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4-[1-(4-Fluorobenzyl)-4-hydroxy-1*H*-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid as a prototype to develop dual inhibitors of HIV-1 integration process

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ABSTRACT

In recent years several potent HIV-1 integrase (IN) inhibitors have been identified and after the successful clinical use of raltegravir, they have gained a definitive place in the treatment of HIV-1 infection. Yet, there is a continuous effort to design newer inhibitors that target different steps in the integration process. Furthermore, the increased understanding of IN structural biology has opened novel approaches to inhibit IN, such as targeting its multimerization or interaction with cellular cofactors. On these bases, we have concentrated our research on the identification of small molecules able to inhibit two different stages of the integration process: the IN strand-transfer phase and the IN–LEDGF/p75 interaction. We found that the 4-[1-(4-fluorobenzyl)-4-hydroxy-1*H*-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid (CHI-1043) is an interesting anti-HIV agent exhibiting dual inhibitory effects. This work has suggested the possibility of also constructing an integration dual inhibitor using a design-in strategy.

1. Introduction

The current treatment regimen for HIV-1-infected individuals involves highly active anti-retroviral therapy (HAART) which typically consists of a cocktail of several potent drugs that includes inhibitors of reverse transcriptase, protease and recently, integrase and viral entry (Ramkumar et al., 2010). Its success is limited by severe side effects, drug–drug/food interactions, and by the development of multidrug resistant viral strains. Therefore, alternative strategies and new targets need to be considered to combat the growing AIDS pandemic.

In recent years the enzyme HIV-1 integrase (IN) has increasingly become an attractive target from the viewpoint of drug design because it has no known human homolog (Barbaro et al., 2005; De Clercq, 2002, 2005; Sechi et al., 2009) and therefore its inhibition has a lower risk of disrupting normal cellular processes. However, because of the rapid emergence of resistance mutations observed with the use of raltegravir (MK-0518, Isentress®, Merck Inc.), the first and only IN inhibitor approved by FDA (Summa et al., 2008), the design of inhibitors against IN can be challenging and therefore there is a continuous effort to identify newer inhibitors that belong to different structural classes or that target different steps in the integration process.

HIV-1 IN is an essential viral enzyme that binds to the double-stranded viral DNA generated by reverse transcription and mediates its integration into the cellular genomic DNA of the infected host to produce a functional provirus (McColl and Chen, 2010).

Based on partial proteolysis experiments, this enzyme can be divided into three domains. The N-terminal domain (residues 1–50) contains two histidines and two cysteines that bind Zn^{2+}. The catalytic core domain (IN_{CCD}, residues 50–212) contains the endonuclease and polynucleotidyl transferase site with its three acidic residues (Asp64, Asp116 and Glu152) which are highly conserved in the integrase superfamily and polynucleotide transferases. They are commonly referred as the 'DDE motif' and bind two divalent metal physiological cofactors of HIV-1 IN, Mn^{2+} or Mg^{2+}, required for catalytic activity. The C-terminus domain (residues 213–288) contains basic amino acid residues and is involved in host DNA binding (Benkhelifa-Ziyyat et al., 2010; Dyda et al., 1994; Pommier et al., 2000).

The IN_{CCD} is essential for two key steps of the integration reaction. In the first step, termed 3′ end processing (3′-EP), IN removes two nucleotides from each viral cDNA end adjacent to a conserved 3′-CA sequence and leads to the formation of new 3′-CA–OH. The second step, named strand transfer (ST), is also a trans-esterification reaction involving a direct nucleophilic attack of the 3′-hydroxyl group of the two newly processed viral 3′-DNA ends on the phosphodiester backbone of the host target DNA (McColl and Chen, 2010; Nair et al., 2006).

Multimeric species of IN are thought to play a role in the integration process. IN dimers appear to be involved in 3′-EP, whereas

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tetrameric structures participate in the ST step (Ramkumar et al., 2010).

IN is supposed to be in equilibrium between its monomeric, dimeric, tetrameric and high order oligomeric states. Only the dimer is able to bind the viral DNA, and thus it represents the active form of the enzyme (Faure et al., 2005; Guiot et al., 2006).

