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Novel Anti-HIV-1 NNRTIs Based on a Pyrazolo[4,3-d]isoxazole Backbone Scaffold: Design, Synthesis and Insights into the Molecular Basis of Action

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Abstract

A series of novel pyrazolo[4,3-d]isoxazoles was synthesized employing the thioamide synthon **3** to obtain the phenyldiazenylthiazolyl derivatives **7a-f**, the thiazolyl derivative **9**, the carbohydrazonamide **12** and the triazinyl counterparts **14a-c.** The prepared compounds were screened for their antiviral activities against two viral strains of HIV-1 (RF and IIIB). All the compounds exhibited highly potent antiviral capacity having submicromolar to subnanomolar EC_{50} values with all derivatives being more active against the tested HIV strains than the reference drug efavirenz. The therapeutic index of these novel pyrazoloisoxazoles was also evaluated against the host cells CEM-SS or MT-4 and they exhibited high therapeutic window. To further investigate the molecular basis of their action, the inhibitory ability of these compounds was bioscreened against the HIV-1 viral enzyme reverse transcriptase (RT). The very potent inhibitory power observed by the pyrazolo[4,3-d]isoxazoles against RT prompted a molecular docking study to try to explore the potential binding mode of these compounds with their respective molecular target. Finally, *in vitro* exploration of the metabolic stability of this series of compounds was evaluated employing rat-plasma half-life assay and they demonstrated reasonable hydrolytic resistance.

Key words: Pyrazoloisoxazole, phenyldiazenylthiazoles, phenyldiazenyltriazines, anti-HIV activity, reverse-transcriptase inhibition, rat-plasma half-life.

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1. Introduction

Human viral infections have been linked with some grave diseases like the human immunodeficiency virus (HIV)-associated acquired immunodeficiency syndrome (AIDS) that is declared by the WHO as a global pandemic.[1] A UNAIDS report demonstrated that the total number of people infected with HIV worldwide in 2012 was 35.3 million, with the number of new infections that year being about 2.3 million and the toll of HIV-related deaths around 1.6 million.[1] Current medical management strategies are based on high active antiretroviral therapy (HAART). HAART options depend on cocktails of at least three medications belonging to at least two types, or "classes," of antiretroviral agents which slow the progress of the disease or improve prognosis.[1] Initially, anti-HIV combinations are typically composed of a non-nucleoside reverse transcriptase inhibitor (NNRTI) plus two nucleoside analogue reverse transcriptase inhibitors (NRTIs). Combinations of agents which include a protease inhibitors (PI) are used if the above regimen loses effectiveness.[1] However, viral resistance to current clinically available antiviral agents has been observed and seems to be inevitable due to the rapid multiple mutations elicited by the various viral strains. On the other hand, vaccination has shown no success in the endeavor for control of this viral infection.[1] Faced with the fact that there is currently no cure or effective HIV vaccine, the quest for novel small molecule antiviral agents is still justifiable.

The majority of the compounds proven to act as anti-HIV agents contain heterocyclic moieties as integral pharmacophoric moieties.[2] Among the pharmaceutically active compounds currently recognized as anti-HIV agents, several heterocyclic moieties have been well established as fundamental pharmacophoric parts of these molecules including thiazoles.[3-11] triazolopyrimidines,[12-15] triazines[16-20] and pyrazoles.[21-25] The use of hydrazonyl halides synthons in the construction of a plethora of heterocyclic biologically active molecules has been much reported in literature. Previous research work from our lab has described the utility of hydrazonyl halides as quite reactive polyfunctional reagents in the synthesis of a variety of novel heterocyclic systems as benzopyranopyrimidotriazine,[26] substituted thiazoles,[27] chromenotriazolo-pyrimidines,[28] and substituted thiadiazole derivatives.[29]

Herein, we report the design and synthesis of new compounds based on the pyrazolo[4,3-d]isoxazole backbone scaffold bearing a phenyldiazenylthiazolyl or phenyldiazenyltriazinyl side chain as potential novel NNRTIs. The aim of performing these structural manipulations is to assess their effect of combination of more than one of these heterocycles in a single molecule on

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their anti-HIV-1 activity. Thus, the synthesized compounds were screened against two viral strains of HIV-1 *viz.* HIV-1_{RF} and HIV-1_{IIIB}. To verify the proposed mode of action of the synthesized derivatives and correlate it with their observed anti-HIV activity, all the target compounds were bioscreened for their inhibitory capacity towards the functional viral enzyme HIV-1 reverse transcriptase (RT). Also, molecular docking of the synthesized compounds was performed with the protein data bank exported co-crystal structure of HIV-1 RT with the well-known clinically used NNRTI efavirenz to predict their possible binding mode with the essential binding pocket amino acids. Finally, metabolic resistance of these compounds was determined by measuring the *in vitro* hydrolytic stability in rat-plasma by measuring their half-lives ($t_{1/2}$).

