Design and synthesis of caffeoyl-anilides as portmanteau inhibitors of HIV-1 integrase and CCR5

Hardik S. Bodiwalla, Sudeep Sabde, Pawan Gupta, Ruchira Mukherjee, Rajender Kumar, Prabha Garg, Kamlesh Kumar Bhutania, Debashis Mitra, Inder Pal Singh

Abstract

Designing multi-functional ligands is a recent strategy by which multiple targets can be inhibited by a single entity. A series of caffeoyl-anilide compounds based on structures of various integrase and CCR5 inhibitors have been designed and synthesized as anti-HIV agents in the present study. Most of the compounds exhibited potent anti-HIV activity at micromolar concentration in CEM-GFP CD4+ T cells infected with HIV-1NL4.3 virus. Compound 14 showed a lower EC50 and better TI as compared to AZT. Mechanism based studies suggest that these compounds inhibit either one or in some cases, both the targets. The experimental data and the docking results showed that these compounds are potential inhibitors for both HIV-1 IN and CCR5.

Keywords:
Caffeoyl-anilide
Anti-HIV
Integrase inhibitor
CCR5 inhibitor
Portmanteau inhibitor

1. Introduction

Human immunodeficiency virus-1 (HIV-1) is the causative agent of the acquired immunodeficiency syndrome (AIDS). Globally, an estimated 33 million people were living with HIV in 2008.1 Almost all FDA approved drugs are either reverse transcriptase (RT) or protease (PR) inhibitors. Recently approved drugs for anti-HIV therapy are Maraviroc (entry inhibitor) and Raltegravir (integrase (IN) inhibitor).2 Currently, highly active antiretroviral therapy (HAART) comprising of a HIV RT with PR or IN inhibitors successfully suppresses HIV viral load to an undetectable level. However, the effect of this therapy is compromised by emergence of resistant HIV strains.

There has been a recent trend of formulations whereby two or more drugs are combined in a single tablet or capsule to make dosing regimen simpler, thereby improving patient compliance. Certain physico-chemical risks like drug-drug and drug-exciptent compatibility are associated with these formulations. Designing multiple ligand is an upcoming drug discovery strategy, where multiple targets can be inhibited or blocked using a single entity. This improves patient compliance and reduces chances of drug resistance. The exploration of such multifunctional ligands has proven valuable for anti-HIV lead discovery.3 Earlier, Vince et al. have proposed the term portmanteau inhibitors for molecules that have two scaffolds merged into one entity and act on different sites or phases of viral life cycle.4

In an ongoing effort to develop novel anti-HIV molecules,5,6 we planned to synthesize portmanteau inhibitors of HIV-1 integrase and CCR5. The purpose of such design was to block the virus at two different stages of life cycle. HIV-1 pol gene encodes three essential enzymes viz. RT, IN and PR. The integrase, which is an intracellular target for HIV, mediates two critical reactions during viral replication. It removes two nucleotides from 3’ end of both cDNA strands. The host genomic DNA is cleaved and processed cDNA strands are joined with host DNA. FDA approved integrase inhibitors block this strand transfer reaction.7 The CCR5 is an extracellular target on the host cell surface and an important mediator of R5 virus entry. Thus, CCR5 is the target at the entry level, when virus attaches to the host cell surface and the integrase, when viral DNA gets integrated into the genome of host cell.

Catechol moiety is a significant structural component in many potent HIV integrase inhibitors such as caffeic acid phenylethyl ester (CAPE, 1) and chicoric acid (CA, 2).8 CA exhibited IC50 values of 0.1–0.5 μM for the end-processing/strand transfer reactions and 0.1–0.2 μM for the disintegration reactions.9 Catechol containing...
inhibitors were proven toxic, perhaps because of nonspecific binding to other targets or due to the formation of oxidized species, such as semiquinones or orthoquinones. Although it was shown in 2000 that chicoric acid inhibits HIV replication by non-specific gp120 interactions, however, Ho et al. have reported in 2005 that CAPE and its analogues acted through distinctly different mechanism. The compounds were considered safe as no mortality was observed in experimental mice. It was also concluded that CAPE like compounds are worth studying further as potential chemotherapeutic agents in HIV infection. Many other recent papers indicate that CAPE and CA derivatives act through inhibition of HIV integrase. Previous studies on SAR of CA indicated that at least one carboxylic acid moiety and bisphenol are necessary for anti-HIV activity. The expected cleavage of its ester linkage by hydrolytic enzymes and the relatively large number of rotatable bonds are limiting steps in its oral bioavailability. It was suggested that optimization of the lead molecule may be achieved by substituting the ester groups by more robust linkages, like amide or ether and preventing free rotation around single bonds by synthesizing conformationally constrained analogs.

