CHAPTER 4

RESULTS AND DISCUSSION

4.1 Cytotoxicity and anti-HIV-1 activity of dimeric chlorogluconol derivatives in TZM-bl cells.

4.1.1 Cell Cytotoxicity Assay using MTT

Compounds were tested for cytotoxic effect in TZM-bl cells using MTT assay kit (Roche) according to the manufacturer’s protocol. Cells cultured in 96well plate were treated with range of concentration of compounds. After 48h of treatment, MTT reagent was added. Mitochondrial enzymes of live cells convert the MTT into insoluble formazan crystals. The crystals were solubilized by addition of isopropanol. Absorbance was measured at 540nm. Percent cytotoxicity was determined by comparing the viability of treated and control cells. CC50 values were calculated. The CC50 values of dimeric chlorogluconols are listed in Table 4.1 and CC50 of quinoline 2,4 diols are indicated in Table 4.2.

4.1.2 Anti-HIV screening in TZM-bl cells

The samples were further tested in TZM-bl (Hela-CD4-LTR-βGal-Luc) cells. Infection in TZM-bl cells with virus pretreated with compounds 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.
Since the compounds showed potent inhibition of infection in TZM-bl cell based assay, IC50 of these compounds was evaluated.
Table 4.1: Anti-HIV-1 activity of selected dimeric phloroglucinol derivatives

<table>
<thead>
<tr>
<th>CODE</th>
<th>Highest non toxic concentration (µM)</th>
<th>% Inhibition by luciferase assay</th>
<th>IC50 (µM)</th>
<th>CC50 (µM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>9.73</td>
<td>95.6 ± 1.2</td>
<td>2.41 ± 0.24</td>
<td>50.89 ± 2.11</td>
<td>21.12</td>
</tr>
<tr>
<td>M2</td>
<td>0.74</td>
<td>44.5 ± 0.7</td>
<td>NA</td>
<td>14.2 ± 0.05</td>
<td>NA</td>
</tr>
<tr>
<td>M3</td>
<td>7.39</td>
<td>73.5 ± 1.5</td>
<td>4.36 ± 0.12</td>
<td>21.01 ± 1.87</td>
<td>4.82</td>
</tr>
<tr>
<td>M4</td>
<td>15.06</td>
<td>60.2 ± 2.3</td>
<td>14.35 ± 0.6</td>
<td>74.74 ± 1.84</td>
<td>5.21</td>
</tr>
<tr>
<td>M5</td>
<td>35.78</td>
<td>99.26 ± 1.6</td>
<td>19.37 ± 0.66</td>
<td>112.2 ± 5.27</td>
<td>5.79</td>
</tr>
<tr>
<td>M6</td>
<td>9.07</td>
<td>99.24 ± 1.5</td>
<td>8 ± 0.27</td>
<td>64 ± 3.43</td>
<td>8.00</td>
</tr>
<tr>
<td>M7</td>
<td>5.75</td>
<td>93.91 ± 1.8</td>
<td>1.49 ± 0.2</td>
<td>28.41 ± 3.73</td>
<td>19.00</td>
</tr>
<tr>
<td>AZT</td>
<td>5</td>
<td>99.9 ± 1.2</td>
<td>1.34 ± 0.02</td>
<td>44.06 ± 0.57</td>
<td>32.88</td>
</tr>
</tbody>
</table>
7 dimeric chloroglucoinol derivatives were evaluated for HIV-1 inhibition activity in TZM-bl cells infected with HIV-1 NL4-3. M3 and M4 had shown moderate anti-HIV-1 activity while M1, M5, M6, and M7 derivatives inhibited HIV-1 infection by more than 90%. M1 having cyclohexanecarbaldehyde substitution and M7 having pyridine-2-yl substitution were most potent with IC50 values 2.41 µM and 1.49 µM. M1 and M7 exhibited better safety index compared to other analogues. Hence, the two compounds M1 and M7 were selected for further evaluation of anti-HIV-1 activity.
Table 4.2 Anti-HIV-1 activity of quinolone 2,4-diol derivatives