2. Results and discussion

2.1. Chemistry

The synthesis of the target novel pyrazolo[4,3-d]isoxazole derivatives is depicted in schemes 1-4. Considering the versatile chemistry of thiosemicarbazide **2** and its ability to react with enormous compounds, it was reacted with the isoxazolone derivative **1** in ethanol catalyzed by sodium hydroxide under reflux to give the pyrazoloisoxazolone derivative **3** instead of the pyrimidinethione **4** (scheme 1). The formation of compound **3** over **4** was elucidated from the fact that the amino group far away from C=S group is more reactive than that adjacent to C=S group. In addition, according to literature reports the reaction of thiosemicarbazide with arylidenelactones resulted in pyrazole formation rather than pyrimidine.[30,31] Although the IR and mass spectra would not be conclusive for assigning the structure in favor of either **3** or **4**, ^{1}HNMR was a useful tool to assign the correct structure of the product. The ^{1}HNMR of the isolated product displayed a D_2O exchangeable signal at δ = 8.13 ppm assigned to the amino group which is in accordance with structure **3**; otherwise it would have appeared more downfield if it was as in structure **4**.

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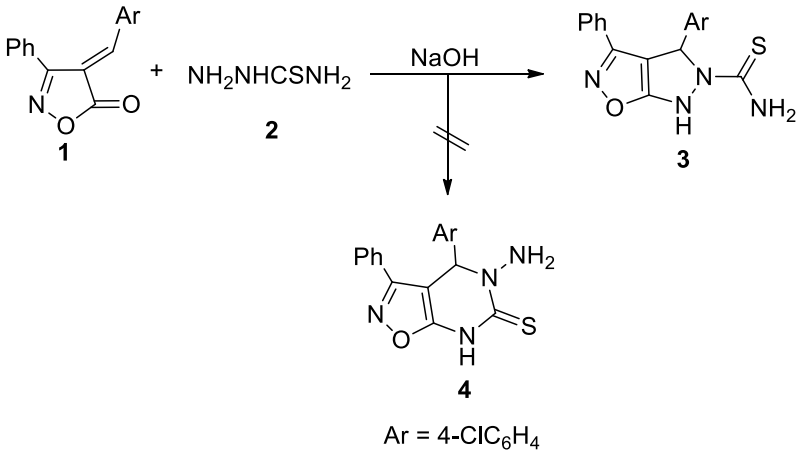

Scheme 1: Synthesis of carbothioamide **3**.

A group of thiazolylpyrazolo[4,3-d]isoxazole **7a-f** was accomplished through 1,3-cycloaddition reaction of nitrilimines (generated *in situ* from hydrazonyl halides **5a-f**) to compound **3** in dioxane containing TEA under reflux temperature as depicted in scheme 2. This reaction itself is an additional proof that the structure of the product obtained from the reaction between isoxazolone **1** and thiosemicarbazide **2** was indeed compound **3** instead of **4**. To eliminate further doubt, it is known that when thiones were brought to react with hydrazonyl halides, they deliver products with concurrent release of hydrogen sulfide which was not the case in our reaction and hence justifying that the obtained structure was certainly **3**. The compounds **7a-f** are absolutely present in the azo form which is indicated from their dark colors and the ^{1}HNMR spectra of these compounds displayed a singlet signal in the range of δ 2.57-2.62 ppm related to the methyl protons (thiazole ring).

