Published in final edited form as:

Eur J Med Chem. 2016 November 10; 123: 673–683. doi:10.1016/j.ejmech.2016.07.077.

Computational and synthetic approaches for developing Lavendustin B derivatives as allosteric inhibitors of HIV-1 integrase

Fatima E. Agharbaoui^{a,b,c}, Ashley C. Hoyte^b, Stefania Ferro^a, Rosaria Gitto^a, Maria Rosa Buemi^a, James R. Fuchs^c, Mamuka Kvaratskhelia^b, and Laura De Luca^{a,*}

^aDipartimento di Scienze Chimiche, Biologiche, Farmaceutiche e Ambientali (CHIBIOFARAM), Polo Universitario SS. Annunziata, Università di Messina, Viale Annunziata, I-98168, Messina, Italy

^bCenter for Retrovirus Research and College of Pharmacy, The Ohio State University, Columbus, OH, 43210, USA

^cDivision of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH, 43210, USA

Abstract

Through structure-based virtual screening and subsequent activity assays of selected natural products, Lavendustin B was previously identified as an inhibitor of HIV-1 integrase (IN) interaction with its cognate cellular cofactor, lens epithelium-derived growth factor (LEDGF/p75). In order to improve the inhibitory potency we have employed *in silico*-based approaches. Particularly, a series of new analogues was designed and docked into the LEDGF/p75 binding pocket of HIV-1 IN. To identify promising leads we used the Molecular Mechanics energies combined with the Generalized Born and Surface Area continuum solvation (MM-GBSA) method, molecular dynamics simulations and analysis of hydrogen bond occupancies. On the basis of these studies, six analogues of Lavendustine B, containing the benzylamino-hydroxybenzoic scaffold, were selected for synthesis and structure activity-relationship (SAR) studies. Our results demonstrated a good correlation between computational and experimental data, and all six analogues displayed an improved potency for inhibiting IN binding to LEDGF/p75 *in vitro* to respect to the parent compound Lavendustin B. Additionally, these analogs show to inhibit weakly LEDGF/p75-independent IN catalytic activity suggesting a multimodal allosteric mechanism of action. Nevertheless, for the synthesized compounds similar profiles for HIV-1 inhibition and cytotoxicity were highlighted. Taken together, our studies elucidated the mode of action of Lavendustin B analogs and provided a path for their further development as a new promising class of HIV-1 integrase inhibitors.

Keywords

HIV-1; Integrase; LEDGF/p75; ALLINI; Computational studies; IN-LEDGF/p75 binding

*Corresponding author. fagharbaoui@unime.it (F.E. Agharbaoui), ldeluca@unime.it (L. De Luca).

1. Introduction

An essential step of the retroviral lifecycle is the insertion of the reverse-transcribed viral genome into the host chromosome. This process is catalyzed by HIV-1 integrase (IN), that has gained popularity as a promising target for the discovery of novel anti-HIV drugs.

IN is comprised of three domains: the N-terminal domain (NTD), the catalytic core domain (CCD) that coordinates two catalytic Mg^{2+} ions and the C-terminal domain (CTD) [1,2]. Initial drug discovery efforts for IN inhibitors have focused on small molecules able to inhibit the catalytic activity of IN and have resulted in three FDA approved IN inhibitors currently in clinical use, raltegravir, elvitegravir and dolutegravir [3–8]. These compounds all share a similar mechanism of action in that all bind at the IN active site in the presence of the viral DNA and inhibit the strand transfer (ST) activity. While these inhibitors have been highly effective against HIV, resistance mutations have emerged in patients [9–11]. Therefore, there is a continual need for the development of new IN inhibitors with innovative scaffolds that target alternative sites of the enzyme.

The integration process comprises two catalytic steps: the first is a hydrolytic reaction termed 3' processing (3' P), followed by a transesterification reaction (also referred as to ST) [12–14]. The cellular chromatin associated protein lens epithelium-derived growth factor (LEDGF/p75) associates with IN and significantly enhances integration efficacy by tethering preintegration complexes to active genes during integration [15–19]. LEDGF/p75 is a transcriptional co-activator strongly associated with chromatin throughout the cell cycle [20–22]. Its C-terminal domain contains the IN-binding domain (IBD), allowing it to not only interact with natural cellular binding partners, but also HIV-1 IN [18,19,23,24].

Recent efforts have led to the discovery of a new class of allosteric IN inhibitors (ALLINIs, also known as LEDGINs, NCINIs, INLAIs, or MINIs) [25–30] targeting the IN dimer interface at the principal LEDGF/p75 binding pocket. Interestingly, two alternative approaches have identified a similar class of quinoline-based ALLINIs: a high throughput screen was used to discover compounds inhibiting 3'-processing activity of IN Ref. [26] and the rational drug design was exploited to develop small molecules to block the IN-LEDGF/p75 interaction [25]. Of note, the rational design approach was made possible by the crystal structure of a CCD-CCD dimer bound to the IBD [31]. Furthermore, the ability to solve the structures of ALLINIs bound at the CCD-CCD dimer [25–28] has facilitated the rapid expansion of this class of inhibitors.

ALLINIs exhibit a multimodal mechanism of action in that they not only inhibit IN-LEDGF/p75 interaction but they also promote higher order aberrant IN multimerization, resulting in inactive protein [2,32,33]. Surprisingly, ALLINIs exhibit higher potency when present during virion morphogenesis compared with the early stage of viral replication [29,34–37]. In the virions, where due to the lack of competing LEDGF/p75 binding to the IN dimer, ALLINIs potentially induce aberrant IN multimerization and result in eccentric, non-infectious virions; whereas during the early stage of HIV-1 replication LEDGF/p75 effectively competes with ALLINI binding to IN and reduces the inhibitor potency [38]. Selection of HIV-1 phenotypes under the genetic pressure of various ALLINIs have

identified substitutions at the IN dimer interface at the inhibitor binding sites that confer resistance to these compounds [25,28,39,40]. Collectively, the studies with ALLINIs have shown that the potent inhibitors that target IN sites distinct from the active site can be developed. At the same time, there is a need to further improve these compounds to overcome the resistance seen in cell culture assays.

Natural Products (NPs) have historically been an extraordinary source for new medicines and are continuing to be the origin of lead compounds for drug discovery [41,42]. Previously, we have reported the application of a structure-based virtual screening strategy for the identification of NPs as potential protein-protein interaction inhibitors (PPIIs) targeting the IN-LEDGF/p75 protein complex [43]. Among them, we focused our interest on the Lavendustin B (Fig. 1A), which inhibited IN binding to LEDGF/p75 in Alphascreen assay [43]. This novel scaffold is unique from all reported ALLINIs and could represent an encouraging new hit compound warranting further improvement and investigation. Therefore, to exploit this novel scaffold and improve its potency as an IN inhibitor, we have employed *in silico* approaches to identify promising Lavendustin B derivatives and examine inhibitory activities using *in vitro* and *cell based* assays.

2. Results and discussion

2.1. Rational design

By using a combination of docking and ultrashort molecular dynamics (MD), we have generated a weighted ensemble of protein-ligand configurations for IN-LEDGF/p75 protein-protein interaction inhibitors. Therefore we estimated their binding affinities by averaging snapshots taken from the MD trajectories, together with the presence of fundamental hydrogen bonds [44]. These *in silico* studies, followed by experimental analysis of selected compounds, have led to the identification of Lavendustin B with an IC_{50} of 94.07 μ M for inhibiting IN binding to LEDGF/p75 *in vitro* [43]. *In silico* docking studies (Fig. 1 B) have highlighted the following interactions: the carboxylic group of Lavendustin B establishes H-bond interactions with the backbone nitrogen atoms of Glu170 and His171 residues, similar to the interactions seen with LEDGF/p75 hotspot residue of Asp366 [24]. Additionally, there is the formation of a potential hydrogen bond with the hydroxyl group side chain of the Thr174 residue. The remaining portion of Lavendustin B is housed within the dimer interface cleft comprised of IN subunit A residues of Thr174, Gln168, Ala169 and Met178 and IN subunit B residues of Ala128, Ala129, Trp131 and Trp132 allowing the molecule to establish hydrophobic contacts with both subunits. We have used these *in silico* predicated interactions as the basis for our current study.

