Inhibition of the Interaction between HIV-1 Integrase and its Cofactor LEDGF/p75: A Promising Approach in Anti-Retroviral Therapy

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Abstract: Although 25 compounds are currently licensed as anti-HIV drugs, the development of multidrug-resistant viruses, as well as their severe side effects, compromise their efficacy and limit treatment options.

The search for new targets in order to cure AIDS has revealed that the inhibition of some protein-protein interactions in the HIV life cycle may provide an important new approach to fight this disease.

The interaction between HIV-1 integrase (IN) and Lens Epithelium-Derived Growth Factor (LEDGF/p75) has increasingly gained attention as a valuable target for a novel anti-retroviral strategy.

This article reviews the discovery and development of molecules capable of interrupting the LEDGF/p75-IN interaction reported to date.

Keywords: AIDS, anti-retroviral strategy, HIV-IN, IBD, LEDGF/p75, PPIs.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a major causative agent of acquired immune deficiency syndrome (AIDS). The current treatment regimen for HIV-1-infected individuals utilises highly active anti-retroviral therapy (HAART). This is, however, linked to severe side effects [1]. Moreover, the suppression of HIV-1 replication is incomplete and the development of multidrug-resistant viruses compromises the efficacy of the drugs currently in use, therefore new different targets need to be identified [2].

HAART typically consists of a cocktail of several potent drugs that inhibit reverse transcriptase, protease and, more recently, also integrase (IN) and viral entry [3].

IN contains 288 amino acids and consists of three functional domains: the N-terminal domain (NTD, residues 1–50), which contains a zinc-binding motif; the catalytic core domain (CCD, residues 51–212), which contains the catalytic triad Asp64, Asp116, and Glu152 (DDE motif) that is essential for catalysis; and lastly, the C-terminal domain (CTD, residues 213–288), which binds DNA non-specifically [4-5].

This enzyme is responsible for proviral DNA integration, an essential step in the viral replication cycle. The process of DNA incorporation occurs in two spatially and temporally distinct steps known as 3'-processing and strand-transfer [6]. IN functions as a multimer; a dimeric species is required for 3'-processing, whereas a tetrameric arrangement is needed for strand-transfer [7]. IN is presumed to be in equilibrium between its monomeric, dimeric, tetrameric and high order oligomeric states. As only the dimer is able to bind the viral DNA, it therefore represents the active form of the enzyme [6].

IN interacts with different host cell proteins, including Lens Epithelium-Derived Growth Factor (LEDGF/p75) which recent results have highlighted as the dominant cellular binding partner of HIV-1 IN [8].

The IN-DNA complex is transported into the nucleus where two DNA-bound dimers form a tetramer in the presence of the cellular protein LEDGF/p75 thereby proceeding to the strand-transfer step which joins the viral DNA to the host chromosomal DNA forming a functional integrated proviral DNA.

Although protein–protein interactions are traditionally more challenging than enzyme active sites for targeted drug development, [9] specific molecules occupying a pocket at the IN dimer interface could prevent LEDGF from binding, and thereby affect HIV-1 replication. Compounds with this mechanism of action, i.e. one capable of interrupting the LEDGF/p75-IN interaction are reported herein.

BIOLOGY OF LEDGF/p75 AND ITS ROLE IN HIV INTEGRATION

Transcriptional coactivator p75, or LEDGF, is a pro-survival protein that is closely associated with condensed chromatin in the nucleus and is essential for nuclear localization and chromosomal association of HIV-1 IN [10].

LEDGF/p75 is 530 amino acids in length (Fig. **1**). It contains several domains with different functions (some of them unknown): a conserved N-terminal PWWP domain (residues 1–93) which is thought to mediate protein–protein and/or protein–DNA interactions [11-12] and three segments of relatively polar amino acids, known as CR (charged region) amino acids: CR1 (residues 94-142), CR2 (residues 208-265)

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and CR3 (residues 266-325) [13]. A putative transferable nuclear-localization signal (NLS) has been mapped to residues 146–156 [14-15] followed by AT-hook motifs (residues 178–197).

All of these above-described domains (residues 1-325) impart a preferential interaction with minor-groove AT-rich DNA regions and are thought to structurally affect accessibility for transcriptional machinery [16-17]. Some studies have indicated a cooperative role in chromatin association for multiple LEDGF/p75 domains, which, importantly, are upstream from the IN-binding domain (IBD), presenting a clear functional picture for LEDGF/p75-mediated chromatin tethering of IN [18-19].

The IBD of LEDGF/p75 ($LEDGF_{IBD}$) has been mapped to residues 347–429 in the C-terminal domain [14, 20], and interacts specifically with the IN catalytic core domain (IN_{CCD})[21].

It is well known that retroviruses, in common with some large DNA viruses, need a chromatin tethering mechanism to allow the viral cDNA to remain attached to chromatin as the latter undergoes spatial and/or compositional change. Moreover, mammalian genomic DNA is a vast target, a significant proportion of which is not transcriptionally active. Lentiviruses, including HIV (both type 1 and type 2), are strongly biased towards integration into transcription units (TUs), with a preference for highly expressed genes [23].

In particular, LEDGF/p75 binds strongly to chromatin in a cell cycle-resilient manner acting as a "chromosomal receptor" for IN [23].

In addition, the stability and nuclear accumulation of HIV-1 IN in human cells are both drastically impaired by LEDGF depletion. These results suggest that LEDGF might tether IN to host cell chromatin and that it may also be involved in its nuclear import and protection from proteasomal degradation [24-25].

LEDGF also strongly modulates the dynamic structure of HIV-1 IN by stabilizing subunit-subunit interactions and promotes IN tetramerization [26-27].

These studies thus confirm the essential role of LEDGF/p75 during HIV integration [13, 24, 28].

As regards the cell biology of LEDGF/p75, it has been reported to be a member of the hepatoma-derived growth factor (HDGF) related protein (HRP) family. Six human HRP family members have been described: HDGF, HRP1, HRP2, HRP3, LEDGF/p75 and LEDGF/p52 [20, 29-30] of which two, LEDGF/p75 and HRP2, possess affinity for HIV-1 IN [25]. LEDGF/p75 and HRP2 are significantly larger than the other HRPs and contain, within their extended C-terminal domains, a second evolutionarily conserved domain, the IBD, which mediates the interaction with HIV-1 IN [14]. LEDGF/p75 and LEDGF/p52 are expressed from the same gene (human PSIP1) [31]; the smaller p52 isoform is produced by alternative RNA splicing and lacks the IBD and fails to engage HIV-1 IN *in vitro* or in live cells [21, 24].

The development of anti-HIV drugs targeting integrase-LEDGF/p75 interaction requires a profound knowledge of the cell biology of LEDGF/p75 to avoid cellular toxicity. Thus, considering that the role of LEDGF in the lentiviral life cycle is well characterized, whereas far less is known about its natural cellular functions, the aim of drug discovery should be the inhibition of protein–protein interaction with HIV IN without affecting the cellular function of LEDGF/p75. [13, 24, 31-33]

The $LEDGF/p75_{IBD}$ has been shown to interact with many other proteins in cells and its overexpression has been reported to upregulate a subset of genes involved in the stress response and to protect cells from apoptosis [34]. Hence, LEDGF appears to act as a multifunctional adaptor and to have the ability to tether to chromatin a plethora of cellular machinery involved in genome expression and maintenance [35].

INTERACTIONS BETWEEN LEDGF/p75 AND HIV INTEGRASE

LEDGF/75 specifically interacts with the HIV-1 IN catalytic core domain (IN_{CCD}) *via* its small domain (IBD), approximate 80-residue, within its C-terminal region [14, 20], but some investigations have revealed that all three IN domains (CCD, NTD and CTD) play a key role.

