

Pharmacophore-Based Discovery of Small-Molecule Inhibitors of Protein–Protein Interactions between HIV-1 Integrase and Cellular Cofactor LEDGF/p75

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The cellular protein lens epithelium-derived growth factor, or transcriptional coactivator p75 (LEDGF/p75), plays a crucial role in HIV integration. The protein–protein interactions (PPIs) between HIV-1 integrase (IN) and its cellular cofactor LEDGF/p75 may therefore serve as targets for the development of new anti-HIV drugs. In this work, a structure-based pharmacophore model for potential small-molecule inhibitors of HIV-1 IN–LEDGF/p75 interaction was developed using the LigandScout software. The 3D model obtained was used for virtual screening of our in-house chemical database, CHIME, leading to the identification of compound CHIBA-3002 as an interesting hit for further optimization. The rational design, synthesis and bio-

logical evaluation of four derivatives were then carried out. Our studies resulted in the discovery of a new and more potent small molecule (7, CHIBA-3003) that is able to interfere with the HIV-1 IN–LEDGF/p75 interaction at micromolar concentration, representing one of the first compounds to show activity against these specific PPIs. Docking simulations were subsequently performed in order to investigate the possible binding mode of our new lead compound to HIV-1 IN. This study is a valid starting point for the identification of anti-HIV agents with a different mechanism of action from currently available antiviral drugs.

Introduction

Although anti-HIV drugs typically target viral proteins, the manipulation of specific protein–protein interactions (PPIs) involved in the HIV life cycle could potentially result in potent drugs that lack cellular toxicity. With this in mind, the objective of our research is hit identification and lead development of small-molecule inhibitors that are able to disrupt specific protein–protein interactions (PPIs). In particular, the interaction between HIV-1 integrase (IN) and the cellular protein lens epithelium-derived growth factor or transcriptional coactivator p75 (LEDGF/p75) has increasingly gained attention as a valuable target for a novel antiviral strategy.^[1–4]

The HIV-1 IN enzyme catalyzes an essential step in the retroviral life cycle—insertion of viral DNA into the genome of the host cell through a multistep process. In the “3′-processing” step, HIV-1 IN removes a dinucleotide from each 3′ end of viral cDNA. These two newly processed 3′-viral DNA ends are then inserted into the host cell DNA during the “strand transfer” reaction.

Sequence alignments, mutagenesis and proteolytic digestion suggested that HIV-1 IN is comprised of three structurally and functionally distinct domains: an N-terminal zinc-binding HHCC domain, a central catalytic core domain and a C-terminal DNA-binding domain.^[5]

All three domains bind DNA, and each separate domain forms a homodimer in solution. Even if all three domains are required for full catalytic activity, site-directed mutagenesis experiments have shown that the central core domain is sufficient to promote a reverse integration reaction in vitro, known

as disintegration, indicating that this region contains the enzymatic catalytic center.

Although HIV-1 IN plays a key role in integration, the process takes place in a more complex environment in vivo. Before integration, viral cDNA becomes associated with a number of viral and cellular proteins to form a large nucleoprotein assembly called a preintegration complex. LEDGF/p75 is a cellular protein that has recently been identified and validated as a novel cellular cofactor of HIV integration and replication.^[6,7] LEDGF/p75 binds HIV-1 IN via a small (~80 residues) IN-binding domain (IBD, amino acids 347–429) within its C-terminal region. IBD is both necessary and sufficient for interaction with HIV-1 IN. The crystal structure of the dimeric catalytic core

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domain of HIV-1 IN complexed with the LEDGF/p75 IBD has recently been reported (PDB code 2B4J).^[8]

The aim of our study was thus to use these structural data as the starting point for a pharmacophore modeling approach to identify novel compounds able to disrupt HIV-1 IN–LEDGF/p75 interactions. The computational approaches presented herein could also be useful in general PPI inhibition strategies.

