CHAPTER 3
MATERIAL AND METHODS
PROCUREMENT OF COMPOUNDS

Table 3.1: List of mallotojaponin (phloroglucinol) analogues selected for anti-HIV-1 activity

<table>
<thead>
<tr>
<th>IUPAC names of selected compounds</th>
<th>CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-[3-Acetyl-5-[(3,5-diacetyl-2,4,6-trihydroxyphenyl)cyclohexyl-methyl]-2,4,6-trihydroxyphenyl]ethanone</td>
<td>M1</td>
</tr>
<tr>
<td>1-[3-Isopentanoyl-5-[(3,5-diisopentanoyl-2,4,6-trihydroxyphenyl)-phenyl-methyl]-2,4,6-trihydroxyphenyl]-3-methylbutan-1-one</td>
<td>M2</td>
</tr>
<tr>
<td>1-[3-Isopentanoyl-5-[(3,5-diisopentanoyl-2,4,6-trihydroxyphenyl)-pyridin-2-yl-methyl]-2,4,6-trihydroxyphenyl]-3-methylbutan-1-one</td>
<td>M3</td>
</tr>
<tr>
<td>1-[3-Acetyl-5-[(3,5-diacetyl-2,4,6-trihydroxyphenyl)-furan-2-yl-methyl]-2,4,6-trihydroxyphenyl]ethanone</td>
<td>M4</td>
</tr>
<tr>
<td>1-[3-Acetyl-5-[(3,5-diacetyl-2,4,6-trihydroxyphenyl)-quinolin-4-yl-methyl]-2,4,6-trihydroxyphenyl]ethanone</td>
<td>M5</td>
</tr>
<tr>
<td>1-[3-Acetyl-5-[(3,5-diacetyl-2,4,6-trihydroxyphenyl)-4-N,N-dimethyl-aminophenyl-methyl]-2,4,6-trihydroxyphenyl]ethanone</td>
<td>M6</td>
</tr>
<tr>
<td>1-[3-Isopentanoyl-5-[(3,5-diisopentanoyl-2,4,6-trihydroxyphenyl)-4-benzyloxyphenyl-methyl]-2,4,6-trihydroxyphenyl]-3-methylbutan-1-one</td>
<td>M7</td>
</tr>
</tbody>
</table>
Table 3.2: List of Buchapine and its analogues selected for study

<table>
<thead>
<tr>
<th>IUPAC names of selected compounds</th>
<th>CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,3-Bis(3-methylbut-2-enyl)quinoline-2,4(1H,3H)-dione</td>
<td>B1</td>
</tr>
<tr>
<td>2-Butoxy-4-(prop-2-ynyloxy) quinoline</td>
<td>B2</td>
</tr>
<tr>
<td>3-(4-Methylpent-3-enyl)-4-(4-methylpent-3-enyloxy)quinolin-2-ol</td>
<td>B3</td>
</tr>
<tr>
<td>4-(Isopentyloxy)quinolin-2-ol</td>
<td>B4</td>
</tr>
<tr>
<td>3-(3-methylbut-2-enyl)-4-(3-methylbut-2-enyloxy)quinolin-2-ol</td>
<td>B5</td>
</tr>
<tr>
<td>4-(3-Methylbut-2-enyloxy)quinolin-2-ol</td>
<td>B6</td>
</tr>
<tr>
<td>3-(3-Methylbut-2-enyl)-3-(1,1-dimethylprop-2-enyl)quinoline-2,4(1H,3H)-dione</td>
<td>B7</td>
</tr>
<tr>
<td>3,3-bis(4-methylpent-3-enyl)quinoline-2,4(1H,3H)-dione</td>
<td>B8</td>
</tr>
<tr>
<td>3-(Prop-2-ynyloxy)4-(prop-2-ynyloxy)quinolin-2-ol</td>
<td>B9</td>
</tr>
<tr>
<td>4-(Prop-2-ynyloxy) quinolin-2-ol</td>
<td>B10</td>
</tr>
<tr>
<td>3-Isopentyl-4-(isopentyloxy)quinolin-2-ol</td>
<td>B11</td>
</tr>
</tbody>
</table>
3.1 Cell lines

TZM-bl (HeLaCD4+CCR5+CXCR4+, LTR-Luc-β-Gal), was obtained through the NIHAIDSRPP, US (PlattBilska et al., 2009; TakeuchiMcClure et al., 2008; WeiDecker et al., 2002). HEK-293T (LandryHalin et al., 2007; DevittThomas et al., 2007) and ME180 cells (TanPearce-Pratt et al., 1993) were obtained from the cell repository of National Centre for Cell Science, India. TZM-bland 293T cells were propagated in DMEM (Invitrogen, USA) with 10% FBS (Invitrogen, USA) and 0.1% penicillin and streptomycin (Sigma, USA). ME180 cells were propagated in McKoy’s 5a medium containing 10% FBS (Invitrogen, USA). PBMCs were isolated using histopaque (Sigma, USA) density gradient centrifugation from blood of healthy donors. 2µg/ml PHA used as stimulant was added 36h prior to infection.

