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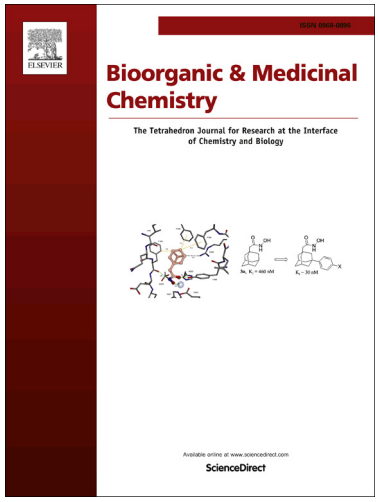

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Graphical Abstract

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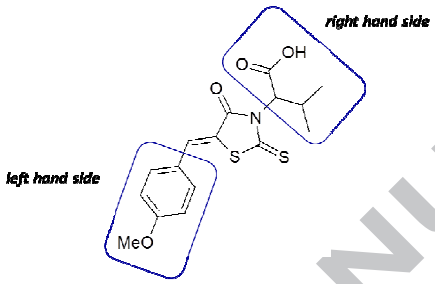

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Optimization of rhodanine scaffold for the development of protein-protein interaction inhibitors.

Stefania Ferro[a,*], Laura De Luca[a], Fatima Ezzahra Agharbaoui[a], Frauke Christ[b] , Zeger Debyser[b] and Rosaria Gitto[a]

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Searching for novel protein-protein interactions inhibitors (PPIs) herein we describe the identification of a new series of rhodanine derivatives. The selection was performed by means virtual-screening, docking studies, Molecular Dynamic (MD) simulations and synthetic approaches. All the new obtained compounds were tested in order to evaluate their ability to inhibit the interaction between the HIV-1 integrase (IN) enzyme and the nuclear protein lens epithelium growth factor LEDGF/p75.

1. Introduction

Since the first description of the acquired immune deficiency syndrome (AIDS), worldwide more than 25 million people have fallen victim to HIV-1 infections. For the treatment of this disease the Food and Drug Administration (FDA) has approved several drugs, often used in combination regimens in the well-known cART (combinatorial AntiRetroviral Therapy). They belong to different categories: reverse transcriptase inhibitors (RTIs), protease inhibitors (PIs); fusion inhibitors (FIs), coreceptor inhibitors (CRIs) and integrase inhibitors (INIs).[1-4]

Although this current therapy decreases viral load and provides a significant improvement in the life expectancy of HIV-1/AIDS patients, the incidence of infection remains one of the major problems in public health. Moreover, the long-term treatment anti-AIDS drugs induces drug-resistant viral variants and emergence of unwanted side effects.[5-8] Therefore, the quest of new more potent and less toxic anti-HIV agents, as a complement for the existing treatment strategies, remains one of the major goals in HIV drug discovery.

Currently, small-molecule inhibitors of the direct protein-protein interactions (PPIs), that play auxiliary roles in the HIV-1 life cycle mediating important biological processes, are an emerging and interesting area in anti HIV-1 drug design.[8, 9]

Particularly, the disruption of association between HIV-1 IN and the ubiquitous lens epithelium growth factor p75 (LEDGF/p75) is currently the most promising target for the design of protein-protein inhibitors (PPIs) as potential anti-HIV drugs.[10-16] LEDGF/p75, a transcriptional co-activator, is a co-factor of HIV-integrase (IN) and is required for the tethering and correct integration of the viral genome into the host chromatin. Several studies highlighted that cells lacking of LEDGF/p75 showed a severe defect in HIV-1 infection characterized by decreased levels of integrated viral DNA.[9, 17-19] Taking into account the pivotal role of the IN-LEDGF/p75 interaction for the HIV-1 infection, in the past years we focused on this attractive and innovative antiviral target. Our studies led us to the identification of several small molecule protein-protein interaction inhibitors (SMPPIIs) able to block the binding between the IN enzyme and its cofactor.[8, 14, 20-26]

Recently, considering that natural products (NP) represent the most productive source of leads for new drugs, we reported the application of a structure-based virtual screening strategy for the discovery of hit structures of natural origin useful in the antiretroviral therapy. Nine compounds have been selected and tested to evaluate their ability to prevent IN-LEDGF interaction. Among them, a rhodanine derivative, the 2-(5-(4-methoxybenzylidene)-2,4-dithioxothiazolidin-3-yl)-3-methylbutanoic acid (**A**) (Figure 1) showed interesting efficacy, displaying an IC_{50} value of 41.28 μM in AlphaScreen assay.[25]

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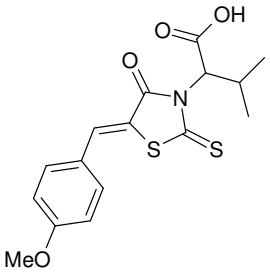