In order to design new analogs, we utilized a published X-ray crystal structure of the active compound KF115 (PDB-400J) which shares a similar binding mode with Lavendustin B (Fig. 1A and C) [28]. KF115 is a pyridine-based inhibitor in the class of ALLINIs that preferentially promoted aberrant IN multimerization over inhibiting the IN-LEDGF/p75 interaction. The superimposition of the crystal structure of KF115 bound to the CCD-CCD dimer with the docked model of Lavendustin B (Fig. 1B) reveals a high degree of similarity with the carboxylic groups of the both compounds interacting with Glu170, His171 and Thr174. In addition, the 4-chlorophenyl and the 3,4-dimethylphenyl groups of KF115

occupy the hydrophobic pockets in a similar manner of the two 2-hydroxyphenyl portions of Lavendustin B. Considering these results, structural modifications on Lavendustin B were introduced *in silico*: the 2-hydroxy group was removed, and halogen atoms (chlorine and fluorine) and methyl substituent were added to explore the hydrophobic areas. The planned modifications on Lavendustin B are depicted in Fig. 1 D for compounds (1–10).

2.2. Docking and molecular dynamics (MD)

Before carrying out the synthesis of the designed compounds (1–10) we wanted to predict the potential binding mode of the analogs by means of the reported computational procedure [43,45]. First a docking simulation into the principal LEDGF/p75 binding pocket on IN [24] was performed using GOLD (Genetic Optimization for Ligand Docking) [46]. In order to take into account the flexible side chain of residue Gln95 two different conformations of IN CCD were used (PDB ID: 3LPU [25] and 2B4J [32]). More than two clusters were taken for additional analysis. To eliminate potentially unfavorable contacts, the geometry of the system was minimized using the steepest descent algorithm followed by a conjugate gradient. The solvent effects were considered through the generalized Born implicit solvent model. The output complex was employed to estimate ligand binding free energy using the MM-GBSA method. The obtained results for both CCD conformations (complex 1 and 2) are shown in Table 1.

Since the calculated binding energy of the complex 1 and 2 were similar we decided to consider only the conformation of the protein retrieved by 2B4J (complex 1) for further or more complete analysis. Fig. 2 shows the binding orientations of the designed analogs. We observed that six compounds, namely (1–3, 5, 6 and 8), share the binding mode with parent Lavendustin B. The other derivatives (4, 7, 9 and 10) assume a binding pose for which they mimic the carboxylic functionality of Lavendustin B but adopt a different orientation for the aromatic portion.

The docked complexes were further analyzed using both Ultrashort Molecular Dynamics simulations and sander module of AMBER 11 [47]. Additionally, the models were used to estimate the binding affinities by averaging snapshots taken from the MD trajectories using the MM-GBSA method (Table 1).

By comparing the binding energies compound (2) was predicted as the most potent derivative of the series, followed by derivatives (1) and (5). By contrast, compounds (4, 7, 9 and 10), which were predicted to bind differently than KF115 or Lavendustin B, displayed the weakest binding energies. Therefore, these compounds were predicted to be less active. To explore this hypothesis, analysis of hydrogen bond occupancies and distance calculations from the hot spot amino acids in the hydrophobic pocket were carried out using Ptraj module from AMBER11 [47].

The obtained results (Table 2) show that (1) and (2) established an extra hydrogen bond interaction with Gln95 residue. Moreover, the carboxylic acid is closer to the hot spot amino acids and has the stronger hydrogen bond occupancies in comparison with Lavendustin B. In contrast, compounds (4, 7, 9 and 10) showed the weaker hydrogen bonding abilities, especially with residue His171. Collectively our *in silico* approach, in combination with our

previous studies [48], suggested the following criteria for the synthesis. The analogs need to bind to the CCD-CCD dimer in a similar mode to Lavendustin B and KF115. Furthermore, the derivatives are expected to exhibit improved binding energy and higher hydrogen bond occupancies in the binding pocket than parent Lavendustin B. This criterion allowed us select compounds (**1–3**, **5**, **6** and **8**) for chemical synthesis and *in vitro* evaluation.

2.3. Chemistry

The picked 5-(dibenzylamino)-2-hydroxybenzoic acids (**1–3**, **5**, **6** and **8**) were synthesized following the multistep procedure depicted in Scheme 1.

Commercially available 2-hydroxy-5-nitro-benzoic acid (**11**) was reduced, using zinc dust in acid medium, to form the corresponding 5-amino-2-hydroxy-benzoic acid (**12**) with high yield and successively converted into the 5-amino-2-hydroxybenzoate (**13**) by esterification reaction.

In the next step, methyl 5-(benzylamino)-2-hydroxybenzoates (**14–19**) were obtained by reaction of intermediate **13** with the suitable benzaldehyde, by means of a reductive amination, in the presence of sodium cyanoborohydride.

For the synthesis of 5-(dibenzylamino)-2-hydroxybenzoates (**20–25**) the obtained intermediates (**14–19**) were treated in dimethylformamide with the appropriate benzyl bromide and sodium hydride, under argon atmosphere. Finally, the target compounds (**1–3**, **5**, **6**, and **8**) were prepared by hydrolysis in basic medium.

2.4. In vitro screening of synthesized compounds **1–3**, **5**, **6** and **8**

All the synthesized derivatives were tested in homogeneous time-resolved fluorescence (HTRF)-based assays to evaluate their inhibitory effects on the IN-LEDGF/p75 binding, LEDGF/p75 dependent integration, and LEDGF/p75 independent 3'-processing activity (Table 3).

As result all the target compounds were able to inhibit LEDGF/p75 dependent IN activity displaying IC₅₀ values ranging from 3.78 µM to 18.50 µM (Table 3 and Fig. 3B) with (**2**) being the most potent in the series. Since this assay does not delineate whether the inhibitor affected IN binding to LEDGF/p75 or impaired IN activity in a LEDGF/p75 independent manner we conducted additional assays as follow. We firstly tested our analogs for their ability to inhibit IN-LEDGF/p75 binding (Table 3 and Fig. 3C). All the tested compounds were able to disrupt IN binding to LEDGF/p75 with IC₅₀ values ranging from 3.28 µM to 27.59 µM and among them compound (**2**) showed the best activity. In addition, we also analyzed the analogs for their ability to inhibit IN 3'-processing activity in the absence of LEDGF/p75. The obtained data showed a weak inhibitions for compounds **1,2,5** and **6**. The results, summarized in Table 3, show that our compounds were able to impair IN activity. Specifically, derivative (**2**) again displayed the best IC₅₀ value (25.67 µM), Fig. 3D. Collectively, these results lead us to think that Lavendustin B and its analog 5-[bis(2-chlorobenzyl)amino]-2-hydroxybenzoic acid (**2**) can share a common mechanism of action with pyridine- or quinoline-based ALLINIs. For example, similarly to reported ALLINIs [26–29,32,33] compound (**2**) inhibits both LEDGF/p75 dependent and independent activities

of IN indicating a multimodal mechanism of action. At the same time we note the following differences. The quinoline-based ALLINIs inhibit IN-LEDGF/p75 binding and IN activity in LEDGF/p75 independent reactions with comparable IC_{50} values [2,27,32,33]. Pyridine-based ALLINIs are significantly more potent (up to 58-fold) for inducing aberrant IN multimerization in the absence of LEDGF/p75 compared with inhibition of IN-LEDGF/p75 binding [28]. In contrast, compound (**2**) was ~8-fold more potent for inhibiting IN-LEDGF/p75 interactions compared with affecting IN activity in the LEDGF/p75 independent manner. Future studies are warranted to clarify the observed differences between these structurally distinct classes of inhibitors.

2.5. Antiviral and cytotoxicity assays

Antiviral activities and cellular toxicity of the most potent analog (**2**) were examined founding similar profiles for HIV-1 inhibition and cytotoxicity (see Fig. 4). Unfortunately, these findings do not allow us to delineate any potential antiviral activity of **2** from its cellular toxicity.

3. Conclusions

By means of a *in silico* methodology a new series of Lavendustin B analogs were designed.

The selected 5-(dibenzylamino)-2-hydroxybenzoic acids (**1–3**, **5**, **6** and **8**) inhibited IN-LEDGF/p75 binding as well as impaired IN activity through allosteric mechanisms, and their improved inhibitory potency has been confirmed using a combination of HTRF-based assays. Additionally, the most active derivative (**2**) exhibited a multimodal mechanism of action, similar to the reported ALLINIs. Unfortunately, compound (**2**) displayed significant cellular toxicity precluding our efforts to delineate its potential antiviral activity.