$LEDGF_{IBD}$-IN_{CCD}

The $LEDGF_{IBD}$ is both necessary and sufficient for the interaction with HIV-1 IN [20]. Cherepanov *et al*. reported that the crystal structure of the dimeric catalytic core domain of HIV-1 IN complexed to the IBD of LEDGF (PDB code 2B4J) [36].

The asymmetric unit of the crystals contains a dimer of IN_{CCDs} (chains A and B) and a pair of $LEDGF_{IBD}$ molecules (chains C and D) bound at the CCD dimer interface (Fig. **2A**).

Typically, the formation of protein complexes is not accompanied by major structural changes in the interacting

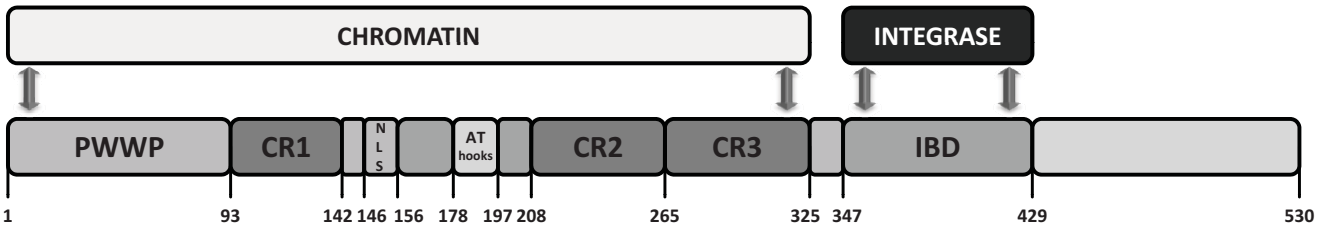


Fig. (1). Domain organization of LEDGF/p75: specific interactions with chromatin and integrase. LEDGF/p75 is essential for the chromosomal targeting of HIV-1 IN and represents an example of a host factor controlling the integration sites of HIV-1 in human cells [22].

proteins [37]. Similarly, binding of the $LEDGF_{IBD}$ does not significantly alter the structure of the CCD. Rotation of the Trp131 side chain is the only significant adjustment found to occur upon complex formation [38].

The side chain of LEDGF residue Ile365 projects into a hydrophobic pocket formed by the IN B-chain residues Leu102, Ala128, Ala129, and Trp132 and A-chain residues Thr174 and Met178.

LEDGF residues Phe406 and Val408 contact and occlude solvent from the exposed Trp131 in the IN B-chain. In addition,LEDGF Asp366 forms a bidentate hydrogen bond to the main chain amides of IN residues Glu170 and His171 in the A-chain. These interactions presumably neutralize the negative charge of the Asp366 carboxylate in an environment that is largely excluded from solvent.

The backbone amide of LEDGF residue Ile365 also establishes a hydrogen bond to the backbone carbonyl group of Gln168 in the A-chain.

Of the three water molecules that are buried within the IN_{CCD}-$LEDGF_{IBD}$ interface, one is ideally positioned to make bridging hydrogen bonds between the backbone carbonyl groups of LEDGF residue Ile365 and IN B-chain residue Thr125.

A well-defined salt bridge exists between Lys364 of LEDGF and A-chain IN residue Glu170. In addition, Lys360 of LEDGF is in close proximity to A-chain residue Asp167 (Fig. **2B**) [36].

These contacts are in agreement with some mutagenesis studies and a number of single amino acid substitutions within the IN_{CCD} and $LEDGF_{IBD}$ have shown the importance of these residues. It has been reported that the mutation of LEDGF residues Ile365, Asp366, or Phe406 ablates the interaction with HIV-1 IN and that the substitution of Val408 significantly reduces binding. In particular, LEDGF Asp366Ala and Asp366Asn mutants fail to bind IN, indicating that the double hydrogen bond is essential for recognition [39]. Furthermore, substituting Ala for either Ile365 or Phe406 ablates the IN–LEDGF/p75 interaction *in vitro*, thereby identifying these amino acids together with Asp366 as hotspot contact residues [3, 39].

In contrast, mutant proteins substituted with Ala at either Lys364 or Lys360 retain binding to IN, thus these residues do not appear to contribute significantly to overall binding affinity [39].

The IN_{CCD}-$LEDGF_{IBD}$ interface is likely to represent a portion of the total contact area between full-length IN and LEDGF proteins.

$LEDGF_{IBD}$-IN_{NTD}

Although the IN_{CCD} suffices for binding to LEDGF, the NTD His12Asn mutation or deletion of the entire Zn^{2+}-binding domain of IN greatly reduces the affinity of the interaction [21], suggesting that the NTD of IN (IN_{NTD}) also contributes to interactions with LEDGF.

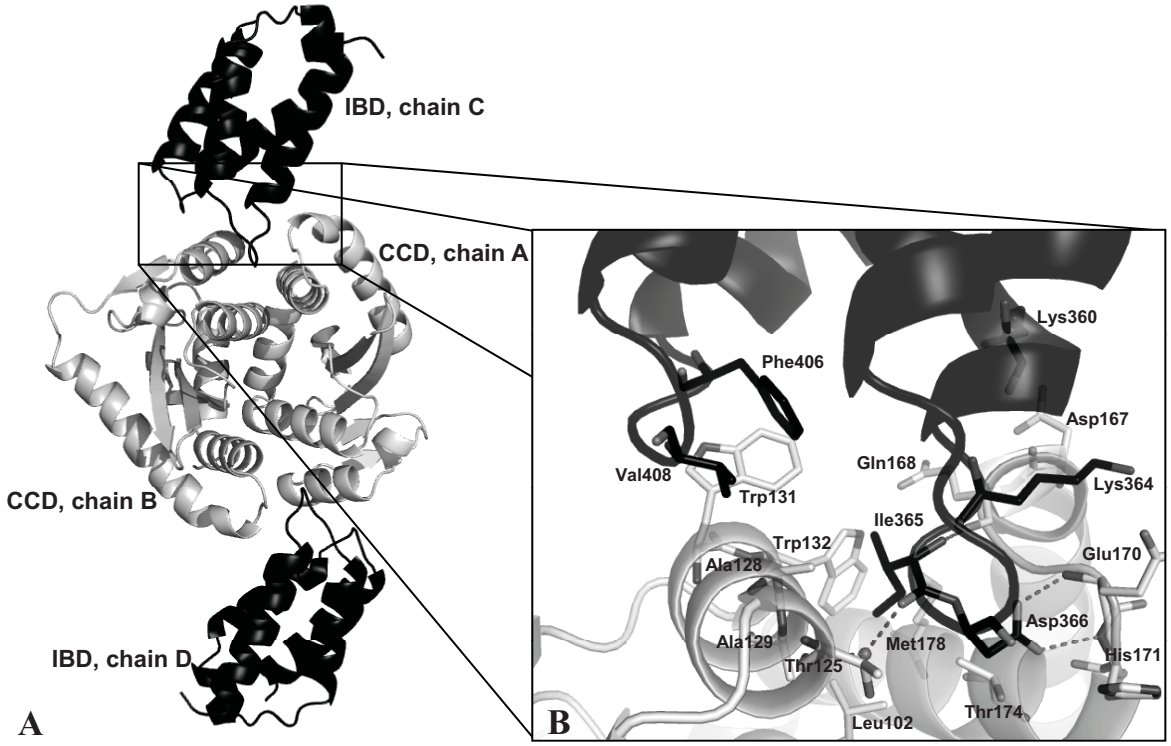


Fig. (2). Structure of the HIV-1 IN_{CCD} in complex with LEDGF/$p75_{IBD}$ (PDB code 2B4J). (**A**) The IN_{CCD} dimer (chains A and B) is coloured grey and the LEDGF/$p75_{IBD}$ subunits (chains C and D) are coloured black. (**B**) Close-up view of the interface: key residues are shown as sticks in two different colours: grey (IN_{CCD} residues) and black ($LEDGF_{IBD}$ residues). Hydrogen bonds are indicated by dotted lines; the water molecule hydrogen-bonded to main chain carbonyl groups of LEDGF residue Ile365 and IN residue Thr125 is shown as a sphere.