Figure 1: 2-(5-(4-Methoxybenzylidene)-2,4-dithioxothiazolidin-3-yl)-3-methylbutanoic acid (**A**)

As a continuation of these researches, herein we report structural modifications and biological screening of new rhodanine analogs designed and synthesized with the aim of achieving further information about the IN-LEDGF/p75 protein interaction. Further our studies are in progress to define the role of the rhodanine scaffold in the antiviral activity also considering that LJ001 rhodanine analogue is effective fighting multiple deadly viruses including HIV-1 and Ebola. Moreover, in *vitro* and in *vivo* assays showed that LJ001 acts on the virus and not on the cells thus lacking of overt cytotoxicity.[27, 28]

2. Results and discussion

With the aim to obtain useful insights for the development of new small-molecules as inhibitors of the IN-LEDGF/p75 interaction (LEDGINs), we previously investigated the most interactions between some PPI inhibitors and IN-LEDGF/p75 complex. In particular, by means of a combination of docking and ultrashort MD, we generated a weighted ensemble of protein-ligand configurations and estimated the binding affinity averaged over snapshots taken from the MD trajectories, together with the presence of fundamental hydrogen bonds.[26] Using this information in a structure-based virtual screening strategy we identified the active compound A. The plausible binding mode of this derivative is showed in Figure 2.

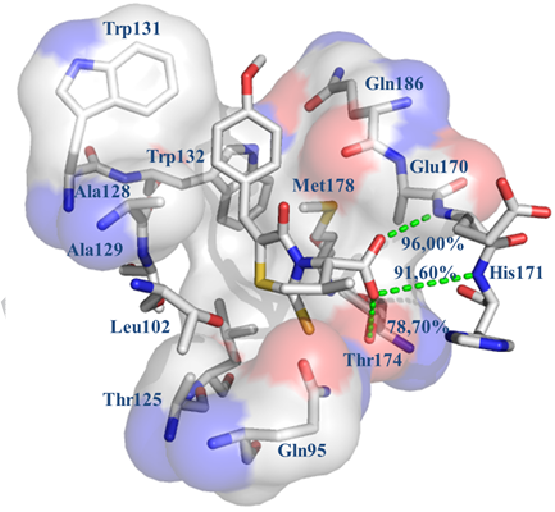


Figure 2: Binding mode of reference compound **A** in complex with IN CCD. Key residues of the pocket are presented. Hydrogen bonds are showed by dotted lines as well as their occupancies during MD simulations in percent. The figure was created using PyMOL.[29]

The p-methoxyphenyl group is accommodated into the binding pocket forming a panel of hydrophobic interactions with the residues Ala128, Ala129, and Trp132, and Met178 of IN; whereas the carboxylate group forms stable hydrogen bonds with the crucial residues (occupancies of 96.00% with Glu170 NH backbone; 91.60% with His171 NH backbone; 78.70% with Thr174 side chain OH). Interactions between IN CCD and compound **A** were examined using PyMOL and LIGPLUS.[30]

We observed that the shape of the pocket occupied by the p-methoxyphenyl group could house larger and lipophilic groups and that there was absence of contact with the crucial Gln168 residue placed near the phenyl group. Furthermore it was not clear the role of isopropyl group that seems not able to create important contacts.

The analysis of these data prompted us to reconsider the class of compound **A** keeping unchanged the rhodanine nucleus and modifying, in this phase of our research, the other two portions of the molecule that we named right hand side (3-methylbutanoic acid moiety) and left hand side (4-methoxybenzylidenic moiety) respectively (Figure 3).

The decision to change a portion at a time is determined by the need to identify the essential pharmacophore of derivatives establishing the structural features that are important for the biological activity.

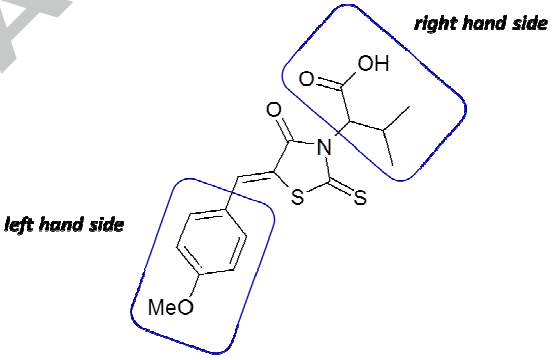


Figure 3: Structural portions of derivative **A**

2.1. Binding site analysis: left hand side modification

In order to identify some representative alternative groups, ZINC database has been browsed to search for molecules that varied only the left hand side portion. The subset of molecules selected from ZINC consisted of 674 compounds. This subset was filtered by Lipinki's rule, redundant structures were removed, then appropriate ionization states and 3D structures were generated. The obtained subset consisted of 64 molecules.