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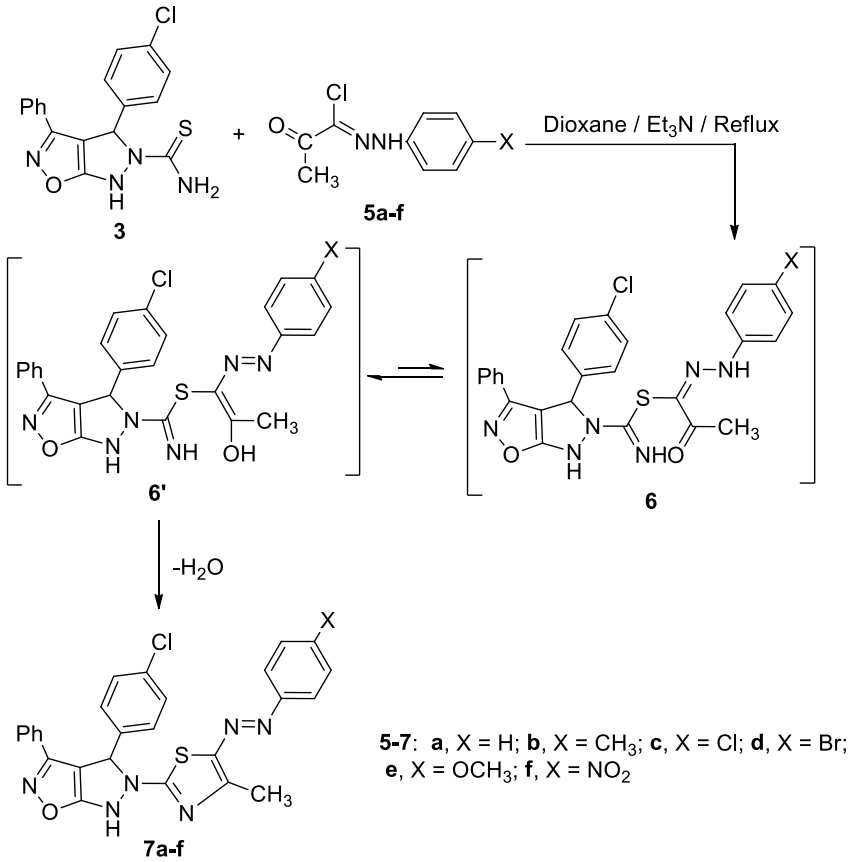

Scheme 2: Synthesis of thiazol-2-ylpyrazolo[4,3-d]isoxazole diazenyl derivatives **7a-f**.

An expeditious alternative route for the synthesis of the azo-derivative **7a** considered as an authentic sample was performed through the classic Hantzsch synthesis between compound **3** and chloroacetone in ethanol to give compound **9** which in turn was coupled to aryl diazonium salts furnishing the target compound **7a** (outlined in scheme 3).

On the other hand, reaction of 2-hydrazinyl-4-methyl-5-(phenyldiazenyl)thiazole **10** with compound **1** afforded a product identical in all aspects (mp., mixed mp. and IR spectra) with **7a** (Scheme 3).

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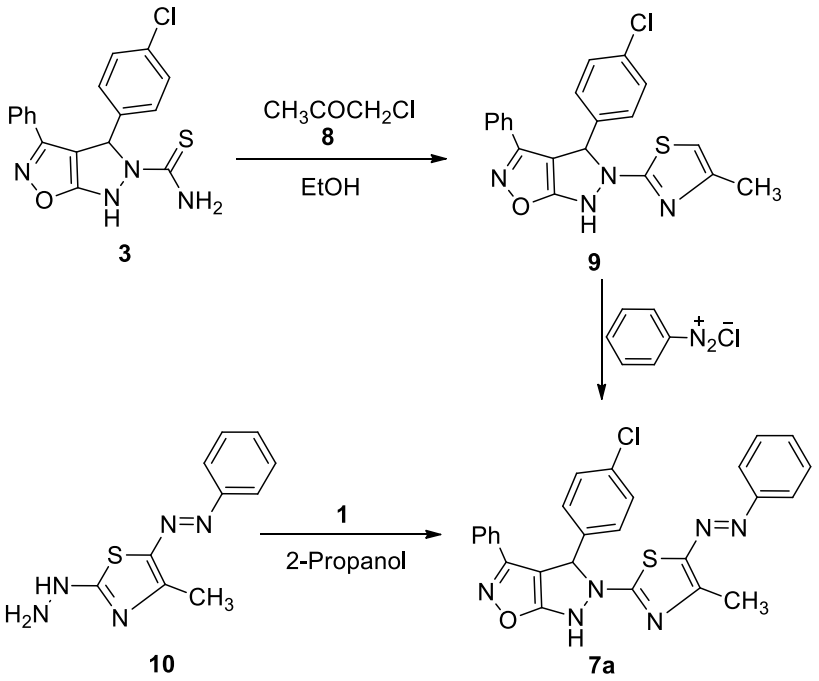

Scheme 3: Alternate routes for the synthesis of **7a**.

The essential idea of the work in this article is mainly dependent on the use of hydrazonyl halides for heterocyclic synthesis. Therefore, the reaction between the title compound **3** with hydrazine hydrate in ethanol under reflux resulted in concomitant evolution of hydrogen sulfide to afford the guanidine derivative **12** (scheme 4). IR spectrum of compound **12** displayed a very broad band absorption in the range of 3429-3178 cm^{-1} due to the NH_2 groups and NH function, thereafter, its mass spectrum showed a molecular ion peak at m/e = 354 which is consistent with the proposed structure.

