Design, Synthesis and Pharmacological Evaluation of HIV-1 Reverse Transcriptase Inhibition of New Indolin-2-Ones

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Abstract: The design, synthesis and anti HIV-1 replication inhibition of 3-(cyclopropylethynyl)-3-hydroxy-indolin-2-ones, analogues of efavirenz (Sustiva™), are described. Different substituted isatins were used to generate final products that contain pharmacophoric features for RT inhibition, such as the oxoindole and cyclopropylethynyl groups. The suitability of the indolin-2-one ring in the planned compounds in replacement to the benzoxazinone ring of efavirenz was proven, since compound **15** presented a greater activity than efavirenz against HIV-1 replication and was not significantly cytotoxic.

Key Words: Isatins, molecular modeling, efavirenz.

INTRODUCTION

HIV/AIDS epidemic is increasing and now represents the third largest cause of death as a result of infectious diseases. Typically, reverse transcriptase (RT) inhibitors serve as a mainstay of most frontline HIV combination therapies. The reverse transcriptase enzyme can be inhibited by two classes of drugs belonging either to the nucleoside reverse transcriptase inhibitors (NRTIs) or to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) [3]. NRTIs act at the active site of RT. They are incorporated in the growing DNA strand and prevent further elongation of the DNA-chain, which terminates DNA synthesis. This class of inhibitors is active in HIV-1 as well as HIV-2 strains. NNRTIs, however, bind to an allosteric site, in the proximity of the active site of the enzyme, in a hydrophobic pocket located 10–15 Å from the active site of the polymerase site of the enzyme, known as the non-nucleoside inhibitor binding pocket, NNIBP [1,2]. This class of drugs is particularly active against HIV-1 and includes compounds such as nevirapine, delavirdine and efavirenz (Fig. **1**).

Efavirenz is one of the most potent and selective second generation RT inhibitors and is the current FDA-approved NNRTI with the best resistance profile to mutations within HIV-1 RT in comparison to the first generation NNRTIs, such as the currently marketed drugs nevirapine and delavirdine [3]. This particularity reinforces the efforts to find new analogues, since drug resistance remains one of the principal limitations associated to current antiviral therapy [4-6].

Earlier works have shown that isatins (1H-indole-2,3-diones) are synthetically versatile substrates used for the synthesis of a large variety of heterocyclic compounds, such as indoles and quinolines, that are present in many marketed drugs. In the last twenty-five years many synthetic isatins have shown a wide range of pharmacological activities [7a], including a recent report of isatin derivatives that act as HIV-1 RT inhibitors [7-11]. This has resulted into a continuous development of new synthetic methodologies to obtain indoles due to their importance in drug discovery programs, [12].

Assuming that the indolin-2-one ring can replace the benzoxazinone ring successfully, the goal of this work was the synthesis of new analogues of efavirenz that retain its main pharmacophoric features, essential for RT inhibition, such as the oxoindole and cyclopropylethynyl groups. The design of the new molecules was also based on pharmacophoric modeling studies of existing NNRTIs [13].

The molecular pharmacophoric features of efavirenz and the designed molecules include the presence of a planar, electron-rich region, a hydrophobic region and hydrogen bonding/acceptor groups (Fig. **2**) [13]. Finally, an OH group was introduced in the isatin derivatives as a replacement for the CF_3 group present in efavirenz, with the aim of achieving extra hydrogen bonding interactions with HIV-1 RT. Molecular modeling of the designed compounds with HIV-1 RT has also been performed. The biological activity was tested on HIV-1 replication *in vitro* and theoretical pharmacokinetic calculations (Lipinski's rule of five) have been made in order to support further *in vivo* studies.

RESULTS AND DISCUSSION

Synthesis

In the synthetic process the starting materials included: isatin (1); 5-bromoisatin (2); 5-methylisatin (3); 5-chloroisatin (4) 1-methylisatin (5); 5-bromo-1-methylisatin (6); 1,5-dimethylisatin (7); 5-chloro-1-methylisatin (8) [14-16].

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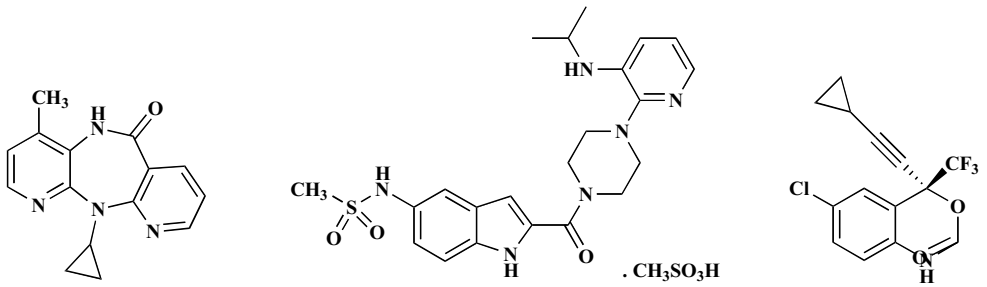


Fig. (1). Structures of nevirapine, delavirdine and Efavirenz®.

Isatins and derivatives can suffer nucleophilic attack at positions C-2 and/or C-3. The chemoselectivity of these reactions depends on the nature of the nucleophile, on the nature of the substituents attached to the isatin ring, and particularly of those bonded to the nitrogen atom, as well as upon the solvent and temperature employed. Nucleophilic addition at position C-3 has been described for the conversion of isatins to other heterocyclic systems [7a]. We have demonstrated that isatins react with diaminosulphurtrifluoride (DAST) giving regioespecific difluoroindoles that act as anti-inflammatory drugs [7b].

The nucleophilic alkynylation of carbonyl compounds is of considerable synthetic and industrial importance [17]. Current methodologies are far from ideal, suffering from both moderate enantioselectivity and limited scope [18]. For the synthesis of the HIV-1 reverse transcriptase inhibitor efavirenz one addition of Li-cyclopropyl acetylide is applied to the corresponding ketoaniline [19].

Syntheses of the new class of 3-(2-cyclopropylethynyl)-3-hydroxy-indolin-2-ones were carried out using cyclopropylacetylene with 1.2 molar equivalents of *N*-buthyl lithium in hexane followed by addition of substrates **1-8** in tetrahydrofuran (THF), resulting in derivatives **9-16** in 35-80% yields after chromatographic purification (Scheme **1**).