The IN–DNA complex is then transported into the nucleus where two DNA-bound dimers form a tetramer in the presence of the cellular protein LEDGF/p75 (Lens Epithelium Derived Growth Factor) prior to the strand-transfer step (Tintori et al., 2010).

LEDGF/p75 is a cellular protein that has been identified and validated as a cellular cofactor of HIV integration and replication (Busschots et al., 2007; Greene et al., 2008). It binds HIV-1 IN via a small (~80 residues) IN-binding domain ($LEDGF_{IBD}$, residues 347–429) within its C-terminal region. IBD is both necessary and sufficient for interaction with HIV-1 IN (Cherepanov et al., 2005).

The targeted disruption of direct cellular IN cofactors required for viral replication has become a heavily sought-after approach for the development of novel first-in-class allosteric IN inhibitors for clinical development. In particular the association between IN and the cellular cofactor LEDGF/p75 is currently the most promising IN interaction for the design of protein–protein disrupting therapeutics (Al-Mawsawi and Neamati, 2011).

This information suggests that the HIV-1 integration is a multistep process and that compounds that target different stages in the integration phase would be attractive candidates for future inhibitor design, as they could have a lesser likelihood for cross-resistance and a possible synergistic effect when combined with the current IN inhibitors and/or with other anti-HIV drugs (Ramkumar et al., 2010). On this basis we have directed our research towards the discovery of candidates with dual site binding ability, i.e. able to inhibit both the IN strand-transfer step and IN–LEDGF/p75 interaction.

2. Materials and methods

2.1. Molecular docking experiments

All inhibitors used for these studies were constructed using Discovery Studio 2.5 (Accelrys, San Diego, CA) and energy minimized using the Smart Minimizer protocol (1000 steps) which combines the Steepest Descent and the Conjugate Gradient methods.

Docking calculations into the $LEDGF/p75_{IBD}$ binding pocket on IN_{CCD} were carried out using the same protocol that we successfully applied in our previous papers (De Luca et al., 2009, 2010).

Docking studies for INSTIs were performed using for the first time the crystal structure of PFV–IN with DNA and two Mg ions complexed with the inhibitor raltegravir. This structural information was retrieved from the Protein Data Bank (PDB code 3OYA) (Hare et al., 2010a,b). Hydrogen atoms were added using Discovery Studio 2.5. The structure of raltegravir was extracted from X-ray complex and used as a control of program performance. Inhibitors were docked using GOLD 4.1.2 (Jones et al., 1997). A 15 Å radius active site was defined considering the coordinates of raltegravir in the X-ray position as the center of the binding pocket. CHEMPLP scoring function was chosen as the fitness function, default parameters were used and the ligands were submitted to 100 genetic algorithm runs (De Luca et al., 2011).

2.2. Minimization process

All of the protein–ligand complexes obtained by docking studies were minimized using the CHARMM force field in Discovery Studio 2.5 with the Generalized Born implicit solvent model. 1000 steps of the Steepest Descent method were followed by 1000 steps of the Conjugate Gradient method for faster convergence toward a local minimum. The two Mg ions within the active site were kept fixed during the minimization process.

2.3. Experimental chemistry

All 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.

2.3.1. Synthesis of 3-acetyl-4-methoxy-1H-indole (**a**)

See synthetic procedure reported by Ferro et al. (2010).