Consequently, the behavior of compound **12** towards 1,3-dipolar cycloaddition reaction was investigated. The reaction of **12** with hydrazonyl halides **5a-c** was conducted in ethanol using TEA as a catalyst to deliver the triazines **14a-c** (scheme 4). The products formed may be present in the hydrazo (**14'**) or the azo (**14**) tautomer but since they were dark in color, this suggested their existence mainly in the azo (**14**) form. Structure elucidation of the products was asserted through the IR, mass and NMR spectral data. In the ^1H NMR spectrum of compounds **14a-c**, a singlet signal at the range of δ 2.55 ppm assigned to the CH_3 protons was observed as well as three more downfield singlet signals (D_2O exchangeable) attributed to the NH functions.

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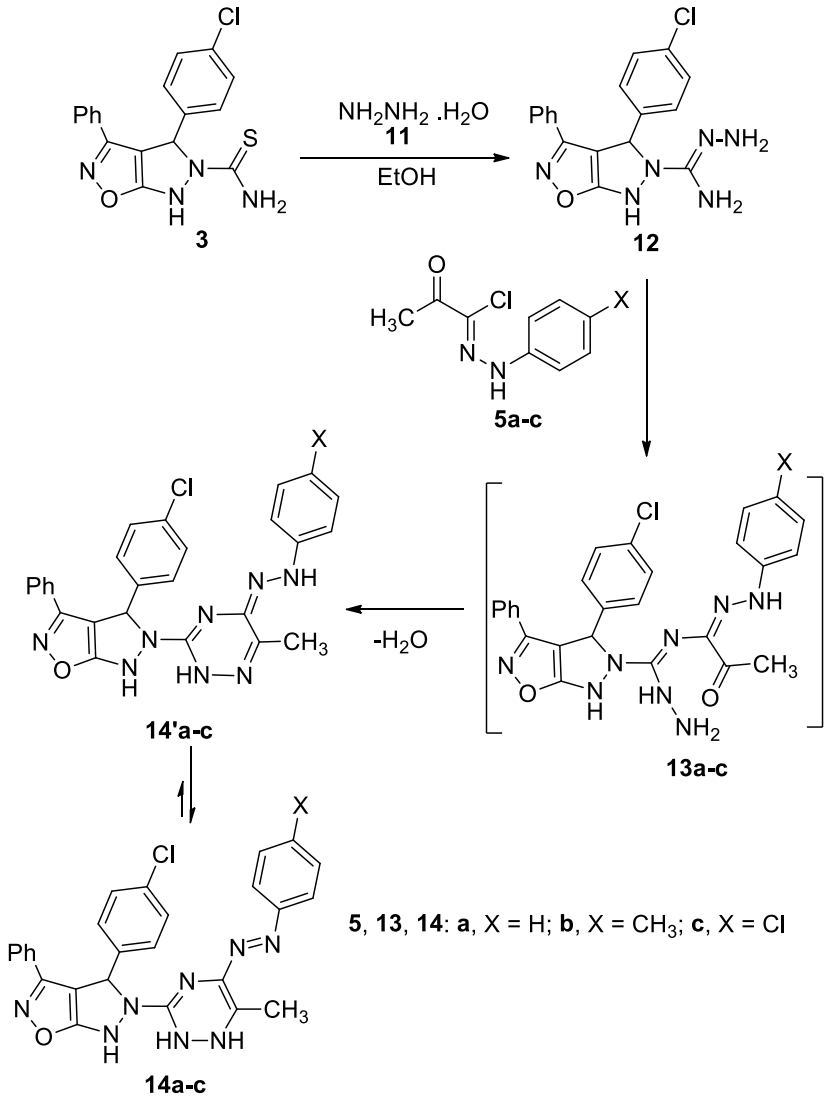

Scheme 4: Synthesis of 1,2,4-triazin-3-ylpyrazolo[4,3-d]isoxazole diazenyl derivatives **14a-c**.

2.2. Anti-HIV and RT inhibitory activities and structure-activity relationship (SAR) findings

Human immunodeficiency virus (HIV) is the causative organism behind the acquired immunodeficiency syndrome (AIDS). The rapid emergence of resistance towards current clinically useful NNRTI anti-HIV agents encourages the search for new molecules with anti-HIV capacity. Therefore, the ability of the newly synthesized compounds to inhibit the cytopathic effect of HIV-1 in cell culture was assessed against the three viral strain HIV-1_{RF} on CEM-SS cells and HIV-1_{IIIB} strain on MT-4 cells indicated as effective concentration of the test agent to inhibit viral cytopathicity by 50% (EC_{50} values) compared to efavirenz as reference compound. Cytotoxicities of these derivatives were also determined on the CEM-SS and MT-4 cell expressed as the concentration of the test compound to induces 50% cytotoxicity (CC_{50} values) along with

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calculating their therapeutic indices (TI = CC_{50}/EC_{50}). Results obtained for these experiments are displayed in Table 1.