Compounds **1**, **2**, **3** and **4** produced the corresponding cyclopropylethynyl alcohols (**9-12**) with crude yields of 83, 74, 85 and 66%, respectively, and have been characterized by NMR spectra. For spectral analysis the products were purified by semi preparative HPLC to separate impurities from the desired products. Compounds **9-12** was obtained in 80, 67, 80 and 60% yields and with 99% purity.

The reaction using *N*-methylisatin derivatives **5**, **6**, **7** and **8**, resulted in the corresponding products with conversion rates of of 80, 85, 75 and 76% (**13-16**) (Scheme **2**). Although the desired products have been obtained in good yields the analysis by NMR spectra showed that these reactions afforded unexpected byproducts generated by nucleophilic attack of the cyclopropylethynyl group on position C-2, resulting in the formation of compounds **17-20** in 5-15% yields. This fact can be explained by the low electrophilicity of the amide carbonyl group. For spectral analysis the products were purified by semi preparative HPLC to separate compounds **17-20** from the desired products (**13-16**).

Purification by semi-preparative HPLC furnished compounds **13**, **14**, **15** and **16** in 60, 35, 47, 50% yields, respectively. These compounds were characterized by NMR spectra. The syntheses of compounds **9-12** have not been reported previously.

MOLECULAR MODELING OF THE LIGAND-PROTEIN COMPLEXES

Molecular modeling of the indolin-2-one HIV-1 reverse transcriptase inhibitor candidates **9-16** (Schemes **1** and **2**) was performed. Manual construction of the molecules using efavirenz co-crystallized with HIV-1 RT and further molecular mechanics calculations of the ligand-protein complexes were made, in order to check if the indolin-2-one would fit the non-nucleoside inhibitor-binding pocket (NNIBP) of

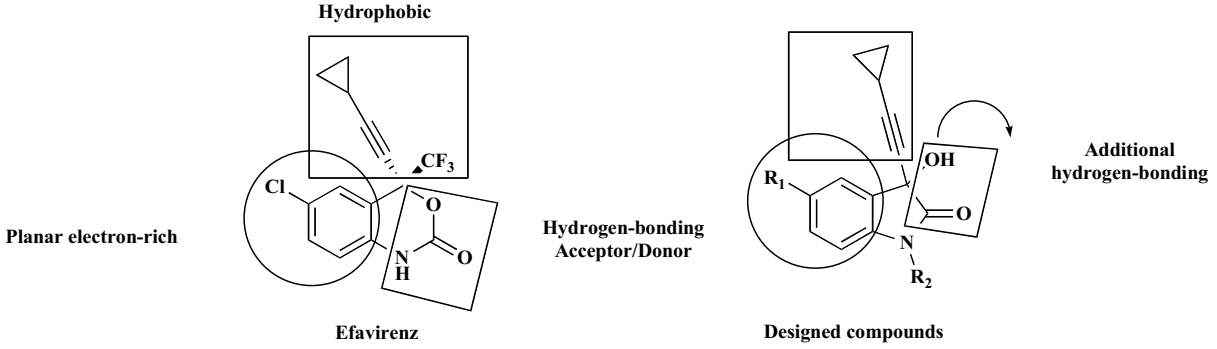
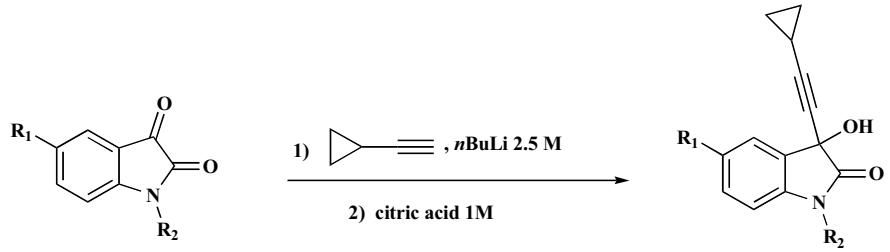


Fig. (2). Corresponding interacting regions of **Efavirenz®** and the similar pharmacophores observed in the designed compounds.



(1) $R_1 = R_2 = H$
(2) $R_1 = Br$; $R_2 = H$
(3) $R_1 = CH_3$; $R_2 = H$
(4) $R_1 = Cl$; $R_2 = H$

(9) $R_1 = R_2 = H$
(10) $R_1 = Br$; $R_2 = H$
(11) $R_1 = CH_3$; $R_2 = H$
(12) $R_1 = Cl$; $R_2 = H$

Scheme 1. Synthesis of compounds **9-12**.

HIV-1 RT. We have evaluated both *R*/*S* stereoisomers of **9-16** in order to gain some insight on the steric requirements of the protein binding site and get a clue on which isomer would preferably bind. We considered this simple approach a good tool to visualize possible interacting sites of the new compounds with the protein, since we have introduced few degrees of freedom in their structure in comparison to efavirenz**,** and would not expect a great perturbation of the protein structure on binding.

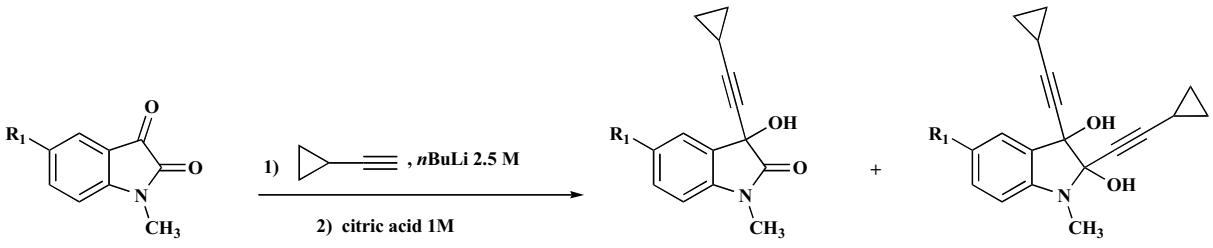
Theoretical binding energy values, measured as the energy variation between bound and unbound states (ΔE, in kcal/mol) were obtained, aiming at the selection of molecules for biological testing (Table **1**). In this procedure, we used molecular mechanics calculations in Sybyl6.8 [20] with Tripos force field [21] using the crystal structure of wild HIV-1 RT in complex with efavirenz [22], available in the Protein Data Bank (PDB) [23]. According to the results obtained, *R* isomers bearing the -N-CH_3 moiety have the most favorable binding energies, compared to efavirenz (ΔE= -42.51 kcal/mol) (Table **1**).