These compounds were docked into the LEDGF/p75 binding site on HIV-1 integrase considering the protein retrieved by complex 2B4J that was used for reference compound **A** (see Experimental Section).[25] After visual examination of the highest scoring molecules, 13 protein-ligand complexes were retrieved, minimized and rescored using MM-GBSA method. Based on these results, ultrashort MD simulations (100 ps) were performed obtaining the best results for the eight compounds (**1**-**8**) showed in Figure 4.

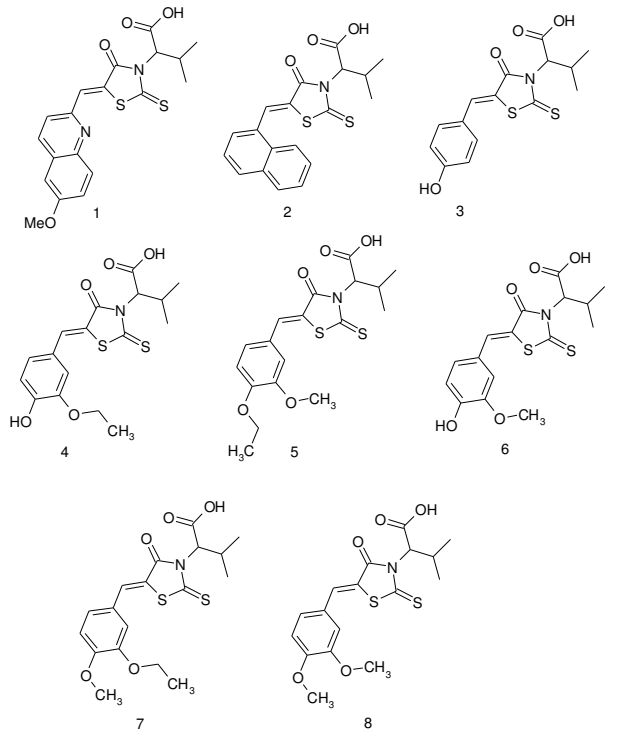
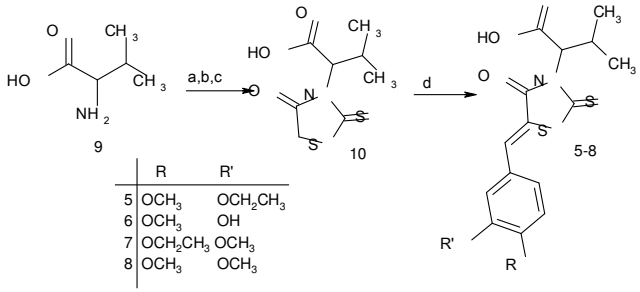


Figure 4: Designed left hand side modifications

To investigate the impact on the inhibitory effect of the left hand side modifications derivatives **1-4** were initially purchased. Successively, after we developed a suitable chemical pathway, compounds **5-8** were synthesized to build a small SAR library for SAR studies. The synthetic approach employed for analogues **5-8** is depicted in Scheme 1.

Scheme 1



Reagents and conditions: a) NaOH, CS_2, 23°C, overnight; b) $ClCH_2CO_2Na$, 23 °C, 3h; c) HCl, reflux, 12h, d) suitable benzaldehyde, NH_4OAc, toluene, reflux, 1-2h.

At first valine (**9**) was cyclized with CS_2 and α-chloroacetate to give the rhodanine nucleus (**10**). In the second step, following Knoevenagel condensation of rhodanine with suitable substituted benzaldehyde, the final desired analogues **5-8** were obtained. As expected the condensation generated two isomers (E and Z); the major product was the thermodynamically stable Z isomer as characterized by the down-field shift of its methylene proton when compared to that of the E isomer.

2.2. Binding site analysis: right hand side modification

Starting from the reference compound **A**, several modifications on the right hand side of the molecule were also taken into account.

Considering the space of the pocket in which the isopropyl group of reference compound A is located (Figure 2) and the commercial availability and price of some amino acids we selected 5 of them. The substitution of isopropyl moiety with these groups generated compounds **11**-**15** that were submitted to the same computational protocol to predict substitutions influence on the inhibitory activity.

Based on computational results the five compounds were selected and synthesized. (Figure 5)

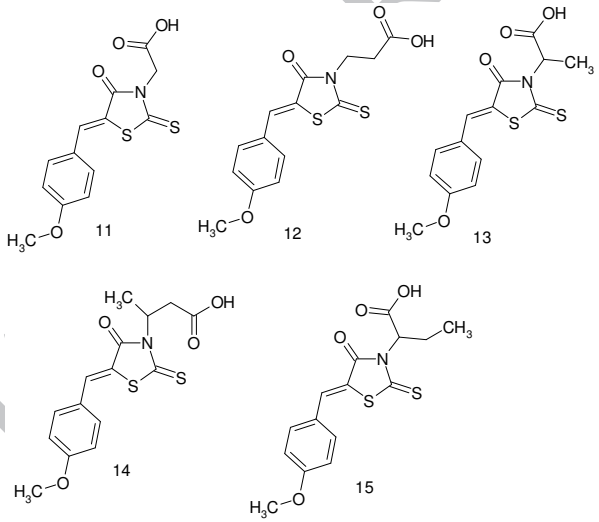
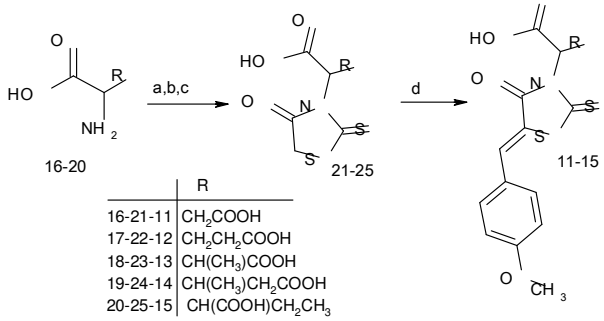