<table>
<thead>
<tr>
<th>Code Assigned</th>
<th>Highest non toxic concentration (µM)</th>
<th>% Inhibition by p24 ELISA</th>
<th>IC50 (µM)</th>
<th>CC50 (µM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>20.2</td>
<td>95.6</td>
<td>4.04 ±0.3</td>
<td>55.89 ±0.7</td>
<td>13.83</td>
</tr>
<tr>
<td>B1</td>
<td>8.42</td>
<td>68.18</td>
<td>2.12 ±0.01</td>
<td>48.22 ±1.2</td>
<td>22.75</td>
</tr>
<tr>
<td>B2</td>
<td>19.7</td>
<td>80.3</td>
<td>8.78 ±0.02</td>
<td>116.26 ±1.3</td>
<td>13.24</td>
</tr>
<tr>
<td>B3</td>
<td>15.38</td>
<td>94.63</td>
<td>8.46 ±0.01</td>
<td>110 ±1.2</td>
<td>13.00</td>
</tr>
<tr>
<td>B4</td>
<td>43.29</td>
<td>94.9</td>
<td>3.64 ±0.01</td>
<td>114.29 ±1.4</td>
<td>31.40</td>
</tr>
<tr>
<td>B5</td>
<td>20.2</td>
<td>82.9</td>
<td>4.07 ±0.1</td>
<td>72.05 ±1.1</td>
<td>17.70</td>
</tr>
<tr>
<td>B6</td>
<td>26.2</td>
<td>90.2</td>
<td>3.62 ±0.03</td>
<td>77.29 ±0.8</td>
<td>21.35</td>
</tr>
<tr>
<td>B8</td>
<td>12.3</td>
<td>87.6</td>
<td>4.75 ±0.7</td>
<td>51.63 ±2.1</td>
<td>10.87</td>
</tr>
<tr>
<td>B9</td>
<td>24.1</td>
<td>84.3</td>
<td>11.6 ±0.23</td>
<td>58.32 ±1.25</td>
<td>5.03</td>
</tr>
<tr>
<td>B10</td>
<td>35.2</td>
<td>83.6</td>
<td>15.23 ±0.56</td>
<td>88.3 ±2.5</td>
<td>5.80</td>
</tr>
<tr>
<td>B11</td>
<td>33.22</td>
<td>65</td>
<td>7.64 ±0.14</td>
<td>128.24 ±1.4</td>
<td>16.79</td>
</tr>
<tr>
<td>AZT</td>
<td>5</td>
<td>99.9</td>
<td>1.34 ±0.02</td>
<td>44.06 ±0.57</td>
<td>32.88</td>
</tr>
</tbody>
</table>
Cytotoxicity of quinoline 2,4 diols was assessed in TZM-bl cells as described earlier. Parent compound buchapine exhibited 50% cytotoxicity at 55.89 µM. Compounds B2, B3, B4 and B11 were less toxic than buchapine. Nafees et al had shown that quinoline 2,4 diols inhibited infection/replication of HIV-1 in CEMGFP cells. As shown in Table 4.2, all 10 derivatives of buchapine have shown potent anti-HIV-1 activity in TZM-bl cells. B1 was found to be potent compared to buchapine and other derivatives as well, with IC50 2.12 µM. B4 has shown a safety index of 31.48 close to that of AZT i.e. 32.88. B1 has a safety index greater than that of buchapine but less than AZT. The prenyl substituted quinoline 2,4 diol (B1) has shown better activity than other compounds. Based on the safety and efficacy data, it seems that B1 and B4 are more potent inhibitors of HIV-1 as compared to buchapine (B7). Hence for further evaluation of anti-HIV-1 efficacy study, B1, B4 and B7 (buchapine) were selected.
4.1.3 Anti-HIV-1 activity of dimeric phloroglucinols in TZM-bl cells infected with different strains of HIV-1

The CXCR4 tropic clones HIV-1NL4-3, HIV-1IIIB, CCR5 tropic clones HIV-1 ADA, HIV-1 NLAD8, JRCSF and dual tropic HIV-1 YU2, HIV-1 89.6 molecular clones were obtained from NIH AIDS RRP, USA. HIV-1 Subtype C virus Indiec1 was gifted by M. Tatsumi, NIID, Japan. Inhibition of HIV-1 infection by compounds was assessed against these viruses in TZM-bl cells. Viruses stocks were prepared and infectivity of each virus was evaluated as explained earlier.

To evaluate efficacy of the compounds against HIV-1 isolates each virus was diluted in DMEM to get 0.05 MOI infections of TZM-bl cells. Treatment of virus with compounds was followed by infection of cells for 4h with treated and untreated virus. Removal of free virus was accomplished by washing cells with SF medium. Then complete medium was added along with compounds to respective wells. Cells were incubated for 48 h at 37°C in CO2 incubator. Media was removed from each well. Cells were washed twice with sterile PBS. Cells were lysed and assayed for luciferase activity. Based on relative luciferase activity of treated and untreated cells, inhibition by the compounds was calculated.