Hydrophobic substituents (Br, CH_3 and Cl, compounds **14-16**) in position 5 of the indolin-2-one ring had a better performance (ΔE= -40.90, -40.46 and –39.96 kcal/mol, respectively), which makes sense if one remembers that the NNIBP is filled with hydrophobic amino acid residues. The proximity of these groups to hydrophobic amino acid residues in the NNIBP pocket can be visualized in Fig. **3** that depicts the putative interactions of the structural subunits of compounds **14** and **15** in NNIBP. From the pictures, it is clear that the hydrophobic substituents in position 5 are within van der Waals contact with Leu234, while the -CH_3 group of the -N-CH_3 function of the indolin-2-one nucleus is in van der Waals contact with Leu100.

Table 1. Binding Energy (ΔE) of the Ligand-Protein Complexes Obtained Using Molecular Mechanics Calculations

Compound (*R* isomer)	ΔE (Kcal/mol)	Compound (*S* isomer)	ΔE (kcal/mol)
9	-34.93	9	-25.51
10	-37.00	10	-26.35
11	-38.00	11	-25.40
12	-37.32	12	-27.23
13	-38.26	13	-25.11
14	-40.90	14	-28.22
15	-40.46	15	-30.11
16	-39.96	16	-28.04
Efavirenz	-42.51	-	-

This result may be interesting for a good fit in the allosteric site of the protein and compensate for the absence of a



(5) $R_1 = H$
(6) $R_1 = Br$
(7) $R_1 = CH_3$
(8) $R_1 = Cl$

(13) $R_1 = H$
(14) $R_1 = Br$
(15) $R_1 = CH_3$
(16) $R_1 = Cl$

(17) $R_1 = H$
(18) $R_1 = Br$
(19) $R_1 = CH_3$
(20) $R_1 = Cl$

Scheme 2. Nucleophilic addiction reactions of *N*-methylisatin derivatives.

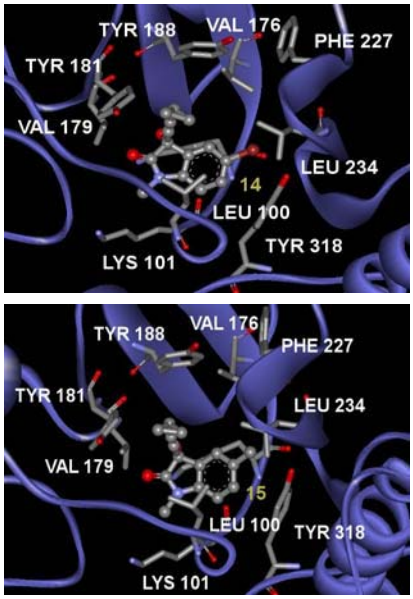


Fig. (3). Positioning of compounds **14**-**15** (*R* isomers, in ball-and-stick display) in NNIBP of HIV-1 RT (amino acid residues in stick display). Hydrogen atoms have been omitted for clarity.

hydrogen bond in that position compared to the one performed by the -NH group of efavirenz with the backbone carbonyl of Lys101 [22]. It is worth mentioning that no extra hydrogen bonding with the –OH group has been observed for this series. The *S* isomers of the above mentioned structures have also performed well in comparison to the other structures in this study, suggesting some degree of stereochemical preference for the designed series of compounds, as it is experimentally observed with the enantiomers of efavirenz. Based on these results, compounds **14** and **15**, two of the structures with the most favorable energy values, and also compound **13**, that bears a hydrogen atom in position 5 of the indolin-2-one ring, were chosen for anti HIV-1 replication testing.

Lipinski's Rule of Five

In addition to ligand-protein complex modeling, *in vivo* absorption capabilities of the designed molecules were tentatively assessed by means of theoretical calculations following Lipinski's rule of five [24], that predict that a compound administered orally will more likely have good absorption or permeation if it satisfies the following criteria:

1. Hydrogen Bond Donors ≤ 5 (OH and NH groups)
2. Hydrogen Bond Acceptors ≤ 10 (N and O atoms)
3. Molecular Weight < 500
4. Calculated log P (ClogP) < 5

This approach has been widely used as a filter in the decision making of which substances should be further developed in drug design programs. The results of the calculations for the molecules designed in this study show that all molecules have a potential for good *in vivo* absorption, since all of them satisfied Lipinski's rule of five with zero violations (Table **2**).

BIOLOGICAL ASSAYS

Toxicity Assays

None of the synthesized compounds tested was toxic to human mononuclear cells when concentrations of 2.5 micrograms/mL or less were employed in 7 day incubations, and only **14** showed a limited toxicity at concentrations of 5 micrograms/ml (Fig. **4**).

Inhibition of HIV-1 replication

Attempts to inhibit HIV-1 replication were carried out on 2 HIV-1 isolates, one being the T-cell line adapted reference strain HIV-1 IIIB and the other a primary HIV-1 isolate of the same genotype B, isolate 2242:1886 (Table **3**). As expected, inhibition of the primary HIV-1 isolate was much less effective than that of the reference TCLA HIV-1 isolate IIIB (Table **3**). Both **13** and **15** inhibited replication of HIV-1 IIIB, and **15** was more effective than the reference antiretroviral drug efavirenz against the reference HIV-1 isolate IIIB (Fig. **5**), while no linear fit could be obtained from the values

Table 2. Lipinski's Rule of Five Calculated with Sybyl 6.8

Compound	CLogP	Molecular Weight	Hydrogen Bond Donors	Hydrogen Bond Acceptors	Satisfies the rule of five?
9	2.45	213.24	2	2	Yes
10	3.57	292.13	2	2	Yes
11	2.95	227.26	2	2	Yes
12	3.42	247.68	2	2	Yes
13	2.96	227.26	1	2	Yes
14	3.96	306.16	1	2	Yes
15	3.46	241.29	1	2	Yes
16	3.81	261.71	1	2	Yes

