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RESEARCH ARTICLE

Synthesis and HIV-1 RT inhibitory action of novel (4/6-substituted benzo[d]thiazol -2-yl)thiazolidin-4-ones. Divergence from the non-competitive inhibition mechanism

Eleni Pitta[1], Athina Geronikaki[1], Sofiko Surmava[2], Phaedra Eleftheriou[2], Vaibhav P. Mehta[3], and Erik V. Van der Eycken[3]

[1]Department of Medicinal Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Greece, [2]Department of Medical Laboratory Studies, School of Health and Medical Care, Alexander Technological Educational Institute of Thessaloniki, Greece, and [3]Laboratory of Organic Synthesis, Department of Chemistry, University of Leuven, Belgium

Abstract

Reverse transcriptase (RT) inhibitors play a major role in the therapy of human immunodeficiency virus type 1 (HIV-1) infection. Although, many compounds are already used as anti-HIV drugs, research on development of novel inhibitors continues, since drug resistant strains appear because of prolonged therapy. In this paper, we present the synthesis and evaluation of HIV-1 RT inhibitory action of eighteen novel (4/6-halogen/MeO/EtO-substituted benzo[d]thiazol-2-yl)thiazolidin-4-ones. The two more active compounds (IC50 : 0.04 μM and 0.25 μM) exhibited better inhibitory action than the reference compound, nevirapine. Docking analysis supports a stable binding of the most active derivative to the allosteric centre of RT. Kinetic analysis of two of the most active compounds indicate an uncompetitive inhibition mode. This is a desired characteristic, since mutations that affect activity of traditional non-competitive NNRTIs may not affect activity of compounds of this series. Interestingly, the less active derivatives (IC50 > 40 μM) exhibit a competitive mode of action.

Keywords RT inhibitors, HIV-1, benzo[d]thiazol-2-yl)thiazolidin-4-ones, competitive, uncompetitive

Introduction

During the past two decades, investigations resulted in development of a great number of potential drugs for the treatment of AIDS, some of which were approved for broad use. Among the different categories of anti-HIV drugs, such as HIV Reverse Transcriptase (RT) inhibitors, Protease Inhibitors, Integrase Inhibitors and Entry Inhibitors, RT inhibitors play the major role in treatment of HIV-infected patients. RT inhibitors structurally belong to two families: the nucleoside/nucleotide RT Inhibitors (NRTI) and the non-nucleoside RT inhibitors (NNRTI). Compared to NRTIs, the commercially available NNRTIs have the advantage of exhibiting less undesired side effects and the disadvantage of easier development of mutations driving to resistant strains. Combination therapies are used to delay development of such mutants. Commercially available NNRTIs are among the ingredients of combination therapies used as a first treatment of infected patients. Though all approved NNRTI have different chemical structures, all of them contact the same site of the molecule of RT. Therefore, a mutation providing resistance to one NNRTI, also provides resistance to all other NNRTIs ("cross resistance")[1,2]. So, the need for developing novel NNRT inhibitors still exists, and development of structures with differences in binding mode is desired[1]. Moreover, achievement of lower toxicity

Address for Correspondence: Prof. A. Geronikaki, Department of Medicinal Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki, 54124, Greece. Tel: +30 2310997616. Fax: +302310997612. E-mail: geronik@pharm.auth.gr; Dr. Phaedra Eleftheriou, Department of Medical Laboratory Studies, School of Health and Medical Care, Alexander Technological Educational Institute of Thessaloniki, Thessaloniki, 57400, Greece. Tel: +302310997616. E-mail: elfther@mls.teithe.grv

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treatments may be an additional expectation of novel agents. Liver toxicity is among the undesired side effects of currently used NNRTIs like nevirapine and efavirenz while central nervous system (CNS) toxicity is associated with treatments containing efavirenz[3].

Commercially available NNRTIs are complex compounds bearing a variety of heterocyclic rings such as benzoxazin-2-one (efavirenz), dipyrido[1,4]diazepin-6-one (nevirapine), pyrimidine (efavirenz)[4], piperazine and indolyl moieties (delavirdine)[5]. Apart from molecules having received FDA acceptance, many compounds have been found to exhibit RT inhibitory action among which derivatives of (hydroxyethoxy-methyl)-6-(phenyl thio) thymine (HEPT)[6], tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepine-2(1H)-one and–thione (TIBO)[7], dipyridodiazepinone like nevirapine[8], 3-[[(1,3-benzoxazol-2-yl) methyl]amino]-5-ethyl-6-methylpyridin-2(1H)-one[9], bis(heteroaryl)piperazines (BHAPs) like delavirdine[10], alpha-anilinophenylacetamide (alpha-APA)[11], 1H,3H-thiazolo[3,4-a]benzimidazole (TBZs)[12], diarylpyrimidin (DAPY)[13] like rilpivirine and 2,3-diaryl-1,3-thiazolidin-4-ones[14,15]. Reverse trancriptase inhibition potency differs among inhibitors. Two commonly used and effective NNRTIs, nevirapine and efavirenz, strongly differ in IC_{50} value with nevirapine exhibiting an indicative IC_{50} of 0.28 μM and efavirenz having an IC_{50} of 47 nm[16,17].

