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The evaluation of statins as potential inhibitors of the LEDGF/p75 – HIV-1 integrase interaction

Running title: Inhibition of the LEDGF/p75–HIV-1 IN interaction by statins

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Abstract

Lovastatin was identified through virtual screening as a potential inhibitor of the LEDGF/p75-HIV-1 integrase interaction. In an AlphaScreen assay, lovastatin inhibited the purified recombinant protein-protein interaction (IC_{50} = 1.97 ± 0.45 μM) more effectively than seven other tested statins. None of the eight statins, however, yielded antiviral activity *in vitro* while only pravastatin lactone yielded detectable inhibition of HIV-1 integrase strand transfer activity (31.65% at 100 μM). A correlation between lipophilicity and increased cellular toxicity of the statins was observed.

The virally-encoded enzyme, HIV-1 integrase (IN), catalyses the insertion of viral DNA into the host chromation through a multi-step process that involves 3'-end processing (3-P) and strand transfer (ST) activity. HIV-1 IN is fundamental to the life cycle of the virus and has been successfully exploited as a target for therapeutic intervention. The IN ST inhibitors (INSTIs) Raltegravir (RAL, Merck and Co, USA), Elvitegravir (EVG, Gilead Sciences, USA) and Dolutegravir (DOL, GlaxoSmithKline, UK) have all been approved by the USA Food and Drug Administration (FDA) and have demonstrated unequivocal clinical relevance.

The integration process is facilitated by a number of host and viral proteins sequestered to HIV-1 IN to form the pre-integration complex (PIC). The interactions between proteins that comprise the PIC represent attractive targets for therapeutic intervention, especially since compounds that engage IN at sites distinct from the catalytic active site should lack cross-resistance to INSTIs. Recent studies have demonstrated the potential of small molecule modulators of the interaction between HIV-1 IN and the human lens epithelium-derived growth factor/p75 (LEDGF/p75) (1-3). These targeted small molecules, categorised as

allosteric IN inhibitors (ALLINIs) and exemplified by LEDGINs (for LEDGF/p75-IN inhibitor) and NCINIs (for non-catalytic IN inhibitor), bind to the LEDGF/p75 binding site formed through the IN catalytic core domain (CCD) dimer interface (reviewed in 4). In addition to disruption of the LEDGF/p75- HIV-1 IN interaction, these inhibitors function independently of LEDGF/p75 to induce multimerization and impair the catalytic activities of HIV-1 IN. Interestingly, ALLINIs exert their full inhibitory activity in late phase of HIV-1 replication impeding particle core maturation and producing non-infectious progeny virus (reviewed in 4). Finally, ALLINI's have been shown to reduce HIV-1 replication *in vitro* at nanomolar levels and present a distinct resistance profile to known INSTIs.

Owing to their relevance as a promising class of HIV-1 therapeutics, we sought novel inhibitors of the LEDGF/p75–HIV-1 IN interaction. This brief study serves to describe the identification of a known statin drug as a potential inhibitor of the protein-protein interaction through virtual screening and the biological evaluation of this, and related, statins.

Methods and Materials

Molecular Modelling

Analysis of the protein-protein interaction between the CCD of HIV-1 IN with the IN-binding domain (IBD) of LEDGF/p75 was conducted using Discovery Studio™ 3.1 (Accelrys, USA). As a preliminary to docking, the LEDGF/p75- HIV-1 IN complex (PDB code 2B4J) was prepared utilizing a CHARMM based force field function contained in Macromolecule's Protein Prepare function to build missing loops, add hydrogens, assign bond orders, and optimize bond lengths, bond angles, torsion angles, and non-bonded interactions. The LEDGF/p75 protein was removed and the LEDGF/p75 binding site was defined on the IN dimer using the Receptor-Ligand Interaction function. Small molecules for

docking were prepared utilizing a CHARMM based force field function contained in Small Molecules' Prepare and Filter Ligands function to add hydrogens, assign bond orders, and optimize bond lengths, bond angles, torsion, change ionization and generate isomers and tautomers of the ligands. Compounds described in literature as disrupting the LEDGF/p75 – HIV-1 IN interaction with varying potency were utilised as training molecules (1-5). Ligands were docked into the predefined LEDGF/p75 binding site utilizing Libdock and CDOCKER docking algorithms and scoring functions (LigScore1, LigScore2, PLP1, PLP2, Jain, PMF, PMF04) contained in the Receptor-Ligand Interaction module were used to evaluate goodness of fit of the docking runs. The top 1000 ligands from both the Libdock docking function and the Jain scoring function were subjected to the CDOCKER docking function. The top 100 results as defined by CDOCKER binding energy were analysed for compounds successfully docked as well as number of times that isomers and tautomers of these specific compounds appeared in the top 100 results.