Table 1. Antiviral data of the studied compounds against different types of HIV and cytotoxicity against host cells.

Comp. no.	HIV-1_{RF}	HIV-1_{IIIB}	CEM-SS	MT-4	CEM-SS	MT-4
	EC_{50} (nM)[a]		CC_{50} (μM)[b]		TI[c]	
3	0.78	0.28	0.36	0.34	461.54	1214.29
7a	0.54	0.3	0.45	0.26	833.33	866.67
7b	0.25	0.55	0.25	0.45	1000	818.18
7c	0.36	0.46	0.33	0.34	916.67	739.13
7d	0.34	0.27	0.14	0.39	411.76	1444.44
7e	0.35	0.44	0.34	0.46	971.43	1045.45
7f	0.25	0.18	0.25	0.28	1000	1555.56
9	0.47	0.35	0.44	0.23	936.17	657.14
12	0.46	0.29	0.34	0.17	739.13	586.21
14a	0.44	0.33	0.23	0.45	522.73	1363.64
14b	0.43	0.49	0.36	0.33	837.21	673.47
14c	0.67	0.37	0.27	0.45	402.99	1216.22
Efavirenz	0.92	0.98	0.55	0.32	347.82	326.53

[a] EC_{50} is the 50% effective concentration for inhibition of cytopathicity of HIV-1_{RF} in CEM-SS cells, and HIV-1_{IIIB} in MT-4 cells.

[b] The CC_{50} is the 50% cytotoxic concentration for mock-infected CEM-SS cells or MT-4 cells.

[c] TI is the therapeutic index: CC_{50}/EC_{50} (HIV-1_{RF} for CEM-SS and HIV-1_{IIIB} for MT-4).

All the tested compounds inhibited the cytopathic effect of HIV with excellent EC_{50} values for HIV-1_{RF} in CEM-SS cells and HIV-1_{IIIB} in MT-4 cells, being more potent than the reference standard efavirenz in both cases.

Concerning the anti-HIV-1_{RF} efficacy, the best activity was displayed by the 4-methylphenyldiazenylthiazole **7b** and its 4-nitro analog **7f** with equivalent EC_{50} values of 0.25 nM. SAR findings demonstrated that cyclization of the carbothioamide **3** (EC_{50} = 0.78 nM) to the thiazole derivative **9** (EC_{50} = 0.47 nM) or the phenyldiazenylthiazole derivatives **7a-d** led to significant enhancement in their anti-HIV potency (EC_{50} range from 0.25 to 0.54 nM). Within the series of the phenyldiazenylthiazoles **7a-d**, it was observed that substitution on the para position of the phenyl ring (compounds **7b-f**, EC_{50} range = 0.25-0.36 nM) always gave better potency than

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the unsubstituted analog **7a** (EC_{50} = 0.54 nM). On the other hand, and with regards to the bioisosteric pair **3** and **12**, the carbohydrazonamide **12** (EC_{50} = 0.46 nM) displayed a 1.7 fold increase in anti-HIV activity compared to its carbothiamide counterpart **3** (EC_{50} = 0.78 nM). However, cyclization of **12** to furnish the phenyldiazenyltriazines **14a-c** did not secure an enhancement of activity in favor of the cyclic analogs (EC_{50} range = 0.43-0.67 nM). It is noteworthy that all the synthesized compounds were 1.2 to 3.7 fold more potent than the well-known NNRTI efavirenz against HIV-1_{RF} (EC_{50} = 0.92 nM).

With regards to anti-HIV activity against HIV-1_{IIIB}, SAR investigations showed that the isosteric pair **3** (EC_{50} = 0.28 nM) and **12** (EC_{50} = 0.29 nM) showed almost similar potency against the HIV-1_{IIIB} strain. On the other hand, cyclic derivatives of **3** viz **9** (EC_{50} = 0.35 nM) and **7a-f** (EC_{50} range = 0.18-0.55 nM) demonstrated equal to lower efficacy with the only exception being **7f** (EC_{50} = 0.18 nM) which was also the most potent derivative among the compounds under investigation in the current study against this HIV-1 tested strain. Moreover, and in line with the observations obtained from the anti-HIV-1_{RF} strain results, structural modification of **12** (EC_{50} = 0.29 nM) through cyclization to the phenyldiazenyltriazens **14a-c** (EC_{50} range = 0.33-0.49 nM) did not yield any gain to the anti-HIV activity. Nevertheless, all the tested compounds were 1.8 to 5.4 times more potent than efavirenz (EC_{50} = 0.98 nM) against HIV-1_{IIIB}.