Kinetic studies revealed that currently used NNRT inhibitors have a non-competitive mode of action against both RNA:primer hybrid and dNTPs [18,19] and most of the NNRTIs discovered till now also exhibit the same way of action[8,9,19]. Crystallographic studies revealed that non-competitive NNRTIs bind to an allosteric centre that is located near the RNA depended polymerase active site of the enzyme on subunit p66[20,21]. Aromatic aminoacids Tyr181, Tyr188, Phe227, Trp229, Tyr318 and hydrophobic aminoacids Pro95, Leu100, Val106, Val108, Val179, Leu234, Pro236 form the hydrophobic cleft of the allosteric binding site[22]. Aromatic π→π interactions and hydrophobic interactions are essential for RT-inhibitor complex stabilization. Hydrogen bonds with Lys101 or Lys103 are present in many cases. The strong involvement of Lys103 in complex stabilization may be responsible for the high frequency (>50%) of mutations of this residue in resistant strains. Crystallographic studies of first generation NNRTIs showed that a butterfly conformation of the molecules favoured binding and was mandatory for effective compounds[19,22]. However, inhibitors adapting different conformations, such as the 4-dihydroquinoxalin-2(1H)-thione derivative, HBY097, were found to strongly interact with the active site as well[23]. Moreover, flexible molecules with the capability to acquire multiple conformations, like etravirine, were found to present inhibition activity against more mutated strains[24]. The allosteric centre is not present in pure RT and is created after interaction with the inhibitor[16]. Moreover, although, the same aminoacids are involved in enzyme-compound interaction of all studied complexes, there are slight differences in the volume of the cavity formed depending on the inhibitor.

Interestingly, besides non-competitive inhibitors, NNRTIs with different mode of action were also mentioned. The bis(heteroaryl)piperazine inhibitor (BHAP), U-90152E, acts as a mixed inhibitor with respect to the template: primer and dNTP binding sites for both the RNA- and DNA-directed DNA polymerase domains of the enzyme[25] while chloroxoquinolinic ribonucleoside, 6-chloro-1,4-dihydro-4-oxo-1-(beta-D-ribofuranosyl) quinoline-3-carboxylic acid, was found to inhibit RT with an uncompetitive and a non-competitive mode of action with respect to dTTP incorporation and to RNA:primer template respectively[26].

During the last years, flexible molecules or molecules with unusual conformation or mode of inhibitory action attracted the interest of the scientists since there is increased probability that these molecules retain capability to inhibit RT mutants resistant to commercially available, traditional NNRTIs.

Thiazolyl/thiazolidinone derivatives with various biological activities have been synthesized by many investigators during the last decades. Analgestic, anti-inflammatory, COX, LOX inhibitory action and antimicrobial activity are among the pharmaceutically interesting properties that were detected in different thiazolyl/thiazolidinone derivatives during the past years[27–29]. A number of thiazolidinone derivatives with anti-HIV activity have been found by different investigators[14,15,30–34]. In 2007, Rawal et al. synthesised and tested a number of 2-aryl-3-heteroaryl-1,3-thiazolidin-4-ones with anti-HIV-1 RT inhibitory action[34]. Most recently, two 3-[4-(2-adamantyl)-1,3-thiazol-2-yl]-2-(dihalogenphenyl)-1,3-thiazolidin-4-ones with uncommon mode of inhibitory action were synthesized by our team[21]. The compounds showed uncompetitive or mixed mechanism of inhibition for RNA/primer template and competitive mechanism for dNTPs[21].

In this study, we present the synthesis and evaluation of HIV-1 RT inhibitory activity of nineteen 2-(2,6-dihalophenyl)-3-(4/6-substituted benzo[d]thiazol-2-yl) thiazolidin-4-ones. Eighteen of them are novel compounds, while one of them, the 3-(benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one had been previously synthesized by Rawal et al and found to have low anti-HIV-1 RT activity[34]. This compound is also tested in the present study for comparison reasons, since the novel compounds have the same main scaffold with it but contain a substituted benzo[d]thiazol-2-yl moiety. The novel compounds differentiate with each other in the substituents of the benzo[d]thiazol-2-yl moiety as well as in the halogens of the 2,6-dihalophenyl moiety. The substituted compounds are divided into two categories: 4/6-halogen substituted benzo [d]thiazol-2-yl derivatives and 4/6-MeO/EtO substituted benzo[d]thiazol-2-yl derivatives. Kinetic studies and docking analysis were performed for some of the tested compounds.

Methods

Chemistry

Melting points of the compounds were determined and are uncorrected. IR spectra were recorded on Perkin Elmer Spectrum BX and DR-8001 Shimadzu. ^{1}H, ^{13}C NMR spectra were recorded on 300 MHz instrument. The ^{1}H chemical shifts are reported in ppm relative to tetramethylsilane. High-resolution mass spectra were recorded by using ion source temperature 150–250°C as required. High-resolution EI-mass spectra were performed with a resolution of 10,000. The purity of compounds was proved by combustion method. Elemental analyses were obtained on an acceptable range (±/0.4%) in a Perkin-/Elmer 240B CHN analyzer (The Perkin-/Elmer Corporation Ltd., Lane Beaconsfield, Bucks, UK). For thin-layer chromatography, analytical TLC plates SIL G/UV254 and 70–230 mesh silica gel were used. Reagents were used without further purification.