4. Experimental section

4.1. Docking simulation

3D structure of each ligand was constructed using Discovery Studio 2.5.5 [49] and minimized using CHARMM force field, followed by Smart Minimizer algorithm performing 1000 steps of Steepest Descent with a root mean square (RMS) gradient tolerance of 3, followed by Conjugate Gradient minimization, until the RMS gradient for potential energy was less than 0.05 kcal/mol/Å. For docking simulations the crystal structure of the dimeric Catalytic Core Domain (CCD) of HIV-1 IN complexed with the IN-binding domain (IBD) of LEDGF/p75 was retrieved from RCSB Protein Data Bank (PDB:2B4J) [31]. The LEDGF/p75 structure and the water molecules from the X-ray crystallography were removed and the missing hydrogens were replaced. Validation of the docking protocol was performed by docking the native co-crystallized ligands of the two crystal structures with the PDB codes 3LPT and 3LPU, into LEDGF/p75 binding site. The comparison of docking results with the co-crystallized form showed success rates with the docked ligand strictly superimposed with the crystallized conformation with RMSD = 1.01 Å indicating that the used scoring function is successful. These values were small enough and supported the hypothesis that experimental binding modes could be reproduced with accuracy using this protocol. The standard default settings were used in all calculations. 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) [46] and as described in our previous paper [48]. For the prediction of ligand binding positions GoldScore fitness function was used. For 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. Default cutoff values of 2.5 Å for hydrogen bonds and 4.0 Å for van der Waals interactions were employed. Automatic bond settings were used, allowing the torsion angles of all acyclic, rotatable bonds in the ligand to vary except for amide bonds. Results differing by less than 0.75 Å in ligand-all-atom RMSD were clustered together. 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. Binding energy of the minimized complex was calculated using the MM-GBSA method [50] implemented in the AMBER program.

4.2. Molecular dynamic simulation

The starting model for simulations of IN-LEDGF/p75 was prepared as described in our previous paper [48]. In brief, from the X-ray structure 2B4J of IN CCD (chains A and B) in complex with the IBD of LEDGF (chains C and D) [31] was used. First, chain D and water molecules were removed from the structure. Then, the missing residues of the CCD of IN were added by superimposing chain C of the HIV-1 IN 1BL3 [51] structure and energy-minimized using Maestro [52] with a RMSD of 0.30 Å. From the resulting complex, the chain C of IBD of LEDGF/p75 was castoff in order to simulate IN-inhibitor complexes. MD simulations were carried out using the sander module of AMBER 11 [53] and parm 99.dat and frcmod.ff03 parameter files [54,55]. These 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 contacts 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 [53]. 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 [50] implemented in the AMBER program was used to evaluate the ligand-protein interaction free energies of the minimized complex and the 100 snapshots extracted at 1 ps intervals. For MM-GBSA analysis, snapshots at 40 ps intervals were extracted from the last 4 ns of the MD trajectory, and the binding free energies were averaged over the ensemble of conformers produced (100 snapshots for each trajectory).

4.3. Chemistry

All starting materials and reagents commercially available (Sigma-Aldrich and Alfa Aesar) were used without further purification. Anhydrous solvents CH₃OH, DMSO, CH₂Cl₂, THF and DMF were used directly from their Sure-Seal bottles and were purchased from Sigma Aldrich. 4 Å molecular sieves also purchased from Sigma Aldrich, were activated by heating to 300 °C under vacuum overnight. All reactions were performed in oven-dried glassware and were monitored for completeness by thin layer chromatography (TLC) using silica gel then visualized by UV light, or developed by treatment with KMnO₄ stain. A 300 MHz Bruker NMR was utilized to obtain ¹H and ¹³C NMR spectra in CDCl₃ or DMSO-*d*₆. All NMR chemical shifts (δ) are reported in parts per million after calibrations to residual isotopic solvent and coupling constants (*J*) are reported in Hz. All exchangeable protons were confirmed by addition of D₂O. Melting points were determined on a BUCHI Melting Point B-545 apparatus and are uncorrected. Mass spectrometry analysis were realized on Bruker MicrOTOF (ESI) equipped with and Agilent 1200 LC.

4.3.1. Procedure for the synthesis of 5-amino-2-hydroxy-benzoic acid (12)—A mixture of 2-hydroxy-5-nitrobenzoic acid (1 mmol, 183 mg) and conc. hydrochloric acid (18 ml) was placed in an ice bath water. Zinc powder (3 mmol, 196 mg) was added dropwise through condenser and the reaction was refluxed for 4 h. Then the mixture was cooled to room temperature, diluted with water and washed with ethyl acetate (3 × 25 ml). The obtained organic solution was evaporated in vacuum to give the crude product. The residue was purified by crystallization with diethyl ether. Yield: 88%, Mp: 280–282 °C. ¹HNMR, (DMSO-*d*₆): δ = 6.70 (dd, *J* = 8.8, *J* = 1.8, 1H, ArH), 6.92 (dd, *J* = 8.8, *J* = 2.9, 1H, ArH), 7.24 (s, 1H, ArH). Anal. Calcd for C₇H₇NO₃: C(54.90%) H(4.61%) N(9.15%). Found: C: 54.80; H: 4.50, N: 9.10.

4.3.2. Procedure for the synthesis of methyl 5-amino-2-hydroxybenzoate (13)—To a solution of 5-amino-2-hydroxybenzoic acid (20 mmol, 3.062 g) in methanol (40 mL) sulfuric acid (4.5 mL) was added at 0 °C. The reaction mixture was refluxed at 80 °C for 24 h. The solvent was then removed, the reaction mixture was neutralized with saturated sodium bicarbonate aqueous solution until pH = 7. The product was extracted with ethyl acetate (3 × 25 ml), the organic layers were combined, dried over anhydrous Na₂SO₄ and concentrated to provide the methyl 5-amino-2-hydroxybenzoate (**13**) as a pale yellow solid. Yield: 90%, Mp: 97–99 °C. ¹HNMR, (DMSO-*d*₆): δ = 3.87 (s, 3H, CH₃), 6.92 (d, *J* = 8.2, 1H, ArH), 7.15 (m, 1H, ArH), 7.39 (m, 1H, ArH), 10.18 (bs, 1H, OH). Anal. Calcd for C₈H₉NO₃: C(57.48%) H(5.43%) N(8.38%). Found: C: 57.67; H: 5.63, N: 8.34.

4.3.3. General procedure for the synthesis of methyl 5-(benzylamino)-2-hydroxybenzoates (14–19)—To a stirred solution of methyl 5-amino-2-hydroxybenzoate (**13**) (1.49 mmol, 250 mg) in MeOH (7 ml), over 4 Å molecular sieves, benzaldehyde (1.49 mmol, 0.151 ml) and AcOH (0.159 ml) were added. The mixture was heated at 40 °C and agitated for 30 min. Then it was stirred for 1 h at room temperature. The solution was cooled to 5–10 °C and NaCNBH₃ (1.94 mmol, 121 mg), was slowly added. The resulted mixture was stirred for 2 h at room temperature before being quenched by the addition of water. The solvent was evaporated under pressure and the crude mixture was taken up in CH₂Cl₂,

washed with water and brine, dried (Na_2SO_4) and evaporated. The residue was purified by chromatography (eluent: Hexane/Ethyl acetate: 8/2) to give intermediates **14–19**.

4.3.3.1. Methyl 5-(benzylamino)-2-hydroxybenzoate (14): Yield: 67%, Oil. ^1H NMR, (CDCl_3): δ = 3.87 (s, 3H, CH_3), 4.25 (s, 2H, CH_2), 6.79–6.87 (m, 2H, ArH), 7.09 (s, 1H, ArH), 7.28–7.36 (m, 5H, ArH), 10.24 (bs, 1H, OH). Anal. Calcd for $\text{C}_{15}\text{H}_{15}\text{NO}_3$: C(70.02%) H(5.88%) N(5.44%). Found: C: 70.12; H: 5.80, N: 5.54.

4.3.3.2. Methyl 5-[(2-chlorobenzyl)amino]-2-hydroxybenzoate (15): Yield: 66%, Oil. ^1H NMR, (CDCl_3): δ = 4.07 (s, 3H, CH_3), 4.57 (s, 2H, CH_2), 7.03 (s, 2H, ArH), 7.21–7.46 (m, 4H, ArH), 7.55 (m, 1H, ArH), 10.38 (bs, 1H, OH). Anal. Calcd for $\text{C}_{15}\text{H}_{14}\text{ClNO}_3$: C(61.76%) H(4.84%) N(4.80%). Found: C: 61.76; H: 4.84, N: 4.80.