4.1.4 Effect of dimeric phloroglucinols on infection of TZM-bl cells with subtype B CXCR4 tropic HIV-1 virus IIIB

Both the phloroglucinol derivatives showed potent activity against HIV-1NL4-3. To confirm the activity we used another CXCR4 tropic HIV-1 strain called HIV-1IIIB.

HIV-1IIIB readily infects T cell lines. It has been demonstrated that virus replicates in T-cells but not in macrophages. Virus was treated with compounds as explained in section 3.1.3. M1 showed 51% reduction in virus production while M7 inhibited virus production up to 0.5% in TZM-bl cells. AZT which was used as standard inhibitor showed 92.93% reduction in virus replication.
4.1.5 Effect of dimeric phloroglucinols on replication of HIV-1 subtype B and subtype C isolates in TZM-bl cells

HIV-1 IndieC1 is a subtype C-CCR5 tropic strain of HIV-1. Virus was diluted to get 0.05 MOI infection of TZM-bl cells. Virus was treated with the compounds for 1h and then cells were infected for 4h with treated and untreated virus. Cells were washed to remove free virus, with PBS. Complete medium was added along with compounds to respective wells. Culture was continued for 48h in CO2 incubator. PBS was used to wash the cells followed by lysis of cells and then assayed for luciferase activity. Based on relative luciferase activity of treated and untreated cells, inhibition by the compounds was calculated.
YK-JR-CSF is an infectious molecular clone of HIV-1JR-CSF. It was isolated from cerebrospinal fluid of an AIDS patient. PBMCs and macrophages get easily infected by this isolate compared to T-cells. The virus is noncytopathic. M1 and M7 were assessed for activity against JRCSF. 0.05 MOI of virus was used for challenge. Initially, virus was treated with compounds and cells were then infected. M7 inhibited virus replication completely while M1 showed 62.11% reduction in infection of TZM-bl cells. AZT showed 100% inhibition of JRCSF.

HIV-1 ADA is a subtype B CCR5 tropic strain of HIV-1 and NLAD8 is the clone of HIV-1 NL4-3 in which envelop of NL4-3 is replaced with that of ADA. Compounds were assessed for inhibition of HIV as described in section 3.1.3. Compound M1 when used at 9.73 µM showed 68.53% inhibition of ADA and 61% inhibition of NLAD8. M7 inhibited infection of TZM-bl cells by ADA up to 80% and by NLAD8 up to 61% at highest nontoxic concentration 5.75 µM. As indicated in Figure 4.1.2 both the compounds showed good activity against ADA and AD8 strain of HIV-1.
Figure 4.1.2: Activity of dimeric chloroglucoinols against a. IndieC and b. JRC SF, c. ADA, d. NLAD8
4.1.6 Inhibition of infection of HIV-1 dual tropic isolates HIV-1 89.6 and HIV-1 YU2 in TZM-bl cells by dimeric phloroglucinol

HIV-1 YU-2 proviral DNA was obtained from brain tissue of patients suffering from AIDS associate dementia. YU2 has very high affinity for primary lymphocytes, cells of macrophage lineage and monocytes. It has been shown that virus is not able to infect productively Sup-T1 or CEMx174 cells.

Macrophages, T lymphocytes and some transformed cell lines CEMx174 and MT-2 can be infected with HIV-189.6. Upon infection virus shows cytopathic effects in lymphocytes and CEM174 and MT2 cells. In addition to CXCR4 and CCR5 receptor, the virus can utilize CCR-3 and CCR-2, as cofactors for fusion and entry.

Both phloroglucinol derivatives have shown potent inhibition of X4 and R5 tropic HIV-1 strains. To assess their activity against the virus strains which can utilize either CXCR4 or CCR5 receptors for entry we used HIV-1 YU2 and HIV-189.6 strains. Both the viruses were treated with compounds as explained in section 3.6. M1 showed reduction of virus production up to 62.25% with 89.6 and 68.26% reduction of virus production was observed with Yu2.