TAK-779 (3) was reported as the first small molecule CCR5 antagonist with an IC50 of 1.4 nM. Thereafter, various groups have exploited anilides as CCR5 antagonists as reported comprehensively by Kazmierski et al. SAR study of 3 suggested that a proper shape of molecule, suitable bulkiness and location of quaternary ammonium moiety is essential for optimal activity. In further studies, 3 exhibited poor oral absorption because of quaternary ammonium moiety. Smithkline Beecham Corp. disclosed various benzanilides as antagonists of receptor. The benzanilides (4 as a representative) have been claimed for treatment of various diseases including HIV by CCR5 antagonism with an IC50 value ranging from 0.0001 to 100 μM.

Based on these reports, we proposed to synthesize and evaluate caffeoyl-anilide compounds as anti-HIV agents (Fig. 1) incorporating caffeoyl moiety of 1 and 2 and anilide moiety of 3 and 4 in the present study. We synthesized molecules in which caffeoyl unit was kept intact and anilide unit was modified with different aromatic and aliphatic substituents to study the changes in anti-HIV activity. In this paper, we describe synthesis, mechanism based (CCR5 and IN) anti-HIV activity and molecular modeling study of newly designed caffeoyl-anilide molecules.

2. Results and discussion

2.1. Chemistry

The target compounds were synthesized as shown in Scheme 1. Commercially available 4-aminocetophenone (5) was reacted with 3,4-dihydroxybenzaldehyde (6) in presence of catalytic amount of H2SO4 in MeOH under reflux for 8 h. However, the yield of the desired product under these conditions was low (~10%). By using 3 equiv of acid the desired product 7 was obtained in a yield of 45–48%.

Various methods for synthesis of chalcones reported in the literature involve protection and deprotection of hydroxyl groups. Further, the acid catalyzed reaction for 7 required refluxing for 8 h. Therefore, in order to improve the reaction conditions, we modified the acid catalyzed coupling using methane sulfonic acid (MSA), which is a clear, colorless liquid with high acid strength (pKa = −1.9). It is less aggressive than H2SO4, easy to use and often recyclable. It is used widely as a catalyst in several reactions (esterification, alkylation) or as catalyst/solvent for condensation or rearrangement reactions.

Higher yield of chalcone 7 was obtained, when reaction was performed in MSA at RT for a short time of 5 min. Reaction performed in various organic solvents such as, MeOH, EtOH, ACN, THF etc. did not give the desired product in good yield. The present method is advantageous over existing acid catalyzed methods for synthesis of chalcones as (i) it does not require protection and deprotection of phenolic hydroxyl groups, (ii) uses cheap, easy to handle and commercially available catalyst (solvent), (iii) room temperature and non-anhydrous reaction conditions, and (iv) shorter reaction times. Further, 7 was dissolved in THF and various acid chlorides were added to the reaction mixture to obtain the final products as shown in Figure 2.

Intermediate (7) and all synthesized compounds (8–21) were identified by Mass, IR, 1H, and 13C NMR spectral data. All compounds showed a typical 1H–1H coupling constant of 15 Hz showing trans stereochemistry of double bond.

2.2. Biology

To measure the effect of synthesized caffeoyl-anilide compounds, these were tested against HIV-1 in a cell based assay where virus infected cells were incubated for 7 days with compounds. The anti-HIV activity assay of compounds was carried out in CEM-GFP T cells infected with HIV-1NL4.3 virus. CEM-GFP
is a human CD4+ reporter T cell line, which expresses GFP upon HIV infection due to transactivation by Tat protein of stably integrated long terminal repeat regulated GFP gene.26 This cell line is widely used for determination of anti-HIV activity due to easy visualization of infected cells.27 HIV-1 NL4.3 is available as a molecular clone from NIH AIDS Repository and is routinely used as a representative isolate of HIV-1. Although it is known to be X4 virus, it has been reported to infect monocytic cell lines like THP1 and U937. Because NL4.3 was used in screening studies, for identification of the mechanism of entry, we used a different X4 isolate and thus IIIB was used as a X4 isolate and Indiec was used as a R5 isolate.