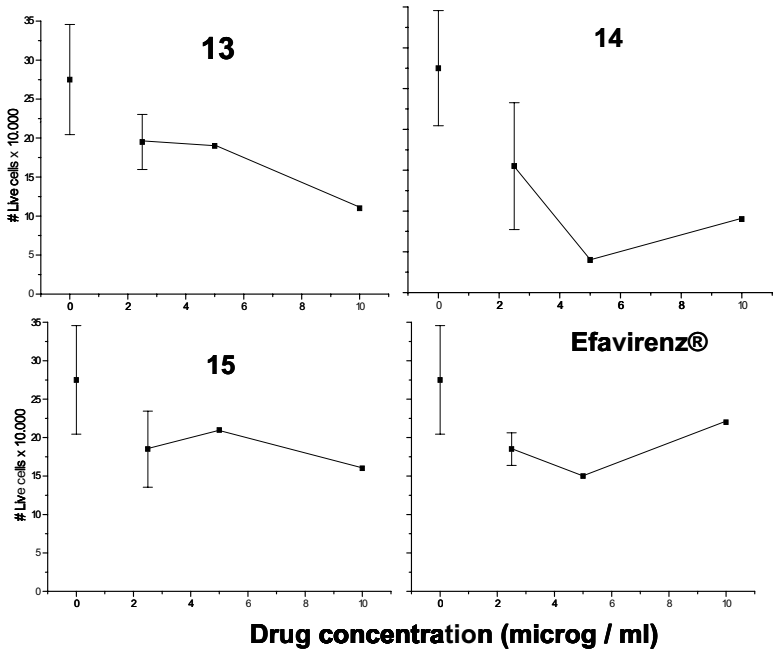


Fig. (4). Cytotoxicity of compounds **13**-**15** and **Efavirenz®** in 7 days incubations with human mononuclear cells.

calculated for compound **14**, although it inhibited replication to some extent (approximately 70%).

Similarly, no dose-response correlation could be established for the inhibition of the primary HIV-1 isolate HIV-1 2242:1886 by efavirenz, as an inhibition above 75% was achieved still at the lowest concentration tested (0.3125 mg/ml). Compound **15** was the most effective compound against replication of this isolate, although 90% inhibition of replication was not reached using concentrations up to 10 microgram per milliliter.

This work has been based on the assumption that the indolin-2-one ring could be a nice replacement for the benzoxazinone ring of the marketed drug efavirenz. Molecular modeling of the new class of inhibitors has allowed the selection of compounds for biological testing and the results corroborated the suggestion that hydrophobic substituents in position 5 of the indolin-2-one ring are necessary for inhibition of HIV-1 replication. This has been proven by the lower anti-HIV-1 replication activity of compound **13** that bears a hydrogen atom in that position, compared to compound **14**, that was more effective than the reference antiretroviral drug efavirenz against the reference HIV-1 IIIB isolate. Additionally, no dose-response correlation could be established for the inhibition of the primary HIV-1 isolate HIV-1 2242:1886 by efavirenz, while compound **15** was the most effective compound against replication of this isolate, although 90% inhibition of replication was not reached using concentrations of **15** up to 10 micrograms per milliliters. These results may be reflecting the formation of molecular aggregates in

Table 3. Concentrations (in Nanogram Per Milliliter) Needed to Inhibit HIV Replication at 50%, 75% and 90% Levels (Isolates 2242:1886 and IIIB) and p Values of Linear Fit Calculations

	P	IC 50% (ng / ml)	IC 75% (ng / ml)	IC 90% (ng / ml)
Efavirenz® x 2242:1886	0.90	not detectable (nd)	500	nd
13 x 2242:1886	0.56	Nd	5000	nd
14 x 2242:1886	0.08419	3000	20000	50000
15 x 2242:1886	0.33	300	20000	300000
Efavirenz® x IIIB	0.15	4.5	450	6500
13 x IIIB	0.03664	550	4000	10500
14 x IIIB	0.98	Nd	nd	nd
15 x IIIB	0.00475	0.2	55	1500

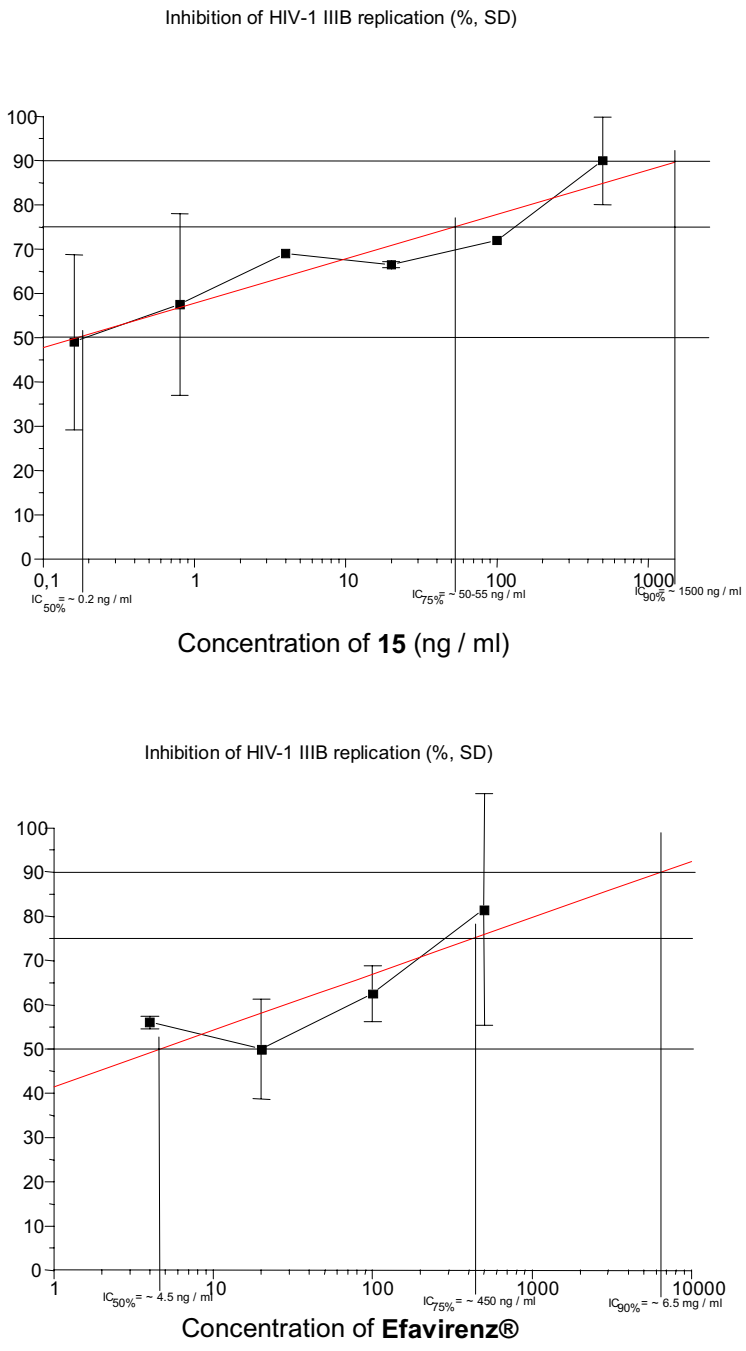


Fig. (5). Inhibition of HIV-1 IIIB replication in normal human pre-activated peripheral blood lymphocytes by the antiretroviral compound **15**, in comparison to inhibition by the reference drug **Efavirenz®**.

the experimental media at certain concentrations, preventing the observation of the biological response at the reported doses.