Results and Discussion

The cellular cofactor LEDGF/p75 plays a unique role during HIV integration and the identification of small molecules that interfere with HIV-1 IN–LEDGF/p75 interactions and block HIV replication might prove an enormous driving force in the field of antiretroviral therapeutics.^[3]

The most critical interacting residues of the IBD are Ile365, Asp366 and Phe406; there have been recent reports that mutation of these LEDGF/p75 residues destroyed interaction with HIV-1 IN.^[8] Therefore, we focused our interest on the LEDGF hotspot residues Ile365 and Asp366, supposing that a small molecule able to mimic this IBD dipeptide might inhibit IN–LEDGF/p75 recognition. This idea was also supported by a recent report showing that a LEDGF/p75-derived peptide (amino acids 355–377), which contains the two HIV-1 IN interacting residues of interest to us (Ile365 and Asp366), could disrupt the IN–LEDGF/p75 interaction.^[9]

In the first step of our rational approach, the X-ray crystal structure of HIV-1 IN in complex with LEDGF/p75 IBD was used to generate a structure-based pharmacophore model. The assumption that this computational methodology could work well in a PPI inhibitor discovery process was partly encouraged by the successful application of the same approach in the identification of new non-nucleoside reverse transcriptase inhibitors.^[10]

The analysis of protein–protein contacts by LigandScout enabled us to highlight the chemical features mainly involved in the interactions between LEDGF residues Ile365–Asp366 and IN, as described by Cherepanov et al.^[8] The hypothesis obtained contained 14 features: one H-bond donor, two H-bond acceptors, two hydrophobic groups and nine excluded volumes (Figure 1). In particular, the H-bond donor reflected one of the two NH groups of Ile365–Asp366 that interacts with the carbonyl oxygen of Gln168. The two H-bond acceptor features were occupied by the Asp366 carboxylate oxygen atoms, which made a bidentate H bond to the main-chain amides of IN residues Glu170 and His171 in chain A. Lastly, the two hydrophobic spheres were occupied by the Ile365 side chain, which projects into a hydrophobic pocket formed by IN chain B residues Leu102, Ala128, Ala129 and Trp132 and chain A residues Thr174 and Met178. The nine excluded volumes reflected potential steric restriction and corresponded to the positions that are sterically claimed by the macromolecular environment surrounding the LEDGF dipeptide (residues Ala128, Ala129, Gln168, Trp132, Glu170, His171, Thr174 and Met178).

The 3D pharmacophore model obtained was then used as a query in a virtual screening approach to filter the CHIME data-

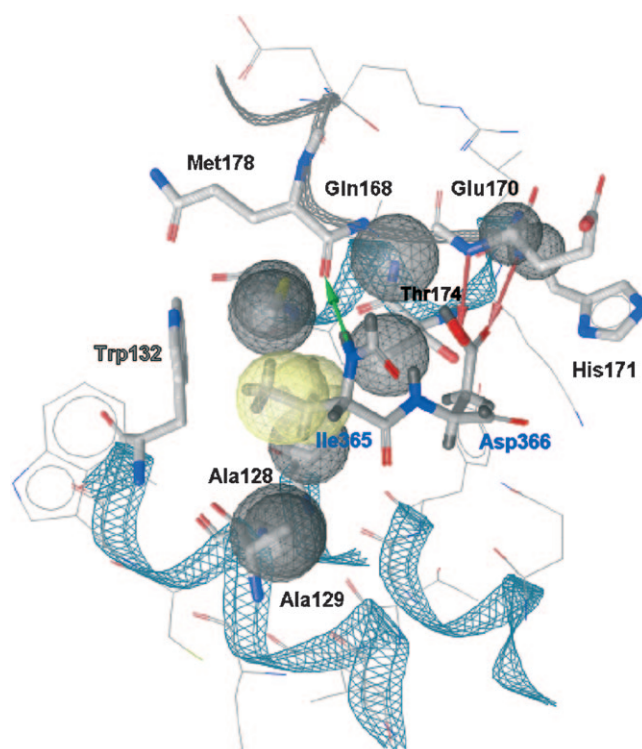
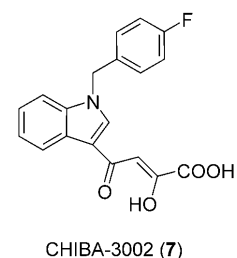