Though M7 inhibited 89.6 virus replication by 100% its activity is reduced to 64.44% against YU2. AZT inhibited replication of both the viruses by 100%.
Figure 4.1.3: Anti-HIV-1 activity of dimeric phloroglucinols against HIV-1 dual tropic isolates HIV-1 89.6 and HIV-1 YU2 in TZM-bl cells

4.1.7 Anti-HIV-1 activity of quinoline 2,4-diol analogues in TZM-bl cells infected with HIV-1 IIIB

Two derivatives of buchapine (B7) were selected for further assessment of HIV inhibition. Both B1 and B4 showed potent inhibition of replication of HIV-1 NL4-3 in TZM-bl cells. Hence we assessed anti-HIV-1 potential of the two analogues and buchapine at their highest nontoxic concentration using another CXCR4 tropic clone HIV-1 IIIB. The parent compound buchapine (B7) showed 82.73% reduction of virus replication while the two analogues B1 and B4 showed 67.75% and 99.5% reduction of virus replication. AZT inhibited virus replication by 99.21%. 
4.1.8 Effect of quinoline 2,4diol analogues on infection of TZM-bl cells with HIV-1 clade B and C isolates

Quinoline 2, 4 diols were evaluated for anti-HIV-1 activity against subtype B HIV-1 isolates ADA, AD8 and YK JRCSF. B1 inhibited replication of ADA and AD8 by 75.71% and 82.43%. B4 showed inhibition up to 96.37% for ADA and 92% for AD8. Buchapine showed 63.69% inhibition of ADA and inhibited AD8 replication up to 89%.

When JRCSF infected TZM-bl were treated with quinoline 2,4 diol the activity of B1 and B7 was found to be 91.7% and 67.89% respectively. B4 showed potent inhibition of up to 96.21% in TZM-bl cells (Fig. 4.1.5).
To further assess the anti-HIV-1 potential of the quinoline 2,4-diols, subtype CCCR5 tropic strain HIV-1 Indie C1 was used to infect TZM-bl cells. The analogues B1, B4 and the parent compound (B7) showed potent activity against Indie C1 and inhibited its replication by 92.5% (B1) 100% (B4 and B7).

Figure 4.1.5 Activity of quinoline 2,4 diols against R5 tropic viruses in TZM-bl cells. (Inhibition of a. ADA, b. AD8, c. YK-JRCSF, d. Indie C1)
4.1.9 Anti-HIV-1 activity against HIV-1 dualtropic isolates HIV-189.6 and HIV-1 YU2 in TZM-bl cells, by quinoline 2,4 diol analogues

HIV-1 89.6 and YU-2 isolates can bind to X4 or R5 receptor for fusion. These viruses were treated with buchapine and its analogues. Replication of 89.6 in TZM-bl cells was inhibited up to 66.23%, 100% and 62.54% by B1, B4 and B7 respectively. In case of Yu2B1 showed 58.95% inhibition of replication. For B4 and B7, 96.21% and 70.8% reduction in virus production was observed respectively.

![Figure 4.1.6: Inhibition of dualtropic virus infection of TZM-bl by quinoline 2,4, diols](image-url)
4.2 Anti-HIV-1 activity of compounds in primary culture of human peripheral blood mononuclear cells

4.2.1 Anti-HIV-1 activity of dimeric phloroglucinol analogues against HIV-1 NL4-3 in PBMCs

Dimeric phloroglucinol derivatives showed potent anti-HIV-1 activity in TZM-bl cells. The derivatives were then tested in Phytohemagglutinin (PHA, 2 µg/ml) activated human PBMCs infected with HIV-1 NL4-3 virus at 0.05 MOI. The infection was continued for 8 to 10 days in the presence and absence of M1 (9.73 µM) and M7 (5.75 µM). The treatment with M1 decreased the HIV-1 production to 78% in hPBMCs. Whereas M7 showed 80% of viral replication. The experimental results with hPBMCs with three healthy donors showed that M1 and M7 inhibit HIV-1 replication in hPBMCs with IC50 3.3 µM and 2.2 µM respectively.
Table 4.3: IC50 and CC50 of phloroglucinols in hPBMCs.

<table>
<thead>
<tr>
<th>Code</th>
<th>Highest Noncytotoxic Concentration</th>
<th>% Inhibition</th>
<th>IC 50 µM</th>
<th>CC 50 µM</th>
<th>Safety Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>9.73</td>
<td>78.6 ± 4.5</td>
<td>3.3 ± 0.1</td>
<td>39.23 ± 1.3</td>
<td>10</td>
</tr>
<tr>
<td>M7</td>
<td>5,7</td>
<td>80.66 ± 1.7</td>
<td>2.5 ± 0.3</td>
<td>21.65 ± 1.7</td>
<td>8.6</td>
</tr>
<tr>
<td>AZT</td>
<td>5</td>
<td>96.28 ± 1.5</td>
<td>1.17 ± 0.07</td>
<td>44.06 ± 0.57</td>
<td>37.6</td>
</tr>
</tbody>
</table>
4.2.2 Dimeric phloroglucinol analogues inhibit HIV-1IIIB infection of PBMCs

In case of PBMCs infected with HIV-1IIIB, M1 and M7 showed inhibition of up to 68% and 72% at the highest non toxic concentration. AZT showed 93% inhibition of viral replication.