Due to the complexity of cellular systems, the greater activity of compound **15** as compared to **14** could not be explained, since both molecules are similar, except that one bears a bromine (compound **14**), while the other bears a methyl group (compound **15**) in position 5 of the indolin-2-one ring. This result emphasizes the difficulties in reproducing theoretical results experimentally due to the inherent complexity of cellular systems, although molecular modeling has been reported as a useful guide in the design and development of biologically active molecules [25]. So, aiming at verifying possible steric restraints in NNIBP, additional molecular dynamics calculations of **13**, **14** and **15** with HIV-1 RT have provided some insight on the possible explanation to the difference in activity among these compounds, since

the average distances along the simulation between selected atoms of compounds **13** and **14** to Leu234 (5.6 Å, data not labeled), were significantly higher than the ones observed for compound **15** (4.5 Å, data not labeled). This observation led us to consider a possible deleterious stereo electronic effect caused by the halogen and should be considered in the future design of other isatin derivatives. Further visual inspection of the molecular trajectories showed that the OH group that has been introduced in substitution for CF_3 group of efavirenz is able to hydrogen bond to the backbone carbonyl of Tyr188 in all the three studied compounds, even though the average heteroatom-heteroatom distances found for compounds **13** and **14**, 3.6 and 3.4 Å, respectively), are higher than the average found for compound **15**, that was 3.2 Å.

Finally, the biological results reported herein showed that compound **15** had no significant cytotoxicity in the system used and presented a better inhibition pattern than efavirenz on HIV-1 replication. Also, the calculated Lipinski's properties showed that these molecules have a good potential for *in vivo* absorption, since they satisfy the rule of five. These data suggested a possible molecular mechanism on HIV-1 RT, and encouraged further studies with the isolated enzyme, in order to establish structure-activity relationships of these new anti-HIV compounds.

CONCLUSIONS

In summary, this paper presented a combination of molecular modeling, synthesis and biological assays that helped gain further insights into the molecular requirements for HIV-1 RT inhibition by 3-(cyclopropylethynyl)-3-hydroxy-indolin-2-one derivatives.

An efficient and practical synthesis of the new class of HIV-1 RT inhibitors, analogues of efavirenz, is now available. The introduction of the cyclopropylethynyl group *via* additional nucleophilic reactions to form 3-(cyclopropylethynyl)-3-hydroxy-indolin-2-ones was achieved in good yields, and led to a new class of efavirenz analogues. The synthesis provided analytically pure compounds (**9**-**16**) in overall yields of 35-80%, in two steps from isatin derivatives. *N*-methylisatin derivatives were poor substrates when the indicated methodology was used.

Overall, eight new compounds were obtained in this study. Molecular modeling of the new class of inhibitors has allowed the selection of compounds for biological testing and the results corroborated the suggestion that hydrophobic substituents in position 5 of the indolin-2-one ring are essential for inhibition of HIV-1 replication, since compound **15** presented a greater activity than efavirenz on HIV-1 replication. The calculated Lipinski's properties showed a good potential for absorption *in vivo* of these molecules since they satisfy the rule of five. Additionally, the biological results showed that compound **15** was not significantly cytotoxic. Further inhibition studies of the new compounds against the purified RT enzyme will be performed in order to establish structure-activity relationships.

EXPERIMENTAL

Chemistry

All solvents and reagents used were obtained from commercial sources, unless otherwise indicated. Dry tetrahydrofuran was purchased from Vetec and used after redistillation. N-BuLi was previously titulated. All reactions were monitored by thin-layer chromatography over precoated Merck Silica gel 60 F254 plates and visualized by UV irradiation. Melting points were measured on a Büchi B-545 instrument. The GC-MS spectra were obtained on a Hewlett Packard 5960 MS connected to a Hewlett Packard 5890 gas chromatograph. NMR spectra were recorded on a Bruker Avance 500 spectrometer. Chemical shifts were reported as δ values (ppm) downfield from internal Me_4Si in the solvent shown. Coupling constants (*J*) are expressed in Hertz (Hz). The following NMR abbreviations are used: s (singlet), d (doublet), dd (double-doublet), t (triplet), q (quartet), m (multiplet). Infrared (IR) spectra were recorded on KBr pellets with a Nicolet 670 FT-IR spectrometer. HPLC was carried out using a Merck-Hitachi LaChrom model equipped with a 7100 pump and a 7455 photodiode array detector. All analytical assays and semi preparative chromatography were performed under the conditions given previously. The abbreviation THF refers to tetrahydrofuran. Elemental analyses for C, H, N, were performed on a Perkin-Elmer Model EA 2400 CHN and the results agreed within ± 0,4% of the calculated values.

Isatin derivatives **1** and **2** are commercially available by Aldrich and derivatives **3** and **4** were obtained using Sandmeyer's methodology [15]. Isatins **5-8** were prepared from their corresponding isatins by reaction of each starting material with CaH_2 in DMF [16] in the presence of iodomethane. The reaction mixture was heated to 100°C for 4 hours. After treatment with 0.5 M hydrochloric acid, the isatins were obtained in 90%, 85%, 92% and 88% yields [14-16]. *N*-methylisatins were characterized by GC-MS, NMR and melting point measurements.

The HPLC analysis was performed with a Shim-pack CLC-C8 column (250mm x 4.6mm i.d.), injection volume 20 μL, using acetonitrile: TFA 0,05% at 30°C and a gradient of these solvents varying between 30-90% in 40 minutes, flow rate between 0.7-1.0 mL/min, and run time of 60 minutes. The spectral data were acquired between 190-800 nm and the effluent was monitored at 215 nm. From these results the method was modified to allow for scaling up to a semi preparative method. The semi preparative HPLC was performed with a Shim-pack PREP-C8 column (250mm x 20mm i.d.). The mobile phase was acetonitrile: TFA 0,05% at 30°C with an average gradient of 30 to 90% during 40 minutes, flow rate between 5 and 7 mL/min, with an average run time of 60 minutes. The injection volume was 400 μL of concentrated product.