Hare *et al*. presented a crystal structure containing the IN_{CCD} and IN_{NTD} of HIV-2 IN in complex with the $LEDGF_{IBD}$ (PDB code 3F9K), extending the known lentiviral IN-LEDGF interface (Fig. **3A**) [40].

The IN_{NTD}-$LEDGF_{IBD}$ interface is essential for high affinity binding and stimulation of concerted DNA integration.

HIV-1 and HIV-2 INs share ~60% amino acid sequence identity over the span of their CCDs. Consequently, the contacts between the HIV-2 IN_{CCD} and the $LEDGF_{IBD}$ are very similar to those observed in the HIV-1 IN_{CCD}-$LEDGF_{IBD}$.

In agreement with prior biochemical analyses [21], the HIV-2 IN_{NTD} also makes extensive contacts with LEDGF. A constellation of acidic residues on the first helix (α1) of the HIV-2 IN_{NTD} (Glu6 [Asp6 for HIV-1], Glu10, and Glu13) faces positively charged residues on the α4 helix of the $LEDGF_{IBD}$ (Lys401, Lys402, Arg404, and Arg405). Side chains of LEDGF residues Lys401, Arg404, and Arg405 are well-ordered, and a well-defined salt bridge involves the IN residues Glu10 and Arg405 of LEDGF. (Fig. **3B**)

The closely positioned and highly conserved IN residue Glu1 is not involved in the interface and instead interacts with Lys25 and Lys186 of the same IN chain, supporting NTD structural integrity and hence overall stability of the IN dimer in complex with LEDGF. Moreover, the domain–domain interfaces observed in the crystal structure have been targeted by mutagenesis to prove their functional relevance [40].

$LEDGF_{IBD}$-IN_{CTD}

A study has recently been published concerning the involvement of IN_{CTD} in the interaction with LEDGF/p75, in which the authors observe that the presence of $LEDGF/p75_{IBD}$ in infected cells prevents access to the IN_{CTD} because it is masked in the presence of $LEDGF/p75_{IBD}$ [41].

Tintori *et al*. performed Molecular Dynamics (MD) simulations both on the free tetrameric form of IN and on the tetrameric form of IN bound to two $LEDGF/p75_{IBD}$ molecules [6]. Taking into account that the structure of full-length HIV-1 IN had yet to be determined, a whole IN tetramer was constructed by assembling the available structures in the Protein Data Bank, as already reported by other authors [42]. This study suggested that the two molecules of LEDGF/p75 were able to stabilize the tetramer by reducing its C-terminal fluctuations [6].

LEDGF-IN INTERACTION INHIBITORS

The significant reductions in HIV-1 infectivity observed in cells that are extensively depleted of LEDGF/p75 highlighted protein–protein interaction (PPI) as a novel target for the development of antiviral drugs. Indeed, some peptides and several small molecules have been reported to be inhibitors of the PPI between IN and LEDGF/p75.

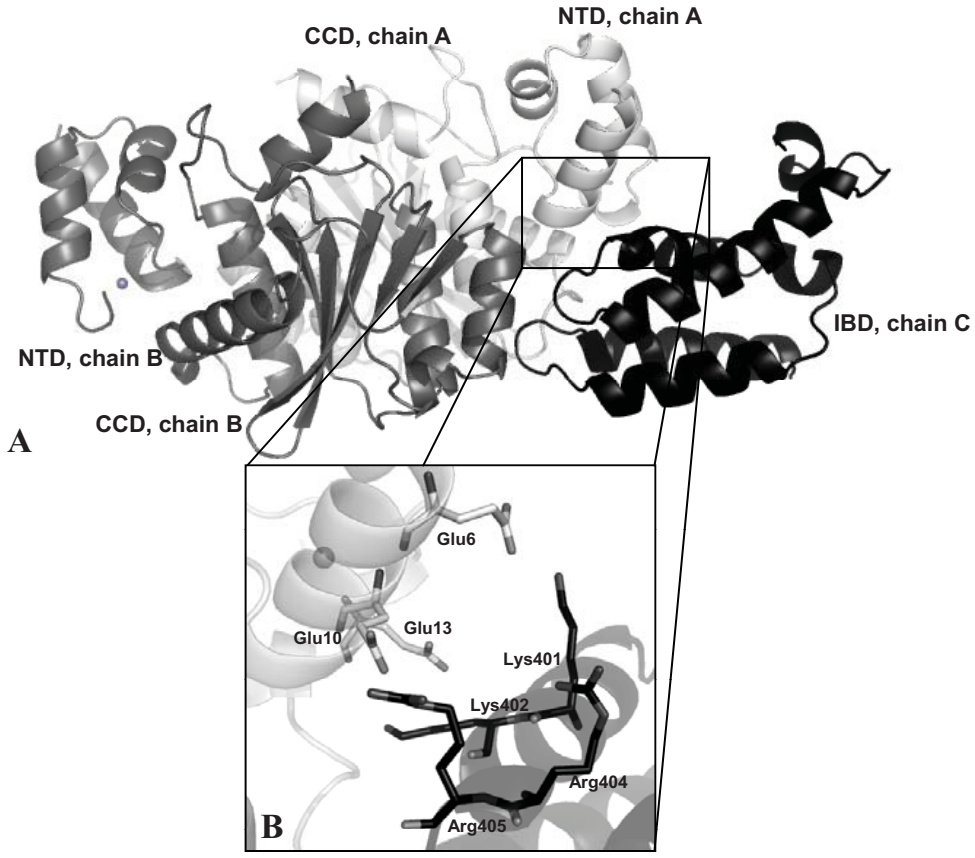


Fig. (3). Structure of the HIV-2 $IN_{CCD+NTD}$ in complex with $LEDGF/p75_{IBD}$ (PDB code 3F9K). (**A**) The $IN_{CCD+NTD}$ dimer (chains A and B) is coloured grey and the $LEDGF/p75_{IBD}$ subunit (chains C) is coloured black. (**B**) Close-up view of the interface: key residues are shown as sticks in two different colours: grey (IN_{NTD} residues) and black ($LEDGF_{IBD}$ residues). Zinc atoms are shown as grey spheres.

Linear Peptide Inhibitors

LEDGF$^{355–377}$

Al-Mawsawi *et al*. examined the biological activity of the LEDGF/p75 peptide ^{355}IHAEIKNSLKIDNLDVNRCIEAL^{377}, which contains two IN interacting residues (Ile365 and Asp366) first identified in the IN binding domain solution structure [43].

The LEDGF/p75 peptide modestly inhibited the IN catalytic activity dependent on the state of IN–DNA complex formation. It appears that the entire LEDGF/p75 protein may be required for strand-transfer stimulation, and the one LEDGF/p75–IN hotspot represented by this peptide is not sufficient for stimulation [43]. The LEDGF/p75 peptide was completely inactive when tested against Ca^{2+} induced pre-assembled IN–DNA complexes indicating that the peptide may exert its inhibitory mechanism of action by disrupting initial DNA binding necessary to IN for 3'-processing catalysis.

Peptide IC_{50} values were 165 μM and 153 μM, for WT 3'-processing and strand-transfer, respectively and were also effective in inhibiting the direct interaction between IN and LEDGF with an IC_{50} value of 25 μM [43].

The activity of the LEDGF/p75 peptide on IN mutant proteins, which are unable to catalyze the DNA strand-transfer reaction, has also been investigated. The LEDGF/p75 peptide displayed increased potency on these IN proteins, from five-fold to over ten-fold, indicating that the IN multimeric state greatly influences the peptide inhibitory effects [44-45]. These results shed light on IN–DNA multimeric formation, and how this process influences the LEDGF/p75–IN interaction.