All synthesized compounds were tested for their cytotoxicity in MTT assay for 48 h before testing for cell based anti-HIV activity. Based on the results of MTT assay, initially all the synthesized derivatives were tested for anti-HIV activity at highest non-toxic concentration. Six out of the 15 compounds showed more than 70% inhibition of HIV-1 replication in CEM-GFP cells at less then 6 μM concentration. These six compounds were then analyzed further for determination of EC50 and CC50.28 The results of activity are shown in Table 1.

The six active compounds were further tested in vitro against HIV-1 integrase and for HIV-1 entry inhibitory activity in TZM-bl cells. For the entry inhibition assay, one CCR5 tropic (Indiec) and one CXCR4 tropic (IIIB) virus was used to identify the mechanism of inhibition. All active compounds showed potent integrase inhibitory activity (Table 2). The entry inhibition assay in TZM-bl cells clearly indicated that the active compounds potently inhibited the entry of CCR5 utilizing virus isolate but did not inhibit the entry of CXCR4 utilizing virus isolate (Table 3). None of the compound was active against HIV-1 RT (data not shown) suggesting that the designed compounds have specificity towards the designated targets.

2.3. SAR

To study the structure–activity relationship, small aliphatic groups like acetyl, isobutyryl, isovaleryl, bulkier aliphatic group like adamantyl, five-member heteroaromatic groups with O and S as heteroatoms, naphthyl, phenyl and mono- and di-substituted phenyl groups were introduced.

Compounds 8–10 which were devoid of aromatic benzanilide moiety were found to be inactive for anti HIV activity. Compound 11 having bulkier adamantyl substitution was active in the anti HIV assay. It showed moderate anti HIV IN activity with an IC50 of 36.8 μM and inhibited virus entry through CCR5.

Out of five-membered heteroaromatic anilides with O and S as heteroatom (12–14), compound 13 having 2-thiophene moiety was inactive. Interestingly compound 14 having thienophene-2-acetyl moiety was found to be the most active compound in cell based assay with IC50 of 0.9 μM and TI of 59.1. This suggested that chain length might be important for anti HIV activity. It showed 53% inhibition of virus entry at 2.6 μM concentration.

Three benzanilides (15–17) similar to 4 were synthesized. As expected, caffeoyl-benzanilide derivatives 15 and 17 were found to show good anti HIV activity with IC50 of 1.6 and 1.9 μM, respectively. In the IN assay, 17 was found be more active (IC50 of

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc μM</th>
<th>% Inhibition</th>
<th>EC50 μM</th>
<th>CC50 μM</th>
<th>TI</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>3.9</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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<tr>
<td>9</td>
<td>1.5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>1.4</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>11</td>
<td>6.0</td>
<td>86.1 ± 1.2</td>
<td>4.2 ± 0.1</td>
<td>13.5 ± 0.7</td>
<td>3.2</td>
</tr>
<tr>
<td>12</td>
<td>2.9</td>
<td>24.2 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>2.7</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>2.6</td>
<td>71.6 ± 1.4</td>
<td>0.9 ± 0.0</td>
<td>51.4 ± 0.2</td>
<td>59.1</td>
</tr>
<tr>
<td>15</td>
<td>2.8</td>
<td>73.0 ± 2.1</td>
<td>1.6 ± 0.0</td>
<td>20.3 ± 0.3</td>
<td>13.0</td>
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<tr>
<td>16</td>
<td>2.4</td>
<td>0</td>
<td>ND</td>
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<tr>
<td>17</td>
<td>6.4</td>
<td>92.4 ± 2.7</td>
<td>1.9 ± 0.0</td>
<td>32.3 ± 0.5</td>
<td>17.4</td>
</tr>
<tr>
<td>18</td>
<td>6.3</td>
<td>16.0 ± 1.1</td>
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<td>ND</td>
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<tr>
<td>19</td>
<td>2.5</td>
<td>77.5 ± 3.2</td>
<td>1.6 ± 0.0</td>
<td>15.7 ± 0.5</td>
<td>9.8</td>
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<td>20</td>
<td>2.5</td>
<td>83.2 ± 2.1</td>
<td>1.8 ± 0.0</td>
<td>18.6 ± 0.1</td>
<td>10.6</td>
</tr>
<tr>
<td>21</td>
<td>2.2</td>
<td>10.9 ± 0.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AZT</td>
<td>5.0</td>
<td>89.8 ± 7.1</td>
<td>1.1 ± 0.0</td>
<td>24.1 ± 0.6</td>
<td>22.9</td>
</tr>
</tbody>
</table>