2.3.2. Synthesis of 3-acetyl-4-methoxy-1-(3,5-dimethylbenzyl)-1H-indole (**b**)

According to the synthetic procedure previously reported by us (Ferro et al., 2009a,b), 3-acetyl-4-methoxy-1*H*-indole (189 mg, 1 mmol) was dissolved in DMF (1 mL) at 0 °C and dry sodium hydride (120 mg, 5 mmol) was added. After stirring for 2 min, 3,5-dimethylbenzyl bromide (298 mg, 1.5 mmol) was added dropwise and the resulting solution was placed in a cylindrical quartz tube (diam. 2 cm), stirred at r.t., and irradiated in a microwave oven at the following conditions: 100 W, 50 °C, 5 min. After addition of a saturated $NaHCO_3$ solution, the reaction mixture was extracted with ethyl acetate (10 mL × 3) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude purified by flash chromatography using a mixture of cyclohexane/ethylacetate (6:4) to give a white solid. Yield: 73%; mp: 110–112 °C. ^{1}HNMR ($CDCl_3$): δ = 2.26 (s, 6H, 2 × CH_3), 2.69 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), 5.20 (s, 2H, CH_2), 6.69 (d, J = 7.9, 1H, ArH), 6.77 (s, 1H, ArH), 6.93–6.97 (m, 2H, ArH), 7.19 (*t*, J = 7.97, 1H, ArH), 7.72 ppm (s, 1H, ArH); Anal. calcd for $C_{20}H_{21}NO_2$: C 78.15, H 6.89, N 4.56, found: C 78.23, H 6.71, N 4.38.

2.3.3. Synthesis of 4-[1-(3,5-dimethylbenzyl)-4-methoxy-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoate (**c**)

According to the synthetic procedure previously reported by us (Ferro et al., 2009a,b), a mixture of 3-acetyl-4-methoxy-1-(3,5-dimethylbenzyl)-1*H*-indole (307 mg, 1 mmol) diethyl oxalate (219 mg, 1.5 mmol) and a catalytic amount of $NaOCH_3$ was suspended in anhydrous THF (2 mL). The reaction mixture was placed in a cylindrical quartz tube (diam. 2 cm), stirred and irradiated at continuous temperature in a microwave oven for two subsequent periods at the same conditions (250 W, 2 min, 50 °C). The solvent was concentrated under reduced pressure and the collected yellow solid was crystallized from ethanol/diethyl ether. Yield: 89%; mp: 270 °C (dec). ^{1}HNMR (DMSO): δ = 1.23 (*t*, J = 7.26, 3H, CH_3), 2.18 (s, 6H, 2 × CH_3), 3.82 (s, 3H, OCH_3), 4.11 (*q*, J = 7.26, 2H, CH_2), 5.28 (s, 2H, CH_2), 6.58–7.65 ppm (m, 8H, 7ArH and CH); Anal. calcd for $C_{24}H_{25}NO_5$: C 70.75, H 6.18, N 3.44, found: C 70.63, H 6.24, N 3.53.

*2.3.4. Synthesis of 4-[1-(3,5-dimethylbenzyl)-4-methoxy-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid (**2**)*

According to the synthetic procedure previously reported by us, (Ferro et al., 2009a,b) 4-[1-(3,5-dimethylbenzyl)-4-methoxy-1*H*-indol-3-yl]-2-hydroxy-4-oxobut-2-enoate (407 mg, 1 mmol) was dissolved in methanol (5 mL) and treated with 2 N NaOH (5 mL, 50 mmol). The reaction mixture was stirred at room temperature for 1.5 h and then acidified with conc. HCl to afford the hydrolyzed derivative as a yellow precipitate. Recrystallization from ethanol/diethyl ether afforded the pure compound **2** as a yellow solid. Yield: 52%; mp: 120–122 °C. ^{1}HNMR (DMSO): δ = 2.19 (s, 6H, 2 × CH_3), 3.89 (s, 3H, OCH_3), 5.41 (s, 2H, CH_2), 6.78–7.59 (m, 7H, 6ArH and CH), 8.48 ppm (s, 1H, ArH); Anal. calcd for $C_{22}H_{21}NO_5$: C 69.65, H 5.58, N 3.69, found: C 69.45, H 5. 41, N 3.75.

2.4. LEDGF/p75–HIV-1 Integrase interaction screening (AlphaScreen technology)

The AlphaScreen assay was performed as described previously (Christ et al., 2010). 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. His_6-tagged integrase (300 nM final concentration) was incubated with the compounds at 4 °C for 30 min. The compounds were added at varying concentrations from 1 up to 100 μM. Afterwards 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.