Figure 4.2.1: Anti-HIV-1 activity of phloroglucinols in PBMCs infected with HIV-1 IIIB
4.2.3 Dimeric phloroglucinolanalogues exhibit potent activity against CCR5 tropic subtype B and subtype C strains of HIV-1 in PBMCs

Phloroglucinol derivatives inhibited replication of subtype B CXCR4 tropic viruses efficiently in hPBMCsculture. To assess the activity against R5 tropic viruses, hPBMCs were infected with subtype BADA, NLAD8, YK-JRCSF and subtype C IndieC1 virus. Infected cells were treated with the compounds and cultures were continued till day 8 and supernatants were assayed for presence of P24 antigen. As shown in Figure 4.2.2, M1 inhibited replication of both subtype B and C viruses up to 81% (ADA), 68% (AD8), 76% (YK-JRCSF) and 75% (IndieC1).

M7 showed 74.3% inhibition of ADA, 61% inhibition of AD8, 84.65% of YK-JRCSF and 81.3% inhibition of IndieC1.

![Activity of phloroglucinols against R5 tropic viruses in hPBMCs](image)

Figure 4.2.2: Activity of phloroglucinols against R5 tropic viruses in hPBMCs. (Inhibition of a. ADA, b. AD8, c. IndieC1, d. YK-JRCSF)
4.2.4 Anti-HIV-1 activity of dimeric phloroglucinol analogues against dual tropic HIV-189.6 and HIV-1YU2 in PBMCs

Infection of PBMCs by both CXCR4 and CCR5 tropic viruses was effectively inhibited by phloroglucinols. To see the effect of these compounds on infection by dual tropic viruses, PBMCs were infected with 89.6 and YU2 strains of HIV-1. HIV-189.6 replication in hPBMCs was inhibited by M1 and M7 up to 68.86% and 71.93% respectively. Both the compounds showed inhibition of YU2 up to 735 and 78% respectively.

Figure 4.2.3: Inhibition of dualtropic isolates HIV-1Yu2 and HIV-189.6 by dimeric phloroglucinol inhPBMCs
4.2.5 Dimeric phloroglucinol analogues are effective against primary isolates of HIV-1 in PBMCs

Two primary isolates VB51 and VB52 were used to infect PBMCs. Cells were treated with M1 and M7 for 8 days. M1 and M7 showed potent activity against both the primary isolates with inhibition of VB51 up to 91.75% and 94% by M1 and M7 respectively. VB52 virus production was inhibited up to 96% by both the compounds (Figure 4.2.4).

Figure 4.2.4: Activity of phloroglucinol against subtype C primary isolates a. VB51 b. VB52 in hPBMCs
4.2.6 Anti-HIV-1 activity of quinoline 2,4-diols against HIV-1 NL4-3 in hPBMCs

Buchapine and its two analogues were evaluated for anti-HIV-1 activity in human PBMCs infected with HIV-1 NL4-3 as explained in section 2.7. As shown in Table 4.3, Buchapine (B7) and its analogue B4 showed inhibition of HIV-1 NL4-3 replication in hPBMCs culture up to 90.45% and 82.31% while B1 showed 65.4% inhibition. IC50 concentrations of the compounds were determined. B1 showed IC50 at 4.1 µM, for B4 IC50 was 13.7 µM and B7 showed 50% inhibition at 11.4 µM. AZT inhibited virus replication up to 50% at 1.17 µM.