General procedure for the synthesis of thiazolidinones by conventional method[15,21,31,33]

The appropriate (hetero) aromatic amine (1.0 mmol) and 2,6-dihalosubstituted benzaldehyde or monosubstituted benzaldehyde (1.2 mmol) were stirred in dry toluene under reflux condition followed by addition of mercatoacetic acid (2.0 mmol). The reaction mixture was refluxed for an additional 20–26 h, concentrated to dryness under reduce pressure and the residue was taken up in ethyl acetate. The organic layer was washed with 5% aq citric acid, water, 5% aq sodium hydrogen carbonate, and finally with brine. The organic layer was dried over sodium sulfate and removed under reduced pressure to give a crude product. The later was purified by column chromatography on silica-gel using hexane-ethyl acetate (8:2) as eluent. The structures of synthesized compounds were characterized by TLC, ^{1}H-NMR, ^{13}C-NMR and HRMS.

Microwave irradiation experiments

All microwave irradiation experiments were carried out in a dedicated CEM-Discover monomode microwave apparatus, operating at a frequency of 2.45GHz with continuous irradiation power from 0 to 300 W with utilization of the standard absorbance level of 300 W maximum power. The reagents, aminobenzothiazole, equimolar amount of 2,6-dihalosubstituted benzaldehyde (1.5 mmol) and mercaptoacetic acid were placed in a 10 mL reaction vial containing a stirring bar. The vial was kept under argon for 30 s and then sealed tightly with a Teflon septum and placed into the microwave cavity. It was irradiated at a required ceiling temperature of 80–100°C using 100 W as maximum power for 10–60 min. Then the reaction mixture was rapidly cooled with gas jet cooling to ambient temperature.

3-(Benzo[d]thiazol-2-yl)-2-(2,6-difluorophenyl)thiazolidin-4--one (1). Yield: 43%, m.p. 143-4 °C, R_f: 0.62 (petroleum ether/ethylacetate 8:2). IR (Nujol), cm^{-1}:1700 (C=0), 1610, 1590, 1096.^{1}HNMR (300 MHz, DMSO): δ = 8.02 (d, *J* = 8.2 Hz, 1H), 7.63 (d, *J* = 9.1 Hz, 1H), 7.43– 7.31 (m, 3H), 7.16– 7.06 (m, 3H), 4.32– 4.16 (m, 2H). ^{13}C NMR (75 MHz, DMSO): δ = 171.5, 161.8, 161.7, 158.5, 156.3, 158.0, 147.8, 131.5, 131.2, 126.4, 124.8, 122.3, 121.6, 117.1, 112.8, 112.5, 54.0, 33.4, 31.0. HRMS (EI): m/z calcd. for $C_{16}H_{10}ON_2S_2F_2$ [M^+]: 348.0203; found 348.0217. Anal $C_{16}H_{10}ON_2S_2F_2$: C, H, N. Calc.: C: 55.16%, H: 2.89,% N: 8.04%. Found: C: 54.74%, H: 2.98%, N: 7.99%.

3-(Benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one (2). Yield: 59%, m.p. 174-6 °C, R_f: 0.62 (petroleum ether/ethylacetate 8:2). IR (Nujol), cm^{-1}:1720, 1600, 1520, 1086. ^{1}HNMR (300 MHz, DMSO): δ = 8.01 (d, *J* = 8.2 Hz, 1H), 7.62– 7.56 (m, 1H), 7.46– 7.31 (m, 4H), 7.20– 7.14 (m, 2H), 4.31– 4.16 (m, 2H). ^{13}C NMR (75 MHz, DMSO): δ = 171.7, 156.4, 147.8, 132.9, 131.1, 126.8, 126.4, 124.8, 122.3, 121.6, 116.4, 116.1, 58.1, 31.0. HRMS (EI): m/z calcd for $C_{16}H_{10}ON_2S_2ClF$ [M^+]: 363.9907; found 363.9901. Anal. $C_{16}H_{10}ClFN_2OS_2$: C, H, N. Calc.: C: 52.67%, H: 2.76%, N: 7.68%. Found:C: 52.44%, H: 2.42%, N: 7.62%.

Evaluation of RT inhibitory action[35]

HIV-1 Reverse Transcriptase Activity was measured using the colorimetric photometric immunoassay kit provided by Roche. Estimation of RT activity is achieved by quantification of the DNA synthesized by RT in a known period of time. Incubation was performed for 2 h at 37°C. PolyA:oligodT RNA template:primer hybrid at a concentration of 2.2 μg/ml was incubated with 20 nM of purified recombinant HIV-1 reverse transcriptase in a 50 μL incubation mixture containing 50 mM Tris-HCl pH 7.8, 160 mM KCl, 11 mM $MgCl_2$, 5.3 mM DTT, 0.5 mM EDTA, 0.3% Triton-X 100 and 10 μM dTTP (unless otherwise mentioned) in the presence of digoxigenin-conjugated dUTP (dUTP-DIG) and biotin-conjugated dUTP (dUTP-biotin). In a following step, the synthesised biotin-DIG-labelled DNA was bound to the surface of streptavidin-coated ELISA-plate wells. After washing out the unbound material, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), was added and bound to the digoxigenin-labelled nucleotides. After a washing step and addition of the appropriate substrate, peroxidase catalyzed the transformation of the substrate, ABTS, to a yellow coloured reaction product. 15 min after ABTS addition, the coloured product was measured using a microtitre plate (ELISA) reader. For the estimation of inhibitory action of the compounds, reverse transcriptase was incubated in the presence of the potential inhibitor for 45 min at room temperature before addition of its substrates (pre-incubation). The activity measured was compared with the total activity of the enzyme in the absence of inhibitor. For total-activity measurement, equal volume of the compound solvent was added in the reaction mixture. Incubation in the absence of enzyme was performed and used as blank for the determination of enzyme activity. The absorbance of the blank was