ND = Not determined.

* Highest noncytotoxic concentration.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc μM</th>
<th>% Inhibition</th>
<th>IC50 μM</th>
</tr>
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<tr>
<td>11</td>
<td>20</td>
<td>67.7</td>
<td>36.8 ± 2.0</td>
</tr>
<tr>
<td>14</td>
<td>20</td>
<td>66.5</td>
<td>44.6 ± 1.4</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>56.0</td>
<td>50.2 ± 1.7</td>
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<tr>
<td>17</td>
<td>20</td>
<td>66.8</td>
<td>25.2 ± 1.9</td>
</tr>
<tr>
<td>19</td>
<td>20</td>
<td>74.0</td>
<td>28.5 ± 0.9</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>69.7</td>
<td>39.3 ± 1.0</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>5%</td>
<td>98.8</td>
<td>1.2 ± 0.07%</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc μM</th>
<th>% Inhibition with R5 tropic virus</th>
<th>% Inhibition with X4 tropic virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>6.0</td>
<td>59.6 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>2.6</td>
<td>52.9 ± 4.7</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>2.8</td>
<td>58.6 ± 3.6</td>
<td>27.8 ± 0.6</td>
</tr>
<tr>
<td>17</td>
<td>5.1</td>
<td>55.8 ± 10.8</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>2.5</td>
<td>71.5 ± 4.4</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
<td>73.4 ± 0.3</td>
<td>44.7 ± 0.8</td>
</tr>
</tbody>
</table>

* Highest noncytotoxic concentration.
In order to explore the effect of substitution pattern on the phenyl ring, electron withdrawing groups like F and CF3 were introduced (18–21). Compounds having 2,4-disubstitution (18 and 21) were inactive. Compounds having 3,4- and 3,5-disubstitution (19 and 20) were found to be active as anti HIV agents with IC50 of 1.6 and 1.8 μM and TI of 9.8 and 10.6, respectively. Both these compounds inhibited entry of R5 tropic virus at a concentration of 2.53 μM. Compound 19 was more active than 20 in HIV-1 IN assay (IC50 of 28.5 μM Vs 39.3 μM). The above observations suggest that substitution at 3, 4 and 5-positions of phenyl ring of anilide part might be important for anti HIV activity. Compounds with adamantyl group and phenyl group showed better activity than the others.

2.4. Molecular modeling

The main objective of docking studies is to explore the binding mode of these compounds against active site of HIV-1 IN and CCR5 proteins. Several computational docking studies using the crystal structure of the IN core domain and inhibitors have been reported.29–35 The docking study of raltegravir (marketed drug) was performed in to the active site of HIV-1 IN. It was found that raltegravir docked near to Mg2+ ion (Fig. 3A) and keto-enol moieties were coordinated by Mg2+ ion (Table S1 in Supplementary data for coordination distance). This coordination is required for chelation of the metal ion.30,36,37 The H-bonding interaction and cation–ion interaction exhibited with Asn155 and Mg2+, respectively. This docking result well supported by published docking results of raltegravir.38 Similarly, synthesized compounds were also docked in to the same active site of HIV-1 IN. All HIV-1 IN active compounds also occupied same space near to Mg2+ ion as raltegravir (Fig. 3B) for best bound conformation of inhibitors into the active site of HIV-1 IN. Similarly, the keto group of caffeoyl moiety was also coordinated by Mg2+ ion (Table S1 in Supplementary data for coordination distance). The reported data also showed similar type of interaction between metal ion of HIV-1 IN and highly electronegative atoms of most of the HIV-1 IN inhibitors.29,36,39,40 The catechol moieties of all compounds were placed near to Lys159. This residue established cation-II and H-bonding interactions with all the compounds. Additionally, ring B of compound 14 also exhibited cation–ion interaction with Mg2+ (Table S1 and Figs. S1–S6 in Supplementary data). The interactions with Lys159 have prime importance in binding of inhibitors into the active site of HIV-1 IN.34 All the compounds consistently exhibited H-bonding with residues Thr66, His67, Gln148, and Lys159 (Table S1 and Figs. S1–S6). The ring A of inhibitors is placed in a hydrophobic cavity formed by active site residues Cys65, Thr66, His67, Asn155, Lys156 and Lys159, ring C was found near to residues Gln62, Leu63, His114, Thr115, Gln148, and Ile151 that exhibited hydrophobic interactions. Series of interactions in docking studies strengthen the binding of inhibitors to the active site of HIV-1 IN.