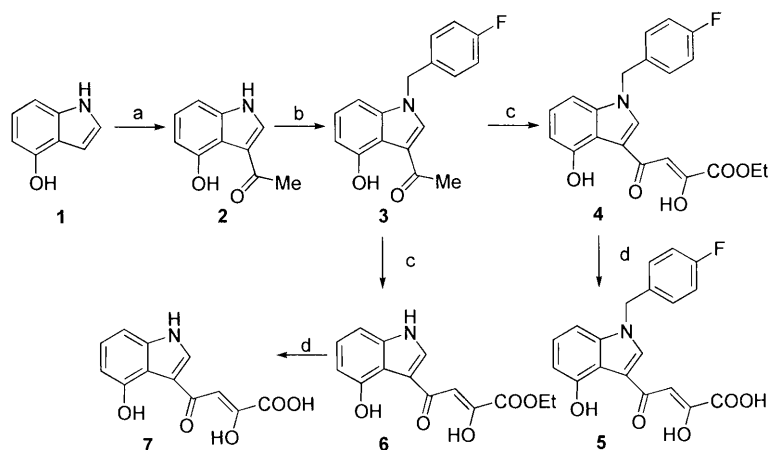
Figure 1. Structure-based pharmacophore model generated using LigandScout^[14] from the X-ray crystal structure of LEDGF (residues Ile365–Asp366) in complex with HIV-1 IN (PDB: 2B4J). Two hydrophobic groups (light yellow spheres), one H-bond donor (green arrow), two H-bond acceptors (red arrow) and nine excluded volumes (gray spheres) are shown.

base, which consists of 3055 small molecules synthesized and/or published by Chimirri and co-workers. The “fast flexible search” option in Catalyst 4.10 was selected. The search returned ~936 compounds, which contained the specified 3D location of chemical functions. A subset of 234 structures was created by considering only compounds with a Catalyst fitness score equal to or greater than 2.00.

The best fitness values were obtained for the series of benzylindoles previously reported by our group as strand-transfer IN inhibitors.^[11] In particular, the best fitness value (Fit = 2.8) was obtained for compound CHIBA-3002 (shown).



As a preliminary assessment, we tested CHIBA-3002 in the AlphaScreen assay and found that it inhibited the IN–LEDGF/p75 interaction by up to 46% at a concentration of 100 μ M. However, the mapping of this molecule into the obtained pharmacophore model lacked the feature needed to form the H-bond interaction with IN residue Gln168. Moreover, we noticed that the CHIBA-3002 phenyl group was very close to a region considered off limits to the ligands. Based on these observations, we reasoned that suitable chemical modifications of CHIBA-3002 might improve the fitness to the pharmacophore hypothesis, leading to improved small-molecule inhibitors of the IN–LEDGF/p75 interaction.



Scheme 1. Reagents and conditions: a) POCl_3 , $\text{CH}_3\text{CON}(\text{CH}_3)_2$, 12 h, RT; b) 4-fluorobenzyl bromide, K_2CO_3 , DMF, 10 min, 100°C , 100 W; c) diethyl oxalate, dry CH_3ONa , THF, two separate steps under the same conditions: 2 min, 50°C , 250 W; d) 2N NaOH, MeOH, 90 min, RT.

We rationally design and synthesized some CHIBA-3002 analogues (4–7, Scheme 1). The 2-hydroxy-4-(4-hydroxy-1*H*-indol-3-yl)-4-oxobut-2-enoic acids 5 and 7 (CHIBA-3003) were prepared following a procedure similar to that employed for hit compound CHIBA-3002.^[5,17] The commercially available 4-hydroxy-1*H*-indole (1) was acylated under Vilsmeier–Haack reaction conditions, using *N,N*-dimethylacetamide and phosphoryl chloride, to give compound 2. Ethyl esters 4 and 6 were synthesized from this key intermediate using microwave-assisted organic synthesis (MAOS). The 4-fluorobenzyl derivative 5 and unsubstituted analogue 7 were subsequently formed by base-catalyzed ester hydrolysis of 4 and 6, respectively.