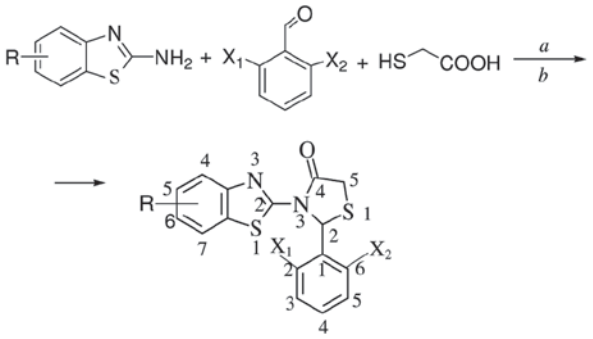
abstracted from the absorbance values obtained from the rest of the samples. Reaction velocity, V, was expressed in arbitary units. 1 Unit equals to product formation which yields 1 O.D. at 405 nm under the testing conditions. Different inhibitor concentrations were added for the calculation of IC_{50} values. For kinetic studies, incubation with different concentrations of dNTPs was performed. Enzfitter for windows 32 bit version was used for the calculations of kinetic analysis results[36]. All experiments were performed in triplicate. Values obtained using nevirapine were used for comparison reasons.

Docking analysis

Based on previously reported molecular docking studies on di-aryl-substituted thiazolidin-4-ones[37], we first used the X-ray crystallographic complex of RT with 9-Cl-TIBO which adopts the common butterfly structure (PDB code 1REV)[38]. Structures of inhibitors were built using Maestro 3D-sketcher[39] minimized with OPLS_2005 force field[40] using the Polak-Ribiere conjugated gradient method (0.001 kJ/Å mol convergence) and finally docked in the protein. As none of the structural waters were conserved in the RT-9-Cl-TIBO complex, all the water molecules were removed from the allosteric site in all the docking simulations. Docking simulations were carried out using the Autodock 4.2[41]. The genetic algorithm-local search (GA-LS) method was used with the following settings: initial population of 300 randomly placed individuals, a maximum number of 2.5 millions energy evaluations and a maximum number of 27,000 generations. The translation step was 1.0 Å, the quaternion and torsion step were set to 5.0 degrees. A mutation rate of 0.02 and a crossover rate of 0.8 were used. Results from Autodock calculations were clustered using an RMSD tolerance of 1.5 Å and the lowest energy conformer of the most populated cluster (the lowest energy cluster in most cases) was selected as the most probable binding conformer. For each compound, 200 docked conformations were generated. To visualize the highest scored conformations into the binding site, we used the Pymol Molecular Graphic System. In a preliminary step, the reliability of the docking protocol was checked by simulations of the binding modes of 9-Cl-TIBO and a comparison of the modelled complex with the available 3D structure derived by X-ray crystallography (1REV) was done. The obtained binding mode of 9-Cl-TIBO was very close to that of the original orientation found in the crystal. The RMS deviation between the docked and crystal ligand coordinates was 0.89 Å. Since, none of the tested compounds obtained the butterfly conformation and it is well known that the binding site of NNRTIs is very flexible[6]; we decided to use another crystallographic complex. Etravirine complex with HIV-1 RT (TMC125, PDB:1SUQ) was selected as the most appropriate for the second phase of the study, since it enables relatively bulky compounds to be freely oriented inside the binding pocket. In case of the TMC125-RT complex (PDB code 1SUQ)[24], the structure of the allosteric pocket presented certain differences compared to the previously used 9-TIBO-RT complex. In particular, the residues Val106, Pro225, Phe227, Leu234, His235, Pro236 and Tyr318 appeared to be moved, making the binding pocket larger and capable to accept bulkier compounds, allowing them to obtain the butterfly conformation. A test docking calculation was also carried out to validate the docking protocol and to confirm that it could be used to reproduce the binding mode of our ligands. The ligand TMC125, in the conformation found in the crystal structure was extracted and docked back into the corresponding binding pocket to determine the ability of Autodock to reproduce the orientation and position of the inhibitor observed in the crystal structure. The results of control docking showed that Autodock was able to determine the orientation and the interactions pattern of the crystallographic inhibitor with a root-mean square deviations (RMSD) of 0.58 Å

Prediction of toxicity[42]

Prediction of toxicity of the tested compounds was made using lazar model through the Open Tox Predict Program. OpenTox is an open source web-service-based framework, that provides unified access to experimental toxicity data, *in Silico* models (including (Q)SAR), and validation/reporting procedures. The Program uses various models to predict toxicity of compounds based on their structure. Lazar model derives predictions from toxicity databases by searching for similar compounds. It is a k-nearest-neighbour approach to predict chemical endpoints from a training set based on structural fragments.