LEDGF$^{361–370}$

Hayouka *et al*. demonstrated that a short LEDGF/p75$_{IBD}$ derived peptide (361–370) inhibited IN catalytic activity *in vitro* (both the 3'-processing and strand-transfer) as well as HIV-1 replication in infected cells [43, 46].

The LEDGF$^{361–370}$ mechanism of action was explored using biophysical, biochemical and cellular assays and demonstrated that all residues of this peptide contribute to IN binding and inhibition [47].

An alanine scan of LEDGF$^{361–370}$ was performed in order to determine precisely which residues participate in IN binding and showed that most of the alanine substituted peptides inhibited IN catalytic activity in the same manner as the parent LEDGF$^{361–370}$ peptide. This indicated that the substitutions had almost no effect on activity, and that no single residue was solely responsible for the inhibitory activity. These results are consistent with fluorescence anisotropy and NMR studies performed on the peptide [47].

A kinetic study of HIV-1 inhibition in cells by LEDGF$^{361–370}$ performed to further establish at which viral replication cycle stage the peptide acts confirmed that it inhibits HIV-1 replication in cells by specifically inhibiting the integration stage. In particular, LEDGF Asp366 is central in the interaction between the LEDGF and IN proteins. When Asp366 was replaced by alanine, the peptide still bound IN and inhibited its activity by 60%. The observed reduction but insignificant inhibition of viral DNA burdens in LEDGF$^{361–370}$ treated mice indicates that this linear peptide does not block virus entry into the cells or other pre-RT steps of virus replication *in vivo*. On the other hand, LEDGF$^{361–370}$ was found to be highly effective in blocking *de novo* synthesis of viral RNA *in vivo* indicating an effect on both the first cycle of virus infection *in vivo* and on subsequent virus spread.

Since viral RNA synthesis occurs after proviral DNA integration, this result is consistent with LEDGF$^{361–370}$ acting during integration or transcription of viral RNA. On this basis, the authors concluded that the linear peptide LEDGF$^{361–370}$ has potential as an anti-HIV drug lead [47].

LEDGF$^{402–411}$

The biological activity of LEDGF$^{402-411}$ was evaluated and it was shown to inhibit the enzymatic activity of IN more potently than LEDGF$^{361–370}$, although its affinity to IN was three-fold weaker (K_d = 3.7 and 12 μM, respectively). This observation is because binding affinity is not the only factor that is responsible for the inhibitory activity of a molecule (K_d and K_i are different in many cases) [46].

Cyclic Peptide Inhibitors

The fact that linear peptides like LEDGF$^{361-370}$ are susceptible to rapid proteolytic degradation limits their use as therapeutic agents [48-49], has led to various chemical modifications in order to improve their stability, selectivity, activity and bioavailability [50-53].

The most important results have been obtained using backbone cyclization (BC), a method that combines N-alkylation and cyclization [50-52, 54-59].

For this reason, a BC conformational library of LEDGF$^{361-370}$ derivatives, in which all peptides have exactly the same amino acid sequence, has been used to identify the cyclization mode that will confer a bioactive conformation on the peptide [60].

The screening of this library resulted in a peptide (Fig. **4**), termed *c*(MZ 4-1), which bound IN with affinity in the low micromolar range, penetrated cells and inhibited IN catalytic activity *in vitro* and HIV-1 replication in cells with the same potency but with significantly improved stability compared to the linear parent peptide LEDGF$^{361-370}$ [60].

The solution NMR structures of *c*(MZ 4-1), solved both in its free form and when interacting with IN, revealed that its bioactive conformation resembles the conformation of the corresponding residues in LEDGF/p75.

Recently, the crystal structure of the Prototype Foamy Virus (PFV)-IN tetramer, which is homologous to the HIV-1 IN, has been solved in the presence and absence of the two potent IN strand-transfer inhibitors currently in clinical use (MK-0518 and GS-9137) [61]. The structural alignment of the docking model of the IN-*c*(MZ 4-1) complex with the structures of the HIV-1 IN-LEDGF/p75$_{IBD}$ complex (PDB code 2B4J) [36] and with the structures of PFV-IN in complex with the MK-0518 and GS-9137 (PDB code 3L2T, 3L2U, respectively) [61] revealed a different binding site for *c*(MZ 4-1). Due to its ability to mimic the bioactive confor-

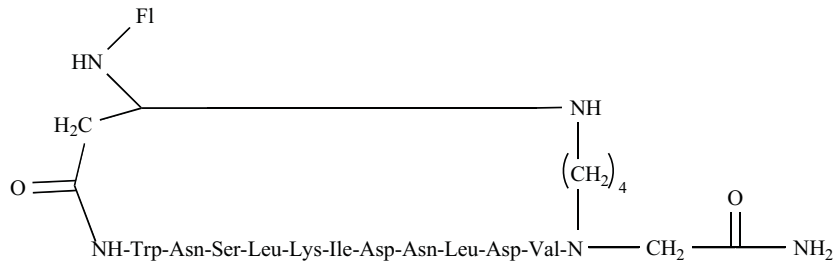


Fig. (4). Chemical structure of the fluorescein-labeled cyclic peptide *c*(MZ 4-1).

mation of the IN-binding loop in LEDGF/p75, *c*(MZ 4-1) is better than its linear parent peptide and has a significantly increased stability *in vitro* and in cells because of the cyclization, as shown by its ability to retain full potency eight days post infection [60].

SMALL MOLECULES

The flatness of protein-protein interfaces usually makes it difficult to identify any small molecules disrupting protein-protein interactions (PPIs). However, $LEDGF_{IBD}$ binds to a defined pocket at the interface of the two IN_{CCDs} therefore it should not be difficult to find small molecules with sufficient binding affinity to occupy the pocket thus preventing LEDGF/p75 from binding [11].

Tetraphenylarsonium and Tetraphenylphosphonium Derivatives

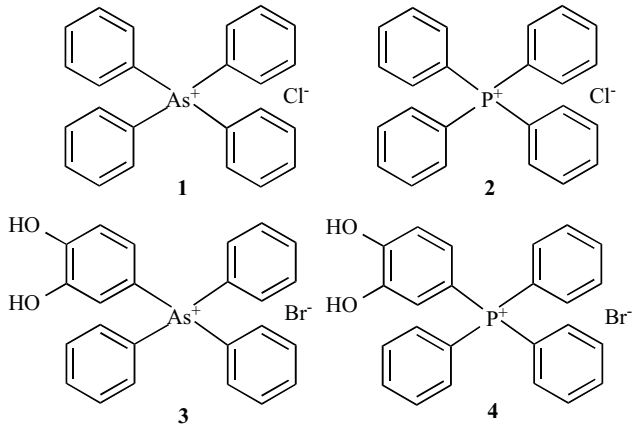
Molteni *et al*. [62] identified compounds **1-3** (Table **1**) that bind at the dimer interface of the HIV-1 IN_{CCD}, in a small cleft of about 5Å deep. They chose compounds that were able to fill a negatively charged pocket located on the protein surface comprising the active site.

In particular, they reported the identification, by X-ray crystallography, of two small molecules, tetraphenylarsonium **1** (PDB code 1HYV) and dihydroxyphenyltriphenylarsonium **3** (PDB code 1HYZ). They have a positively charged arsenic atom surrounded by four aromatic groups that are able to fill the identified pocket.

These compounds were both defined as IN inhibitors, although only compound **3** weakly inhibited *in vitro* enzymatic activity of IN (Table **1**). Nevertheless, the small molecule binding site evidenced in this study is the same binding cleft that we now know to be the interaction site between $LEDGF/p75_{IBD}$ and IN_{CCD}.