4.3.3.3. Methyl 5-[(3-chlorobenzyl)amino]-2-hydroxybenzoate (16): Yield: 62%, Oil. ^1H NMR, (CDCl_3): δ = 3.93 (s, 3H, CH_3), 4.28 (s, 2H, CH_2), 6.81–6.89 (m, 2H, ArH), 7.07 (s, 1H, ArH), 7.25–7.29 (m, 3H, ArH), 7.38 (s, 1H, ArH), 10.21 (bs, 1H, OH). Anal. Calcd for $\text{C}_{15}\text{H}_{14}\text{ClNO}_3$: C(61.76%) H(4.84%) N(4.80%). Found: C: 61.59; H: 4.73, N: 4.68.

4.3.3.4. Methyl 5-[(2-fluorobenzyl)amino]-2-hydroxybenzoate (17): Yield: 65%, Oil. ^1H NMR, (CDCl_3): δ = 4.08 (s, 3H, CH_3), 4.52 (s, 2H, CH_2), 7.02 (s, 2H, ArH), 7.20–7.29 (m, 3H, ArH) 7.39–7.55 (m, 2H, ArH), 10.36 (bs, 1H, OH). Anal. Calcd for $\text{C}_{15}\text{H}_{14}\text{FNO}_3$: C(65.45%) H(5.13%) N(5.09%). Found: C: 65.37; H: 5.01, N: 4.97.

4.3.3.5. Methyl 5-[(3-fluorobenzyl)amino]-2-hydroxybenzoate (18): Yield: 62%, Oil. ^1H NMR, (CDCl_3): δ = 3.91 (s, 3H, CH_3), 4.27 (s, 2H, CH_2), 6.80–6.87 (m, 2H, ArH), 7.06 (s, 1H, ArH), 7.22–7.41 (m, 4H, ArH), 10.19 (s, 1H, OH). Anal. Calcd for $\text{C}_{15}\text{H}_{14}\text{FNO}_3$: C(65.45%) H(5.13%) N(5.09%). Found: C: 65.37; H: 5.01, N: 4.97.

4.3.3.6. Methyl 2-hydroxy-5-[(2-methylbenzyl)amino]benzoate (19): Yield: 63%, Oil. ^1H NMR, (CDCl_3): δ = 2.30 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.50 (s, 2H, CH_2), 6.80–6.86 (m, 2H, ArH), 7.05–7.14 (m, 2H, ArH), 7.30–7.36 (m, 3H, ArH), 10.21 (bs, 1H, OH). Anal. Calcd for $\text{C}_{16}\text{H}_{17}\text{NO}_3$: C(70.83%) H(6.32%) N(5.16%). Found: C: 70.68; H: 6.21, N: 5.29.

4.3.4. General procedure for the synthesis of methyl 5-(dibenzylamino)-2-hydroxybenzoates (20–25)—The appropriate methyl 5-(benzylamino)-2-hydroxybenzoate (**14–19**) (0.3 mmol) was dissolved in anhydrous DMF and was added under argon to a suspension of NaH (0.3 mmol, 29 mg) in anhydrous DMF. The reaction mixture was stirred at 0 °C under for few minutes, followed by the addition of a solution of suitable benzyl bromide (0.4 mmol) in anhydrous DMF. After 3 h, the reaction mixture was extracted by ethyl acetate (3 × 30 ml) and washed with water (2 × 20 ml) then with brine (2 × 20 ml). The organic layer was dried (Na_2SO_4), filtered and the solvent removed under reduced pressure. The resultant product was purified via flash chromatography (Hexane/Ethyl acetate: 98/2) affording the title compounds **20–25**.

4.3.4.1. Methyl 5-(dibenzylamino)-2-hydroxybenzoate (20): Yield: 65%, Mp: 159–161 °C. ¹HNMR, (CDCl₃): δ = 3.68 (s, 3H, CH₃), 4.53 (s, 4H, CH₂), 6.85 (d, *J* = 9.0, 1H, ArH), 7.00 (dd, *J* = 9.0, *J* = 3.2, 1H, ArH), 7.24–7.36 (m, 11H, ArH), 10.18 (bs, 1H, OH). Anal. Calcd for C₂₂H₂₁NO₃: C(76.06%) H(6.09%) N(4.03%). Found: C: 75.09; H: 6.06, N: 4.13.

4.3.4.2. Methyl 5-[bis(2-chlorobenzyl)amino]-2-hydroxybenzoate (21): Yield: 62%, Mp: 175–177 °C. ¹HNMR, (CDCl₃): δ = 3.87 (s, 3H, CH₃), 4.66 (s, 4H, CH₂), 6.83 (m, 2H, ArH), 7.09 (m, 1H, ArH), 7.20–7.28 (m, 6H, ArH), 7.38–7.43 (m, 2H, ArH), 10.17 (bs, 1H, OH). Anal. Calcd for C₂₂H₁₉Cl₂NO₃: C(63.47%) H(4.60%) N(3.36%). Found: C: 63.28; H: 4.54, N: 3.52.

4.3.4.3. Methyl 5-[bis(3-chlorobenzyl)amino]-2-hydroxybenzoate (22): Yield: 60%, Mp: 179–181 °C. ¹HNMR, (CDCl₃): δ = 3.93 (s, 3H, CH₃), 4.50 (s, 4H, CH₂), 6.89 (d, *J* = 9.1, 1H, ArH), 7.00 (dd, *J* = 9.1, *J* = 3.0, 1H, ArH), 7.13–7.18 (m, 2H, ArH), 7.24–7.32 (m, 7H, ArH), 10.25 (bs, 1H, OH). Anal. Calcd for C₂₂H₁₉Cl₂NO₃: C(63.47%) H(4.60%) N(3.36%). Found: C: 63.57; H: 4.50, N: 3.12.

4.3.4.4. Methyl 5-[bis(2-fluorobenzyl)amino]-2-hydroxybenzoate (23): Yield: 61%, Mp: 177–179 °C. ¹HNMR, (CDCl₃): δ = 3.92 (s, 3H, CH₃), 4.63 (s, 4H, CH₂), 6.87 (d, *J* = 9.2, 1H, ArH), 7.00 (dd, *J* = 9.2, *J* = 3.2, 1H, ArH), 7.05–7.13 (m, 5H, ArH), 7.24–7.30 (m, 2H, ArH), 7.28–7.30 (m, 2H, ArH), 10.22 (bs, 1H, OH). Anal. Calcd for C₂₂H₁₉F₂NO₃: C(68.92%) H(5.00%) N(3.65%). Found: C: 68.74; H: 4.93, N: 3.32.

4.3.4.5. Methyl 5-[bis(3-fluorobenzyl)amino]-2-hydroxybenzoate (24): Yield: 58%, Mp: 172–174 °C. ¹HNMR, (CDCl₃): δ = 3.92 (s, 3H, CH₃), 4.51 (s, 4H, CH₂), 6.89 (d, *J* = 9.1, 1H, ArH), 7.00 (dd, *J* = 9.1, *J* = 3.0, 1H, ArH), 7.14–7.17 (m, 2H, ArH), 7.25–7.32 (m, 7H, ArH), 10.27 (bs, 1H, OH). Anal. Calcd for C₂₂H₁₉F₂NO₃: C(68.92%) H(5.00%) N(3.65%). Found: C: 68.79; H: 4.98, N: 3.45.

4.3.4.6. Methyl 5-[bis(2-methylbenzyl)amino]-2-hydroxybenzoate (25): Yield: 61%, Mp: 164–166 °C. ¹HNMR, (CDCl₃): δ = 2.37 (s, 6H, CH₃), 3.95 (s, 3H, CH₃), 4.60 (s, 4H, CH₂), 6.91 (s, 1H, ArH), 6.92 (s, 1H, ArH), 7.19–7.37 (m, 9H, ArH), 10.27 (bs, 1H, OH). Anal. Calcd for C₂₄H₂₅NO₃: C(76.77%) H(6.71%) N(3.73%). Found: C: 76.65; H: 6.68, N: 3.89.