Preparation of test compounds

Mevastatin was purchased in purified form (Santa Cruz Biotechnology, USA) and fluvastatin (Lescol, Novartis Pharmaceuticals Inc., Switzerland) was cherry-picked from a compound library (NIH Clinical Compound, NCC, Evotec, USA). Lovastatin (Mevacor, Merck and Co, USA), simvastatin (Zocor, Merck & Co, Inc., USA), atorvastatin (Lipitor, Pfizer, USA) and pravastatin (Pravachol, Bristol-Myers Squibb, USA) were prepared from whole tablets using methods previously described (7-10). Briefly, each tablet was crushed separately until finely ground and dissolved in distilled water (dH_2O). The mixture was added to an extraction vessel and methanol was passed through repeatedly with slight agitation. The combined methanol solutions were filtered through a 0.22 μM filter following the addition of ammonium sulphate to eliminate water residue and solvent was removed through

evaporation. Lovastatin lactone was hydrolysed to the β-hydroxy acid form from purified lovastatin as previously described (10). Briefly, purified lovastatin was dissolved in dH_20 and 0.1 M NaOH and allowed to stir overnight; after which the solution was adjusted to pH 7.5. A mixture of dH_20, acetonitrile (ACN) and ethyl acetate (1:1:1) was used to precipitate the lovastatin β-hydroxy acid product which was then filtered through a 22μM filter and dried. Pravastatin acid was condensed to the lactone form by suspending purified pravastatin acid in ethyl acetate, adding trifluoroacetic acid (TFA) and stirring overnight as per methods previously described (9,10). The reaction was then dried and a mixture of dH_20, ACN and ethyl acetate (1:1:1) was used to precipitate the product which was then filtered through a 0.22 μM filter and dried. Each extracted product was analysed by nuclear magnetic resonance (NMR, 400 MHz Bruker Avance Spectrometer at 298K equipped with a BBI 5 mm probe) and mass spectroscopy (Dionex Ultimate 3000 Rapid Separation LC system equipped with a C-18 pre-coated column and coupled to a microTOF QII Bruker mass spectrometer fitted with an electrospray source) to confirm structure and purity. Purified compounds (> 95% purity) were dissolved in dimethyl sulphate (DMSO) and stored at -20°C until used.

AlphaScreen Assay

The AlphaScreen assay was performed as previously described (1). Briefly, 300nM biotin-labelled recombinant HIV-1 subtype B IN was incubated with CX05168 (Haoyan Chemexpress Co, LTD, China) or test compounds (final concentrations of 100 μM for single-dose experiments or 8 final concentrations ranging from 100 – 0.78 μM for dose-response experiments) for 30 minutes at 26°C while shaking. Thereafter, 100nM HIS-tagged LEDGF/p75 was added and the mixture was incubated for 60 minutes in assay buffer (150mM NaCl, 25mM Tris, 2mM $MgCl_2$, 1mM DTT, 0.01% Tween 20 and 0.02 % BSA, pH 7.4) at 26°C with gentle shaking. Streptavidin donor beads (Perkin Elmer, USA) and nickel

chelate acceptor beads (Perkin Elmer, USA) at final concentrations of 10µg/ml each were added to the mixture which was then incubated for 60 minutes at 30°C in the dark with gentle shaking. The plate was read at 520 - 620nm using an EnSpire® Multimode Plate Reader (Perkin Elmer, USA). Percentage inhibition was determined for single-dose experiments while IC_{50} values were determined as the concentration required to reduce protein-protein interaction by 50% and calculated using OriginPro® 8.0 software (Origin Lab Corporation, USA). All inhibition values are the average of triplicate experiments.