The X-ray of compound **1** showed a strong ion-dipole interaction with the carbonyl O atom of Gln168 (A-chain of the integrase dimer) which pointed directly towards the arsenic centre. In addition, two Trp residues (Trp131 and

Table 1. HIV-1 Inhibition of Tetraphenylarsonium (1,3) and Teytaphenylphosphonium (2,4)



Compound	Full length IC_{50} (μM)		Core 50-212 IC_{50} (μM)
	TC	ST	
1	> 240	> 240	> 5
2	> 200	> 200	> 5
3	150	150	380
4	13.5	13.5	200

TC= terminal cleavage; ST= strand-transfer; DIS= disintegration.

Trp132) (B-Chain of the integrase dimer) stacked with one of the phenyl rings of derivative **1** (Fig. **5**).

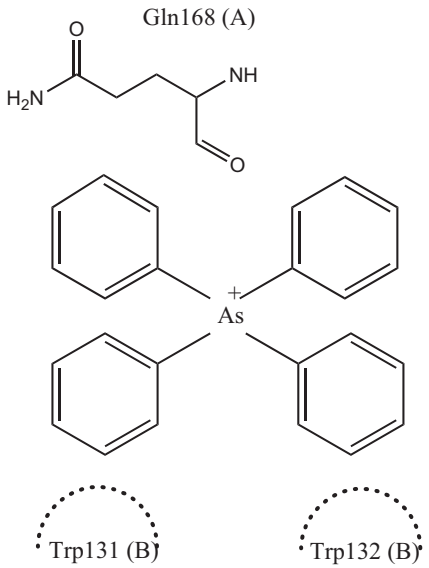


Fig. (5). Interaction of compound **1** with IN_{CCD} based on the crystal structure.

The dihydroxyphenyltriphenylarsonium **3**, in which a catechol group was present, was in the same position as derivative **1** but the four phenyl rings were less well-defined than for compound **1**. It was therefore not possible to establish the position of the catechol. Comparison of the two complexes, however, indicates significant differences between the two compounds. In the case of compound **3**, binding caused a structural change far from its binding site and better activity (IC_{50}= 150 μM).

With the goal of increasing potency, further derivatives were synthesized. Substituting phosphonium for arsonium yielded a compound (**4**) that was tenfold more active with an IC_{50} in the micromolar range (Table **1**) this being the most significant improvement.

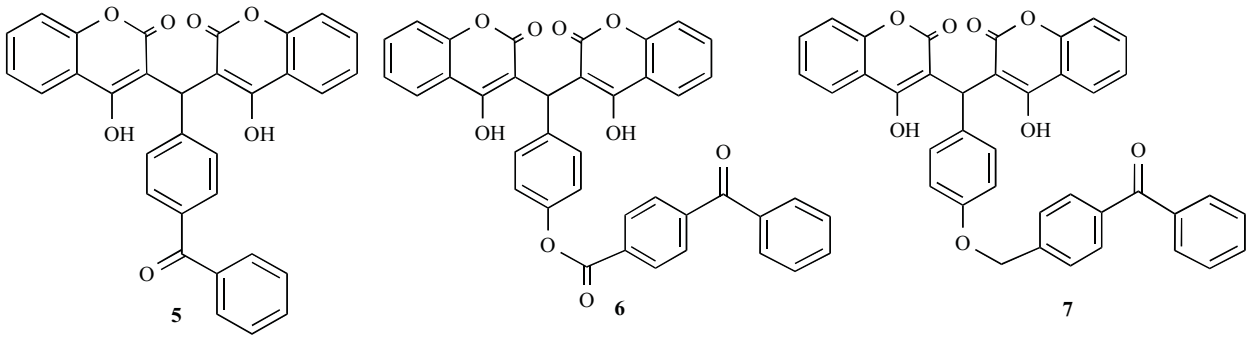
Hydroxycoumarin Derivatives

Using photoaffinity labeling and mass spectrometry, Al-Mawsawi *et al.* [63] identified, for a set of coumarin IN inhibitors, a binding site on HIV-1 IN which, as reported by Busschot *et al*. [13], overlaps with the binding cleft of LEDGF/$p75_{IBD}$.

Compound **5** was covalently photo-crosslinked with the IN_{CCD} and subjected to HPLC purification. Fractions were subsequently analyzed by MALDI-MS (Matrix Assisted Laser Desorption Ionization Mass Spectometry) and electrospray ionization (ESI)-MS to identify photo-crosslinked products. A single binding site for compound **5** located within the tryptic peptide ^{128}AACWWAGIK^{136} was thereby identified. Site-directed mutagenesis followed by *in vitro* inhibition assays resulted in the identification of two specific amino acid residues, Cys130 and Trp132, in which substitutions resulted in a marked resistance to the IN inhibitors **5-7**.

In order to distinguish key binding interactions between the IN core domain, particularly with respect to the peptide

Table 2. IN Inhibitory Effect (IC_{50} μM Values) of Hydroxycoumarin Derivates 5-7



Compound	WT	Cys130Ala	Cys130Ser	Trp132Ala	Trp132Gly	Trp132Arg	Trp132Tyr
3'- Processing							
5	27+/-3	31+/-3	62+/-11	80+/-8	97+/-3	47+/-7	21+/-4
6	14+/-0	9+/-1	35+/-6	35+/-9	77+/-7	22+/-5	5+/-2
7	18+/-1	30+/-3	58+/-9	38+/-11	71+/-10	24+/-2	8+/-1
Strand-transfer							
5	18+/-3	24+/-5	12+/-4	NA	NA	NA	18+/-1
6	11+/-4	6+/-1	2+/-1	NA	NA	NA	6+/-1
7	17+/-<2	10+/-<1	4+/-2	NA	NA	NA	13+/-4

^{128}AACWWAGIK^{136} region and compound **5**, the authors docked this derivative onto the IN_{CCD} dimer (PDB code 1BL3) [64] carrying both WT sequence and two Trp132 mutations (Trp132Gly and Trp132Tyr).

Compound **5** binds to the peptide ^{128}AACWWAGIK^{136} and disrupts the formation of a catalytically functional tetrameric IN. The benzophenone moiety of compound **5** occupies an area close to the backbone of amino acid residues Ala133 and Gly134.

Lastly, molecular modeling studies suggested that the coumarins exhibit their inhibitory effects by causing a steric obstruction at the dimer interface of IN, which could lead to an arrest in the formation of an enzymatically functional multimeric complex.

The biological evaluation of coumarin derivatives showed that these compounds inhibit IN-mediated catalytic activities with similar IC_{50} values, from 14 to 27 μM (Table **2**).

Benzoic Acid Derivative

The first small molecule expressly studied for its inhibitory activity against IN-LEDGF/p75 interaction was a benzoic acid derivative, 4-[(5-bromo-4-{[2,4-dioxo-3-(2-oxo-2-phenylethyl)-1,3-thiazolidin-5-ylidene]methyl}-2-ethoxyphenoxy)methyl]benzoic acid **D77** (**8**) (Fig. **6**) from the SPECS bank [65].

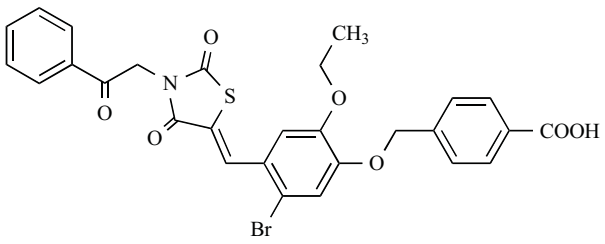


Fig. (6). Chemical structure of **D77** (**8**).

D77 (**8**) showed strong inhibition against IN-LEDGF/p75 interaction (in yeast two-hybrid assay and mammalian two-hybrid assay) as well as antiretroviral activity.