General Procedure

Cyclopropylacetylene (2 mmol) was charged into dry tetrahydrofuran (10 mL). The solution was cooled to –5°C, and *n*-butyllithium in hexane 2.5 M (1,2 mmol) was added, keeping the temperature below 5°C. The mixture was stirred for 30 minutes and the corresponding isatins (1 mmol) were added to the reaction in dry THF (10 mL). The resulting solution was left at room temperature for 24 hours and quenched by addition into 1M citric acid (15 mL). The mixture was warmed to room temperature and the solution was extracted with CH_2Cl_2 (3 x 15 mL). The combined organic phases were washed with water (3 x 10 mL), dried over an-

hydrous sodium sulfate, filtered and then evaporated at reduced pressure.

3-(2-cyclopropylethynyl)-3-hydroxyindolin-2-one (9)

White solid. m.p. 198-199°C; Yield 83%; ^{max} 212.2 nm; IR (KBr) ν 762.48, 918.85, 1110.31, 1183.89, 1336.44, 1471.23, 1620.23, 1718.32, 2227.08, 3204.41; ^{1}H NMR (acetone-d^6) δ 0.57-0.60 (m, 2H), 0.76-0.81 (m, 2H), 1.28-1.33 (m, 1H), 5.17 (s, 1H), 6.92 (d, 1H, *J*= 8.0 Hz), 7.05 (t, 1H, *J*= 7.5 Hz), 7.27 (t, 1H, *J*= 7.5 Hz), 7.41 (d, 1H, *J*= 7.5 Hz), 9.43 (s, 1H); ^{13}C NMR (acetone-d^6) δ 0.06, 8.60, 8.64, 69.95, 74.44, 89.87, 110.92, 123.33, 124.33, 125.35, 130.64, 132.33, 142.20, 175.79; MS (GC) *m/z* 212 (M^+-1). Anal. Calcd. for $C_{13}H_{11}NO_2$: C, 73.23; H, 5.20; N, 6.57. Found: C, 72.94; H, 5.41; N, 6.55.

5-bromo-3-(2-cyclopropylethynyl)-3-hydroxyindolin-2-one (10)

White solid. m.p. 213-215°C; Yield 74%; ^{max} 212.2 nm; IR (KBr) ν 673.88, 816.81, 929.70, 1178.36, 1329.41, 1475.39, 1720.76, 2232.79, 2864.66, 3036.83, 3327.99; ^{1}H NMR (acetone-d^6) δ 0.60-0.61 (m, 2H), 0.79-0.80 (m, 2H), 1.30-1.31 (m, 1H), 5.87 (s, 1H), 6.90 (d, 1H, *J*= 8.5 Hz), 7.44 (d, 1H, *J*= 8.0 Hz), 7.52 (s, 1H), 9.52 (s, 1H); ^{13}C NMR (acetone-d^6) δ 0.09, 8.71, 69.88, 73.71, 90.69, 112.99, 115.15, 128.32, 133.48, 134.56, 141.50, 175.17; MS (GC) *m/z* 292 (M^+). Anal. Calcd. for $C_{13}H_{10}BrNO_2$: C, 53.45; H, 3.45; N, 4.79. Found: C, 53.74; H, 3.30; N, 4.75.

3-(2-cyclopropylethynyl)-3-hydroxy-5-methylindolin-2-one (11)

White solid. m.p. 207-209°C; Yield 85%; ^{max} 212.2 nm; IR (KBr) ν 699.03, 1496.92, 1707.16, 1898.58, 2232.83, 3184.86, 3273.18; ^{1}H NMR (acetone-d^6) δ 0.58-0.59 (m, 2H), 0.78-0.80 (m, 2H), 1.29-1.31 (m, 1H), 2.30 (s, 3H), 5.63 (s, 1H), 6.80 (d, 1H, *J*= 8.0 Hz), 7.08 (d, 1H, *J*= 7.5 Hz), 7.23 (s, 1H), 9.31 (s, 1H); ^{13}C NMR (acetone-d^6) δ -0.05, 8.56, 20.98, 70.01, 74.55, 89.68, 110.59, 125.92, 130.79, 132.25, 132.57, 139.57, 139.68, 175.80; MS (GC) *m/z* 226 (M^+-1). Anal. Calcd. for $C_{14}H_{13}NO_2$: C, 73.99; H, 5.77; N, 6.16. Found: C, 74.29; H, 5.79; N, 6.42.

5-chloro-3-(2-cyclopropylethynyl)-3-hydroxyindolin-2-one (12)

White solid. m.p. 224-226°C; Yield 66%; ^{max} 212.2 nm; IR (KBr) ν 683.64, 819.72, 930.39, 1084.30, 1178.89, 1327.85, 1475.61, 1730.30, 2232.94, 2861.73, 3039.96, 3112.83, 3317.96; ^{1}H NMR (acetone-d^6) δ 0.59-060 (m, 2H), 0.79-0.80 (m, 2H), 1.28-1.32 (m, 1H), 5.87 (s, 1H), 6.94 (d, 1H, *J*= 8.0 Hz), 7.30 (d, 1H, *J*= 8.0 Hz), 7.39 (s, 1H), 9.51 (s, 1H); ^{13}C NMR (acetone-d^6) δ -0.04, 8.68, 69.91, 73.67, 90.63, 100.95, 112.47, 125.49, 127.95, 130.54, 134.15, 141.01, 175.28; MS (GC) *m/z* 246 (M^+-1). Anal. Calcd. for $C_{13}H_{10}ClNO_2$: C, 63.04; H, 4.07; N, 5.66. Found: C, 63.02; H, 3.89; N, 6.01.

3-(2-cyclopropylethynyl)-3-hydroxy-1-methylindolin-2-one (13)

White solid. m.p. 180-181°C; Yield 60%; ^{max} 215.7 nm; IR (KBr) ν 761.85, 920.96, 1091.29, 1164.98, 1350.02, 1375.58, 1466.62, 1611.27, 1703.27, 2226.87, 3287.11; ^{1}H NMR (acetone-d^6) δ 0.57 (m, 2H), 0.77-0.78 (m, 2H), 1.27-1.28 (m, 1H), 3.15 (s, 3H), 5.68 (s, 1H), 6.96 (d, 1H, *J*= 7.5Hz), 7.09 (t, 1H, *J*= 7.5 Hz), 7.34 (t, 1H, *J*= 7.5 Hz), 7.42 (d, 1H, *J*= 7.0 Hz); ^{13}C NMR (acetone-d^6) δ 0.09, 8.62, 8.66, 26.53, 69.72, 74.34, 89.96, 109.58, 123.78, 124.91, 130.77, 131.70, 144.14, 174.29; MS (GC) *m/z* 226 (M^+-1). Anal. Calcd. for $C_{14}H_{13}NO_2$: C, 73.99; H, 5.77; N, 6.16. Found: C, 73.63; H, 5.68; N, 6.45.