Figure 5: Designed right hand side modifications

The new designed compounds were obtained following the synthetic procedure described in Scheme 2.

Scheme 2



Reagents and conditions: a) NaOH, CS_2, MW: 5 min at continuous temperature (100°C), 100 Watt; b) $ClCH_2CO_2Na$, MW: 5 min at continuous temperature (100°C), 100 Watt; c) HCl, MW: 30 min at continuous temperature (120°C), 100 Watt; d) *p*-methoxy benzaldehyde, NH_4OAc, toluene, reflux, 1-2h.

The commercially available amino acids **16-20** were cyclized with CS_2 and α-chloroacetate to form the substituted rhodanine derivatives **21-25**. In this step of the synthetic procedure Microwave Assisted Organic Synthesis (MAOS) was employed thus reducing reaction time (40min vs overnight) and usual thermal reagents degradation.

Successively, intermediates **21-25** were transformed into the final compounds **11-15** by reaction with *p*-methoxy benzaldehyde. Also in this case the spectroscopic characterization of the synthetized derivatives confirmed the major thermodinamical stability of Z isomer.

2.3. Biological results

We tested all new compounds (**1-8, 11-15**) in AlphaScreen assay to evaluate their inhibitory effects on the LEDGF/p75–IN interaction. (Table 1)

Table 1. Inhibition of IN-LEDGF/p75 interaction of compounds **1-8** and **11-15**

Compound	%	IC_{50} (μM)
1	93	15.76±1.51
2	89	18.75±0.6
3	77	43.75±0.75
4	82	24.99±0.8
5	28	-
6	43	-
7	20	-
8	NA	
11	36	-
12	66	>100
13	47	-
14	29	-
15	54	>100
A	88	41.28

% inhibition at 100 μM
IC_{50}: Concentration required to inhibit the HIV-1 IN-LEDGF/p75 interaction by 50%.
NA= Not Active

The biological results showed that all the new obtained derivatives, with the exception of compound **8,** exhibited inhibitory effects at the fixed dose of 100 μM concentration with a percentage ranging from 20% to 93%. Particularly, concerning this new series of IN-LEDGF/p75 SMPPIIs we observed that the best results were displayed for rhodanine-derivatives **1-4** in which left hand side modifications were performed. Among them compounds **1**,**2** and **4** present an improvement of the activity in comparison with the reference compound **A** and are more active than the derivatives characterized by different substitutions on the the right hand side of the parent compound **A**.

In order to explain this enhancement of activity the binding mode of compound **1**, one of the most active rhodanine analog, we show the computational results in Figure 6.

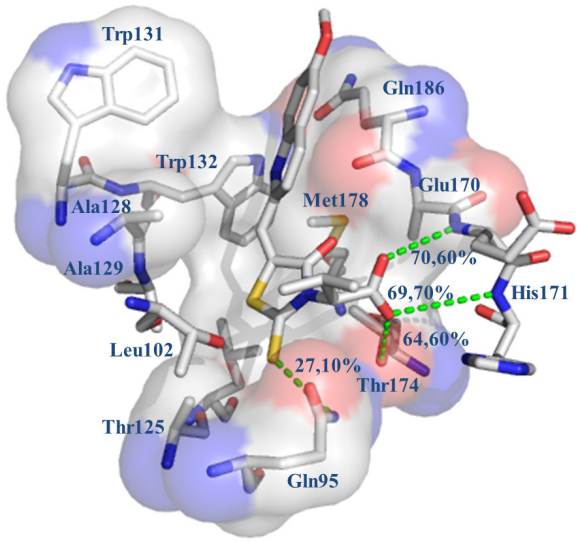


Figure 6: Binding mode of compound **1** in complex with IN CCD. Key residues of the pocket are presented. Hydrogen bonds are showed by dotted lines as well as their occupancies during MD simulations in percent. The figure was created using PyMOL.[29]

