-i)**, five compounds *i.e*. **6a, 6d, 6e, 6g** and **6i** were found to be active against HIV-1 with EC_{50} values less than 20 μM. It was observed that among halo-substituted derivatives electronegativity and atomic size of the substituent play an important role *i.e*. the activity enhanced with decreasing electronegativity and increasing atomic size of the substituent. Among methoxy-substituted compounds, the following order was observed; 2-MeO > 4-MeO > 3-MeO with EC_{50} values less than 20 μM. Moreover other electron donating group *e.g.* methyl substitution at 4-position in compound **6i** showed significant anti-HIV-1 activity with EC_{50} of 9.9 μM whereas **6h** (2-Me) was totally inactive with EC_{50} greater than 100 μM.

In the series of anilides (**7a-f**), compounds **7a, 7b, 7c, 7d and 7e** were effective inhibitors of HIV-1. It was found that compound **7a** bearing an unsubstituted phenyl moiety displayed comparable anti-HIV-1 activity (EC_{50} of 6.8 μM) to the halogen-substituted compounds (**7b**-**7d**) and the mono-methoxy bearing **7e**. Compound **7d** having 3-bromo moiety showed the most potent inhibitory activity against HIV-1 amongst these compounds with an EC_{50} of 6.3 μM, whilst the 2-bromo derivative **7c** and methoxy-substituted compound **7e** both exhibited similar activities with EC_{50} values of 6.5 μM and 7.2 μM, respectively. Whilst Table **1** illustrates that the amide derivatives with either no or one aryl substituent (electron-withdrawing substituents such as chloro and bromo or an electron-donating methoxy group) had significantly higher (at least 10-fold) anti-HIV activities than **7f** containing two electron-donating groups. It is worthwhile to mention here that all anti-HIV compounds from anilide (**7a-e**) series were significantly toxic to normal human PBM, CEM and Vero cell lines.

Compounds were also evaluated for cytotoxicity in primary human PBM, CEM and Vero cells to determine their spectrum of toxicity. CEM cells are a line of lymphoblastic cells originally derived from a child with acute lymphoblastic leukemia whereas Vero cells are derived from African Green monkey kidney cells. In the series **6a-i**, none of compounds showed cytotoxicity in primary human PBM cells except **6f**, which was toxic to all the three cell systems used. In addition, **6c** was toxic to CEM cells and **6a** to Vero cells and **6d** was toxic to both CEM and Vero cells. However, among the series of compounds **7a-f**, five compounds *i.e*. **7a-e** showed acute toxicity in primary human PBM, CEM and Vero cells, with the exception of **7e** which showed no toxicity in Vero cells. Compound **7f** showed no toxicity in the cell systems used with IC_{50} values greater than 100 μM.

To propose a mode of inhibitory action, these synthetic compounds were investigated by docking into the HIV-1 reverse transcriptase NNRTI drug-binding site. The best fit compounds **6e, 6g** and **6i** were selected on the bases of their anti-HIV activity and lower toxicity. The best conformations from docking of the compounds were selected on the basis of their Fred chemguass score[25] shown in **Table 2** and visual inspection. Fred docking tool uses the shapes and implicit solvent interaction terms to pose and score small molecules in protein binding sites. Interestingly, we have found a good relationship between docking scores and anti-HIV activities of these compounds which would be consistent with their predicted plausible binding modes shown in **figure 2**. The compound **6i**, **6e** and **6g** possessed the best scores in descending order**.** Compound **6i** having methyl group substitution at the tail phenyl ring was ranked best with Fred docking score of -13.775. Similarly nevirapine, a potent clinical anti-HIV NNRTI compound, was also docked with the NNRTI binding pocket of HIV-1 reverse transcriptase to validate the docking procedure (**Figure 3**). In docking

simulations, we observed that the compounds efficiently docked in the binding pocket of HIV-1 reverse transcriptase, thus consistent with their experimental anti-HIV activity.

Table 1. Anti-HIV-1 and cytotoxic activity of *N*-substituted benzyl/phenyl-2-(3,4-dimethyl-5,5-dioxidopyrazolo[4,3-*c*][1,2]benzothiazin-2(4*H*)-yl)acetamides.