5-bromo-3-(2-cyclopropylethynyl)-3-hydroxy-1-methylindolin-2-one (14)

White solid. m.p. 222-224°C; Yield 35%; ^{max} 212.2 nm; IR (KBr) ν 813.53, 929.70, 1009.64, 1102.37, 1354.34, 1490.81, 1601.22, 1719.09, 2240.76, 2816.35, 3005.02, 3270.43; ^{1}H NMR (acetone-d^6) δ 0.59 (m, 2H), 0.79 (m, 2H), 1.30 (m, 1H), 3.16 (s, 3H), 5.89 (s, 1H), 6.96 (d, 1H, *J*= 8.0 Hz); 7.52 (s, 1H), 7.54 (s, 1H); ^{13}C NMR (acetone-d^6) δ -1.03, 7.73, 25.72, 68.59, 72.56, 89.76, 110.67, 114.68, 126.90, 132.55, 132.81, 142.40, 172.74; MS (GC) *m/z* 306 (M^+). Anal. Calcd. for $C_{14}H_{12}BrNO_2$: C, 54.92; H, 3.95; N, 4.58. Found: C, 54.74; H, 3.89; N, 4.59.

3-(2-cyclopropylethynyl)-3-hydroxy-1,5-dimethylindolin-2-one (15)

White solid. m.p. 166-168°C; Yield 47%; ^{max} 212.2 nm; IR (KBr) ν 692.12, 815.48, 933.17, 1010.37, 1099.33, 1361.03, 1503.18, 1702.28, 2237.36, 2863.84, 2920.65, 3275.06; ^{1}H NMR (acetone-d^6) δ 0.56-0.57 (m, 2H), 0.77-0.78 (m, 2H), 1.27-1.29 (m, 1H), 2.32 (s, 3H), 3.13 (s, 3H), 5.66 (s, 1H), 6.85 (d, 1H, *J*= 7.5Hz), 7.15 (d, 1H, *J*= 7.5Hz), 7.25 (s, 1H); ^{13}C NMR (acetone-d^6) δ -0.02, 8.64, 8.66, 21.06, 26.60, 69.88, 74.36, 89.92, 109.40, 125.61, 130.93, 131.66, 133.30, 141.64, 174.52; MS (GC) *m/z* 241 (M^+). Anal. Calcd. for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27; N, 5.81. Found: C, 74.64; H, 6.34; N, 6.05.

5-chloro-3-(2-cyclopropylethynyl)-3-hydroxy-1-methylindolin-2-one (16)

White solid. m.p. 204-206°C; Yield 50%; ^{max} 212.2 nm; IR (KBr) ν 683.50, 726.21, 815.03, 932.96, 1008.31, 1074.86, 1102.41, 1351.72, 1429.93, 1492.67, 1611.65, 1718.64, 2238.90, 2815.53, 3013.59, 3273.79; ^{1}H NMR (acetone-d^6) δ 0.59-0.60 (m, 2H), 0.79-080 (m, 2H), 1.27-1.31 (m, 1H), 3.17 (s, 3H), 5.48 (s, 1H), 7.01 (d, 1H, *J*= 8.0 Hz), 7.39 (d, 1H, *J*= 8.5Hz), 7.42 (s, 1H); ^{13}C NMR (acetone-d^6) δ -0.07, 0.07, 8.69, 26.71, 69.59, 73.50, 90.69, 111.15, 125.08, 128.45, 130.58, 133.43, 142.90, 173.83; MS (GC) *m/z* 261 (M^+).

2,3-bis(2-cyclopropylethynyl)-1-methylindoline-2,3-diol (17)

Yellow solid. m.p. 128-130°C; Yield 15%; ^{max} 365.3 nm; IR (KBr) ν 741.07, 791.70, 915.98, 958.05, 1097.44, 1128.85, 1355.06, 1387.68, 1457.63, 1486.14, 1539.44, 1630.89, 2241.19, 2341.78, 2360.76, 3007.95, 3086.11, 3286.76; ^{1}H NMR (acetone-d^6) δ 0.48-0.49 (m, 2H), 0.71-0.73 (m, 2H), 0.86-0.87 (m, 2H), 0.96-0.99 (m, 2H), 1.16-1.18 (m, 1H), 3.82 (s, 3H), 5.78 (s, 1H), 6.97 (d, 1H, *J*= 8.0 Hz), 7.02 (t, 1H, *J*= 7.5), 7.31 (t, 1H, *J*= 7.5 Hz, *J*= 8.0 Hz), 7.40 (d, 1H, *J*= 7.0 Hz), 8.23 (s, 1H); ^{13}C NMR (acetone-d^6) δ 0.07, 8.58, 8.66, 10.53, 10.98, 21.99, 73.87, 75.48, 87.42,

95.72, 109.61, 123.34, 123.83, 130.57, 133.41, 145.50; MS (GC) *m/z* 293 (M^+).

5-bromo-2,3-bis(2-cyclopropylethynyl)-1-methylindoline-2, 3-diol (18)

Yellow solid. m.p. 186-188°C; Yield 5%; ^{max} 371.8 nm; IR (KBr) ν 714.56, 737.86, 926.05, 956.75, 1112.21, 1131.69, 1355.48, 1489.79, 1537.58, 1607.77, 1631.07, 1732.04, 2236.89, 3005.83, 3095.15, 3332.04; ^{1}H NMR (acetone-d^6) δ 0.50 (m, 2H), 0.73-0.75 (m, 2H), 0.89-1.00 (m, 5H), 1.19-1.22 (m, 1H), 3.28 (s, 3H), 5.83 (s, 1H), 6.96 (d, 1H, *J*= 8.0 Hz), 7.47-7.49 (m, 2H), 8.21 (s, 1H); ^{13}C NMR (acetone-d^6) δ 0.01, 0.06, 8.61, 8.69, 10.78, 11.23, 22.09, 68.37, 73.57, 74.77, 88.19, 96.47, 111.52, 114.99, 127.71, 133.34, 135.53, 144.82; MS (GC) *m/z* 371 (M^+-1).

2,3-bis(2-cyclopropylethynyl)-1,5-dimethylindoline-2,3-diol (19)

Yellow solid. m.p. 137-139°C; Yield 10%; ^{max} 365.3 nm; IR (KBr) ν 789.36, 946.92, 1146.90, 1349.90, 1493.18, 1543.03, 1720.07, 2241.04, 2915.22, 3006.24; MS (GC) *m/z* 307 (M^+).