Cytotoxicity assay

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: MT-4 from Dr. Douglas Richman (catalogue number 120). Peripheral blood mononuclear cells (PBMCs) were isolated from pooled buffy coats (South African National Blood Service, SANBS, RSA) by means of the Ficol-Hypaque density gradient separation method. The cytotoxicity assay was performed as per standard method and as described previously (12). Briefly; MT-4 cells or 3 day PHA-stimulated PBMCs were plated in 96-well microtiter plates at $2x10^5$ cells/ml and allowed to stabilize for 2 hours at 37 ºC and 5% CO_2. Thereafter, test compounds were added to the plate through serial dilution (two-fold) to allow for 8 final compound concentrations ranging from 200 - 1.56 µM in a total volume of 200 µl/well. The cells and compounds were then incubated for 96 hours at 37 ºC and 5% CO_2. To each well, 20 µl CellTiter 96® AQueous One Solution (Promega, USA) was added; the plates were incubated for 4 hours and absorbance was read at 490 nm on a multiplate reader (xMark™, Bio-Rad, USA). CC_{50} values were determined as the concentration of the test compound required to reduce the cell viability by 50% and were calculated using OriginPro® 8.0 software (Origin Lab Corporation, USA). The values obtained were averages of at least three separate experiments.

Antiviral activity

To determine antiviral activity, 50 µl of HIV-1_{NL4-3} virus was added to 2 x10^5 MT-4 cells/ml at a multiplicity of infection of 0.1 and the mixture was spinoculated at 3000 xg for 90 minutes. After washing off unbound virus, cells were plated in 96 well microtiter plates at 100 µl/ well and allowed to stabilise for 1 hour at 37 ºC and 5% CO_2. Thereafter, test compounds were added to the plate through serial dilution (two fold) to allow for 8 final compound concentrations ranging from 200 - 1.56 µM in a total volume of 200 µl/well. The cells and compounds were then incubated for 96 hours at 37 ºC and 5% CO_2. Cell-free supernatants were collected from each well and p24 concentration was determined using the Vironostika HIV-1 p24 Antigen ELISA (bioMérieux, France) as per manufacturer's instructions. EC_{50} values were determined as the concentration of the test compound required to reduce p24 concentration by 50% and were calculated using OriginPro® 8.0 software (Origin Lab Corporation, USA). The values obtained were averages of at least three separate experiments. Selectivity index (SI) values were calculated as the ratio of CC_{50}/EC_{50}.

Strand transfer assay

The assay for the detection of HIV-1 IN ST inhibition was adapted from previously described methods (13). Briefly, 0.15 µM double-stranded biotinylated donor DNA (5'-biotin-GTGTGGAAAATCTCTAGCA-3' and 5'-ACTGCTAGAGATTTTCCACAC-3') was added to the wells of streptavidin-coated 96-well microtiter plates (R&D Systems, USA). Following incubation at room temperature for 60 minutes and a stringent wash step, 1 µM purified recombinant HIV-1 subtype B IN (in the presence of $MgCl_2$ and $MnCl_2$) was assembled onto the pre-processed donor DNA through incubation for 30 minutes at 22 ºC. Following a wash step, the test compounds were titrated into individual wells either at a final concentration of 10 µM (for single-dose inhibition assays) or at a minimum of 8 different concentrations

ranging from 50 – 0.39 μM (for dose-response inhibition assays). The microtiter plates were incubated for 30 minutes at 37 ºC, washed and the ST reaction was initiated through the addition of 0.25 μM double-stranded FITC-labelled target DNA (5'-TGACCAAGGGCTAATTCACT-FITC-3' and 5'-AGTGAATTAGCCCTTGGTCA-FITC-3') in Hepes buffer containing $MgCl_2$ and $MnCl_2$. After an incubation period of 60 minutes at 37 ºC, the plates were washed as before and an alkaline phosphatase (AP) - conjugated anti-FITC secondary antibody (Sigma, USA) was added. Finally, the plates were washed and chromogenic substrate (BluePhos, KPL, USA) was added to allow for photometric measurement at 620nm using a microplate reader (xMark^{TM}, Bio-Rad, USA). Percentage inhibition was calculated utilising the formula: % inhibition = (1 - (A_{620} Compound – A_{620} No IN control) / (A_{620} No inhibitor control – A_{620} No IN control))*100. IC_{50} values were determined as the concentration required to reduce activity of HIV-1 IN by 50% and were calculated using OriginPro® 8.0 software (Origin Lab Corporation, USA). All inhibition values are the average of triplicate experiments.