1-19

1. R = H, $X_1 = X_2 = F$
2. R = H, $X_1 = F$, $X_2 = Cl$
3. R = 6-Cl, $X_1 = X_2 = Cl$
4. R = 6-Cl, $X_1 = F$, $X_2 = Cl$
5. R = 6-Cl, $X_1 = X_2 = F$
6. R = 4-Cl, $X_1 = X_2 = Cl$
7. R = 4-Cl, $X_1 = F$, $X_2 = Cl$
8. R = 4-Cl, $X_1 = X_2 = F$
9. R = 6-F, $X_1 = X_2 = Cl$
10. R = 6-F, $X_1 = F$, $X_2 = Cl$
11. R = 6-F, $X_1 = X_2 = F$
12. R = 6-OMe, $X_1 = X_2 = Cl$
13. R = 6-OMe, $X_1 = F$, $X_2 = Cl$
14. R = 6-OMe, $X_1 = X_2 = F$
15. R = 4-OMe, $X_1 = X_2 = Cl$
16. R = 4-OMe, $X_1 = F$, $X_2 = Cl$
17. R = 6-OEt, $X_1 = X_2 = Cl$
18. R = 6-OEt, $X_1 = F$, $X_2 = Cl$
19. R = 6-OEt, $X_1 = X_2 = F$

Scheme 1. General synthetic procedure for benzothiazolyl-4-thiazolidinones. Reagents and conditions: (a) Conventional method: toluene, reflux for 20–26 h, (b) microwave-assisted technic: 80–100 °C, power 100 W, 10–60 min.

It uses a SMILES file and pre-computed fragments with occurrences as well as target class information for each compound as training input. It also features regression, in which case the target activities consist of continuous values. Lazar model uses activity-specific similarity (i.e. each fragment contributes with its significance for the target activity) that is the basis for predictions and confidence index for every single prediction.

Results and discussion

Chemistry

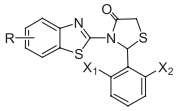
Synthesis of the compounds was performed both by the conventional one pot method previously described[15,21] and/or by microwave assisted one-pot synthesis (Scheme 1). According to one-pot classical procedure, reaction of the suitable aminobenzothiazole with equimolar amount of 2,6-dihalosubstituted benzaldehyde was performed in the presence of excess amount of mercaptoacetic acid in reflux toluene for 20–26h. When classical procedure was used, products were obtained as racemates in low to moderate yields. Using microwave assisted one-pot synthetic procedure the reaction time was reduced from 20–26 h needed for the classical method to 10–60 min, depending on compounds. The final products were obtained as pure crystals in satisfactory to good yields, higher than those achieved by the traditional method. All synthesized compounds were characterized by elemental analysis and spectroscopic methods (^{1}H NMR, ^{13}C-NMR, HRMS).

The IR spectra showed >C=O,–C-C- and C-Cl, C-F stretching absorption band at 1700 cm^{-1} (strong) 1600 and 1540 cm^{-1} as well as 1096–1089 cm^{-1}, respectively. In ^{1}H-NMR spectra in DMSO the δ values range (8.10–7.00 ppm) is in agreement with the theoretical presence of aromatic protons while the signal at 4.40–4.10 ppm is attributed to a proton of the position 2 (CH) of thiazolidinone moiety. The other protons appeared at the expected chemical shifts. Furthermore in ^{13} C-NMR spectra peaks were observed at δ 172–170 ppm due to the presence of >C=O group as well as peaks at δ 161–165 ppm, that attributed to C-2 of benzothiazole ring. Signals at 158–141 ppm were accounted for C-F of aromatic ring. The presence of signals at 53–60 ppm, as well as at 30–34 ppm were attributed to C-2 and C-5 of thiazolidinone moiety respectively.

HIV-1 RT inhibitory action

The main observation obtained from the measurement of HIV-1 RT inhibitory action (Table 1) is that the presence of the appropriate halogen or alkoxy-substituent at positions 4 or 6 of the benzyl ring of the benzothiazolyl moiety may convert a practically inactive non-substituted derivative to a relatively strong inhibitor. So, while the

Table 1. HIV-1 RT Inhibitory action.



	R	X_1	X_2	Inhibition % (4 μM)	IC_{50}[a] (μM)	Km[b] (μM)	Vmax[b]
1	4-H, 6-H	F	F	19	>40		
2	4-H, 6-H	F	Cl	12	>40		
3	4-H, 6-Cl	Cl	Cl	30	>40		
4	4-H, 6-Cl	F	Cl	33	>40		
5	4-H, 6-Cl	F	F	34			
6	4-Cl, 6-H	Cl	Cl	26	>40		
7	4-Cl, 6-H	F	Cl	83	0.04	5.5 ± 0.3	0.196 ± 0.016
8	4-Cl, 6-H	F	F	30	>40	42.4 ± 3.6	0.465 ± 0.042
9	4-H, 6-F	Cl	Cl	68	0.25		
10	4-H, 6-F	F	Cl	32			
11	4-H, 6-F	F	F	34			
12	4-H, 6-MeO	Cl	Cl	36	>40		
13	4-H, 6-MeO	F	Cl	39	>40		
14	4-H, 6-MeO	F	F	25	>40		
15	4-MeO, 6-H	Cl	Cl	57	3.0		
16	4-MeO, 6-H	F	Cl	21	>40		
17	4-H, 6-EtO	Cl	Cl	40			
18	4-H, 6-EtO	F	Cl	48	3.4	7.7 ± 0.7	0.285 ± 0.023
19	4-H, 6-EtO	F	F	32	>40	25.7 ± 2.5	0.470 ± 0.055
nevirapine					0.3		
-						12.5 ± 0.5	0.473 ± 0.025