5-chloro-2,3-bis(2-cyclopropylethynyl)-1-methylindoline-2, 3-diol (20)

Yellow solid. m.p. 179-181°C; Yield 10%; ^{max} 371.8 nm; IR (KBr) ν 718.45, 795.18, 925.20, 957.64, 1113.90, 1131.90, 1273.79, 1356.71, 1488.81, 1552.05, 1611.65, 1630.21, 2236.89, 3009.71, 3200.00; ^{1}H NMR (acetone-d^6) δ 0.50 (m, 2H), 0.73-0.75 (m, 2H), 0.88-0.99 (m, 5H), 1.18-1.20 (m, 1H), 3.28 (s, 3H), 5.82 (s, 1H), 7.00 (d, 1H, *J*= 8.0 Hz), 7.32-7.36 (m, 2H), 8.22 (s, 1H); ^{13}C NMR (acetone-d^6) δ 0.03, 8.64, 8.70, 10.77, 11.23, 22.10, 73.55, 73.65, 74.83, 88.18, 96.46, 111.01, 124.92, 127.88, 130.44, 135.23, 144.39; MS (GC) *m/z* 327 (M^+).

MOLECULAR MODELING

All calculations and graphical manipulations were performed on a Silicon Graphics Origin workstation using the software package Sybyl version 6.8 [20]. Molecular mechanics minimizations were performed with Tripos force field [21]. The structure of wild HIV-1 RT co-crystallized with Efavirenz [22] used in this work was obtained from the RCSB Protein Data Bank (ID code 1FK9, refined at a 2.5 Å resolution) [23].

Compounds coordinates were created in the Sketcher program, available in Sybyl 6.8 and hydrogens were added with the Build/Add Hydrogens subroutine. The structure of Efavirenz was used as a template fragment for ligand positioning in the binding site of HIV-1 RT, and was modified to build our own set of structures. Tripos atom types were set. Gasteiger-Hückel [25] charges have been assigned to the protein and ligand atoms. Crystallographic water molecules were not considered in the molecular modeling of the ligand-protein complexes and have been deleted from the crystal structure. Additional visual inspection to account for interactions with amino acid residues in NNIBP was performed using Sybyl 6.8 graphical environment.

The ligand-protein complexes built in the previous step were further minimized using molecular mechanics calculations with Tripos force field. Energy minimizations were performed with steepest descents until the calculation reached a RMS gradient of 0.5 Kcal/Å. The cutoff for non-bonded interactions was 8 Å and the dielectric constant ( ) was 4. The termination gradient was set to 0.05. The binding energy was calculated as the energy variation (ΔE) between the bound and unbound states, as follows:

$$\Delta E = E_{comp} - (E_{Prot} + E_{lig})$$

where ΔE= energy variation or binding energy; E_{comp}= energy of the ligand-protein complex; E_{Prot}= energy of the unbound protein and E_{lig}= energy of the unbound ligand (energy values in kcal/mol).

Molecular dynamics simulations (data not shown) have been performed using the minimized complexes of **13**, **14** and **15** with HIV-1 RT. Simulations have been performed at 300 K for 100 ps with steps of 0.001 ps and a dielectric = 3.5. Backbone constraints were applied in this procedure and coordinates were saved every 0.005 ps for the purpose of subsequent geometric analysis.

Lipinski's Rule of Five

CLOGP, the log of the octanol/water partition coefficient, was calculated with the CLOGP software [26]. Molecular Weight calculations have been performed with the MOLPROP facility, available in Sybyl 6.8 [20].

BIOLOGICAL ASSAYS

HIV Isolates

The T-cell line adapted (TCLA) B genotype CXCR4 tropic reference isolate HIV-1 IIIB and the primary B genotype CCR5 tropic isolate 2242:1886 were employed (kindly donated by Dr E.M. Fenyö, Lund University, Lund, Sweden). Both viral stocks were amplified by 2 passages in phytohemaglutinin pre-activated peripheral blood mononuclear cells (PHA-PBMC, mixture of cells from 4 different donors), aliquots stored at -80°C and tissue culture infectious dose 50% [$TCID_{50\%}$] determined in PBMC [28].

HIV Replication Inhibition Assay

The standard assay incubating PHA-PBMC (from 2 to 4 blood donors) with viral stocks at a multiplicity of infection (MOI) of 0.001-0.005 (10-50 infective units per well per 10^5 cells) according to WHO-UNAIDS Guidelines (2002) [29], was used. Drugs were dissolved at 1 mg/ml dimethylsulfoxide and two fold serial dilutions (0.5 mg/ml down to 1.28 pg/ml) in the culture medium were freshly prepared immediately before use. RPMI 1640 culture medium containing 10% fetal bovine serum, 10 U/ml recombinant human IL-2, 2 mM Gluthamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin (all reagents acquired from Sigma-Aldrich, St Louis, MS, USA) was used throughout. Two changes of at least 90% of the medium after 72 hours were carried out after centrifugation, maintaining the same drug concentrations. Evaluation of replication inhibition was carried out by quantification of the HIV-1 p24 antigen (HIV-1 antigen ELISA, ZEPTOMETRIX CORPORATION, BUFFALO, NY) in the culture supernatants on day 7 [28]. Tissue culture infective doses 50% (TCID 50%) were measured simultaneously to determination of replication inhibition, using the same donor

PBMC's, with quadruplicates of sequential five- or two-fold dilutions (isolates IIIB and 2242:1886, respectively). Controls included in each assay consisted of negative controls (non-infected cells; "wash" controls of wells containing virus only, without cells or antibody); as well as at least 10 controls for each virus for 100% replication values in absence of antiretroviral drugs. Percentage of replication inhibition was calculated in reference to the p24 antigen concentration values obtained using the same virus dilution in absence of inhibitors. Inhibition of replication was considered positive when a reduction of at least 50% of viral input was detected as measured by p24 concentration. However, previous experience using this assay indicates that only values above 75% are highly reproducible [30].

Toxicity Assay

Toxicity of the antiretroviral drugs was assessed for 3 concentrations of each compound, after 7 days of incubation, in absence of HIV-1, in comparison to cells incubated in culture medium only for the same period of time, by Trypan Blue exclusion [31]. In brief, a freshly prepared solution of 10 μL trypan blue (0.04%) in culture medium was mixed to 10 μL of each cellular suspension for 5 min, spread onto a microscope slide and covered with a coverslip. Nonviable cells appear blue stained. At least 200 cells were counted per treatment.

Statistical Analyses

Linear regression was plotted using the Micro Cal Origin software (*Micro Cal Origin*, Northampton, MA, USA, 1991-1992).

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