Regarding the cytotoxicity of these derivatives, though their cytotoxic effect against CEM-SS cells (CC_{50} in the range of 0.14-0.45 µM) is apparently higher than the reference drug efavirenz (CC_{50} 0.55 µM), yet their very potent antiviral activity reflected in their nanomolar level EC_{50} values led to better therapeutic indices (TI = CC_{50}/EC_{50}), TI ranges between 402.99 and 1000, compared to efavirenz (TI = 347.82). This was more obvious when studying the cytotoxicity against MT-4 cells, where efavirenz displayed a TI of 326.53 and all the tested compounds had TIs in the range of 586.21-1555.56 (i.e. 1.78 to 4.76 fold improvement in the therapeutic window).

2.3. HIV-1 reverse transcriptase (RT) inhibitory activities and *in vitro* rat-plasma half-life

The inhibition of HIV-1 RT by the compounds as a plausible mechanism of action was determined and displayed in terms of the test compound concentration that causes 50% reduction in the enzyme's polymerase activity with poly(rC)·oligo(dG) as the template primer (IC_{50} values). Finally, the metabolic stability of these derivatives in rat-plasma was also investigated ($t_{1/2}$ values). The results of these experiments are displayed in table 2.

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Table 2. Reverse transcriptase inhibitory activity of the studied compounds and rat plasma half-life.

Comp. no.	HIV-1 RT IC_{50} (nM)[a]	Rat plasma $t_{1/2}$ (min)
3	0.037	132±3.4
7a	0.019	265±3.5
7b	0.027	343±4.4
7c	0.035	454±5.5
7d	0.016	244±3.7
7e	0.036	234±3.6
7f	0.027	365±3.7
9	0.046	343±4.6
12	0.018	274±2.6
14a	0.045	123±2.7
14b	0.019	154±5.4
14c	0.028	243±4.3
Efavirenz	0.166	88.87±1.22

[a] Inhibitory activity *vs.* HIV-1 reverse transcriptase with poly(rC)·oligo(dG) as the template primer.

Regarding the HIV-1 RT inhibitory screening, all the tested compounds were found to inhibit RT employing poly(rC)·oligo(dG) as the template primer with much higher potency than the reference compound efavirenz (IC_{50} 0.166 nM) eliciting IC_{50} values in the range of 0.016-0.045 nM. The most active RT inhibitor amongst all compounds was **7d** (IC_{50} = 0.016 nM). Structure-activity relationship (SAR) inferences from the obtained results demonstrate that while transformation of the carbothioamide **3** (IC_{50} = 0.037 nM) to its carbohydrazonamide **12** congener (IC_{50} = 0.018 nM) significantly enhanced the activity, cyclization of **3** to the thiazolyl derivative **9** reduced its RT inhibitory efficacy (IC_{50} = 0.046 nM). On the other hand, the phenyldiazenylthiazole derivatives **7a-f** were all more potent than their precursor **9** (IC_{50} range 0.016 to 0.036 nM). Finally, while the substituted phenyldiazenyltriazenes **14b**&**c** were more active than their thiazole counterparts **7b**&**c**, the activity in the case of the unsubstituted analogs **7a** and **14a** was in favor of the thiazole **7a** (IC_{50} = 0.019 nM).

On the other hand, to have a preliminary assessment of the plasma hydrolytic stability of the new derivatives, their half-life ($t_{1/2}$) was investigated utilizing rat plasma assay. The assay results showed that all compounds possessed good stability profiles reflected by their long $t_{1/2}$ (123 to 454 min) which were comparable to efavirenz (88.87 min). This could be structurally

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justifiable in terms of the lack of presence of highly metabolically labile bonds in the structures of the new compounds. However, it is quite appreciated that human plasma half-life measurements would be more reflective of the real plasma stability of these novel compounds.

2.4. Molecular docking

It is well documented that NNRTIs do not bind to the active site of RT, instead they allosterically bind to another distinct site away from the active binding site known as NNRTI pocket. In an attempt to investigate the binding mode of this series of NNRTIs with their biological target, molecular docking experiments were launched using MOE software version 2008.10 employing the default parameters. Docking study was performed for all the synthesized compounds to assess their binding interaction mode with their HIV viral enzyme target utilizing the co-crystal structure of the well-known NNRTI efavirenz with HIV-1 RT that was obtained from the protein data bank (PDB ID: 1IKW). For validation purposes the co-crystallized ligand was re-docked within its binding pocket and the rmsd value was determined. The observed rmsd value was 0.6512 Å for efavirenz in the binding site of RT. Molecular binding simulation results of the most active compound in this study, **7d**, with HIV-1 RT is displayed in figures 1 panels A-D. Docking of all the prepared compounds was performed and they all elicited strong binding in the active pocket of HV-RT (data not shown).