2.5. Integrase inhibition

To determine the susceptibility of the HIV-1 integrase enzyme towards our compounds we used enzyme-linked immunosorbent assays. These assays use an oligonucleotide substrate of which one oligonucleotide (5′-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTC-3′) is labeled with biotin at the 3′ end and the other oligonucleotide is labeled with digoxigenin at the 5′ end. For the overall integration assay the second 5′-digoxigenin labeled oligonucleotide is (5′- GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′). For the Strand Transfer assay the second oligonucleotide lacks GT at the 3′ end. The integrase enzyme was diluted in 750 mM NaCl, 10 mM Tris pH 7.6, 10% glycerol and 1 mM β-mercapto ethanol. To perform the reaction, 4 μl diluted integrase (corresponding to a concentration of 1.6 μM) and 4 μl of annealed oligonucleotides (7 nM) were added in a final reaction volume of 40 μl containing 10 mM $MgCl_2$, 5 mM DTT, 20 mM HEPES pH 7.5, 5% PEG and 15% DMSO. The reaction was carried out for 1 h at 37 °C. Reaction products were denatured with 30 mM NaOH and detected by an immunosorbent assay on avidin coated plates (Christ et al., 2010).

2.6. In vitro anti-HIV and drug susceptibility assays

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 (Pauwels et al., 1988). This assay is based on the reduction of the yellow colored 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 fivefold 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.

3. Results and discussion

In previous papers we reported ligand-based approaches useful to generate three-dimensional pharmacophore models which led to the identification of a series of indole DKA analogs (Barreca et al., 2005; De Luca et al., 2008, 2011; Ferro et al., 2004, 2007a,b, 2009a,b, 2010) able to selectively inhibit the ST step of the integration process which represents the major target in the development of anti-HIV-1 IN drugs.

More recently we focused our attention on the interaction between IN and its cellular cofactor LEDGF/p75, the inhibition of which is increasingly recognized as an emerging potential therapeutic strategy. In fact LEDGF/p75 is an IN interacting cellular protein (Van Maele et al., 2006) which plays an important role in chromatin tethering. It interacts with IN via its C-terminal domain, also known as the IN binding domain ($LEDGF_{IBD}$). The biochemical test for the identification of HIV integrase inhibitors, targeting the interaction between HIV–IN and the cellular cofactor LEDGF/p75 is the AlphaScreen assay which has been developed for facilitating the identification of novel inhibitors specific for this protein–protein interaction (De Luca et al., 2010).

Our studies led to the discovery of a series of benzylindoles derivatives active as inhibitors against IN–LEDGF/p75 interaction evaluated in the AlphaScreen assay (De Luca et al., 2009). After a number of rational modifications both on the indole system and the benzyl moiety, suggested by molecular modeling studies (De Luca et al., 2009, 2010), the most potent compound was the 4-[1-(3,5-dimethylbenzyl)-4-hydroxy-1*H*-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid (**1**) (Fig. 1) showing an IC_{50} value of 3.5 μM. So we considered this derivative as a promising lead compound to identify small molecules able to exert a specific blockade of this protein–protein interaction.

The results of the biological assay were in agreement with our computational studies that suggested the positioning as well as the interactions between the inhibitor **1** and IN. Docking studies suggested the ability of compound **1** to interact with the portion of IN dimer (A and B chains) in contact with $LEDGF_{IBD}$ engaging the following contacts (Fig. 2): (a) the carboxylate group interacted with Glu170, His171 and Thr174 of IN A-chain; (b) the hydroxyl group of the diketoacid moiety bound Gln95 of B-chain; (c) the benzene fused-ring of indole nucleus was placed into the IN hydro-

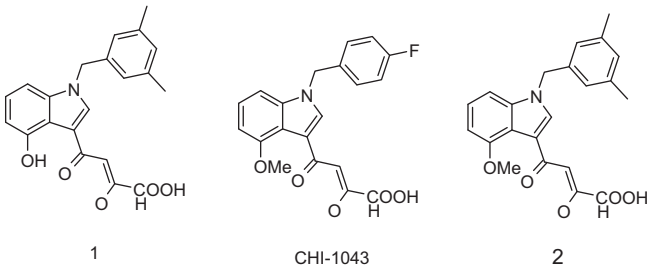


Fig. 1. Chemical structures of compounds **1**, **2** and CHI-1043.