	Anti-HIV-1 Activity in Human PBM cells* (μM)			Cytotoxicity* (IC_{50}, μM)		
Code	**R**	**EC_{50}**	**EC_{90}**	**PBM**	**CEM**	**Vero**
6a	2-Cl	15.9	28.5	74.2	68.4	38.7
6b	2-F	57.5	≥100	87.5	82.0	96.1
6c	4-F	22.4	≥100	>100	41.9	>100
6d	2-Br	16.7	87.5	63.1	38.1	36.5
6e	2-OMe	17.2	60.3	≥100	>100	>100
6f	3-OMe	21.1	92.0	18.7	28.8	31.1
6g	4-OMe	18.6	≥100	>100	>100	73.0
6h	2-Me	≥100		>100	>100	>100
6i	4-Me	9.9	77.7	52.2	>100	63.1
7a	H	6.8	16.3	7.7	<1.0	6.8
7b	2-F	7.6	35.4	18.0	9.5	28.2
7c	2-Br	6.5	15.7	2.4	6.0	8.2
7d	3-Br	6.3	15.3	8.1	5.2	7.2
7e	3-OMe	7.2	17.3	10.5	9.3	≥100
7f	2,4-$(OMe)_2$	>100		>100	>100	>100
AZT		0.0033	0.031	>100	14.3	56.0

***All experiments were conducted in replicate.**

Table 2.
Docking scores of anti-HIV-1 compounds with HIV-1 reverse transcriptase.

Sr. No.	Compound	Fred Chemguass Score
1	**6e**	-13.644
2	**6g**	-12.323
3	**6i**	-13.775
4	**Nevirapine**	-14.104

Post-docking analysis also indicated that compound **6g, 6e** and **6i** had good hydrophobic interactions with Leu100, Val106, Tyr-181, and Tyr-188. All these compounds have been gorged well in the hydrophobic binding pocket of HIV-1 reverse transcriptase, shown in **figure 2**. We have also observed that these compounds have t-type π-π stacking between phenyl ring of head group and Trp-229 residue, which might be responsible for their good binding affinity with HIV-1 reverse transcriptase.

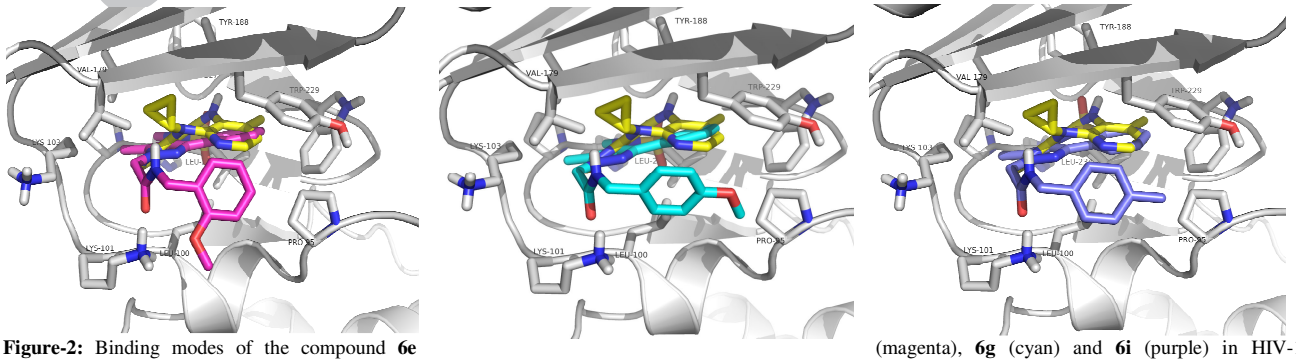


Figure-2: Binding modes of the compound **6e** (magenta), **6g** (cyan) and **6i** (purple) in HIV-1 reverse transcriptase NNRTI binding pocket. Nevirapine (Yellow) is the co-crystal structure with in HIV-1 reverse transcriptase.