Comparing the results of compound **1** with those obtained for the prototype **A** (Figure 2), we highlighted that the overall stability of hydrogen bonds is similar for both derivatives. The methoxyquinoline moiety is accommodated into the hydrophobic pocket formed by residues of, Ala128, Ala129, Trp131, Gln168, Ala169. The carboxylate group is able to establish the same contacts with residues of Glu170, His171 and Thr174 of chain A of IN. Moreover, the sulphur atom of compound **1** forms a hydrogen bond with residue Gln95 providing a better stability of the complex thus suggesting a possible explanation to the higher potency of this new derivative. Interactions between IN CCD and compound **1** were examined using PyMOL and LIGPLUS.[30]

Concerning the right hand side modifications performed on compound **A** we can observe generally a decrease of the activity, for these compounds (**11-15**), when compared with left hand side modified derivatives. This account suggest that the 3-methylbutanoic acid portion, typical of the 2-(5-(4-methoxybenzylidene)-2,4-dithioxothiazolidin-3-yl)-3-methylbutanoic acid (**A**), is the most favorable moiety for the contact with IN.

Derivatives **1-8** and **11-15** were also tested in MT-4/MTT-assay as anti-HIV agents and among them derivative **12** showed antiviral activity (EC_{50}=2.41μM) and a very interesting selectivity index (SI=61). According these results, it is reasonable to think to another mechanism of action for this compound. Further studies are in progress.

3. Conclusions

With the aim of improving the IN-LEDGF inhibition activity of reference compound **A**, we identified and synthesized a new series of small molecules, belonging to the family of rhodanine derivatives. The selection was performed by virtual-screening, docking studies and MD simulations.

The biological results of the selected compounds showed for some of them inhibitory effects at micromolar concentration. Particularly, the best activity was obtained for derivatives in which left hand side modifications of scaffold were performed, thus affording novel SAR information that can be useful for future studies

4. Experimental section

4.1. Docking studies

Docking studies were performed using the genetic optimization for ligand docking (GOLD) software package version 4.1.1 from the Cambridge Crystallographic Data Centre (CCDC).[31]For our docking simulations, we used the crystal structure of the dimeric CCD of HIV-1 IN complexed with the IBD of LEDGF/p75 available from the RCSB Protein Data Bank under the PDB ID: 2B4J.[32] The LEDGF/p75 structure was removed and hydrogen atoms were added to the IN protein in Discovery Studio 2.5.5.[33]

For the prediction of ligand binding positions GoldScore fitness function was used. Per each ligand 100 independent runs and a maximum of 15000 genetic operations were performed using the default operator weights and a population size of 100 chromosomes. Results differing by less than 1.00 Å in ligand-all atom RMSD were clustered together. A 20.0 Å radius active site was drawn on the original position of the LEDGF/p75 IBD dipeptide Ile365-Asp366 and automated cavity detection was used. Two hydrogen bond constraints were used to specify that two protein atoms should be hydrogen-bonded to the ligand, namely NH backbone of Glu170 and His171 with a constraint weight of 5.

4.2. MM-GBSA rescoring and MD simulations

The starting model for simulations were prepared as described in our previous paper.[26] MD simulations were carried out using the sander module of AMBER 11 [34] and parm 99.dat and frcmod.ff03 parameter files.[35] General Amber force field (GAFF) [36] parameters were assigned to the designed ligands, while partial charges were calculated using the AM1-BCC method as implemented in the Antechamber suite of AMBER 11.

The geometry of the system was minimized in order to remove any bad contact using the steepest descent algorithm for the first 250 steps before switching to the conjugate gradient algorithm for the remaining 250 steps.

Solvent effects were taken into account by using the generalized Born implicit solvent model. The minimized structure was the input for MD runs using constant-temperature Langevin dynamics at 300 K for 100 ps with a time step of 1fs and a distance cutoff of 12.0 Å for the nonbonded interactions.

Snapshots of the complexes during the simulations and the average structures, were obtained with the Ptraj module of the AMBER 11 suite. [34]

The hydrogen bonds were detected when the acceptor-donor atom distance was lower than 3.5 Å and the acceptor-H-donor angle was more than 120°. The MM-GBSA method [37] implemented in the AMBER program, was used to evaluate the ligand-protein interaction free energies of both the minimized complex and the 100 snapshots extracted at 1 ps intervals.

4.3. Chemicals

All commercially available reagents and solvents were used without any further purification. The microwave-assisted reactions were carried out in a CEM Focused Microwave Synthesis System, Model Discover, working at the power necessary for refluxing under atmospheric conditions. Melting points were determined on a BUCHI Melting Point B-545 apparatus and are uncorrected. Elemental analyses (C, H, N) were carried out on a Carlo Erba Model 1106 Elemental Analyzer and the results are within ± 0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC; column chromatography was performed on Merck silica gel 60 (230-400 mesh) and Flash Chromatography (FC) on a Biotage SP_1 EXP. ^{1}H-NMR spectra were recorded in $CDCl_3$ with TMS as internal standard or [D_6]DMSO on a Varian Gemini-300 spectrometer. Chemical shifts were expressed in δ (ppm) and coupling constants (*J*) in hertz (Hz). All the exchangeable protons were confirmed by addition of D_2O.