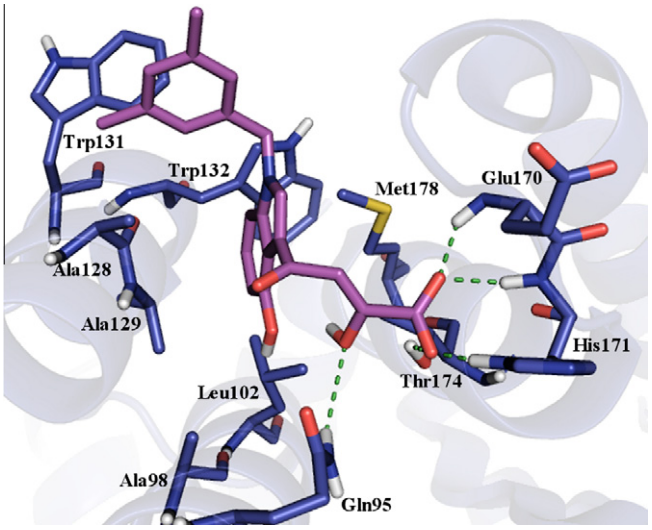


Fig. 2. Docking of compound **1** (magenta), into the LEDGF/p75$_{IBD}$ binding pocket of IN$_{CCD}$. This figure as well as Fig. 3 were produced with PyMol (DeLano and DeLano Scientific LLC).

phobic pocket; (d) the 3,5-dimethylbenzyl N-substituent occupied a large hydrophobic area consisting of Ala128, Ala129, Trp132 and the important residue Trp131.

Unfortunately, when compound **1** was tested in MT4 cells as anti-HIV agent it showed low antiviral activity (EC_{50} = 44 μM) and displayed cytotoxic effects. Obviously, our goal was not only to obtain potent inhibitors of IN–LEDGF/p75 interaction, but also potential antiretroviral candidates and the results in cells were disappointing.

At this point, considering that compound **1** presented a chemical structure similar to that of a series of our indole derivatives active against the IN strand-transfer step (Barreca et al., 2005; De Luca et al., 2008; De Luca et al., 2011; Ferro et al., 2007a,b, 2010, 2009a,b), we chose to select among our INSTIs, some derivatives highly active in cells with low toxicity and to verify their ability to bind not only to the portion of the IN$_{CCD}$ containing the DDE motif but also to the LEDGF$_{IBD}$. Following this idea, our objective was to identify molecules able to inhibit two different integration processes: the ST step and the IN–LEDGF/p75 interaction.

With these thoughts in mind we addressed our attention to CHI-1043 (Fig. 1), considered the most promising compound of our developed INSTIs presenting a good trade-off between the anti-IN and anti-HIV-1 activities (IC_{50} = 0.14 μM; EC_{50} = 0.59 μM; SI = 70) (De Luca et al., 2008). CHI-1043 interferes with the viral replication cycle at the time of retroviral integration and also shows efficacy against HIV-2, SIV and viral strains resistant to other ST inhibitors (Hombrouck et al., 2008).

Table 1
Number of hydrophobic contacts between the derivatives **1** and CHI-1043 and the aminoacids of the LEDGF/p75 binding hydrophobic pocket of IN$_{CCD}$ calculated by Ligplot 4.5.3 program (Wallace et al., 1995) from docked ligand–target complexes.