As a first step, we decided to introduce a potential H-bond functionality, absent in CHIBA-3002, by inserting a hydroxy group at position C-4 of the indole system (compound 5). The alignment of compound 5 to the 3D model provided a higher fit value compared to CHIBA-3002 (3.6 vs 2.8), since, as hypothesized above, derivative 5 was able to fulfill the pharmacophore hypothesis. Derivative 7 was designed, incorporating the hydroxy group at position C-4 of the indole but lacking

the benzyl moiety. The fit value of this analogue (Fit=4.1) surpassed those of both compounds 5 and 7. This improvement could be due to the absence of steric hindrance caused by the benzyl group, and consequently enabling a better positioning of the molecule in the pharmacophore model (Figure 2).

The synthesized compounds 5 and 7 were subsequently tested in the AlphaScreen assay to evaluate their ability to disrupt the HIV-1 IN interaction with LEDGF/p75 IBD, and proved to be inhibitors of that specific PPI. In agreement with our hypothesis, compound 5 showed a higher potency than

CHIBA-3002, while compound 7 proved to be the most active molecule (Table 1). Ester derivatives 4 and 6 were also tested in the assay. Both esters showed lower activity than the corre-

Table 1. Inhibitory activities of CHIBA-3002 and compounds 4–7.

Compound	Inhibition ^[a] [%]	IC_{50} ^[b] [μM]
CHIBA-3002	46 %	–
4	NA	–
5	56 %	76
6	37 %	–
7 (CHIBA-3003)	71 %	35

[a] Measured at $100\ \mu\text{M}$. [b] Concentration required to inhibit the HIV-1 IN–LEDGF/p75 interaction by 50 %. NA = not active.

sponding acids. This result could be due to the steric hindrance of the alkyl group, preventing the molecule from adopting a suitable conformation so as to form H bonds between the ligand oxygen atoms and Glu 170 and His 171.

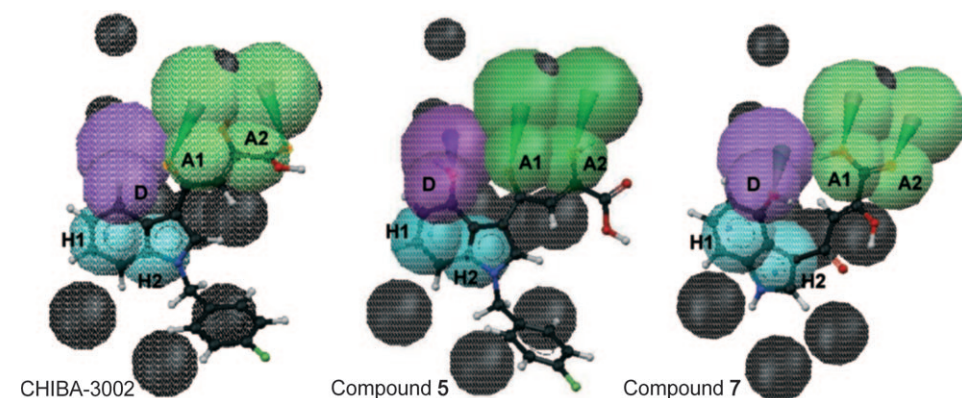


Figure 2. Mapping of compounds CHIBA-3002, 5 and 7 onto the Catalyst^[15] pharmacophore model. Pharmacophoric features: two hydrophobic groups (H1–H2, cyan), one H-bond donor (D, magenta), two H-bond acceptors (A1–A2, green) and nine excluded volumes (black).