3.2 Virus isolates and generation of viral stocks

HIV-1 X4 tropic molecular clone NL4.3 and IIIB, R5 tropic strains HIV-1ADA and JRCSF, X4-R5 dual tropic strains 89.6 and Yu-2 were obtained from NIHAIDSRPP, USA. HIV-1 Indie C1, a full length molecular clone of HIV-1 subtype C Indian isolate was a kind gift of Dr. MTatsumi, Japan (MochizukiOtsuka et al., 1999). Primary subtype C isolates VB51 and VB52 were obtained from NARI, India.

In 293T cells, transfections of clones were carried out to generate/produce virus. Briefly, around 1.5 X 106 293T cells were seeded in 90 mm culture plates and were incubated for 12-16 hours for adherence in CO2 incubator. After the incubation period, cells were transfected with plasmid DNA using calcium phosphate transfection. In this, Calcium-DNA complexes were prepared by mixing 15 to 20 µg of plasmid DNA in 125 mM CaCl2 (Sigma, USA) with HEPES buffer. After 20min of incubation at room temperature, the mix was added over the cells. After 12 hours of transfection, cultures supernatant was collected and was stored at −70 °C. Virus was pelleted from
culture supernatants at 28000 rpm for 2 hours 30 minutes using ultracentrifugation in SW29 rotor. The pellet was resuspended in serum free RPMI with 100 mM HEPES, pH 7.3 filtered, aliquot and was stored at –70 °C.

Subtype C primary isolates VB51 and VB52, isolated from infected patients were obtained from NARIPune, India

3.3 Quantization of Concentrated Virus

Amount of virus in the supernatant was assessed using HIV-1 p24 Antigen Core ELISA according to manufacturer’s protocol. Briefly, Virus pellet was serially diluted in serum free RPMI. 200ul of diluted sample was added to the pre-coated wells and 20ul of Lysis buffer was added. Plate was incubated for 2h and then washed to remove unbound protein. Biotinylated detector antibody was then added. The wells were then washed to remove unbound antibody. 100ul Streptavidin HRP was then added. Color development was facilitated by adding substrate to HRP. Absorbance was measured at 490nm. Based on absorbance concentration of virus was calculated.
3.4 Determination of Infectious Virus Concentration of Viral Stocks

The total number of infectious virus in the viral stock was calculated according to the standard protocol using TZM-bl cell line (Kimpton & Emerman, 1992). In this, 2 X 10^5 cells/well were seeded in 12 well plate and were incubated overnight. After the incubation, viral stock was serially diluted in 1 ml of complete media. The medium was discarded and the monolayer of TZM-bl cells were infected with diluted virus for 4 hours. Uninfected cells were used as negative control. The infected cells were incubated for 48 hours and then media was removed and washed with 1X PBS. 0.5% glutaraldehyde was used to fix the cells at room temperature for 10 min. Cells were washed with PBS to remove glutaraldehyde completely.

Then β-galactosidase staining solution [Potassium ferri ferrocyanide (12.5 mM Potassium ferricyanide and 12.5 mM Potassium ferrocyanide); 1:50 dilution of 50 mg/ml 5-bromo-4-chloro-3-indolyl, β-D-galactoside (X-gal) in Dimethylformamide and 1 mM MgCl2 in 1X PBS] was added on top of the monolayer of fixed cells. The infected cells stained blue with β-gal staining solution.

The total population of cells having blue color was counted under the microscope at 10X magnification in five to eight fields and average of all the fields was calculated.