4.3.5. General procedure for the synthesis of 5-(dibenzylamino)-2-hydroxybenzoic acids (1–3, 5–6 and 8)—The appropriate methyl-2-hydroxybenzoate (20–25) was dissolved in a mixture of THF (2 ml) and MeOH (2 ml), and 2 M NaOH (4 ml) was added. The reaction was refluxed for 24 h. After this time the solvent was removed under reduced pressure and the residue was acidified to pH 2 with 2 N HCl. The solution was extracted with ethyl acetate (3 × 10 ml), dried over Na₂SO₄, filtered and the solvent removed under reduced pressure. The final products (1–3, 5–6 and 8) were crystallized with a mixture of Hexane/Ethyl acetate/Ethanol (1/1/1) and some drops of methanol.

4.3.5.1. 5-(Dibenzylamino)-2-hydroxybenzoic acid (1): Yield: 59%, Mp: 140–142 °C. ¹HNMR, (DMSO-*d*₆): δ = 4.60 (s, 4H, CH₂), 6.76 (d, *J* = 9, 1H, ArH), 7.00 (dd, *J* =

9, $J = 3.2$, 1H, ArH), 7.05 (s, 1H, ArH), 7.23–7.35 (m, 10H, ArH), 10.53 (bs, 1H, OH), 13.65 (bs, 1H, OH). Anal. Calcd for $C_{21}H_{19}NO_3$: C(75.66%) H(5.74%) N(4.20%). Found: C: 75.54; H: 5.60; N: 4.15. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 333, $C_{21}H_{19}NO_3$, m/z theory 333,1365, m/z found 334,14326.

4.3.5.2. 5-[Bis(2-chlorobenzyl)amino]-2-hydroxybenzoic acid (2): Yield: 53%, Mp: 148–150 °C. 1H NMR, (DMSO- d_6): $\delta = 4.68$ (s, 4H, CH_2), 6.75–6.84 (m, 2H, ArH), 6.94 (s, 1H, ArH), 7.23–7.31 (m, 6H, ArH), 7.47 (m, 2H, ArH). Anal. Calcd for $C_{21}H_{17}Cl_2NO_3$: C(62.70%) H(4.26%) N(3.48%). Found: C: 62.58; H: 4.35; N: 3.35. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 401, $C_{21}H_{17}Cl_2NO_3$, m/z theory 401,0585, m/z found 402,0651.

4.3.5.3. 5-[Bis(3-chlorobenzyl)amino]-2-hydroxybenzoic acid (3): Yield: 50%, Mp: 147–149 °C. 1H NMR, (DMSO- d_6): $\delta = 4.64$ (s, 4H, CH_2), 6.78 (d, $J = 8.9$, 1H, ArH), 6.97–7.05 (m, 2H, ArH), 7.21–7.39 (m, 8H, ArH), 10.55 (bs, 1H, OH). Anal. Calcd for $C_{21}H_{17}Cl_2NO_3$: C(62.70%) H(4.26%) N(3.48%). Found: C: 62.86; H: 4.10; N: 3.25. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 401, $C_{21}H_{17}Cl_2NO_3$, m/z theory 401,0585, m/z found 402,0651.

4.3.5.4. 5-[Bis(2-fluorobenzyl)amino]-2-hydroxybenzoic acid (5): Yield: 52%, Mp: 137–139 °C. 1H NMR, (DMSO- d_6): $\delta = 4.65$ (s, 4H, CH_2), 6.79 (d, $J = 9.1$, 1H, ArH), 6.99 (dd, $J = 9.1$, $J = 3.2$, 1H, ArH), 7.06–7.34 (m, 9H, ArH), 10.59 (bs, 1H, OH), 13.69 (bs, 1H, OH). Anal. Calcd for $C_{21}H_{17}F_2NO_3$: C(68.29%) H(4.64%) N(3.79%). Found: C: 68.07; H: 4.46; N: 3.58. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 369, $C_{21}H_{17}F_2NO_3$, m/z theory 369,3678, m/z found 370,1241.

4.3.5.5. 5-[Bis(3-fluorobenzyl)amino]-2-hydroxybenzoic acid (6): Yield: 49%, Mp: 141–143 °C. 1H NMR, (DMSO- d_6): $\delta = 4.63$ (s, 4H, CH_2), 6.78 (d, $J = 8.8$, 1H, ArH), 6.99 (dd, $J = 8.8$, $J = 3.2$, 1H, ArH), 7.04 (d, $J = 3.2$, 1H, ArH), 7.20–7.38 (m, 8H, ArH), 10.52 (bs, 1H, OH), 13.69 (bs, 1H, OH). Anal. Calcd for $C_{21}H_{17}F_2NO_3$: C(68.29%) H(4.64%) N(3.79%). Found: C: 68.14; H: 4.59; N: 3.61. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 369, $C_{21}H_{17}F_2NO_3$, m/z theory 369,3678, m/z found 370,1249.

4.3.5.6. 5-[Bis(2-methylbenzyl)amino]-2-hydroxybenzoic acid (8): Yield: 48%, Mp: 136–138 °C. 1H NMR, (CDCl $_3$): $\delta = 2.28$ (s, 6H, CH_3), 4.53 (s, 4H, CH_2), 6.83 (d, $J = 9.2$, 1H, ArH), 7.85 (m, 1H, ArH) 7.14–7.20 (m, 9H, ArH), 9.79 (bs, 1H, OH). Anal. Calcd for $C_{23}H_{23}NO_3$: C(76.43%) H(6.41%) N(3.88%). Found: C: 76.26; H: 6.30; N: 3.95. ESI(+), CH_3OH , HR-MS: ion $[M + H]^+$, m/z 361, $C_{23}H_{23}NO_3$, m/z theory 361,1678, m/z found 362,1745.

4.4. Recombinant proteins and HTRF-based assays

His-tagged LEDGF/p75, FLAG-tagged IN and His-tagged IN were constructed and purified as described previously [32]. Homogeneous time resolved fluorescence energy transfer (HTRF) based LEDGF/p75 dependent IN activity, IN-LEDGF/p75 binding, and LEDGF/p75 independent 3' processing assays were carried out as previously described [32]. The HTRF signal was recorded using a Perkin Elmer Multimode EnSpire plate reader. The fitted dose-

response curves were generated to calculate IC₅₀ using Origin software (OriginLab, Inc.). All fitted curves displayed a R² of 0.99 or greater.

4.5. Antiviral activity and cytotoxicity assays

The indicated concentrations of the test compounds or diluent control (DMSO) were added directly to the target cells and the cells were infected with untreated virions. HeLa TZM-bl cells (2×10⁵ cells/well of a 6-well plate in 2 ml of complete medium) were pre-incubated with the indicated concentrations of the test inhibitor or diluent control (DMSO) for 2 h. The cells were then infected with HIV-1 virions equivalent to 4 ng of HIV-1 p24 as determined by HIV-1 Gag p24 ELISA (ZeptoMetrix) following manufacturer's protocol. Two hours post-infection the culture supernatant was removed, washed once with complete medium, and then fresh complete medium was added with the inhibitor concentration maintained. The cells were cultured for 48 h and the cell extracts were prepared using 16 reporter lysis buffer (Promega). Luciferase activity was determined using a commercially available kit (Promega). The cytotoxicity assays were performed as described previously [28]. The fitted dose-response curves were generated to calculate EC₅₀ or CC₅₀ using Origin software (OriginLab, Inc.). All fitted curves displayed a R² of 0.97 or greater.

Acknowledgments

We are grateful to Dr. Ross C. Larue for critical reading of the manuscript and helpful comments. These studies were in part supported by University of Messina Research & Mobility 2015 Project (project code RES_AND_MOB_2015_DE_LUCA) and by National Institutes of Health grant R01AI110310 (to M.K. and J.R.F.)