4.4. *Synthesis of 3-methyl-2-(4-oxo-2-thioxotetrahydrothiophene-3-yl)butanoic acid* ***(10)***

L-valine (**9**) (1 mmol, 117,14 mg) was dissolved with sodium hydroxide (2 mmol, 80 mg) in water (10 ml). Then, carbon disulfide (1 mmol, 60µl) was added to the reaction mixture and it was stirred overnight. After the addition of an aqueous solution (1M) of $ClCH_2CO_2Na$ (1ml) the stirring was continued at room temperature for 3h. Successively hydrochloric acid solution (3ml, 5.5N, 16.5mmol) was added ad the reaction mixture was refluxed overnight. The mixture was neutralized with a saturated $NaHCO_3$ aqueous solution, extracted with ethyl acetate (10 mL x 3) and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the crude mixture was purified by flash chromatography using a mixture of CH_2Cl_2/CH_3OH (90:10) as eluent. Yield: 68%; mp: 130-132°C. ^{1}HNMR ($CDCl_3$): δ = 0.82 (d, J=7.1, 3H, CH_3), 1.25 (d, J=6.4, 3H, CH_3), 2.75-2.90 (m, 1H, CH), 4.02 (s, 2H, CH_2), 5.27 (d, J=9.4, 1H, CH), 7.53 (bs, 1H, COOH). Anal. calcd for $C_8H_{11}NO_3S_2$: C 41.19, H 4.75, N 6.00, found: C 41.32, H 4.88, N 5.91.

4.5. General procedure for the synthesis of 2-[(benzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylbutanoic acids ***(5-8)***

Suitable benzaldehyde (3 mmol) and ammonium acetate (3 mmol, 231,24 mg) were added to a solution of cyclized rhodanine intermediated **10** (1 mmol, 233,31 mg) in toluene (20 ml). The reaction was refluxed overnight and a saturated $NaHCO_3$ solution was added. The mixture was extracted with ethyl acetate (10 mL x 3), washed with BRINE (10 mL x 2), and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the crude mixture was purified by flash chromatography using a mixture of CH_2Cl_2/CH_3OH (90:10) as eluent.

2-[5-(4-ethoxy-3-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylbutanoic acid (**5**) Yield: 55%; mp: 182-184°C. ^{1}HNMR (DMSO-d_6): *δ* = 0.67 (d, *J*=6.6, 3H, CH_3), 1.21 (d, *J*=6.6, 3H, CH_3), 1.32 (t, *J*=7.2, 3H, CH_3), 2.65-2.85 (m, 1H, CH), 3.81 (s, 3H, OCH_3), 4.09 (q, *J*=7.2, 2H, CH_2), 4.85-5.00 (m, 1H, CH), 7.09-7.21 (m, 3H, ArH), 7.68 (s, 1H, CH). Anal. calcd for $C_{18}H_{21}NO_5S_2$: C 54.67, H 5.35, N 3.00, found: C 54.81, H 5.12, N 3.21.

2-[5-(4-hydroxy-3-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylbutanoic acid (**6**) Yield: 61%; mp: 218-220°C. ^{1}HNMR (DMSO-d_6): *δ* = 0.68 (d, *J*=6.4, 3H, CH_3), 1.22 (d, *J*=6.5, 3H, CH_3), 2.65-2.80 (m, 1H, CH), 3.81 (s, 3H, OCH_3),

4.90-5.10 (m, 1H, CH), 6.92-7.16 (m, 3H, ArH), 7.65 (s, 1H, CH). Anal. calcd for $C_{16}H_{17}NO_5S_2$: C 52.30, H 4.66, N 3.81, found: C 52.18, H 4.78, N 3.95.

2-[5-(3-ethoxy-4-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylbutanoic acid (**7**) Yield: 68%; mp: 192-194°C. ^{1}HNMR (DMSO-d_6): δ = 0.67 (d, *J*=6.5, 3H, CH_3), 1.24 (d, *J*=6.5, 3H, CH_3), 1.34 (t, *J*=7.0, 3H, CH_3), 2.68-2.82 (m, 1H, CH), 3.83 (s, 3H, OCH_3), 4.07 (q, *J*=7.0, 2H, CH_2), 4.90-5.00 (m, 1H, CH), 7.12-7.23 (m, 3H, ArH), 7.66 (s, 1H, CH). Anal. calcd for $C_{18}H_{21}NO_5S_2$: C 54.67, H 5.35, N 3.00, found: C 54.78, H 5.44, N 3.16.