Results and Discussion

Identification of lovastatin

Screening of virtual compound libraries through the LEDGF/p75-binding site on HIV-1 IN identified several molecules of interest of which the top ten compounds, as defined by CDOCKER binding energies, were selected for further investigation. These ten compounds identified *in silico* were biologically evaluated in a previously described AlphaScreen assay which had been validated with CX05168. Five of the ten compounds were observed to inhibit the interaction between LEDGF/p75 and the HIV-1 IN complex at greater than 50% at a single-dose concentration of 100 μM. These compounds were subjected to a dose-response evaluation and the most potent molecule yielded an IC_{50} within the low micromolar range

(IC_{50} = 1.97 ± 0.45 μM, Table 1). Cross-referencing of this inhibitor, which appeared once in top 100 results and produced a CDOCKER binding energy of -36.821 using the Jain scoring function, identified the compound as the HMG-Coenzyme A reductase inhibitor and type 1 statin; lovastatin. Several previously published studies have examined the anti-HIV effect of different statins *in vitro* and *in vivo* (14,15). For lovastatin, in particular, anti-HIV activity has been attributed to pleiotropic actions, including the depletion of lipid rafts (16), the down regulation of Rho activity (17), the suppression of LFA-1/ICAM-1 interaction (18) and the inhibition of CCR5 coreceptor and RANTES expression (19) while other studies have conversely reported no or negligible anti-HIV activity (20,21). Interestingly, Hu and co-workers identified the type 2 statin, atorvastatin, as an inhibitor of the LEDGF/p75 – HIV-1 IN interaction *via* molecular docking and then determined an IC_{50} of 8.90 μM (22). In the same study, they evaluated fluvastatin but found this type 2 statin to be lacking notable inhibitory activity. To the best of our knowledge, no previous study has identified or investigated lovastatin as a potential modulator of the LEDGF/p75 – HIV-1 IN interaction.

Preparation and biological evaluation of statins

For further evaluation of type 1 and type 2 statins, as well as of the hydroxy acid and lactone forms we purified lovastatin, pravastatin, atorvastatin and simvastatin from whole tablets. Mevastatin was purchased in purified form and fluvastatin was cherry-picked from a compound library. Lovastatin was hydrolysed to its β-hydroxy acid form with NaOH while pravastatin was condensed into its lactone form with TFA. All structures were validated through nuclear magnetic resonance and mass spectroscopy and were shown to be > 95% pure (results not shown).

We then evaluated the activity of these additional statins in the AlphaScreen assay, with the findings reported in Table 1. Briefly, for the type 1 statins, deviations from the lovastatin structure served to reduce inhibitory activity. Pravastatin (lactone form), which differs from lovastatin in that the six-alphamethyl group on the hexahydronaphthalene ring is replaced with a hydroxy group, inhibited the interaction by 25.77% at a single-dose concentration of 100 μM. Simvastatin, which retains the alphamethyl group on the naphthalene ring but has an additional methyl on the side chain, yielded 28.78% inhibition in the single-dose test. Mevastatin, which differs from lovastatin only by the absence of the six-alphamethyl group, inhibited the interaction by 13.25%. Variation from the lactone form to the hydroxy acid form of lovastatin reduced the inhibition observed at 100 μM to 42.48%. Similarly, pravastatin in its administered hydroxy acid form yielded a minimal 8.55% inhibition. From this preliminary comparison of inhibition and statin structure it appears that a closed lactone ring in addition to a methyl group is requisite for inhibitory activity, while minor alterations to the side chain adversely affect inhibitory activity of type 1 statins within the described assay. For the type 2 statins; atorvastatin inhibited the interaction by 54.26% in the 100 μM single-dose evaluation and produced an IC_{50} of 10.22 ± 0.80 μM. In contrast, fluvastatin inhibited the interaction by 4.87% at 100 μM. The results obtained for the type 2 statins closely correlated those obtained by Hu and co-workers also using an AlphaScreen assay (22).

In addition to blocking the LEDGF/p75- HIV-1 IN interaction, detailed mechanistic studies have revealed that some LEDGINs also indirectly inhibit the strand transfer function of HIV-1 IN (2,3, reviewed in 4). In our hands, the two pravastatin forms were most effective with pravastatin lactone inhibiting activity at similar levels to CX05168 within a HIV-1 IN ST ELISA-based assay. None of the other statins tested produced appreciable ST inhibition (Table 1).