Abbreviations

ALLINI	allosteric integrase inhibitor
CCD	catalytic core domain
CTD	C-terminal domain
IBD	IN-binding domain
IN	Integrase
INLAIs	Integrase lens epithelium-derived growth factor Allosteric Inhibitors
vDNA	viral DNA
GOLD	Genetic Optimization for Ligand Docking
LEDGF/p75	lens epithelium-derived growth factor
LEDGINs	lens epithelium-derived growth factor Integrase Inhibitor
MD	molecular dynamic
MM-GBSA	Molecular Mechanics energies combined with the Generalized Born and Surface Area continuum solvation
MINIs	Multimerization Integrase Inhibitors

NPs	Natural Products
NTD	N-terminal domain
NCINIs	Non Catalytic Integrase Inhibitors
PPI	protein-protein interaction
PPIIs	protein-protein interaction inhibitors
3'P	3' processing
ST	strand-transfer
VSV-g	vesicular stomatitis virus g

References

1. Jaskolski M, Alexandratos JN, Bujacz G, Wlodawer A. Piecing together the structure of retroviral integrase, an important target in AIDS therapy. *FEBS J.* 2009; 276:2926–2946. [PubMed: 19490099]
2. Feng L, Larue RC, Slaughter A, Kessl JJ, Kvaratskhelia M. HIV-1 integrase multimerization as a therapeutic target. *Curr Top Microbiol Immunol.* 2015; 389:93–119. [PubMed: 25778682]
3. Wills T, Vega V. Elvitegravir: a once-daily inhibitor of HIV-1 integrase. *Expert Opin Investig Drugs.* 2012; 21:395–401.
4. FDA approves raltegravir tablets. *AIDS Patient Care STDS.* 2007; 21:889.
5. Karmon SL, Markowitz M. Next-generation integrase inhibitors : where to after raltegravir? *Drugs.* 2013; 73:213–228. [PubMed: 23413196]
6. Summa V, Petrocchi A, Bonelli F, Crescenzi B, Donghi M, Ferrara M, Fiore F, Gardelli C, Paz O Gonzalez, Hazuda DJ, Jones P, Kinzel O, Laufer R, Monteagudo E, Muraglia E, Nizi E, Orvieto F, Pace P, Pescatore G, Scarpelli R, Stillmock K, Witmer MV, Rowley M. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. *J Med Chem.* 2008; 51:5843–5855. [PubMed: 18763751]
7. van Lunzen J, Maggiolo F, Arribas JR, Rakhmanova A, Yeni P, Young B, Rockstroh JK, Almond S, Song I, Brothers C, Min S. Once daily dolutegravir (S/GSK1349572) in combination therapy in antiretroviral-naïve adults with HIV: planned interim 48 week results from SPRING-1, a dose-ranging, randomised, phase 2b trial. *Lancet Infect Dis.* 2012; 12:111–118. [PubMed: 22018760]
8. Pandey KK. Critical appraisal of elvitegravir in the treatment of HIV-1/AIDS. *HIV, AIDS (Auckl).* 2014; 6:81–90. [PubMed: 24876793]
9. Sichtig N, Sierra S, Kaiser R, Daumer M, Reuter S, Schuler E, Altmann A, Fatkenheuer G, Dittmer U, Pfister H, Esser S. Evolution of raltegravir resistance during therapy. *J Antimicrob Chemother.* 2009; 64:25–32. [PubMed: 19447792]
10. Steigbigel RT, Cooper DA, Kumar PN, Eron JE, Schechter M, Markowitz M, Loutfy MR, Lennox JL, Gatell JM, Rockstroh JK, Katlama C, Yeni P, Lazzarin A, Clotet B, Zhao J, Chen J, Ryan DM, Rhodes RR, Killar JA, Gilde LR, Strohmaier KM, Meibohm AR, Miller MD, Hazuda DJ, Nessly ML, DiNubile MJ, Isaacs RD, Nguyen BY, Teppler H, B.S. Teams. Raltegravir with optimized background therapy for resistant HIV-1 infection. *N Engl J Med.* 2008; 359:339–354. [PubMed: 18650512]
11. Metifiot M, Vandegraaff N, Maddali K, Naumova A, Zhang X, Rhodes D, Marchand C, Pommier Y. Elvitegravir overcomes resistance to raltegravir induced by integrase mutation Y143. *AIDS.* 2011; 25:1175–1178. [PubMed: 21505303]
12. Li M, Mizuuchi M, Burke TR Jr, Craigie R. Retroviral DNA integration: reaction pathway and critical intermediates. *EMBO J.* 2006; 25:1295–1304. [PubMed: 16482214]
13. Krishnan L, Engelman A. Retroviral integrase proteins and HIV-1 DNA integration. *J Biol Chem.* 2012; 287:40858–40866. [PubMed: 23043109]

14. Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. *Nat Rev Microbiol.* 2012; 10:279–290. [PubMed: 22421880]
15. Cherepanov P, Maertens G, Proost P, Devreese B, Van Beeumen J, Engelborghs Y, De Clercq E, Debyser Z. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *J Biol Chem.* 2003; 278:372–381. [PubMed: 12407101]
16. Ciuffi A, Llano M, Poeschla E, Hoffmann C, Leipzig J, Shinn P, Ecker JR, Bushman F. A role for LEDGF/p75 in targeting HIV DNA integration. *Nat Med.* 2005; 11:1287–1289. [PubMed: 16311605]
17. Llano M, Saenz DT, Meehan A, Wongthida P, Peretz M, Walker WH, Teo W, Poeschla EM. An essential role for LEDGF/p75 in HIV integration. *Science.* 2006; 314:461–464. [PubMed: 16959972]
18. Shun MC, Raghavendra NK, Vandegraaff N, Daigle JE, Hughes S, Kellam P, Cherepanov P, Engelman A. LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes Dev.* 2007; 21:1767–1778. [PubMed: 17639082]
19. Busschots K, Vercammen J, Emiliani S, Benarous R, Engelborghs Y, Christ F, Debyser Z. The interaction of LEDGF/p75 with integrase is lentivirus-specific and promotes DNA binding. *J Biol Chem.* 2005; 280:17841–17847. [PubMed: 15749713]
20. Llano M, Morrison J, Poeschla EM. Virological and cellular roles of the transcriptional coactivator LEDGF/p75. *Curr Top Microbiol Immunol.* 2009; 339:125–146. [PubMed: 20012527]
21. Al-Mawsawi LQ, Neamati N. Blocking interactions between HIV-1 integrase and cellular cofactors: an emerging anti-retroviral strategy. *Trends Pharmacol Sci.* 2007; 28:526–535. [PubMed: 17888520]
22. Engelman A, Cherepanov P. The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. *PLoS Pathog.* 2008; 4:e1000046. [PubMed: 18369482]
23. Cherepanov P, Devroe E, Silver PA, Engelman A. Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. *J Biol Chem.* 2004; 279:48883–48892. [PubMed: 15371438]
24. Cherepanov P, Ambrosio AL, Rahman S, Ellenberger T, Engelman A. Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. *Proc Natl Acad Sci U S A.* 2005; 102:17308–17313. [PubMed: 16260736]
25. Christ F, Voet A, Marchand A, Nicolet S, Desimmi BA, Marchand D, Bardiot D, Van der Veken NJ, Van Remoortel B, Strelkov SV, De Maeyer M, Chaltin P, Debyser Z. Rational design of small-molecule inhibitors of the LEDGF/p75-integrase interaction and HIV replication. *Nat Chem Biol.* 2010; 6:442–448. [PubMed: 20473303]
26. Fader LD, Malenfant E, Parisien M, Carson R, Bilodeau F, Landry S, Pesant M, Brochu C, Morin S, Chabot C, Halmos T, Bousquet Y, Bailey MD, Kawai SH, Coulombe R, LaPlante S, Jakalian A, Bhardwaj PK, Wernic D, Schroeder P, Amad M, Edwards P, Garneau M, Duan J, Cordingley M, Bethell R, Mason SW, Bos M, Bonneau P, Poupart MA, Faucher AM, Simoneau B, Fenwick C, Yoakim C, Tsantrizos Y. Discovery of BI 22436, a Noncatalytic site integrase inhibitor (NCINI) of HIV-1. *ACS Med Chem Lett.* 2014; 5:422–427. [PubMed: 24900852]
27. Tsang M, Jones GS, Niedziela-Majka A, Kan E, Lansdon EB, Huang W, Hung M, Samuel D, Novikov N, Xu Y, Mitchell M, Guo H, Babaoglu K, Liu X, Geleziunas R, Sakowicz R. New class of HIV-1 integrase (IN) inhibitors with a dual mode of action. *J Biol Chem.* 2012; 287:21189–21203. [PubMed: 22535962]
28. Sharma A, Slaughter A, Jena N, Feng L, Kessl JJ, Fadel HJ, Malani N, Male F, Wu L, Poeschla E, Bushman FD, Fuchs JR, Kvaratskhelia M. A new class of multimerization selective inhibitors of HIV-1 integrase. *PLoS Pathog.* 2014; 10:e1004171. [PubMed: 24874515]
29. Le Rouzic E, Bonnard D, Chasset S, Bruneau JM, Chevreuil F, Le Strat F, Nguyen J, Beauvoir R, Amadori C, Brias J, Vomscheid S, Eiler S, Levy N, Delelis O, Deprez E, Saib A, Zamborlini A, Emiliani S, Ruff M, Ledoussal B, Moreau F, Benarous R. Dual inhibition of HIV-1 replication by integrase-LEDGF allosteric inhibitors is predominant at the post-integration stage. *Retrovirology.* 2013; 10:144. [PubMed: 24261564]