As a final step, the possible binding mode of the most active derivative 7 against HIV-1 IN was investigated by performing automated docking studies using the software GOLD.^[12] Firstly, test docking calculations using the LEDGF dipeptide Ile365–Asp366 were carried out with the aim of comparing experimental and predicted binding modes and validating the docking protocol. The best

docking pose of the 19 clusters found for the LEDGF dipeptide agreed well with the experimental binding mode with a root-mean square deviation (RMSD) of 0.82.

The best docked conformation of compound **7** is shown in Figure 3a, together with the experimental position of the LEDGF residues Ile365–Asp366. Compound **7** interacted with the HIV-1 IN residues in a similar fashion to that of the LEDGF dipeptide. Indeed, in agreement with the mapping of the designed molecule to the 3D pharmacophore model, the carboxylate group interacted with the main chains of Glu170 and His171 by H-bond interactions, while the hydrophobic pocket formed by IN chain B residues was occupied by the fused ben-

zene ring of the indole system. Moreover, compound **7** formed a H bond with the backbone carbonyl group of IN amino acid Gln168 as expected, due to the presence of the hydroxy group at C-4 position. (Figure 3b) The docking results confirmed that CHIBA-3003 (**7**) could inhibit the IN–LEDGF/p75 interaction by mimicking some specific PPIs between the viral enzyme and its cellular cofactor.

Recently, a benzoic acid derivative was reported as an inhibitor of the IN–LEDGF/p75 interaction,^[2] although the proposed IN-binding site seems to be different from the one proposed in this manuscript.

Conclusions

This study identified small molecules able to inhibit IN–LEDGF/p75 interactions, through pharmacophore modeling, virtual library screening and rational drug design.

In particular, compound **7** could prove to be a valid starting point for the discovery of new and more potent derivatives able to disrupt PPIs between IN and its intracellular cofactor LEDGF/p75, potentially blocking HIV replication through a mechanism of action that differs to those of antiviral agents used currently. Furthermore, some derivatives were also active in the IN inhibition test suggesting a possible dual mode of action may be responsible for their potency. This could be particularly important considering the need for combined therapy targeting the different stages of the HIV life cycle to effectively suppress infection.

Further studies are in progress to improve the affinity of the inhibitors for the protein–protein interface, and to obtain anti-HIV agents active against multiple targets.

Experimental Section

Pharmacophore modeling and in silico screening

The software LigandScout^[14] was used for the detection and interpretation of crucial interaction patterns between IN and specific cofactor LEDGF/p75 residues. LigandScout allows the automatic construction and visualization of 3D pharmacophores from structural data of macromolecule–ligand complexes. Detected chemical features include H-bond donors and acceptors as directed vectors, positive and negative ionizable regions, as well as lipophilic areas represented by spheres. In order to increase selectivity, the LigandScout model also includes spatial information about potentially inaccessible areas for any ligand, thus taking into account any potential steric restrictions. In particular, excluded volume spheres placed in positions that are sterically forbidden are automatically added to the generated pharmacophore model.

We have used the crystal structure of the dimeric catalytic core domain of HIV-1 IN complexed with the recently reported LEDGF/p75 IN-binding domain (IBD)