2-[5-(3,4-dimethoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylbutanoic acid (**8**) Yield: 59%; mp: 159-161°C. ^{1}HNMR (DMSO-d_6): δ = 0.69 (d, *J*=7.00, 3H, CH_3), 1.21 (d, *J*=6.4, 3H, CH_3), 2.66-2.78 (m, 1H, CH), 3.82 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 4.95-5.09 (m, 1H, CH), 7.13-7.26 (m, 3H, ArH), 7.73 (s, 1H, CH). Anal. calcd for $C_{17}H_{19}NO_5S_2$: C 53.53, H 5.02, N 3.67, found: C 53.44, H 4.86, N 3.72.

For compounds **1-4**, purchased by means of the Chemical Sourcing Service of Sigma-Aldrich, all physical and spectral data were in accordance with the literature.

*4.6. General procedure for the synthesis of (4-oxo-2-thioxo-1,3-thiazolidin-3-yl)dioic acids **(21-25)***

A suspension of suitable amino acid (**16-20**) (1 mmol) and carbon disulfide (1 mmol, 60 µl) in a solution of NaOH (2mmol) and water (10 ml) was reacted in a microwave reactor for 5 min at 100°C. After automated cooling chloroacetic acid (1mmol) was added and the mixture was reacted again at 100°C for 5 min. After cooling HCl (3ml, 6N) was added and the reaction was finished at 120 °C for 30 min. The mixture was neutralized with a saturated $NaHCO_3$ aqueous solution, extracted with ethyl acetate (10 mL x 3) and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the crude mixture was purified by flash chromatography using a mixture of CH_2Cl_2/CH_3OH (90:10) as euent.

2-(4-Oxo-2-thioxo-1,3-thiazolidin-3-yl)butanedioic acid (**21**) Yield: 45%; mp: 148-150°C. ^{1}HNMR (DMSO-d_6): δ = 4.40 (s, 2H, CH_2), 4.54 (s, 2H, CH_2), 13.39 (bs, 1H, OH). Anal. calcd for $C_5H_5NO_3S_2$: C 31.41, H 2.64, N 7.32, found: C 31.02, H 2.83, N 7.58.

2-(4-Oxo-2-thioxo-1,3-thiazolidin-3-yl)pentanedioic acid (**22**) Yield: 56%; mp: 152-154°C. ^{1}HNMR (DMSO-d_6): δ = 3.98-4.12 (m, 2H, CH_2), 4.45 (d, *J*=7.0, 2H, CH_2), 4.52 (s, 2H, CH_2). Anal. calcd for $C_6H_7NO_3S_2$: C 35.11, H 3.44, N 6.82, found: C 35.42, H 3.22, N 7.01.

2-Methyl-3-(4-oxo-2-thioxo-1,3-thiazolidin-3-yl)butanedioic acid (**23**) Yield: 67%; mp: 118-120°C. ^{1}HNMR (DMSO-d_6): δ = 1.43 (d, *J*=7.0, 3H, CH_3), 4.30 (s, 2H, CH_2), 5.42 (q, *J*=7.0, 1H, CH). Anal. calcd for $C_6H_7NO_3S_2$: C 35.11, H 3.44, N 6.82, found: C 35.34, H 3.12, N 6.68.

3-Methyl-2-(4-oxo-2-thioxo-1,3-thiazolidin-3-yl)pentanedioic acid (**24**) Yield: 58%; mp: 125-127°C. ^{1}HNMR (DMSO-d_6): δ = 1.33 (d, *J*=7.0, 3H, CH_3), 2.82-2.96 (m, 2H, CH_2), 4.11 (s, 2H, CH_2), 5.25-5.31 (m, 1H, CH), 12.42 (bs, 1H, COOH). Anal. calcd for $C_7H_9NO_3S_2$: C 38.34, H 4.14, N 6.39, found: C 38.52, H 4.27, N 6.20.

3-Ethyl-2-(4-oxo-2-thioxo-1,3-thiazolidin-3-yl)pentanedioic acid (**25**) Yield: 61%; mp: 141-143°C. ^{1}HNMR (DMSO-d_6): δ = 0.79 (t, *J*=7.7, 3H, CH_3), 2.02-2.14 (m, 2H, CH_2), 4.35 (s, 2H, CH_2), 5.28-5.33 (m, 1H, CH). Anal. calcd for $C_7H_9NO_3S_2$: C 38.34, H 4.14, N 6.39, found: C 38.48, H 4.31, N 6.22.

*4.7. General procedure for the synthesis of 2-[(5Z)-5-(4-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]dioic acids **(11-15)***

The suitable cyclized rhodanine nucleus (1mmol) (**21-25**) was reacted with p-methoxy benzaldehyde (3 mmol, 408,45 mg) following the same synthetic approach employed to obtain derivatives **5-8**.