[a]Values are means of three determinations and deviation from the mean is <10% of the mean value. adNTP concentration: 10 μM. [b]Each experiment was done in triplicate. Enzfitter for windows 32 bit version was used for the calculations. V was expressed in arbitary units. 1 Unit equals to product formation which yields 1 O.D. at 405 nm under the testing conditions.

non-substituted 3-(benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one, 2, exhibits practically no activity under experimental conditions, as also mentioned by Rawal et al.[34], its 4-Cl-benzo[d]thiazol-2-yl analogue, **7**, is a potent RT inhibitor with an IC_{50} value of 0.04 μM (0.016 μg/ml). Compound 18, the 6-EtO-benzo[d]thiazol-2-yl analogue of compound 2, also exhibits considerable activity with an IC_{50} value of 3.4 μM (1.39 μg/ml). Although, both, halogen or alkoxy-substitution of the benzo[d]thiazol-2-yl moiety seems to have a positive influence on inhibitory action in all cases, this influence is not substantial for most of the compounds. Only four of the tested compounds exhibited considerable inhibition activity under the testing conditions (IC_{50}: 0.04– 3.4 μM). Two halogen substituted derivatives, the 3-(4-Cl-benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluorophenyl)thiazolidin-4-one, **7**, and the 3-(6-F-benzo[d]thiazol-2-yl)-2-(2,6-dichloro-phenyl)thiazolidinone-4-one, 9, were the most active compounds, followed by two alkoxy-substituted derivatives, the 3-(4-MeO-benzo[d]thiazol-2-yl)-2-(2,6-dichloro-phenyl)thiazolidin-4-one, 15, and the 3-(6-EtO-benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluoro-phenyl)thiazolidin-4-one, 18.

Different combination of halogens at positions 2- and 6- of the 2-6-dihalophenyl moiety resulted in remarkable difference in inhibitory action. This had been observed in the series of compounds tested previously by Rawal et al., as well. Moreover, it was found that the presence of different halogen substituents at the same position of the benzyl ring of the benzo[d]thiazol-2-yl moiety also resulted in great difference in activity as concluded by comparison of the 6-F-benzo[d]thiazol-2-yl derivative, 9 (IC_{50}: 0.25 μM), with the 6-Cl-benzo[d]thiazol-2-yl analogue, 3 (IC_{50} > 40 μM).

Docking analysis was performed for some of the compounds in order to elucidate the differences observed in IC_{50} values.

Docking analysis of selected compounds

Although, only crystallographic results may provide a certain prove about the binding of the compounds to the enzyme, docking analysis may give an indication of probable binding mode and help us to propose an explanation of the results.

All synthesized compounds posses the thiazolidinone heterocyclic ring as well as a second benzo[d]thiazolyl heterocyclic moiety offering the possibility of hydrogen bond formation with Lys101 or Lys103 which stabilizes most NNRTI complexes. Moreover, they have two hydrophobic moieties, the dihalogen substituted phenyl group at position 2 of the thiazolidin-4-one ring and the benzothiazolyl moiety. Both these moieties offer to the molecule the possibility to occupy the hydrophobic cleft of Trp229, Tyr181 and Pro95 present at the allosteric site where traditional NNRTIs bind. However, only some of the compounds can take the orientation allowing these interactions.

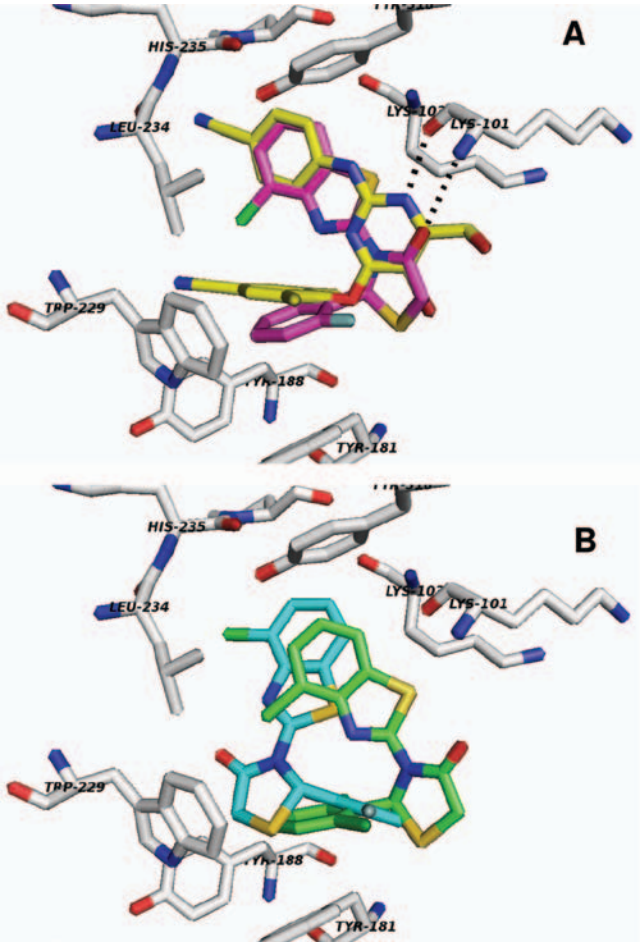


Figure 1. Docking of (A) compounds 7 (magenta) and etravirine (yellow) and (B) compounds 6 (green) and 8 (blue) in the allosteric centre of HIV-1 RT.