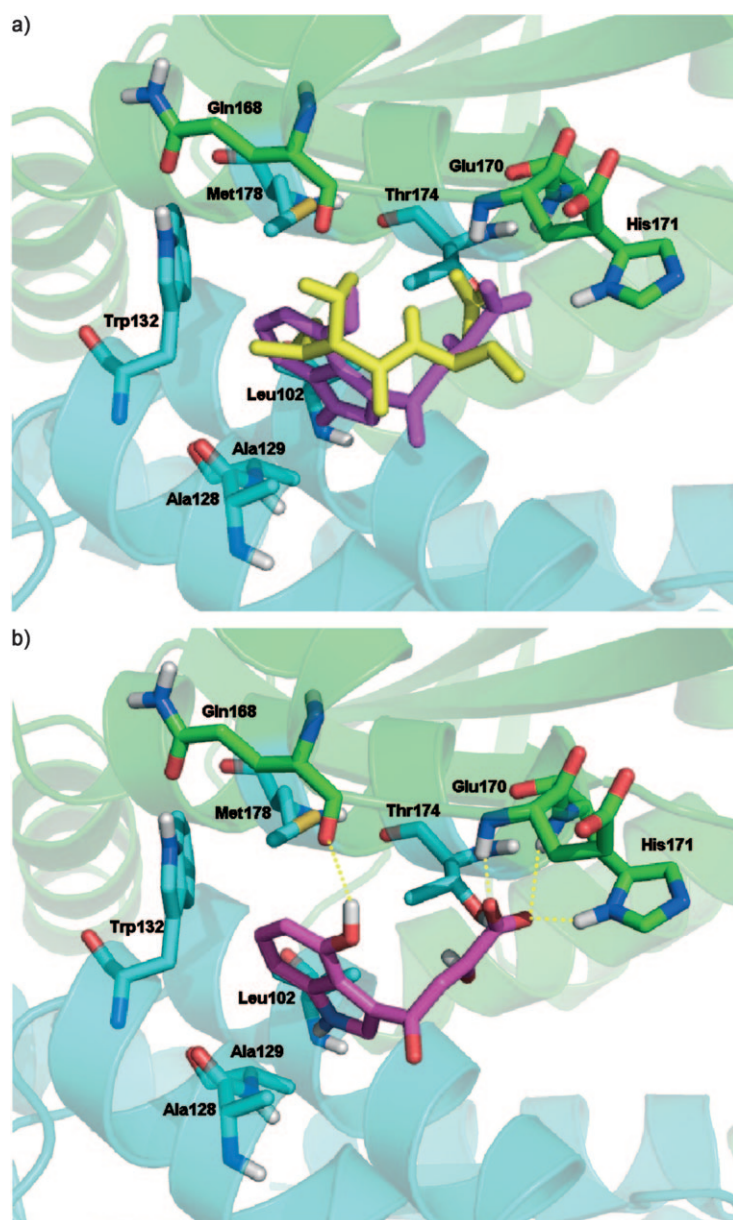


Figure 3. a) Predicted binding mode of compound **7** (violet) compared to the crystallized position of LEDGF residues Ile365–Asp366 (yellow). b) Compound **7** (colored by atom type) is involved in H-bond interaction (yellow dashed lines) with three IN residues (green), while IN residues constituting the hydrophobic pocket are in cyan. This figure was prepared using the PyMOL program.^[13]

(PDB code 2B4J).^[8] The macromolecule was represented by IN chains A and B, whereas the “ligand” was comprised of two important LEDGF/p75 IBD amino acids: Ile 365 and Asp 366 (chain C). The pharmacophore model obtained was converted into the correct format for the Catalyst software package version 4.10^[15] and then used as a query to screen the CHIME database containing 3055 of compounds. The structures in the virtual library CHIME were built using the 2D/3D editor sketcher and minimized using the CHARMM-like force field implemented within Catalyst 4.10. The best fit value for each compound in the CHIME database was calculated, allowing for a maximum of two features to be omitted. Finally, the molecules were ranked according to their best fit values. The designed molecules 4–7 were built as previously described for the CHIME compounds.

Molecular Docking experiments

The crystal structure of the dimeric catalytic core domain of HIV-1 IN complexed with the LEDGF/p75 IBD was retrieved from the RCSB Protein Data Bank (entry code 2B4J)^[8] and used as our docking simulation target. The LEDGF structure was removed and hydrogen atoms were added to the IN protein using the Biopolymer module in Sybyl 8.0.^[16] The structures of the ligands were constructed using standard bond lengths and angles from the Sybyl 8.0 fragment library and fully optimized by the semiempirical quantum mechanical method AM1. Coordinates for the LEDGF dipeptide Ile 365–Asp 366 were taken directly from chain C of the crystal structure (PDB: 2B4J) and used as a control of program performance. Docking studies were performed using the genetic optimization for ligand docking (GOLD) software package version 3.1.1 from the Cambridge Crystallographic Data Centre (CCDC).^[12] ChemScore was chosen as a fitness function and the standard default settings were used in all calculations. For each of the 100 independent genetic algorithm runs, a default maximum of 100 000 genetic operations was performed using the default operator weights and a population size of 100 chromosomes. Default cutoff values of 2.5 Å for H bonds and 4.0 Å for van der Waal interactions were employed. All single bonds were treated as rotatable. Results differing by less than 1.5 Å in ligand-all atom RMSD were clustered together. A 15.0 Å radius active site was drawn on the original position of the LEDGF/p75 IBD dipeptide Ile 365–Asp 366 and automated cavity detection was used.