Pertaining to the HIV-1 RT docking results, **7d** was found to bind more strongly to the binding pocket of the target enzyme than the native ligand efavirenz as judged by their binding energy scores (S values = -16.5061 Kcal/mol and -10.9087 Kcal/mol, respectively). Also, it was observed that while efavirenz binds to RT with only one H-bond between its oxazine NH and Lys101, **7d** was able to display two pi-cationic interactions between the thiazolyl and the 4-chlorophenyl rings of the molecule and Lys101 and Lys103 of the active site, respectively (Figure 1, panels A, B and D). The 2D overlay study showed that **7d** binds in the same region as the native ligand efavirenz indicating that **7d** recognizes the NNRTI binding pocket of RT like the well-known NNRTI efavirenz (Figure 1, panel C).

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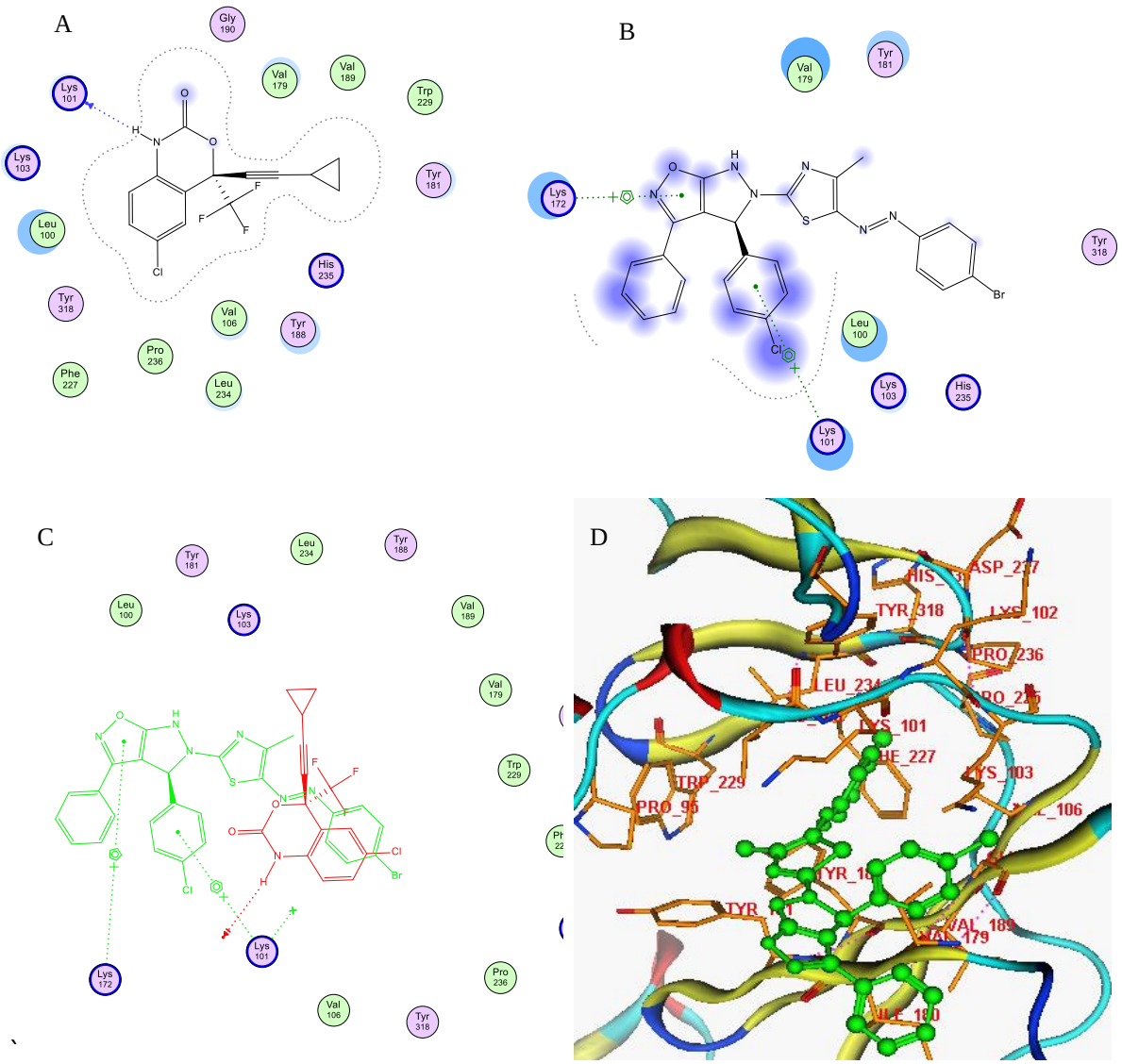