30. van Bel N, van der Velden Y, Bonnard D, Le Rouzic E, Das AT, Benarous R, Berkhout B. The allosteric HIV-1 integrase inhibitor BI-D affects virion maturation but does not influence packaging of a functional RNA genome. *PLoS One*. 2014; 9:e103552. [PubMed: 25072705]
31. Cherepanov P, Ambrosio AL, Rahman S, Ellenberger T, Engelman A. Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. *Proc Natl Acad Sci U S A*. 2005; 102:17308–17313. [PubMed: 16260736]
32. Kessl JJ, Jena N, Koh Y, Taskent-Sezgin H, Slaughter A, Feng L, de Silva S, Wu L, Le Grice SF, Engelman A, Fuchs JR, Kvaratskhelia M. A multimode, cooperative mechanism of action of allosteric HIV-1 integrase inhibitors. *J Biol Chem*. 2012; 287:16801–16811. [PubMed: 22437836]
33. Jurado KA, Engelman A. Multimodal mechanism of action of allosteric HIV-1 integrase inhibitors. *Expert Rev Mol Med*. 2013; 15:e14. [PubMed: 24274067]
34. Jurado KA, Wang H, Slaughter A, Feng L, Kessl JJ, Koh Y, Wang W, Ballandras-Colas A, Patel PA, Fuchs JR, Kvaratskhelia M, Engelman A. Allosteric integrase inhibitor potency is determined through the inhibition of HIV-1 particle maturation. *Proc Natl Acad Sci U S A*. 2013; 110:8690–8695. [PubMed: 23610442]
35. Balakrishnan M, Yant SR, Tsai L, O'Sullivan C, Bam RA, Tsai A, Niedziela-Majka A, Stray KM, Sakowicz R, Cihlar T. Non-catalytic site HIV-1 integrase inhibitors disrupt core maturation and induce a reverse transcription block in target cells. *PLoS One*. 2013; 8:e74163. [PubMed: 24040198]
36. Desimie BA, Schrijvers R, Demeulemeester J, Borrenberghs D, Weydert C, Thys W, Vets S, Van Remoortel B, Hofkens J, De Rijck J, Hendrix J, Bannert N, Gijssbers R, Christ F, Debyser Z. LEDGINs inhibit late stage HIV-1 replication by modulating integrase multimerization in the virions. *Retrovirology*. 2013; 10:57. [PubMed: 23721378]
37. Fontana J, Jurado KA, Cheng N, Ly NL, Fuchs JR, Gorelick RJ, Engelman AN, Steven AC. Distribution and redistribution of HIV-1 Nucleocapsid protein in immature, mature, and integrase-inhibited virions: a role for integrase in maturation. *J Virol*. 2015; 89:9765–9780. [PubMed: 26178982]
38. Feng L, Dharmarajan V, Serrao E, Hoyte A, Larue RC, Slaughter A, Sharma A, Plumb MR, Kessl JJ, Fuchs JR, Bushman FD, Engelman AN, Griffin PR, Kvaratskhelia M. The competitive interplay between allosteric HIV-1 integrase inhibitor BI/D and LEDGF/p75 during the early stage of HIV-1 replication adversely affects inhibitor potency. *ACS Chem Biol*. 2016; 11:1313–1321. [PubMed: 26910179]
39. Feng L, Sharma A, Slaughter A, Jena N, Koh Y, Shkriabai N, Larue RC, Patel PA, Mitsuya H, Kessl JJ, Engelman A, Fuchs JR, Kvaratskhelia M. The A128T resistance mutation reveals aberrant protein multimerization as the primary mechanism of action of allosteric HIV-1 integrase inhibitors. *J Biol Chem*. 2013; 288:15813–15820. [PubMed: 23615903]
40. Slaughter A, Jurado KA, Deng N, Feng L, Kessl JJ, Shkriabai N, Larue RC, Fadel HJ, Patel PA, Jena N, Fuchs JR, Poeschla E, Levy RM, Engelman A, Kvaratskhelia M. The mechanism of H171T resistance reveals the importance of N inverted question mark -protonated His171 for the binding of allosteric inhibitor BI-D to HIV-1 integrase. *Retrovirology*. 2014; 11:100. [PubMed: 25421939]
41. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod*. 2012; 75:311–335. [PubMed: 22316239]
42. Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. *AAPS J*. 2006; 8:E239–E253. [PubMed: 16796374]
43. De Luca L, Morreale F, Christ F, Debyser Z, Ferro S, Gitto R. New scaffolds of natural origin as Integrase-LEDGF/p75 interaction inhibitors: virtual screening and activity assays. *Eur J Med Chem*. 2013; 68:405–411. [PubMed: 23994868]
44. De Luca L, Ferro S, Morreale F, Christ F, Debyser Z, Chimirri A, Gitto R. Fragment hopping approach directed at design of HIV IN-LEDGF/p75 interaction inhibitors. *J Enzyme Inhib Med Chem*. 2013; 28:1002–1009. [PubMed: 22803661]
45. Ferro S, De Luca L, Agharbaoui FE, Christ F, Debyser Z, Gitto R. Optimization of rhodanine scaffold for the development of protein-protein interaction inhibitors. *Bioorg Med Chem*. 2015; 23:3208–3214. [PubMed: 25999203]

46. Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. *J Mol Biol.* 1997; 267:727–748. [PubMed: 9126849]
47. Case, DA., Darden, TA., Cheatham, TE., III, Simmerling, CL., Wang, J., Duke, RE., Luo, R., Walker, RC., Zhang, W., Merz, KM., Roberts, B., Wang, B., Hayik, S., Roitberg, A., Seabra, G., Kolossváry, I., Wong, KF., Paesani, F., Vanicek, J., Liu, J., Wu, X., Brozell, SR., Steinbrecher, T., Gohlke, H., Cai, Q., Ye, X., Wang, J., Hsieh, M.-J., Cui, G., Roe, DR., Mathews, DH., Seetin, MG., Sagui, C., Babin, V., Luchko, T., Gusarov, S., Kovalenko, A., Kollman, PA. AMBER 11. University of California; San Francisco: 2010.
48. De Luca L, Morreale F, Chimirri A. Insight into the fundamental interactions between LEDGF binding site inhibitors and integrase combining docking and molecular dynamics simulations. *J Chem Inf Model.* 2012; 52:3245–3254. [PubMed: 23194297]
49. D.S. Accelrys.
50. Kollman PA, Massova I, Reyes C, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y, Wang W, Donini O, Cieplak P, Srinivasan J, Case DA, Cheatham TE 3rd. Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc Chem Res.* 2000; 33:889–897. [PubMed: 11123888]
51. Maignan S, Guilloteau JP, Zhou-Liu Q, Clement-Mella C, Mikol V. Crystal structures of the catalytic domain of HIV-1 integrase free and complexed with its metal cofactor: high level of similarity of the active site with other viral integrases. *J Mol Biol.* 1998; 282:359–368. [PubMed: 9735293]
52. v.S.L.N.Y. Maestro, NY, 2009.
53. D.A.D. Case, T. A.; Cheatham, T. E.; III; Simmerling, C. L.; Wang, J.; Duke, R. E.; Luo, R.; Walker, R. C.; Zhang, W.; Merz, K. M.; Roberts, B.; Wang, B.; Hayik, S.; Roitberg, A.; Seabra, G.; Kolossváry, I.; Wong, K. F.; Paesani, F.; Vanicek, J.; Liu, J.; Wu, X.; Brozell, S. R.; Steinbrecher, T.; Gohlke, H.; Cai, Q.; Ye, X.; Wang, J.; Hsieh, M.-J.; Cui, G.; Roe, D. R.; Mathews, D. H.; Seetin, M. G.; Sagui, C.; Babin, V.; Luchko, T.; Gusarov, S.; Kovalenko, A.; Kollman, P. A.; University of California: San Francisco, 2010.
54. Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, Yang R, Cieplak P, Luo R, Lee T, Caldwell J, Wang J, Kollman P. A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *J Comput Chem.* 2003; 24:1999–2012. [PubMed: 14531054]
55. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. Development and testing of a general amber force field. *J Comput Chem.* 2004; 25:1157–1174. [PubMed: 15116359]
56. DeLano, WL. DeLano Scientific LLC. The PyMOL Molecular Graphics System; San Carlos, CA, USA: 2008.