The total number of infectious virions/ng of the viral stock was calculated using the following equation.

\[
\text{Average of total number of blue cells } \times 150 = \frac{\text{Amount(ng) of virus}}{\text{Amount(ng) of virus}}
\]

The MOI can be calculated as the ratio between the number of infectious virions to the total number of cells used in the infection assay.
3.5 Cell Viability assay

Viability of TZM-bl, ME180 cells and PBMCs (104 cells/well in 96 well plate) after 48 h post-incubation with different concentration of compounds was assayed using MTT Kit (Roche) according to the manufacturer’s protocol as described earlier (Bodiwala Sabde et al., 2011b; Vassilev Kazmer et al., 2001). Briefly, Cell Proliferation Kit (Roche) was used to see the effect of compounds on proliferation of TZM-bl cell line, according to the manufacturer’s protocol. Briefly, 1×104 cells/well were cultured in flat bottom 96 well culture plate. Cells were treated with different concentrations of compounds.

In controls cells were kept nontreated. After 48 hrs incubation, addition of 10 µl of MTT Reagent (5 mg/ml) to the wells initiated reaction. Isopropanol was used to solubilize the crystals of formazan produced. Absorbance was read at 540 nm. For assessing cytotoxicity of compounds in primary culture of hPBMCs, 50×104 cells/well were added in 96-well plate and assay was carried out as described above.
3.6 In vitro efficacy studies using TZM-bl cells

TZM-bl cells express Escherichiacoli lacZ and fireflyluciferase gene under LTR promoter. Upon infection, Tat produced by provirion causes activation/amplification of the reporter gene, resulting ingeneration of β-galactosidase as well as luciferase inthese cells. After 48hof culture, the luciferase activity was assessed, as described earlier (TakeuchiMcClureet al., 2008; Bodiwala, Sabde et al 2011a).

3.6.1 Anti-HIV assay in TZM-bl cells

The compounds were assessed in TZM-bl(Hela-CD4-LTR-βGal-Luc)cells. CD4CXCR4 and CCR5 are expressed in abundance on cell membrane of TZM-bl cells. It also has luciferase and β-galactosidase genes inserted/clone downstream of the HIV-1 LTR. Infection of TZM-bl cells with HIV-1 pretreated with samples is a method used by many laboratories to study the potential of antivirals as a microbicide candidate. The cells were infected with 0.05 MOI of NL4.3 viruses either with or without sample pretreatment, followed by incubation for 48 hours with the samples. The antiviral activity was assayed by luciferase assay of the cell lysate after 48 hours. Zidovudine (Azt) was used as positive control.
3.7 Anti-HIV activity assay in human PBMCs

Human PBMCs (PBMC) were used for anti-HIV activity assay as described previously (Ulrich Busch et al., 1988a; Slusher Kuwahara et al., 1992). Briefly, 5 X 10^6 activated normal huPBMCs were infected with HIV-1 NL4.3 at a MOI of 0.05 in serum free RPMI containing polybrene. After 4 h of infection, cells were washed twice with serum free RPMI and then cells were resuspended in complete medium. Cells were stimulated with 20 U/ml recombinant human interleukin-2 (Roche, Germany). Cells were added to 24-well plates and compounds were added at different concentrations. AZT was used as a positive control.

Culture was continued for 7 days. Supernatants were collected. Virus inhibition by compound was assessed by analyzing levels of p24 protein in the culture supernatant by using HIV-1 p24 antigen capture ELISA (PerkinElmer USA). For anti-HIV-1 activity against other X4R5 and dual tropic viruses same method was followed. For infection of PBMCs with primary isolates, 5 ng of virus were used to infect 5 X 10^6 cells.

3.8 Time of Addition experiment

In order to identify the step at which the compound might be inhibiting HIV infection, we have performed infection in TZM-bl cells with addition of compounds at different timepoints. For pretreatment with virus, HIV-1 NL4-3 virus was mixed with compounds and incubated for 1 h. Thereafter, the virus + compound mixture was added to TZM-bl cells in 96-well plates and incubated for 4 h. Cells were then washed to remove unbound virus and compounds. Cells were cultured for 48 h and then assessed for luciferase activity. Percent inhibition of virus infection was calculated by comparing relative luciferase units of compound treated and nontreated cells. For during infection condition, virus and compounds were added simultaneously to TZM-bl cells followed by incubation and luciferase assay as described above. For post-infection condition, TZM-bl cells were infected with
virus for 4h. Unbound virus was removed by washing with serum free medium. Compounds were added to the wells after 0h, 2h, 6h and 10h of infection. Cells were then assessed for luciferase activity after 48h as described above.

3.9 In vitro Reverse Transcriptase activity

Inhibition of HIV-1 RT activity by compounds was assessed using Reverse Transcriptase Assay kit (Roche, Germany) as described earlier (Bodiwala Sabde et al., 2011b). Nevirapine, a potent NNs RTI was used as positive control.