Molecular docking with a site-directed mutagenesis investigation and surface plasmon resonance (SPR) assays provided a possible binding mode. The crystal structure of IN_{CCD} in complex with IBD (PDB code 2B4J) was used to perform docking studies of **D77**. The results revealed that compound **8** was located at the interface of IN_{CCD} dimer with $LEDGF_{IBD}$, but this did not influence IN dimerization, suggesting that the inhibition of **D77** against IN-LEDGF interaction was not caused by the disruption of IN dimerization.

Docking results highlighted that **D77** (**8**) was able to occupy the hydrophobic pocket located between IN B-chain residues Ala98, Leu102, Ala128, Ala129, Trp131, and Trp132 and IN A-chain residues Ala169 and Met178.

The carboxylate group of **D77** formed hydrogen bonds with the side chain of IN residue Thr174 in A-chain and the side chain of IN residues Gln95 and Thr125 in B-chain.

There were hydrophobic interactions between the indole group of Trp131 in B-chain and benzene rings 1 and 2 (Fig. **7**). From the docking results, residues Trp131, Thr125, Gln95 in B-chain and Thr174 in A-chain were discovered to play important roles in the binding of D77 (**8**) to IN_{CCD}.

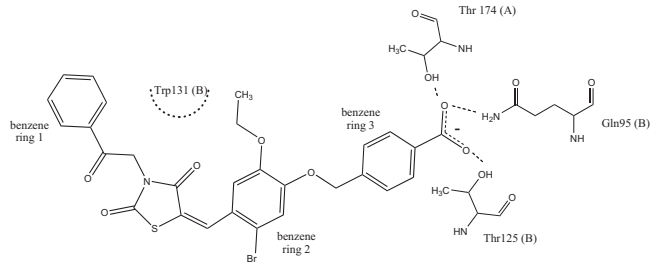


Fig. (7). Schematic view of protein-**D77** interactions based on the docking studies.

Site-directed mutagenesis technique with SPR assays were performed in order to validate the binding site of D77 (**8**) against HIV-1 IN. During the assays, four important residues, Gln95, Thr125, Trp131 and Thr174, were substituted by alanine. Substitutions of alanine for Thr125 were able to significantly reduce D77 (**8**) binding to IN, and the mutation of Gln95Ala, Trp131Ala and Thr174Ala were able to almost abolish D77 binding to IN.

Compound **8** was able to bind to HIV-1 IN and $IN^{52–210}$ by K_D value at 5.81 and 6.83 μM, respectively, which strongly supported that D77 (**8**) interacted with HIV-1 IN_{CCD}.

The results also showed that compound **8** was able to inhibit HIV-1 replication with EC_{50} value of 39.90 μM in MT-4 cell (8.43 μM for C8166 cells) [66] and cytotoxicity was observed with 50% cytotoxic concentration (CC_{50}) value of 128.79 μM in mock-infected MT-4 cells (44.19 μM for C8166 cells)

Indole Derivatives

Recently De Luca *et al*. reported the identification of several molecules acting as inhibitors of protein-protein interactions between HIV-1 IN_{CCD} and $LEDGF_{IBD}$ [67-68]. This work focused on the crystal structure of the dimeric HIV-1 IN_{CCD} complexed to the $LEDGF_{IBD}$ (PDB code 2B4J) that highlighted the most important interactions between these two domains [36]. In particular, it found the $LEDGF_{IBD}$ residues involved in the interaction to be located in its interhelical loop regions and four relevant amino acid residues, Ile365, Asp366, Phe406, and Val408, to be essential for direct contact with IN_{CCD} residues. These data were used to generate a structure-based pharmacophore model consisting of 14 features: one hydrogen bond donor (reflecting NH of Ile365 of $LEDGF_{IBD}$, that interacts with the oxygen atom of carbonyl group of Gln168 of IN_{CCD}), two hydrogen bond acceptors (mimicking two oxygen atoms of the carboxylate group of Asp366 of $LEDGF_{IBD}$ making a bidentate hydrogen bond to the main chain amides of Glu170 and His171 of IN_{CCD}), two hydrophobic groups (Ile365 side chain of $LEDGF_{IBD}$) and nine excluded volumes (potential steric restrictions) (Fig. **8**) [67].

The 3D pharmacophore model was therefore used as a query in a virtual screening approach to filter the in-house CHIME database (consisting of a collection of small molecules synthesized in Chimirri's laboratory) and some derivatives were identified. The best result was obtained with derivative (2*Z*)-4-[1-(4-fluorobenzyl)-1*H*-indol-3-yl]-2-hydroxy-4-oxo-but-2-enoic acid **CHIBA-3002** (**9**) (Fig. **9**), previously reported as an active IN strand-transfer inhibitor [70-71]. When tested with AlphaScreen assay, derivative **9** inhibited the IN-LEDGF/p75 interaction by up to 46%, at 100 μM concentration.

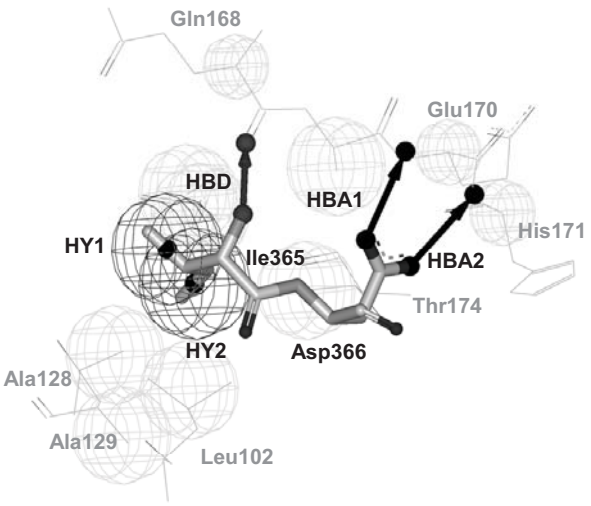


Fig. (8). Structure-based pharmacophore model generated using LigandScout program [69] from the X-ray crystal structure of LEDGF (residues Ile365–Asp366) in complex with HIV-1 IN (PDB code 2B4J). Two hydrophobic groups (HY1 and HY2 spheres), one H-bond donor (HBD arrow), two H-bond acceptors (HBA1 and HBA2 arrows) and nine excluded volumes (grey spheres) are shown [67].

The AlphaScreen test is a robust biochemical assay for the identification of HIV integrase inhibitors, targeting the interaction between HIV IN and the cellular cofactor LEDGF/p75, thereby effectively promoting the identification of novel inhibitors specific for this protein-protein interaction [43].

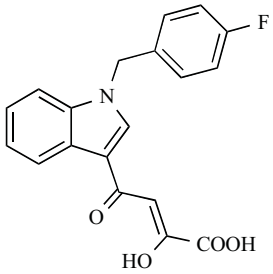
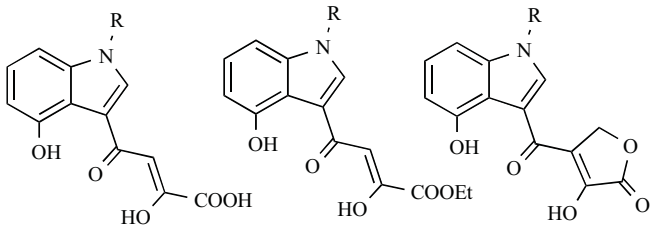


Fig. (9). Chemical structure of lead compound **CHIBA-3002** (**9**).

With the aim of optimizing the activity of the hit molecule **CHIBA-3002** (**9**) and in order to gain SAR information on potential IN-LEDGF interaction inhibitors, the authors followed the suggestions of their molecular modeling studies and planned some suitable chemical modifications: the introduction of a hydroxyl group at C4 position of the benzene fused ring, a hydrogen atom or some moieties with different hydrophobicity at N1 of the indole skeleton, and the cyclization or esterification of the diketoacid portion (Table **3**) [68].