IN, chain B	Derivative 1	CHI-1043
Ala 128	4	2
Ala 129	3	2
Trp 131	6	5
Trp 132	6	5

Firstly, we carried out docking experiments to explore the ability of CHI-1043 to establish those contacts considered necessary to block the interaction between the targeted proteins IN and LEDGF/p75. Docking studies were performed on the LEDGF/p75 binding pocket of IN$_{CCD}$ using the GOLD program (Jones et al., 1997) and the docking protocol that we successfully applied in our previous papers (De Luca et al., 2009, 2010). The result of this study is shown in Fig. 3A, revealing that CHI-1043 occupies the same binding pocket of derivative **1** thus implying its potential ability to inhibit the IN–LEDGF/p75 interaction.

Once selected, CHI-1043 was tested in the AlphaScreen assay and proved to be able to inhibit the IN–LEDGF/p75 interaction with an IC_{50} value of 36.16 μM. These results suggested that this molecule inhibits both the IN ST step (IC_{50} 0.14 μM) and IN–LEDGF/p75 interaction (IC_{50} 36 μM), thus affording, to our knowledge, the first dual inhibitor of HIV-1 integration process.

In particular the inhibitory activity of CHI-1043 on the IN–LEDGF/p75 interaction could be due, as expected, to its ability to engage a set of interactions similar to those of **1**, which had proved to be a potent IN–LEDGF/p75 inhibitor. (Fig. 3A).

Docking studies highlighted that both the carboxylate group and the hydroxyl group of the diketoacid moiety of CHI-1043 interacted with the same aminoacids of compound **1** and also the benzene-fused ring of indole system was placed into the same IN hydrophobic pocket. Moreover, the 4-methoxy group of CHI-1043 could create an accessory hydrophobic contact with residue Leu102 chain B. However, the presence of the 4-fluorobenzyl substituent, instead of the 3,5-methylbenzyl one, reduced the possibil-

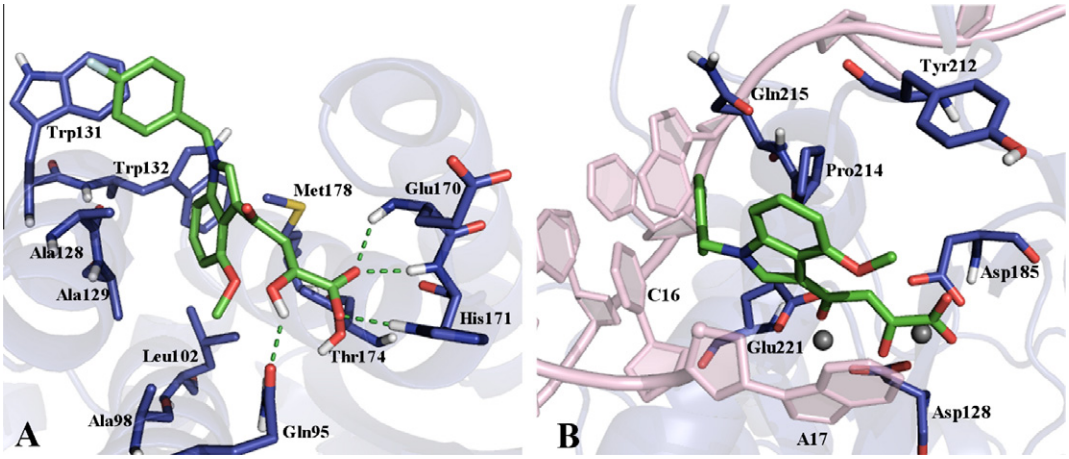
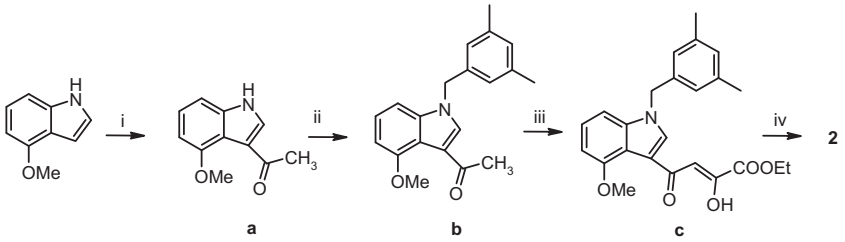


Fig. 3. Docking of CHI-1043 (green) into (A) LEDGF/p75$_{IBD}$ binding pocket of IN$_{CCD}$ and (B) IN–DNA active site. The divalent metal ions are shown as gray spheres, while the viral DNA is depicted in violet(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).