2-[(5Z)-5-(4-Methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]butanedioic acid (**11**) Yield: 64%; mp: 243-245°C. ^{1}HNMR (DMSO-d_6): δ = 3.84 (s, 3H, OCH_3), 2.02-2.14 (m, 2H, CH_2), 4.72 (s, 2H, CH_2), 7.12 (d, *J*= 9.1, 2H, ArH), 7.62 (d, J= 9.1, 2H, ArH) 7.85 (s, 1H, CH). Anal. calcd for $C_{13}H_{11}NO_4S_2$: C 50.47, H 3.58, N 4.53, found: C 50.54, H 3.67, N 4.89.

2-[(5Z)-5-(4-Methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]pentanedioic acid (**12**) Yield: 64%; mp: 222-224°C. ^{1}HNMR (DMSO-d_6): δ = 2.61 (t, *J*=7.9, 2H, CH_2), 3.83 (s, 3H, OCH_3), 4.21 (t, *J*=7.9, 2H, CH_2), 7.12 (d, J= 9.0, 2H, ArH), 7.61 (d, J= 9.0, 2H, ArH) 7.77 (s, 1H, CH). Anal. calcd for $C_{14}H_{13}NO_4S_2$: C 52.00, H 4.05, N 4.33, found: C 51.84, H 4.18, N 4.14.

2-[(5Z)-5-(4-Methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylpentanedioic acid (**13**) Yield: 59%; mp: 130-132°C. ^{1}HNMR (DMSO-d_6): δ = 1.53 (d, *J*=7.0, 1H, CH), 3.84 (s, 3H, OCH_3), 5.60 (q, *J*=7.0, 3H, CH_3), 7.12 (d, J= 8.5, 2H, ArH), 7.61 (d, J= 8.5, 2H, ArH) 7.79 (s, 1H, CH). Anal. calcd for $C_{14}H_{13}NO_4S_2$: C 52.00, H 4.05, N 4.33, found: C 52.23, H 4.21, N 4.19.

2-[(5Z)-5-(4-Methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]-3-methylpentanedioic acid (**14**) Yield: 65%; mp: 176-178°C. ^{1}HNMR (DMSO-d_6): δ = 1.42 (d, *J*=7.0, 2H, CH_2), 2.90 (q, *J*=7.1, 3H, CH_3), 3.83 (s, 3H, OCH_3), 5.40-5.47 (m, 1H, CH), 7.12 (d, J= 8.8, 2H, ArH), 7.59 (d, J= 8.8, 2H, ArH) 7.70 (s, 1H, CH). Anal. calcd for $C_{15}H_{15}NO_4S_2$: C 53.40, H 4.48, N 4.15, found: C 53.52, H 4.36, N 4.07.

3-Ethyl-2-[(5Z)-5-(4-methoxybenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl]pentanedioic acid (**15**) Yield: 86%; mp: 164-166°C. ^{1}HNMR (DMSO-d_6): δ = 0.81 (t, *J*=7.5, 3H, CH_3), 2.13-2.23 (m, 2H, CH_2), 3.83 (s, 3H, OCH_3), 5.48 (t, *J*= 7.5, 1H, CH), 7.12 (d, *J*= 8.8, 2H, ArH), 7.62 (d, *J*= 8.8, 2H, ArH) 7.79 (s, 1H, CH). Anal. calcd for $C_{15}H_{15}NO_4S_2$: C 53.40, H 4.48, N 4.15, found: C 53.29, H 4.61, N 4.33.

4.8. LEDGF/p75-HIV-1 Integrase interaction screening

The AlphaScreen assay was performed as previously describe.[38] Reactions were performed in 25 µl final volume in 384-well Optiwell™ microtiter plates (Perkin–Elmer). The reaction buffer contained 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.01% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin. His6-tagged integrase (300 nM final concentration) was incubated with the compounds at 4°C for 30 min. The compounds were added in varying concentrations from 1 up to 100 nM. Afterward 100 nM of recombinant flag-LEDGF/p75 was added and incubation was extended by another

hour at 4°C. Subsequently, 5 µl of Ni-chelate-coated acceptor beads and 5 µl of anti-flag donor beads were added to a final concentration of 20 µg/ml of both beads. Proteins and beads were incubated at 30°C for 1 h in order to allow association to occur. Exposure of the reaction to direct light was prevented as much as possible and the emission of light from the acceptor beads was measured in the EnVision plate reader (Perkin–Elmer, Benelux) and analyzed using the EnVision manager software.

4.9. In vitro anti-HIV and drug susceptibility assay

The inhibitory effect of antiviral drugs on the HIV-induced cytopathic effect (CPE) in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT-assay.[39] This assay is based on the reduction of the yellow coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose ($CCID_{50}$) of the HIV(III_B) strain was determined by titration of the virus stock using MT-4 cells. For the drug-susceptibility assays, MT-4 cells were infected with 100-300 $CCID_{50}$ of the virus stock in the presence of five-fold serial dilutions of the antiviral drugs. The concentration of the various compounds that achieved 50% protection against the CPE of the different HIV strains, which is defined as the EC_{50}, was determined. In parallel the 50% cytotoxic concentration (CC_{50}) was determined.

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