Table 3. CHIBA-3002 (9) Analogues and Biological Evaluation of the Most Representative Derivatives



10 (a-j) **11 (a-j)** **12 (a-j)**

	R		R
a	H	**f**	CH_2-2,3$(CH_3)_2C_6H_3$
b	CH_2-4FC_6H_4	**g**	CH_2-2,4$(CH_3)_2C_6H_3$
c	CH_2-2$CH_3C_6H_4$	**h**	CH_2-2,5$(CH_3)_2C_6H_3$
d	CH_2-3$CH_3C_6H_4$	**i**	CH_2-2,6$(CH_3)_2C_6H_3$
e	CH_2-4$CH_3C_6H_4$	**j**	CH_2-3,5$(CH_3)_2C_6H_3$

Compound	% Inhibition 100 μM	*IC_{50} μM
9 (CHIBA-3002)	46	-
10a (CHIBA-3003)	71	35
10b (CHIBA-3000)	56	76
10e	86	68
10f	93	28.5
10j	94	3.5
11e	60	170.7
12d	61	53.3
12e	81	8.7
12g	96	11.7
12i	70	7.5
12j	69	122.8

*Concentration required to inhibit the HIV-1 IN-LEDGF/p75 interaction by 50%.

The designed compounds were synthesized and tested in the AlphaScreen assay and all of them produced inhibitory effects at 100 μM concentration at rates ranging from 16% to 96%. The analysis of available IC_{50} values indicated that the ester moiety negatively affected the efficacy of this series of PPI inhibitors and that the most active molecules were the derivatives **10j**, **12e**, **12g**, and **12i** active at low micromolar

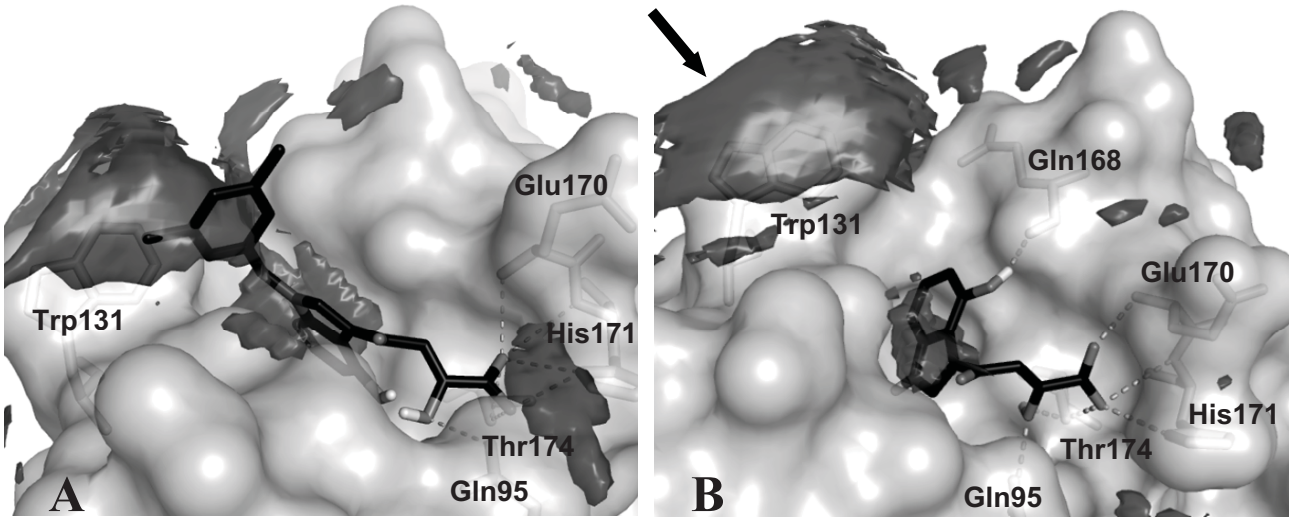


Fig. (10). MIFs calculations results of selected DRY probe (-0.5 kcal/mol, dark gray contours) with the plausible docking mode (using PDB code 2B4J) of compounds (**A**) **10j** (after minimization) and (**B**) **CHIBA-3003** (**10b**) [68]. This figure was prepared using the PyMOL program [73].

concentration (IC_{50}= 3.5 μM, 8.7 μM, 11.7 μM and 7.5 μM respectively).

The best biological results (Table **3**) were obtained for compounds in which both a hydroxyl substituent on C-4 and, in particular, a highly hydrophobic portion on N-1 were present.

In fact, as shown in Table **3**, the most potent derivative of the series **10j** was tenfold more efficacious than the N1 unsubstituted derivative **CHIBA-3003** (**10a**) (3.5 μM vs 35.0 μM).

The above-mentioned modifications were suggested by a combination of docking studies and molecular interaction fields (MIFs) calculated by GRID program [72] on HIV IN_{CCD}. This software afforded more information about the binding mode of our derivatives, and allowed us to explore new binding pocket regions that could be useful to identify new important chemical frames that would be able to improve their inhibitory potency.

The probes selected for GRID study were the DRY probe representing steric and hydrophobic interactions; the N1 (amide nitrogen) probe to represent hydrogen-bond-donor groups; and the O (sp2 carbonyl oxygen) probe to represent hydrogen-bond-acceptors. Fig. **10** shows the results of the MIFs calculations of selected probe DRY with the plausible docking mode (using PDB code 2B4J) of compounds **CHIBA-3003** (**10b**) and **10j**, into the region of protein–protein interaction between HIV-1 IN and cofactor LEDGF/p75.

The most active compound **10j** displayed the following interactions: (a) the carboxylate group was able to interact with Glu170, His171, and Thr174 (b) the hydroxyl group of the diketoacid moiety interacted with Gln95, (c) the benzene fused-ring of indole nucleus was placed into IN hydrophobic pocket, (d) the 3,5-dimethylbenzyl N-substituent occupied the most relevant area calculated by DRY probe (Fig. **11A** and Fig. **11**).

However, for unsubstituted derivative **CHIBA-3003** (**10a**) this last area (indicated by a black arrow and coloured dark gray in Fig. **10B**) was vacant, suggesting that an alternative explanation for the highest inhibitory efficacy of compound **10j** could be due to its ability to map well the DRY area related to the crucial residue Trp131.

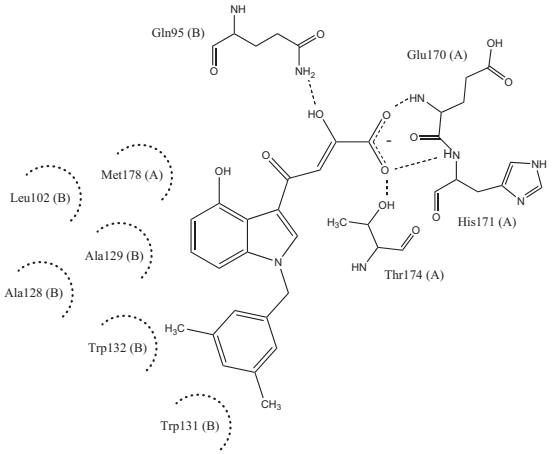


Fig. (11). Schematic view of interactions between **10j** and IN_{CCD} based on the docking studies.

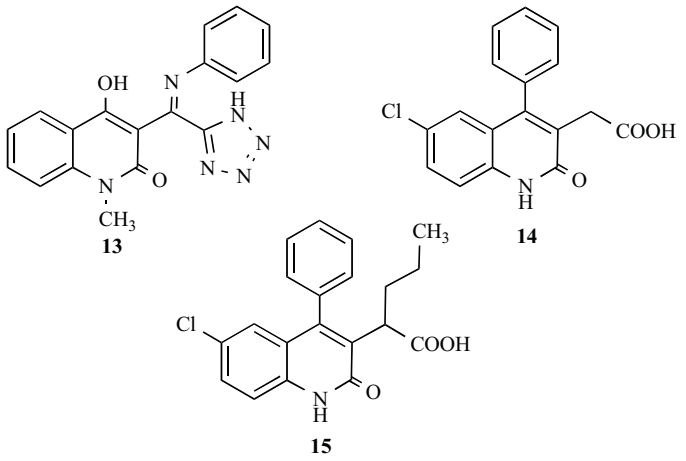
Quinoline Derivatives

In the same period in which the results on the indole derivatives, reported in the preceding section, were published, the crystal structures of other small molecules active as PPIs inhibitors in LEDGF/p75-binding pocket were solved [74].