Scheme 1. Reagents and conditions: (i) $POCl_3$, $CH_3CON(CH_3)_2$, RT, 12 h; (ii) 3,5-dimethylbenzyl bromide, NaH, DMF, mw: 5 min at continuous temperature (50 °C), 100 W; (iii) diethyl oxalate, dry CH_3ONa, THF, two separate steps under the same conditions mw: 2 min, at continuous temperature (50 °C), 250 W; (iv) 2 N NaOH, MeOH, RT, 1.5 h.

ity to create some important contacts in the hydrophobic area, as described above for derivative **1** (Table 1).

This last observation could explain the lower potency of CHI-1043 as protein–protein interaction inhibitor when compared with **1** (36.16 μM versus 3.5 μM) in the AlphaScreen assay.

Even though the 3,5-dimethylbenzyl substituent seems important for inhibition of IN–LEDGF/p75 interaction, the 4-fluorobenzyl one, according to our experience, appears crucial for the inhibition of the INST step.

This hypothesis is in accordance with the evidence that highly potent INSTIs present a halogen-substituted aromatic functionality. Recently Hare et al. (2010a) reported cocrystal structures containing prototype foamy virus (PFV) intasome complexed with an expanded set of first and second-generation INSTIs evidencing that an important common and conserved structural feature of INSTIs is a halogenated benzyl group.

In fact this moiety is able to displace the 3' adenine of bound viral DNA, inserting between the base of the penultimate cytidine, with which it forms a face-to-face π–π interaction, and Pro214 of IN, thus contributing to the inhibition of strand-transfer step.

On this basis, and while realizing that the structure of integrase with DNA and two Mg ions from PFV–IN can be considered realistic, we used the complex with raltegravir (PDB code 3OYA) to perform docking studies for CHI-1043 in order to explore its binding mode (see Section 2).

After the validation of the docking protocol, CHI-1043 was docked in the same binding site of raltegravir. The results revealed that CHI-1043 showed a similar binding mode compared to other INSTI-bound crystal structures. (Fig. 3B) (Hare et al., 2010b).

In particular, the common metal-coordinating pharmacophore of the INSTIs, that usually comprises three specifically positioned heteroatoms (oxygen or, less commonly, nitrogen), is represented by the diketo acid moiety coordinating the two metal cofactors within the active site of IN. The second conserved structural feature of INSTIs is a halogenated benzyl group, in our case the 4-fluorobenzyl group which interacts with the residues C16 of viral DNA and Pro214. Furthermore the fluorine atom seems to establish van der Waals interactions with Gln 215, thus confirming the key role of the halogen atoms in INSTIs.

In order to confirm that the elimination of the halogenated benzyl group negatively influenced the inhibition of IN ST step, we synthesized. A new CHI-1043 analog (**2**) in which the 4-fluorobenzyl portion at N-1 has been replaced with a 3,5-dimethylbenzyl moiety.

For the synthesis of derivative **2** we followed our previous synthetic procedures (Ferro et al., 2010, 2009a,b) outlined in Scheme 1.

Derivative **2** was then evaluated as INSTI and, as expected, this modification drastically reduced the ST inhibitory effects with respect to CHI-1043 (IC_{50} 5.49 μM versus IC_{50} 0.14 μM). Moreover, compound **2** showed a poorer anti-HIV activity (EC_{50} 4.63 μM versus EC_{50} 0.59 μM) in MT4 cells and cytotoxicity at the same concentration.

In conclusion, this study suggests that CHI-1043 could be considered a good starting point towards our goal of identifying dual inhibitors of the HIV-1 integration processes with antiretroviral activity and safety profile. The design of inhibitors that can accommodate binding to two distinct biological targets or two different sites remains an intriguing yet challenging scientific endeavor. We are working in this direction and suitable modifications are in progress; the results of our efforts together with mutation studies will be reported in due course.

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