Figure 1. The binding mode of compound 7d with HIV RT (PDB ID: 1IKW). Panels A and B represent the 2D binding mode of the co-crystallized ligand (efavirenz) and **7d** with essential active site amino acids of HIV-RT, respectively. Panels C and D represent the 2D overlay of the co-crystallized ligand (efavirenz, red) and docked **7d** (green) and 3D docking pose of **7d** in the active site of HIV-RT, respectively.

3. Conclusion

In conclusion, we have synthesized a series of pyrazolo[4,3-d]isoxazole derivatives bearing a substituted phenyldiazenylthiazolyl or phenyldiazenyltriazinyl side chain that have been screened against three different strains of HIV. The synthesized compounds have dispalyed potent anti-HIV efficacy with EC_{50} values in the subnanomolar level against HIV. It was observed that in most of the cases the phenyldiazenylthiazolyl derivatives showed better activity compared

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to the phenyldiazenyltriazinyl ones. In fact, the most active antiviral agent in this study was the p-chlorophenyldiazenylthiazolyl derivative **7d** constantly displaying the lowest EC_{50} value in all antiviral assays. All these derivatives also displayed high therapeutic indices (TI values) associated with their low cytotoxicity towards CEM-SS and MT-4 cells thus reflecting selective toxicity towards the viral cells and a high safety margin for non-viral cells. To investigate the possible molecular basis behind these molecules observed antiviral capacity, all compounds were tested against HIV-1 RT. The high inhibitory activity displayed by all the test compounds against the screened enzymes might in great part explain their detected biological activities. Docking study results for the tested compounds were explanatory to the enzyme inhibitory assay results where the *in silico* drug-ligand binding interaction poses observed clarified the binding modes and binding energy scores of these derivatives. Lastly, based upon all the above mentioned findings, the synthesized pyrazolo[4,3-d]isoxazoles furnish a promising molecular scaffold for potent anti-HIV agents thus meriting further investigation which is currently underway.

4. Experimental

4.1. Chemistry

Methods and characterization for all novel compounds are detailed in the ESI.

4.2. Biology

4.2.1. *In vitro* anti-HIV-1 assay and HIV-1 RT inhibition assay

Evaluation of the antiviral activity of the new pyrazolo[4,3-d]isoxazole derivatives against the viral strain HIV-1_{RF} infection in CEM-SS cells was performed using the MTS cytoprotection assay as previously described.[32,33] Evaluation of the antiviral activity of the compounds against the viral strain HIV-1_{IIIB} in MT-4 cells was performed using the MTT assay as previously described.[32,33] For control experiments, mock-virus stocks were prepared by irradiation (20 Gy of 250-KV x-rays) or heat-inactivation for 30 minutes at 59°C.

Analysis of the effects of the compounds on recombinant HIV-1 RT enzyme (p66/51 dimer) was performed as previously described.[34] Briefly, inhibition of purified recombinant reverse transcriptase enzyme was measured by the incorporation of [^{32}P]GTP into poly(rC).oligo(dG) (rCdG) homopolymer template primers.

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4.2.2. *In vitro* hydrolytic stability study in rat plasma

All the compounds were tested for their hydrolytic stability, utilizing rat plasma *in vitro* using 1,1-diphenylethylene as an internal standard and employing previously described experimental protocols.[33]

4.3. Molecular modeling

The crystal structure of the viral enzyme HIV-1 RT (PDB ID: 1IKW) with its respective co-crystallized ligand was obtained from the protein data bank for performing the docking study. All docking procedures were achieved by MOE (Molecular Operating Environment) software 2008.10 provided by chemical computing group, Canada. Docking in the binding pocket of the selected enzyme was performed for all synthesized derivatives for the sake of comparison. Docking protocol was verified by re-docking of the co-crystallized ligand in the vicinity of the binding pocket of the enzyme by specifying a 5 Å grid. The best conformation for each compound was chosen based on the binding energy score (S) for drug-ligand interaction pose analysis.

Abbreviations used

HIV, human immune-deficiency virus; RT, reverse transcriptase; rmsd, root mean square deviation; S, binding energy score; SSPE.

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