Fig. 1.

A) Chemical structures of 5-[bis(2-hydroxybenzyl)amino]-2-hydroxybenzoic acid (Lavendustin B) B) Superimposition of Lavendustin B (green) and compound KF115 (cyan) in complex with IN CCD (PDB code 2B4J). Key residues of the pocket are presented. The figure was created using PyMOL [56]. C) Chemical structures of KF115. D) Substitutions on the parent molecule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Superimposition of IN CCD with A) Lavendustin B (violet) and compound **1** (cyan); B) compounds **2** (green), **3** (cyan) and **4** (magenta); C) compounds **5** (green), **6** (cyan) and **7** (magenta); D) compounds **8** (green), **9** (cyan) and **10** (magenta). Key residues of the pocket are presented. The figure was created using PyMOL [56]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

A) chemical structure of compound 2; B) Curve fitting of inhibition of LEDGF/p75 dependent IN activity by compound 2 (black squares); C) Curve fitting of dose-dependent inhibition of IN-LEDGF binding by compound 2 (black squares); D) Curve fitting of dose-dependent inhibition of 3' processing by compound 2 (black squares). The average values from three independent experiments are shown for each assay.

Fig. 4.

A) Luciferase quantification to measure the inhibition by compound **2** over the indicated concentrations. VSV-g pseudotyped virus was used to infect HEK293T cells under drug treatment. B) Cytotoxicity effect of compound **2** over the indicated concentrations without viral infection.

Scheme 1.

Reagents and conditions: (i) Zinc dust, 0–5 °C, HCl_{conc.}, reflux, 4 h; (ii) MeOH, H₂SO₄, 0 °C, 80 °C, 24 h; (iii) MeOH, AcOH, 30', 40 °C, rt 1 h, 5–10 °C NaCNBH₃, 2 h rt; (iv) NaH, DMF, Argon 3 h rt; (v) NaOH, MeOH, THF, 60 °C, 24 h, HCl 2 N.

Table 1

Binding free energy estimation.

Compound	R	G _{bind} (complex 1) kcal/mol 2B4J-Ligand	G _{bind} (complex 2) kcal/mol 3LPU-Ligand	G _{bind} (Snapshots) 2B4J-Ligand kcal/mol
Lavendustin B		-15,39	-14,77	-18,09
1	H	-21,54	-19,41	-22,41
2	2-Cl	-25,73	-25,73	-24,99
3	3-Cl	-24,74	-24,41	-21,68
4	4-Cl	-21,33	-19,02	-17,75
5	2-F	-20,45	-20,64	-22,47
6	3-F	-20,10	-20,51	-20,08
7	4-F	-15,87	-16,00	-15,54
8	2-CH ₃	-22,24	-23,35	-21,40
9	3-CH ₃	-16,29	-15,09	-14,00
10	4-CH ₃	-12,86	-11,38	-12,65

G_{bind} is the calculated binding free energy.

Table 2

Hydrogen bonds analysis from the results of MD simulation for IN in complex with the designed compounds.

Compound	IN in complex with	Donor	Acceptor	Occupancy (%) ^a	Distance (Å) ^b
Lavendustine B	Lavendustin B	Thr174(A) OH	O3	11.80	3.092 (0.21)
		Glu170(A) NH	O1	78.20	2.930 (0.18)
		Glu170(A) NH	O2	36.60	3.051 (0.22)
		His171(A) NH	O2	63.30	3.230 (0.18)
1	H	Thr174(A) OH	O	21.90	3.077 (0.21)
		Thr174(A) OH	O1	10.40	3.093 (0.26)
		Glu170(A) NH	O2	90.40	2.923 (0.17)
		Glu170(A) NH	O1	64.30	3.108 (0.22)
2	2Cl	His171(A) NH	O1	76.70	3.102 (0.19)
		Gln95(A) NH	O	10.40	3.140 (0.18)
		Thr174(A) OH	O	76.60	3.029 (0.20)
		Thr174(A) OH	O1	34.90	2.896 (0.25)
3	3Cl	Glu170(A) NH	O2	98.80	2.966 (0.18)
		Glu170(A) NH	O1	78.80	3.070 (0.20)
		His171(A) NH	O1	84.30	3.086 (0.19)
		Gln95(A) NH	O	37.20	3.102 (0.19)
4	4Cl	Thr174(A) OH	O2	72.30	3.024 (0.20)
		Glu170(A) NH	O1	86.40	2.907 (0.16)
		Glu170(A) NH	O	61.20	3.083 (0.20)
		His171(A) NH	O	77.70	3.073 (0.18)
5	2F	Thr174(A) OH	O1	21.30	2.958 (0.26)
		Glu170(A) NH	O1	63.60	2.944 (0.17)
		His171(A) NH	O1	25.60	3.206 (0.18)
		His171(A) NH	O	24.50	3.168 (0.20)
5	2F	Thr174(A) OH	O2	24.30	3.043 (0.27)
		Glu170(A) NH	O1	95.60	2.960 (0.18)
		Glu170(A) NH	O2	76.70	3.060 (0.20)
		His171(A) NH	O1	82.10	3.093 (0.18)

Compound	IN in complex with	Donor	Acceptor	Occupancy (%) ^a	Distance (Å) ^b
6	3F	Thr174(A) OH	O1	78.40	2.999 (0.26)
		Thr174(A) OH	O	17.90	3.093 (0.18)
		Glu170(A) NH	O1	57.40	2.933 (0.16)
		His171(A) NH	O1	21.20	3.173 (0.18)
		His171(A) NH	O	10.40	3.267 (0.17)
7	4F	Thr174(A) OH	O	45.50	2.978 (0.27)
		Glu170(A) NH	O	32.40	2.970 (0.20)
		His171(A) NH	O1	23.70	3.076 (0.19)
		Thr174(A) OH	O2	54.80	2.950 (0.26)
		Thr174(A) OH	O	17.40	3.164 (0.19)
8	2CH ₃	Glu170(A) NH	O1	96.80	2.866 (0.14)
		Glu170(A) NH	O2	49.00	3.173 (0.21)
		His171(A) NH	O2	46.00	3.210 (0.18)
		Glu170(A) NH	O	21.60	2.970 (0.19)
		His171(A) NH	O1	10.30	3.243 (0.17)
9	3CH ₃	Thr174(A) OH	O	28.57	3.206 (0.19)
		Thr174(A) OH	O1	23.81	3.316 (0.19)
		Glu170(A) NH	O1	50.00	2.884 (0.16)
		His171(A) NH	O	28.57	3.065 (0.18)
		His171(A) NH	O1	26.19	3.273(0.14)
10	4CH ₃				

^aThe listed donor and acceptor pairs correspond to the hydrogen bonds occupancies during the simulation.

^bThe average distance with standard error (SE = standard deviation/N^{1/2}) in parentheses between hydrogen-acceptor atom and hydrogen-donor atom in the investigated time period.

Table 3

Inhibition of IN-LEDGF/p75 binding, LEDGF/p75 dependent integration, and LEDGF/p75 independent 3'-processing activity of the synthesized compounds.

Compound	R	LEDGF/p75 dependent IN Activity (IC ₅₀ μM)	IN-LEDGF/p75 binding (IC ₅₀ μM)	3'P (IC ₅₀ μM)
1	H	7.93 ± 0.79	8.75 ± 2.36	69.97 ± 10.27
2	2-Cl	3.78 ± 0.35	3.28 ± 0.80	25.62 ± 1.88
3	3-Cl	13.48 ± 4.45	19.50 ± 2.55	>70
5	2-F	9.00 ± 0.24	17.84 ± 4.25	63.05 ± 5.16
6	3-F	18.50 ± 2.19	20.83 ± 2.46	68.56
8	2-CH₃	14.66 ± 0.69	27.59 ± 3.98	>70
^a Lavendustin B	—	—	94.07	—

Data for IC₅₀ are given as the mean ± SD from at least three independent experiments.

^aThe IC₅₀ of Lavendustin B is reported elsewhere and was evaluated by alpha screen assays [43].