We found lipophilicity of the statins to increase *in vitro* cellular toxicity, a phenomenon that has been previously observed (23). Lovastatin yielded CC_{50} values of 5.31 μM and 6.50 μM in the MT-4 cell line and in PBMCs respectively, as determined 96-hours following compound addition. Studies conducted using the xCELLigence System (Roche Applied Sciences, Germany) confirmed the toxic nature of this statin and revealed that lovastatin initiated cell death within 2 hours of introduction to the HeLa cell-line and completed its toxic effect within 24 hours (data not shown). Similarly, atorvastatin, simvastatin, lovastatin acid, mevastatin and fluvastatin yielded low CC_{50} values (< 14 μM) in the MT-4 cell line (Table 1). The two hydrophilic statins were observed to be less cytotoxic. Pravastatin lactone yielded a CC_{50} value of 59.22 μM while no toxicity was observed for pravastatin within the concentration limits defined for our assay. Irrespective of lipophilicity, none of the statins yielded discernible SI values (Table 1). Cell-based antiviral activity could only be detected within the toxic range of the statins and was attributed to cell death rather than to genuine inhibition. In contrast, CX05168 effectively inhibited viral replication as described previously (1,2) and yielded an SI value of 39.97 (Table 1).

Discussion

This report provides the first account of lovastatin as a potential useful inhibitor of the LEDGF/p75-HIV-1 IN interaction. It also corroborates the previous identification of atorvastatin as an inhibitor of the interaction and provides additional information for this and other statins. Data compiled on all eight statins demonstrates the influence of lipophilicity on the toxicity of the statins.

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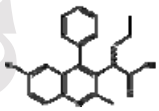
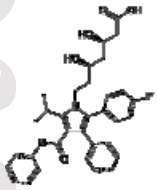
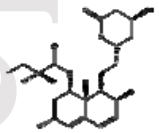
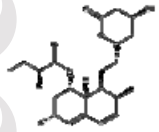
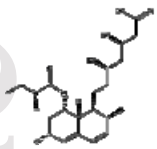
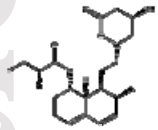
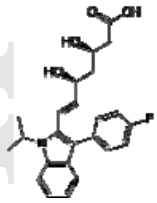
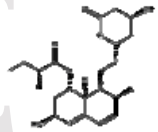
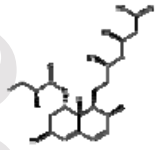
Conflict of Interest

The authors have no conflict of interest to declare.

Table 1. Biological activity of eight statins and the known inhibitor, CX05168

	LEDGF/P75 – HIV-1 IN **% inhibition at 100µM ± SE [a] (IC_{50} [b] ± SE, µM)**	**EC_{50} [c] ± SE (µM)** **MT-4**	**CC_{50} [d] ± SE (µM)** **MT-4 (PBMCs)**	**SI [e]**	**Strand transfer (ST) inhibition** **% inhibition at 100µM ± SE (IC_{50} ± SE ; µM)**
 CX05168	91.37 ± 6.85 (1.33 ± 0.05)	1.78 ± 0.28	71.15 ± 2.33	39.97	31.73 ± 4.89 (70.12 ± 2.26)
 Atorvastatin	54.26 ± 2.03 (10.22 ± 0.80)	23.68 ± 0.21	13.36 ± 2.38	0.56	Enhanced activity
 Simvastatin	28.78 ± 0.70	ND	12.70 ± 0.66	-	9.43 ± 1.22
 Lovastatin	75.51 ± 6.89 (1.97 ± 0.45)	6.95 ± 0.21	5.31 ± 0.19 (6.50 ± 0.31)	0.76	7.82 ± 5.61

 Lovastatin acid	42.48 ± 1.69	ND	6.53±0.77	-	8.60 ± 2.81
 Mevastatin	13.25 ± 3.66	6.70 ± 2.20	3.72 ± 0.25	0.55	0.63 ± 14.12
 Fluvastatin	4.87 ± 1.18	ND	10.64 ± 1.14	-	11.51 ± 4.60
 Pravastatin lactone	25.77 ± 5.53	29.55 ± 0.56	59.22 ± 1.66	2.00	31.65 ± 6.63
 Pravastatin	8.55 ± 3.22	> 100	> 100	-	18.54 ± 1.02

[a] SE, Standard error. Standard error of the mean for at least three separate experiments.
[b] IC_{50}, 50% inhibitory concentration. Calculated as the concentration of compound required to reduce the LEDGF/p75-IN interaction by 50%.
[c] EC_{50}, 50% effective concentration. Defined as the concentration of compound required to reduce HIV-1 replication by 50%.
[d] CC_{50}, 50% cytotoxic concentration. Calculated as the concentration of compound required to reduce cell viability by 50%.
[e] SI, selectivity index. Calculated as the ratio of CC_{50}/EC_{50}

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