Firstly, some of these molecules were identified through virtual screening based on a pharmacophore model, created by an analysis of the different crystal structures of HIV-1 integrase and, more specifically, of the $LEDGF_{IBD}$ complex with the IN_{CCD}, followed by docking experiments.

The 4-hydroxy-1-methyl-3-[(*Z*)-(phenylimino)(1H-tetrazol-5-yl)methyl]quinolin-2(1*H*)-one (**13**) was selected as

Table 4. Biological Activities of Selected 2-(quinoli-3-yl)acetic Derivatives (13-15)



Compound	AlphaScreen (IC_{50} μM)		MTT/MT4 (μM)	
	LEDGF/p75-IN binding	EC_{50}[a]	CC_{50}[b]	SI[c]
13	36%[d]	ND	ND	
14	12.2+/-3.4	41.9+/-1.1	>150	>3
15	1.37+/-0.36	2.35+/-0.28	59.8+/-0.5	25

[a]Effective concentration required to reduce HIV-1-induced cytophatic effect by 50% in MT-4 cells.
[b]Cytotoxic concentration reducing MT-4 cells viability by 50%.
[c]Selectivity index: ratio CC_{50}/EC_{50}.
[d]Percent inhibition at 100 μm of the small molecules.

a hit molecule. This showed 36% inhibition of the LEDGF/p75-IN interaction at 100 μM concentration.

Suitable chemical modifications led to other more active derivatives with increased activity in AlphaScreen [43] and in cell culture assay (Table **4**), e.g. compound **14** (IC_{50}=12.2 μM) and its other close analogs such as **15** (IC_{50}=1.37 μM) (Fig. **14**).

Considering the selectivity of **15** in the cell culture (SI=25) the authors performed a thorough virological characterization, evaluating the cross-resistance profile of this new compound in comparison with established antiviral agents and resistance selection against the new drug candidate.

Notably, compound **15** retained full activity against raltegravir-resistant strains, underlining its divergent mode of action with respect to raltegravir, the only INST inhibitor approved by FDA.

Furthermore, the IBD-resistant strain with the Ala128Thr Glu170Gly double mutation was fully resistant to **15** but it retained sensitivity to all other antiviral agents tested, confirming that Ala128 is an important contact point at the interface IN_{CCD}-$LEDGF_{IBD}$ [74].

In order to validate the pharmacophore model, the authors soaked IN_{CCD} crystals with compounds **13** and **15** and carried out X-ray structure determination. The obtained crystal structures are deposited under PDB codes 3LPT and 3LPU respectively.

The compounds are bound in a cleft between the two monomers of the IN core dimer showing a similar binding mode. The extra propyl group of compound **15** is accommodated in an appropriate void deep inside the cleft and the carboxylate moiety forms hydrogen bonds with the main chain nitrogens of integrase residues Glu170 and His171, similar to the side chain of Asp366 of $LEDGF_{IBD}$. Furthermore, the apolar side chain of Ala128 of integrase packs directly against the chlorine atom of compound **15**; making it clear that HIV-1 mutant Ala128Thr is resistant to **15** (Fig. **12**).

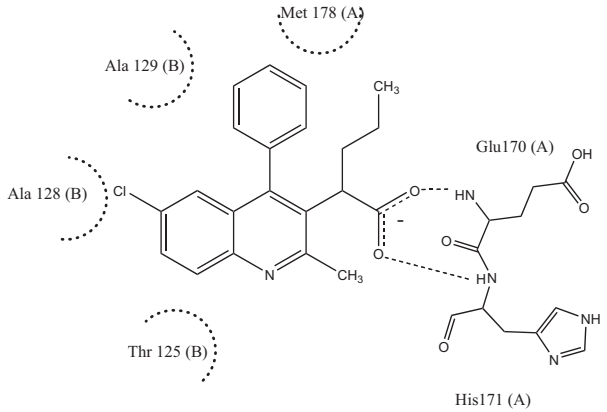
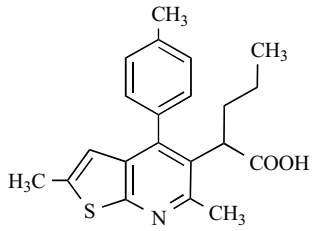


Fig. (12). Schematic view of interactions between compound **15** and IN_{CCD} based on the crystal structure.

In spite of this, it is possible to design new compounds to avoid steric hindrance by Ala128Thr and even to gain a beneficial influence from this mutation.

While the virological profiling of **15** was in progress, chemical synthesis of more congeners of 2-(quinolin-3-yl)acetic acids was carried out. Among the obtained derivatives, compound **16** (Fig. **13**) inhibited LEDGF/p75-integrase interaction in the submicromolar range (IC_{50}=0.58±0.30 μM) and anti-HIV activity increased to the same extent (EC_{50}=0.76±0.08 μM), resulting in a selectivity index of nearly 100.



16

Fig. (13). Chemical structure of quinoline derivative **16**.

Comparison of Docking Poses and Conclusions

De Luca at al. previously reported the comparison of the docking results of the most active compound of the benzylindole series **10j** (3.5 μM) and the X-ray position of quinoline derivative **15** which showed comparable activity (1.37 μM) (Fig. **14**) [68].

Their carboxylate groups are in the same position and the indole nucleus of **10j** and the phenyl group of **15** occupy the same hydrophobic area. An unlike orientation was observed between the dimethylphenyl moiety of **10j** and the benzene fused-ring containing a chlorine atom of **15** which interacted with two different hydrophobic spaces.

The authors of this review also reproduced and herein report the docking position of **D77** (**8**), in comparison with **10j** and **15** (unpublished data).

They observed that although the carboxylic groups of **10j** and **15** were in the same position, one of the **D77** showed a different orientation. In contrast, the indole nucleus of **10j**, the phenyl group of **15** and benzene ring 3 of **D77** (**8**) occupy the same hydrophobic pocket.

Finally, the dimethylphenyl moiety of **10j** and the benzene ring 2 of **D77** (**8**) bind the same hydrophobic area differently from the benzene fused-ring containing a chlorine atom of **15** which interacts with a dissimilar hydrophobic region.

In summary, all these three representative compounds **(10j, 15 and D77)** show similar interactions but also show different dispositions of some peculiar features.

These observations could be particularly useful, not only to identify new potential IN-LEDGF/p75 interaction inhibitors but also to optimize those already known by suitable chemical modifications.

In conclusion, the above reported studies highlight that LEDGF plays a fundamental role in HIV-1 replication promoting the viral integration by tethering the preintegration complex to the chromatin. Thus, blocking interactions between IN and cellular cofactor LEDGF/p75 could be a challenging and promising strategy in AIDS therapy.

Fig. (14). Docking poses of compound **D77 (8)** (dark gray) and **10j** (light gray) compared to the crystallized position of **15** (black) into the LEDGF/p75 binding pocket of IN_{CCD}. This figure was prepared using the PyMOL program [73].

Although only few inhibitors of IN-LEDGF/p75 are known to date, it seems clear that the development of new types of clinically useful allosteric inhibitors of IN could effectively complement currently used antiretroviral therapies, both inhibiting HIV replication as well as acting against virus strains resistant to drugs with a different mechanism of action.

ACKNOWLEDGEMENT

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