Docking studies were also performed on CCR5 modelled protein using AUTODOCK 4.2 program. The putative binding modes of different compounds were published in last few years using modelled CCR5 protein. Based on published results, the active site residues were considered during docking studies.41–44 The binding site of CCR5 is hydrophobic in nature due to multiple aromatic residues present in pocket. This property imparted tight binding to the inhibitors.45 Figure 4 shows best docked conformation of inhibitors into the active site of CCR5. Most of the compounds consistently exhibited H - bonding interaction with backbone carbonyl of Ser160 and amino hydrogen of Lys197 (Table S1). The interaction with Lys197 was supported by literature study on site directed mutagenesis.46 Interestingly, the aromatic side chain of Phe245 interacted via II–II stacking with aromatic rings (see Table S1 and Figs. S7–S12 for II–II interaction in Supplementary data) of all the inhibitors except compound 11 which only exhibited II–II stacking with Phe109 and Trp248. Additionally, other compounds 17 and 20 also exhibited II–II stacking with aromatic side chain of Phe109 and Trp248. The ring B of all inhibitors interacted with Lys197 (–NH3+) via cation–ion interaction (Table S1). Altogether, hydrophobic nature of CCR5 active site and multiple ring system of inhibitors imparted hydrophobic interactions with active site residues (Phe109, Phe112, Gly163, Thr167, Ser169, Ser180, Phe182, Lys197, Ile200, Leu203, Leu207, Phe245, Leu246, Trp248, Ala249, Tyr251 and Asn252). The compound 11, unlike other compounds did not superimpose into the active site. (Fig. 4 for compound 11 in magenta color). Low inhibitory activity of compound
4.1.1. General

4.1. Chemistry

of this, it is expected that generation of drug resistant viruses will compared to only RT inhibition in case of previous reports. Because by two distinct mechanisms, integrase and entry inhibition as trend in docking studies with good binding score that was sup-

11 is reinforced by different orientation into active site of CCR5 with low binding energy. All the compounds followed the same trend in docking studies with good binding score that was sup-
ported by experimental inhibitory activity (Table S1).

3. Conclusion

In conclusion, we have synthesized caffeoyl-anilides as port-
manteau inhibitors of HIV-1 IN and cellular CCR5. The intermediate 4-amino-3′,4′-dihydroxychalcone was prepared by MSA in good yield in very short time. Compounds 11, 14, 15, 17, 19 and 20 were found to be active in cell based anti-HIV assay with EC50 values of 4.1, 1.9, 1.6, 1.8, 1.6 and 1.8

4.1.2. Procedure for synthesis of (E)-3-(4-aminophenyl)-1-(3,4-
dihydroxyphenyl)acryloyloxy)phenyl)-acet-

amide (8). 77%; Olive green solid; mp >280

°C (Buchi, Switzerland) operating at a reduced pressure. HPLC analy-
ses were performed using Shimadzu HPLC system with a PDA detector on Phenomenex Luna C18 column (250 × 4.6 mm, 5µ) with a mixture of solvents A and B (condition 1: A/B = water/aceto-
nitrite 30:70, flow rate 1.0 mL/min; condition 2: A/B = 0.2% acetic acid in water/acetonitrile 50:50, flow rate 1.0 mL/min). All compounds showed >95% purity by HPLC.