3.10 In vitro Integrase assay

To evaluate the compounds for integrase inhibition activity HIV-1 Integrase Assay kit (Xpressbio LifeScience Products USA) was used according to manufacturer’s protocol. In this assay, a double stranded HIV-1LTR U5 donorsubstrate (DS) oligonucleotide containing an end-labeled-biotin was added to streptavidin coated 96 well plate. The integrase is then added to the oligo. The mixture is treated with different concentrations of compounds. A dsOligo target substrate containing 3'-end modifications was then included in the reaction mix. Integrase has inherent property to cleave the two oligos from the 3'-end of the HIV-1 LTR DS. A strandtransfer reaction catalyzed by IN causes integration of DS oligo into TS oligo strand. The 3'end modification was then detected colorimetrically by addition of an antibody-HRP labeled against target substrate.

Absorbance was measured. Absorbance of compound treated wells and controls were compared to calculate the percent inhibition by compounds. Raltegravir was used as a reference compound in this experiment.
3.11 Cell-Cell fusion assay

1ug of pYK-YU2-gp120 and 500ng of pcTat were introduced in 293T cells by transfection, using Lipofectamine2000 (Invitrogen, USA) and incubated for 24h. Meanwhile 104 TZM-bl cells/well were cultured in a 96 well plate. On day 2 post-transfection, 293Tcells were trypsinized and mixed in 1:2 ratio with TZM-bl cells in presence or absence of compounds. The co-culture was incubated for 24h in CO2 incubator. On day 4, cells were washed with PBS twice and fixed using 0.2% glutaraldehyde and then stained for β-galactosidase enzyme activity using substrate beta-gal (5-bromo-4-chloro-3-indolyl-β-D galactopyranoside Sigma, USA). After 12h, numbers of stained cells in untreated and treated wells were counted and percent of inhibition by the compounds was calculated.

3.12 Evaluation of compounds as potential HIV-1 microbicide

3.12.1 Measurement of transepithelial resistance:

5 X 105 ME180 cells were added to laminin coated trans-well inserts of plate (0.4µm polycarbonate, 6.5mm inserts, Costar). Transepithelial electrical resistance was measured on day 9 after addition of fresh medium, in presence or absence of compounds, using MillicelERSpotentiometer (Millipore USA). Fluorescent beads were also used to check confluence of epithelial layer of ME180 cells as described earlier(GaliArien et al., 2010).
3.12.2 Effect of compounds on secretion of proinflammatory cytokines

2×10⁵ ME180 cells were cultured overnight at 37°C in CO₂ incubator. Medium was replaced and cells were treated with nontoxic concentrations of compounds for 24 h. Culture supernatants were collected and then assayed for presence of pro-inflammatory and anti-inflammatory cytokines by using multiplex bead based array in Bioplex 200 system (BioRad, USA) according to manufacturer’s protocol.

3.12.3 Transepithelial migration of HIV-1 NL4.3 virus in the dual chamber model system

Once ME180 cells reached confluence, HIV-1 NL4.3 virus (0.05 MOI) and the compound mixture was added in laminin-coated (200 ng/ml) apical chambers of trans-well plate. In basal chamber, 2×10⁵ PBMCs were added. The apical chamber with cells is then put into basal well of trans-well plate. After 24 h, trans-well was removed from the plate and culture of PBMCs was continued for 10 days in complete medium. At day 10, culture supernatants were collected to assess p24 gag protein levels produced in control and treated wells. Tenofovir was used as a positive control in this experimental system.

3.12.4 Anti-HIV activity against cell associated virus:

To infect ME180 cells, we followed protocol described by Ulrich et al. 1995 (Ulrich Busch et al., 1988b), with slight changes. Briefly, 10⁴ ME180 cells/well were cultured in a 96 well plate and incubated overnight. PBMCs infected with 0.05 MOI of HIV-1 NL4.3 virus for 7 days were centrifuged and washed 2 times with SFRPMI. Cells were then suspended in complete medium so as to get 5 X 10⁴ cells/ 50 µl. Compounds were added. Tenofovir was used as a positive control. 5 X 10⁴ cells of infected PBMCs were co-cultured for 4 h with ME180 cells.
ME180 cells were then washed thrice to remove PBMCs and culture was continued till 5 days without addition of compounds. Cell free supernatants were collected to assess levels of p24 produced by antigen capture ELISA (Perkin Elmer, USA).