Chemistry

General: All microwave-assisted reactions were carried out in a Discover focused microwave synthesis system (CEM, Matthews, NC, USA) working at 250–300 W. 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 $\pm 0.4\%$ of the theoretical values. Merck silica gel 60 F₂₅₄ 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 SP4 EXP. ¹H NMR spectra were recorded on a Varian Gemini-300 spectrometer in CDCl₃ with TMS as internal standard, or with DMSO as a reference solvent peak. Chemical shifts are expressed in δ (ppm) and coupling constants (*J*) are given in Hz.

3-Acetyl-4-hydroxy-1H-indole (2): POCl₃ (0.92 mL, 10 mmol) was added to *N,N*-dimethylacetamide (2.79 mL, 30 mmol) at 0 °C and stirred. 4-Hydroxy-1H-indole 1 (133.15 mg, 1 mmol) was added to the mixture and the reaction was stirred at RT for 12 h. The reaction mixture was poured over ice and basified with aq NaOH (4 N).

The mixture was extracted with EtOAc and the organic phase was dried (Na₂SO₄), filtered and concentrated in vacuo. The residue was crystallized from CH₂Cl₂ to give the title compound as a white solid (70%): mp: 200–202 °C; ¹H NMR: δ = 2.52 (s, 3H, CH₃), 6.46 (d, 1H, *J* = 7.96 Hz, ArH), 6.88 (d, 1H, *J* = 7.96 Hz, ArH), 7.06 (t, 1H, *J* = 7.96 Hz, ArH), 8.41 (s, 1H, ArH), 11.49 (s, 1H, OH), 12.02 ppm (br s, 1H, NH). Anal. calcd for C₁₀H₉NO₂: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.72; H, 5.34; N, 7.51.

3-Acetyl-1-(4-fluorobenzyl)-4-hydroxy-1H-indole (3): 3-Acetyl-4-hydroxy-1H-indole (2) (159 mg, 0.001 mol) was dissolved in DMF (2 mL) and K₂CO₃ (138 mg, 0.001 mol) was added. The mixture was stirred for 2 min and then 4-fluorobenzyl bromide (568 mg, 0.003 mol) was added dropwise. The resulting solution was placed in a quartz tube (\varnothing 2 cm) and irradiated in a microwave oven (100 W) at 100 °C for 10 min with stirring. A saturated NaHCO₃ solution was added and the mixture was extracted with EtOAc. The combined organic extracts were dried (Na₂SO₄), filtered and concentrated in vacuo. The residue was powdered by treatment with Et₂O and crystallized from EtOH or purified by flash chromatography (cyclohexane/EtOAc, 6:4) to give the title compound as a white solid (77%): mp: 138–140 °C; ¹H NMR: δ = 2.53 (s, 3H, CH₃), 5.28 (s, 2H, CH₂), 6.72–7.18 (m, 7H, ArH), 7.66 (s, 1H, ArH), 11.50 ppm (s, 1H, OH). Anal. calcd for C₁₇H₁₄FNO₂: C, 72.07; H, 4.98; N, 4.94. Found: C, 72.34; H, 5.13; N, 5.16.