Docking of nevirapine with HIV-1 reverse transcriptase showed that it had interactions with Leu100, Val106, Glu138, Tyr181, Tyr188, Gly190, Phe227 and Trp229 (**Figure 3**). These results are consistent with prior work and with the X-ray data for this drug and RT. [26,27,28]

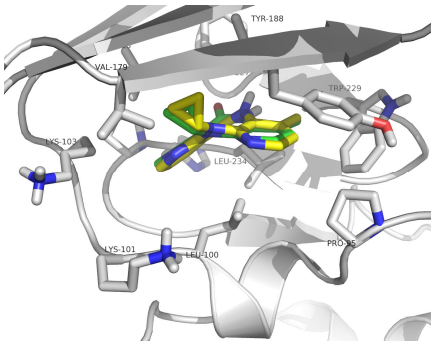


Figure 3: Superposition of docked pose (Green) of Nevirapine over its co-crystal structure (Yellow).

In conclusion, most of the compounds evaluated were active as anti-HIV-1 agents *i.e*., **6a, 6d, 6e, 6g and 6i** from the series **6a-i** of benzylamides and **7a, 7b, 7c, 7d, 7e** from the series **7a-h** of anilides. Among these compounds that displayed anti-HIV-1 activity, **6e, 6g** and **6i** showed no toxicity in primary human PBM, CEM and Vero cells. Furthermore, the mechanism of effective antiviral compounds was proposed from molecular docking and interaction analysis studies with HIV-1 RT as the target of drug action. These evaluations indicate the potential of the pyrazolobenzothiazine ring system as a lead structure group for the potential development of new anti-HIV agents, and in particular would select compounds of type **6** over type **7** based on the differential toxicity data.

Experimental Protocols

Anti-HIV-1 In order to evaluate the synthesized compounds for their *in vitro* antiviral effects in primary human peripheral blood mononuclear (PBM) cells, we used a standardized assay as previously described by Schinazi *et al*. (**1990**).[29] Cells were obtained from Life South Community Blood Centers (Atlanta, GA), were isolated by Histopaque (Sigma-Aldrich, St. Louis, MO) and discontinuous gradient centrifugation from healthy seronegative donors. The median effective concentration (EC_{50}) was determined using a reported method (Belen'kii and Schinazi, **1994**)[30] Assays were conducted using at least two different donor cells in duplicate or triplicate. The results are presented in Table **I**.

Cytotoxicity Evaluation All the titled compounds were also evaluated for cytotoxicity in primary human PBM, CEM and Vero cells, to determine their spectrum of toxicity. CEM cells are a line of lymphoblastic cells originally derived from a child with acute lymphoblastic leukemiawhereas, Vero cells are derived from African Green monkey kidney cells. These cells (i.e. PBM, CEM and Vero cells) were cultured in 96-well plates ($5\text{-}10^4$ cells per well) along with increasing concentrations of the test compound.[31] Cell viability was measured after 5-day incubation period using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, Wis.) by incubating in an incubator at 37 °C with 5% CO_2 for human PBM cells. The results are also summarized in Table **I**.

Molecular Docking To further evaluate the mechanism of antiviral activity of the reported compounds, a cheminformatics based approach was used employing molecular docking simulations to understand the mechanism of antiviral activity. The three-dimensional crystal structure of the HIV-1 reverse transcriptase was obtained from the Protein Data Bank using PDB ID: 3V81[32]. The 3D structure of the downloaded protein was prepared by removing the water molecules and other heteroatoms from the structure using PDB2RECEPTOR utility implemented in Openeye software suits. Similarly Openeye Fred docking tool was used for docking simulations and its FRED Chemgauss4 score was used for ranking the poses of each docked compound. Maestro software was used for the drawing and 3D conversion of the structures of synthesized compounds. Prior to docking simulations, 200 conformations of each compound were generated using the OMEGA software. The binding modes of the compounds were analyzed using the Pymol visualization program.

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Supplementary Material

Supplementary data (representative experimental procedures, characterization data, and copies of spectra) associated with this article can be found, in the online version, at http://….

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