4.1.2. Procedure for synthesis of (E)-3-(4-aminophenyl)-1-(3,4-
dihydroxyphenyl)prop-2-en-1-one (7)

4-Aminoacetophenone (5, 500 mg, 3.70 mmol) was allowed to stir with 3,4-dihydroxybenzaldehyde (6, 391 mg, 2.84 mmol) in MSA (1.42 mL, 14.85 mmol) for 5 min at room temperature. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous sodium sulfate. Solvent was removed under vacuum and crude product was purified by column chromatography to yield 7. Yield: 60%; yellow solid; mp 184–185 °C; IR (Neat) νmax 3441, 3357, 1653, 1598, 1529, 1441, 1277 cm⁻¹; ¹H NMR (CD3OD) δ 7.88 (d, 2H, J = 8.2 Hz), 7.60 (d, 1H, J = 15.4 Hz), 7.48 (d, 1H, J = 15.4 Hz), 7.16 (d, 1H, J = 2.1 Hz), 7.07 (d, 1H, J = 8.2 Hz), 6.81 (d, 1H, J = 8.1 Hz), 6.68 (d, 2H, J = 8.6 Hz); ¹³C NMR (CD3OD) δ 190.8, 155.7, 149.9, 147.2, 145.6, 132.7, 129.1, 128.2, 123.6, 120.2, 117.0, 116.0, 114.7; MS (ESI) m/z 256 [M+1]⁺; HRMS (ESI) m/z calcd for C18H13NNaO3 [M+Na]⁺: 278.0793, found: 278.0794.

4.1.3. General procedure for synthesis of 8–21

To the solution of 7 in THF, various acid chlorides (1.0 equiv) were added. Reaction mixture was allowed to stir for 4 h at room temperature. Solvent was evaporated under vacuum and crude product was washed with n-hexane to yield 8–21 in 74–97% yield.

4.1.3.1. (E)-N-(4-(3-(3,4-Dihydroxyphenyl)acryloyloxy)phenyl)-acet-

amide (8). 77%; Olive green solid; mp >280 °C (Buchi, Switzerland) operating at a reduced pressure. HPLC analyses were performed using Shimadzu HPLC system with a PDA detector on Phenomenex Luna C18 column (250 × 4.6 mm, 5µ) with a mixture of solvents A and B (condition 1: A/B = water/acetonitrile 30:70, flow rate 1.0 mL/min; condition 2: A/B = 0.2% acetic acid in water/acetonitrile 50:50, flow rate 1.0 mL/min). All compounds showed >95% purity by HPLC.

4.1.3.2. (E)-N-(4-(3-(3,4-Dihydroxyphenyl)acryloyloxy)phenyl)-iso-

butyramide (9). 75%; Brown dark oil; IR (KBr disc) νmax 3323, 1646, 1593, 1521 cm⁻¹; ¹H NMR (CD3OD) δ 7.94 (d, 2H, J = 8.4 Hz), 7.64 (d, 2H, J = 8.4 Hz), 7.57 (d, 1H, J = 15.4 Hz), 7.41 (d, 1H, J = 15.4 Hz), 7.10 (s, 1H), 7.01 (d, 1H, J = 8.1 Hz), 6.72 (d, 2H, J = 8.6 Hz), 2.0 (s, 3H); ¹³C NMR (CD3OD) δ 189.8, 170.6, 148.6, 145.5, 143.0, 133.4, 129.3, 127.0, 122.3, 118.8, 118.7, 118.0, 115.2, 114.3, 22.6; MS (APCI) m/z 298 [M+1]⁺; HRMS (ESI) m/z calcd for C18H13NNaO3 [M+Na]⁺: 320.0899, found: 320.0901.