Among the different available crystallographic complexes of NNRT Inhibitors with HIV-1 RT, the etravirine complex (TMC125, PDB:1SUQ) was selected as the most appropriate, since it enables relatively bulky compounds to be freely oriented inside the binding pocket.

Using this docking system, docking analysis of the most active derivative, **7**, (Figure 1A) showed that this compound adopts a conformation similar to that of etravirine (TMC125) in the PDB:1SUQ complex. More precisely, the 2-Cl-6-F-phenyl ring of compound **7** is oriented in the pocket consisted by the aminoacids Tyr181, Pro95, Tyr188 and Trp229 while the substituted benzothiazolyl moiety interacts with the aminoacids Leu234, Tyr318 and His235. These hydrophobic interactions may contribute to complex stabilization. The part of thiazolidinone moiety containing the sulfur atom is placed in a cleft consisted of the aminoacids Lys101 and Val179 of the p66 subunit and Glu138 of p51 subunit of RT while oxygen of the carbonyl group of the thiazolidinone ring forms a hydrogen bond (Hb) with hydrogen of the amino group of the backbone of Lys101. Hb formation with Lys101 is a common characteristic of NNRTIs although CO of the

main-chain of the amino acid is usually involved in the bond, as also observed in case of ertavirine (Figure 1A)[43]. Lys103 which is involved in Hb formation in most cases of traditional NNRTIs and is mutated in most resistant strains does not seem to be involved in complex stabilization of compound **7**.

The Hb with Lys101, present in case of derivative **7**, is not observed in docking results of the less active compounds 6 and 8 (Figure 1B). Compound 8, seems to obtain a totally different orientation in the RT allosteric centre. The 2,6-difluoro-phenyl ring is placed in vicinity to aminoacids Val179, Tyr188 and Tyr181 while the benzothiazolyl moiety interacts with the aminoacids Phe227 and Tyr318. The part of thiazolidinone moiety containing the sulfur atom is placed in a cavity consisted of the aminoacids Trp227, Tyr188 and Tyr181. This orientation does not favour Hb formation.

Compound 6, the 2,6-dichloro-phenyl analogue, can be placed in the allosteric centre in a way very similar to that of derivative **7**. However, a slight change in the orientation also disables the Hb formation, thus leading to a less stable complex.

The absence of Hbs in the complexes of the less active inhibitors may explain the difference in activity. This probable explanation is not supported by the differences in binding energies (compound 6:-9.3 kcal/mol, compound 7:-9.32 kcal/mol, compound 8:-9.0 kcal/mol) which are very similar. This implies that the enzyme probably obtains a much different and more convenient conformation in case of binding with compound **7** which can not be approached by the structures obtained by crystallographic data of etravirine.

Moreover, compounds 9, 15 and 18 which are also active, do not adopt an orientation that favours Hb formation or ensures a high binding capacity to the allosteric centre. This also implies that another binding mode or structural alteration of the allosteric centre may exist in the presence of these inhibitors.

In order to explore this hypothesis, we proceeded to kinetic studies of two of the active compounds, derivatives **7** and 18 and two of the practically inactive compounds, derivatives 8 and 19.

Kinetic properties of selected compounds

RT activity of compounds **7,** 18, 8 and 19 was measured in the presence of stable concentrations of RNA:primer substrate and increasing concentrations of dNTP (dTTP) in the absence and in the presence of stable concentration (4 μM) of inhibitors.

None of the tested compounds exhibited the common non-competitive inhibition mechanism. The more active compounds (**7** and 18) follow an uncompetitive inhibition mode while the less active compounds (8 and 19) adopt a competitive inhibition mechanism (Figure 2, Table 1). This means that, under the testing conditions, compounds **7** and 18 bind exclusively to the enzyme after dNTP binding. It is well known that the structure of RT is modified after binding of dNTPs[43].