Ethyl 4-[1-(4-fluorobenzyl)-4-hydroxy-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoate (4): Prepared following a previously reported procedure^[11,17] giving the title compound as a white solid (92%): mp: 264–266 °C dec; ¹H NMR: δ = 1.35 (t, *J* = 7.1 Hz, 3H, CH₃), 4.08 (q, *J* = 7.1 Hz, 2H, CH₂), 5.34 (s, 2H, CH₂), 6.21–8.15 (m, 9H, ArH and CH), 14.64 ppm (s, 1H, OH). Anal. calcd for C₂₁H₁₈FNO₅: C, 65.79; H, 4.73; N, 3.65. Found: C, 65.93; H, 4.62; N, 3.84.

4-[1-(4-Fluorobenzyl)-5-hydroxy-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoic acid (5): Prepared following a previously reported procedure^[11,17] giving the title compound as a white solid (90%): mp: 223–225 °C; ¹H NMR: δ = 5.45 (s, 2H, CH₂), 6.56–9.07 (m, 9H, ArH and CH), 11.26 ppm (s, 1H, OH). Anal. calcd for C₁₉H₁₄FNO₅: C, 64.23; H, 3.97; N, 3.94. Found: C, 64.48; H, 4.22; N, 3.78.

Ethyl-2-hydroxy-4-(1H-indol-3-yl)-4-oxobut-2-enoate (6): Prepared from intermediate 2 following the previously reported procedure^[11,17] used to obtain compound 4, giving the title compound as a white solid (84%): mp: 263–265 °C dec; ¹H NMR: δ = 1.24 (t, *J* = 7.1 Hz, 3H, CH₃), 4.08 (q, *J* = 7.1 Hz, 2H, CH₂), 5.37 (s, 1H, OH), 6.18–7.88 (m, 5H, ArH and CH), 11.25 (br s, 1H, NH), 14.45 ppm (s, 1H, OH). Anal. calcd for C₁₄H₁₃NO₅: C, 61.09; H, 4.76; N, 5.09. Found: C, 61.32; H, 4.91; N, 4.89.

2-Hydroxy-4-(4-hydroxy-1H-indol-3-yl)-4-oxobut-2-enoic acid (7): Prepared following a previously reported procedure^[11,17] used to obtain 5, giving the title compound as a white solid (82%): mp: 208–210 °C; ¹H NMR: δ = 6.54 (d, 1H, *J* = 8.11 Hz, ArH), 6.91 (d, 1H, *J* = 8.11 Hz, ArH), 7.11 (t, 1H, *J* = 8.11 Hz, ArH), 8.77 (s, 1H, ArH), 11.17 (s, 1H, OH), 12.63 ppm (br s, 1H, NH). Anal. calcd for C₁₂H₉NO₅: C, 58.30; H, 3.67; N, 5.67. Found: C, 58.46; H, 3.82; N, 5.44.

HIV-1 IN–LEDGF/p75 interaction screening

The AlphaScreen assay was performed as described previously.^[9] Reactions were performed in 25 μ L 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 MgCl₂, 0.01% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin. His₆-tagged inte-

grase (300 nM final concentration) was incubated with the compounds at 4 °C for 30 min. The compounds were added in varying concentrations (1–100 μM). Afterwards 100 nM of recombinant flag-LEDGF/p75 was added and incubation was extended by 1 h 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 $\mu\text{g mL}^{-1}$ 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.

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Keywords: antiviral agents • inhibitors • HIV-1 integrase • protein–protein interactions • molecular modeling

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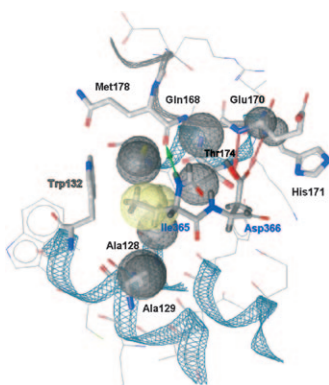
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FULL PAPERS

Anti-HIV agents: The identification of potential antiviral agents discovered using crystal data pharmacophore modeling, virtual library screening and rational drug design is described. These compounds are one of the first examples of small molecules able to inhibit HIV integrase–LEDGF/p75 interactions.



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**Pharmacophore-Based Discovery of
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Protein Interactions between HIV-1
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LEDGF/p75**