4.1.3.3. (E)-N-(4-(3-(3,4-Dihydroxyphenyl)acryloyloxy)phenyl)-3-

methylbutanamide (10). 78%; Dark brown oil; IR (Neat) νmax 3516, 3309, 1665, 1592, 1564, 1524 cm⁻¹; ¹H NMR (CD3OD) δ 8.05 (d, 2H, J = 8.4 Hz), 7.76 (d, 2H, J = 8.4 Hz), 7.68 (d, 1H, J = 15.0 Hz), 7.52 (d, 1H, J = 15.4 Hz), 7.20 (s, 1H), 7.11 (d, 1H, J = 8.2 Hz), 6.83 (d, 1H, J = 8.2 Hz), 2.29 (d, 2H, J = 7.1 Hz), 2.18 (sept, 1H, J = 6.7 Hz), 1.02 (d, 6H, J = 6.4 Hz); ¹³C NMR (CD3OD) δ 189.8, 173.0, 148.6, 145.5, 145.0, 143.0, 133.5, 129.4, 127.0, 122.3, 119.0, 118.0, 115.2, 114.3, 45.9, 26.1, 21.3; MS (APCI) m/z
7.67 (d, 1H, J = 8.4 Hz), 7.77 (d, 2H, J = 8.8 Hz), 7.67 (d, 1H, J = 15.2 Hz), 7.49 (d, 1H, J = 15.6 Hz), 7.18 (d, 1H, J = 6.7 Hz), 7.09 (d, 1H, J = 8.0 Hz), 6.81 (d, 1H, J = 8.0 Hz), 1.69–2.10 (15H, protons of adamantyl); 13C NMR (CD3OD) δ 189.8, 178.0, 148.6, 145.5, 145.4, 141.3, 133.6, 129.1, 127.0, 122.3, 120.0, 118.1, 115.2, 114.3, 36.8, 36.2, 28.0; MS (ESI) m/z 418 [M+1]*; HRMS (ESI) m/z calcd for C32H27NNaO4 [M+Na]^+: 432.1212, found: 432.1200.

1.1.1.9. (E)-N-(4-(3,4-Dihydroxyphenyl)acryloyl)phenyl)-2-naphthamide (16). 80%. Buff white solid; mp 187–189 °C; IR (Neat) νmax 3184, 3099, 2924, 1690, 1659, 1659 cm⁻¹; 1H NMR (CD3OD) δ 10.75 (s, 1H), 9.71 (s, 1H), 9.13 (s, 1H), 8.62 (s, 1H), 8.17 (d, 2H, J = 8.4 Hz), 8.10–7.99 (m, 6H), 7.68–7.57 (m, 4H), 7.26 (s, 1H), 7.18 (d, 1H, J = 8.0 Hz), 6.81 (d, 1H, J = 8.0 Hz); 13C NMR (DMSO-d6) δ 187.9, 166.5, 149.1, 146.0, 144.9, 134.9, 134.9, 133.5, 132.4, 132.3, 130.0, 129.5, 128.7, 128.6, 127.4, 126.8, 124.9, 120.0, 118.8, 116.2, 116.0; MS (ESI) m/z 432 [M+Na]^+: HRMS (ESI) m/z calcd for C32H27NNaO4 [M+Na]^+: 468.0835, found: 468.0828.
4.2. Biology

4.2.1. Cell cytotoxicity assay using MTT

Cytotoxicity of potential candidates was assayed using MTT Kit (Roche) in the CEM-GFP cell line according to the manufacturer's protocol. Briefly, 2 × 10^4 cells/well were seeded in 96-well plate; samples were then added into the wells at different concentrations keeping untreated and vehicle treated wells as controls. After 48 h incubation, 10 μL of MTT Reagent (5 mg/mL) were added in to the wells to allow the reaction. Formazan crystals produced during the reaction were solubilized and color development was read at 540 nm. The highest noncytotoxic concentration was determined by first measuring % viability at three different concentrations, and then making further dilutions to determine the concentration that gave 95% viability of the cells.

4.2.2. Anti-HIV screening in CEM-GFP cells

Human CD4+ T cell line, CEM-GFP cells were infected with HIV-1NL4.3 virus at a multiplicity of infection (MOI) of 0.05 using the standard protocol previously published from the lab.27,28 The infection was monitored by GFP visualization under the microscope. The cells were then incubated with samples for up to 7 days post infection. Preliminary screening was performed by GFP quantitation by microfluorometry. Virus production was assayed in the culture supernatant on day-7 post infection by p24 antigen capture ELISA (Perkin–Elmer Life Science, USA). AZT was used as a positive control. The activity is reported as% decrease in p24 antigen in the culture supernatant of HIV-1 infected cells treated with compound as compared to culture supernatant of untreated HIV-1 infected cells.