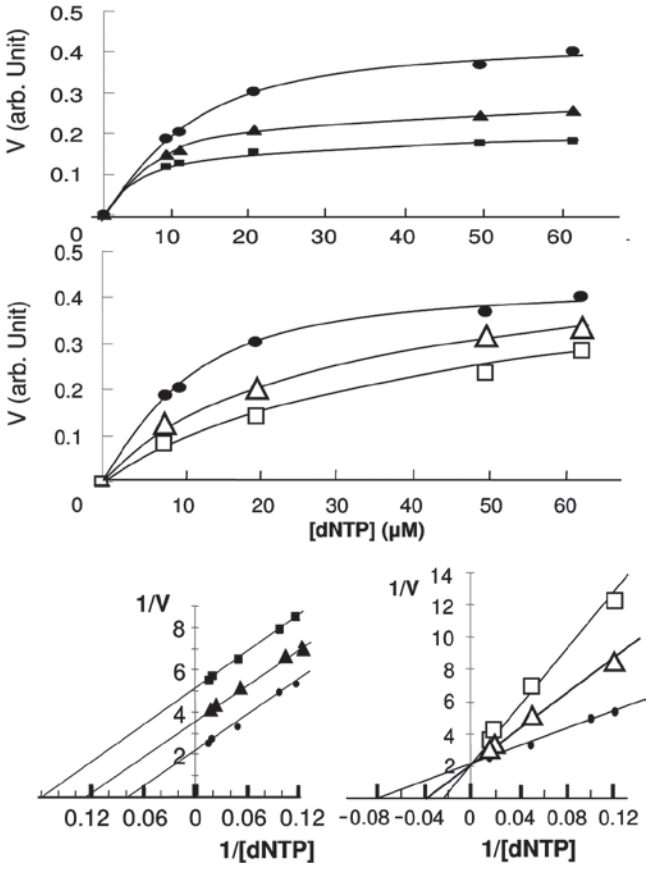


Figure 2. Michaelis-Menten and double reciprocal curves in the absence of inhibitor (●) and in the presence of 4μM of compound 7 (■), compound 18 (▲), compound 8 (□)and compound 19 (△).

These structural changes may enable a more stable interaction between RT allosteric centre and active inhibitors of this series, although only crystallographic studies may confirm this hypothesis. One more example of this kind of RT inhibition by a quinolinic derivative which can bind to the allosteric site of NNRTIs in a different than the classic way, was mentioned recently by Souza et al.[26].

However, inhibitors 8 and 19 probably fail to stably associate to the allosteric centre of the enzyme and compete with dNTPs for binding to the active centre. Perhaps this difference in inhibition mechanism may explain the great differences observed in IC_{50} values of the studied molecules.

The Km value of RT in the absence of inhibitor was found to be 12.5 μM under the testing conditions (Table 1). This is higher than the values calculated when other methods were used but is close to the values referred by investigators who used the same method[44]. The presence and partial incorporation of conjugated-dUTPs according to the method may be responsible for the relatively high Km value obtained in the absence of inhibitor, since the presence of modified dNTPs in the reaction mixture was found to increase Km values in many cases[20,21,45–48]. In the presence of compounds **7** and 18 which showed uncompetitive inhibition mode, Km values as well as Vmax

were decreased (compound **7**: Km = 5.5, Vmax = 0.196; compound 18: Km = 7.7, Vmax = 0.285). Vmax remained practically the same, while Km values were increased in the presence of compounds 8 and 19 (compound 8: Km = 42.4, Vmax = 0.465; compound 19: Km = 25.7, Vmax = 0.470).

Kinetic results show that compound **7** probably binds after dNTPs binding to the active site. This necessary condition implies that change in enzyme structure after dNTPs binding is a requirement for stable binding of compound **7**. This may explain the inability of docking studies based on the structure of etravirine to support the experimental results.

Toxicity prediction

According to Lasar model, the predicted LC50 values for the most active compounds, derivatives **7** and 9, are 0.11945591 mM (confidence: 0.11945591) and 0.11945591 mM (confidence: 0.105261415) respectively. Predicted LC50 values for nevirapine and efavirenz, two currently in use NNRTIs are 0.8378014 mM (confidence: 0.17544146) and 0.015711168 mM (confidence: 0.1532123), respectively. Predicted toxicity of the tested compounds is lower than that of efavirenz, but higher than nevirapine. Prediction results only give a first indication about probable toxicity and cannot replace *in vitro* and *in vivo* experiments. However, prediction programs are highly recommended in order to reduce *in vivo* experiments.

Conclusion

Among the 18 novel (4/6-substituted benzo[d]thiazol-2-yl) thiazolidin-4-ones, synthesized and tested for anti-HIV-1 RT inhibitory action, four derivatives exhibited IC_{50} values lower than 3.4 μM (1.39 μg/ml) and two of them showed much better inhibition than the reference compound, nevirapin.

The main conclusion obtained from the evaluation of HIV-1 RT inhibitory action (Table 1) is that the presence of the appropriate halogen or alkoxy- substituent at positions 4 or 6 of the benzyl ring of the benzothiazolyl moiety may convert a practically inactive non-substituted derivative to a relatively strong inhibitor. Two halogen substituted derivatives, the 3-(4-Cl-benzo[d]thiazol-2-yl)-2-(2-chloro-6-fluoro-phenyl)thiazolidin-4-one, **7** (IC_{50} = 0.04 μM) and the 3-(6-F-benzo[d]thiazol-2-yl)-2-(2,6-dichloro-phenyl) thiazolidinone-4-one, 9 (IC_{50} = 0.25 μM), were the most active compounds.

Docking analysis based on the crystallographic data of etravirin-RT complex, indicates that compound **7**, which is the most active derivative, could be stabilized in the allosteric centre of RT by Hb formation, although the increased activity cannot be explained by the binding energy of the compound. However, kinetic analysis of two of the most active compounds (**7** and 18), showed uncompetitive mode of inhibition, thus suggesting that they bind to the enzyme after dNTP binding. The kinetic studies together with the docking results lead to the conclusion that the active compounds probably need structural characteristics of the allosteric centre that can be acquired only after dNTPs binding. So, they bind at a binding site structurally altered and different of the commonly used NNRTIs like etravirine. The results differentiate these compounds from the traditional non-competitive NNRTIs, which is a desired characteristic, since mutations that affect activity of non-competitive NNRTIs may not affect activity of compounds of this series.

Interestingly, the less active derivatives (IC_{50} > 40 μM) exhibit a competitive mode of action which probably explains the much lower activity of these compounds.

Declaration of interest

The authors report no declarations of interest.

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