4.2.3. HIV-1 integrase inhibition assay

In vitro integrase assay was performed to analyze the HIV-1 IN inhibition by the compounds using HIV-1 Integrase Assay kit (Xpressbio Life Science Products, USA) according to manufacturer's protocol. Briefly, Streptavidin coated 96-well plates were coated with a double-stranded HIV-1 LTR U5 donor substrate (DS) oligonucleotide containing an end-labeled biotin. Full-length recombinant HIV-1 integrase protein was then loaded onto this oligo substrate. Compounds were added to the reaction and then a different double-stranded target substrate (TS) oligo containing 3’-end modifications was added to the plate. The HIV-1 integrase cleaves the terminal two bases from the exposed 3’-end of the HIV-1 LTR DS and then catalyzes a strand-transfer reaction to integrate the DS into the TS. The products of the reaction were detected colorimetrically using an HRP-labeled antibody directed against the TS 3’-end modification. Percent inhibition in the integrase assay was first measured at 20 μg/mL, as all the compounds showed more than 50% inhibition, the IC_{50} values were determined in μg/mL and then converted to μM. Sodium azide was used as a positive control in this experiment.

4.2.4. Entry inhibition assay

TZM-bl cells are reporter cells expressing CD4, CCR5 and CXCR4 along with reporter proteins luciferase and β-galactosidase under the control of HIV-1 long terminal repeat (LTR) promoter.46 TZM-bl cells (10^4) were seeded in each well of a 96 well plate. Cells were incubated overnight at 37°C in a CO_2 incubator. After 16–20 h, medium was removed from the wells and were fed with fresh complete medium (DMEM with 10% FBS; 100 μL/well). Compounds were then added in different concentrations to respective wells. After 1 h of incubation, cells were washed twice with serum free media and were then infected with HIV-1 Indiec (RS isolate) or HIV (X4 isolate). After 4 h of infection, cells were again washed twice with serum free media and were then re-suspended in complete medium. After 48 h, cells were washed twice with PBS and Luciferase assay was performed using SteadyLite® luciferase assay kit (Perkin–Elmer Life Science, USA) according to manufacturer's protocol. Relative Luciferase units were compared between control infected cells and treated infected cells controls to calculate percent inhibition of virus infection.

4.3. Molecular modeling

The six active compounds were selected for docking studies against HIV-1 IN (PDB ID: 1QS4)47 and CCR5 proteins. 1QS4 is used for binding mode analysis of HIV-1 IN inhibitory activity. However, no X-ray crystal structure is available in PDB for CCR5 protein. So, hypothetical model of CCR5 was generated using MODELLER 9v7 program.48 Protein sequence of CCR5 was retrieved from Swissprot ID P51681. The first step was to search for a number of related sequences to find a related three dimensional protein structure as a template by the PSI-BLAST program. Bovine rhodopsin (PDB ID: 1F89)49 was mostly selected as a template molecole for CCR5 model development.41,43,45 The sequence alignment was performed using Align2D module in Modeller program and also alignment was manually checked for error (Fig. S13 in Supplementary data shows sequence alignment between CCR5 and template protein). Protein models were built and minimized using CHARMMg to obtain stable structures. Protein models were validated using PROCHECK50 and finally one model that fits best in Ramachandran plot was used for docking studies (Fig. S14 in Supplementary data shows Ramachandran plot of CCR5 model and Fig. S15 shows super imposition of model with template protein). All the compounds were built using Sybyl7.51 and subsequently minimized using Powell gradient method52 using Gasteiger Huckel charges.53 The protein structures were prepared for docking using AUTODOCK Tool. The docking was performed using AUTODOCK 4.2 program.54 Co-crystallized ligand and all water molecules were removed from crystal protein (1QS4) while a magnesium ion (Mg^{2+}) at the active site of HIV-1 IN (1QS4) was maintained. Polyr hydrogens were added and non polar hydrogens were merged, finally Kallman united atom charge and atom type parameter was added to both 1QS4 and modelled CCR5 proteins. Grid map dimensions (for HIV-1 IN 56 × 68 × 46; for CCR5 64 × 56 × 60) were set surrounding active site. Lamarckian genetic search algorithm54 was employed and docking run was set to 30.

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Supplementary data


References and notes