Quinoline 2,4-diols were also assessed for the cytotoxic effect on hPBMCs. M4 with CC50 87.26 µM was found to be less toxic than B1 and B7 with CC50 46.9 µM and 23.2 µM respectively. The safety index indicated that B1 and B4 are safer than the parent compound B7 buchapine.
Table 4.4: Anti-HIV-1 activity of compounds in hPBMCs infected with 0.05 MOI of HIV-1NL4-3

<table>
<thead>
<tr>
<th>CODE</th>
<th>Conc. µM</th>
<th>% Inhibition</th>
<th>IC50 µM</th>
<th>CC50 µM</th>
<th>Safety Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>20.2</td>
<td>90.45 ± 3.4</td>
<td>11.4 ± 1.1</td>
<td>46.9 ± 0.3</td>
<td>4.11</td>
</tr>
<tr>
<td>B4</td>
<td>32.47</td>
<td>82.31 ± 1.3</td>
<td>13.72 ± 1.6</td>
<td>87.26 ± 1.2</td>
<td>6.3</td>
</tr>
<tr>
<td>B1</td>
<td>5.6</td>
<td>65.4 ± 1.45</td>
<td>4.1 ± 0.4</td>
<td>23.2 ± 0.2</td>
<td>5.3</td>
</tr>
<tr>
<td>AZT</td>
<td>5</td>
<td>94.36 ± 0.78</td>
<td>1.17 ± 0.07</td>
<td>42.8 ± 0.3</td>
<td>36.5</td>
</tr>
</tbody>
</table>

4.2.7 Anti-HIV-1 activity of quinoline 2,4-diols against HIV-1IIIB in hPBMCs

PHA activated PBMCs were infected with X4 tropic HIV-1IIIB isolate.

B1 and B4 showed inhibition of up to 67% and 94% at the highest non toxic concentration. B7 inhibited virus replication in PBMCs up to 68%. AZT showed 93% inhibition of viral replication.
Figure 4.2.5: HIV-1 inhibition by quinoline 2,4-diols in hPBMCs infected with CXCR4 tropic HIV-1 IIIB virus.

4.2.8 Quinoline 2,4-diols inhibit CCR5 tropic subtype B and subtype C HIV-1 viruses in hPBMCs

Anti-HIV-1 activity of buchapine and its analogues was assessed using CCR5 tropic subtype B viruses ADA, AD8, YK-JRCSF and subtype C IndieC1 viruses.

B7 and B4 inhibited replication of both subtype B and C viruses by 80-90% as indicated in Figure 4.2.6. B1 showed anti-HIV-1 activity in the range of 72-82% against these virus isolates in hPBMCs.
Figure 4.2.6: Anti-HIV-1 activity of quinoline 2,4, diols in hPBMCs infected with subtype B and subtype C R5 tropic viruses. (Inhibition of a. ADA, b. NLAD8, c. YK-JRCSF, d. Indie C1)
4.2.9 Anti-HIV-1 activity of quinoline2,4diols against dual tropic HIV-189.6 and HIV-1YU2 in PBMCs

When PBMCs infected with either 89.6 or Yu2 were treated with quinoline 2,4 diols, marked reduction in virus replication was observed. B1 showed 72% and 80% reduction of 89.6 and YU2 virus replication. B4 inhibited production of both viruses by 87% while B7 exhibited 83% activity in case of 89.6 and 80% in case of YU2 (Figure 4.2.7).

![Figure 4.2.7: Inhibition of dual tropic virus infection of hPBMCs by quinoline2,4, diols](image)

Figure 4.2.7: Inhibition of dual tropic virus infection of hPBMCs by quinoline2,4, diols
4.2.10 Effect of quinoline2, 4-diols on infection of hPBMCs with primary isolates

PHA activated hPBMCs were infected with subtype C primary isolates VB51 and VB52 and then treated with buchapine and its analogues for 8 days post infection. When supernatants were assessed for p24 antigen, more than 95% inhibition was seen with B4 and B1 showed 82% and 92% reduction of VB51 of VB52. B7 exhibited 87% activity against VB51 and inhibited VB52 production up to 95% in hPBMCs.

Figure 4.2.8: Activity of compounds B7 (20.2μM), B4 (32.47μM), B1 (5.47μM) and AZT (5μM) against subtype C primary isolates VB51 and VB52 in hPBMCs
4.3 Mechanism of Action

4.3.1 Time of addition experiment in TZM-bl cells

4.3.1.1 Mallotojaponinanalogues inhibit HIV-1 at early phases of infection

HIV-1 virus aliquots and TZM-bl cells were treated with M1 and M7 at different timepoints during the course of infection like before infection, during infection and post infection. When M1 and M7 were added from the start of infection and after infection (PDP), they could block replication of HIV-1 by greater than 90% (Figure 4.3.1).

When M1 is added immediately after infection, the activity of M1 reduced to 30% and lost when M1 was added 6 hrs post infection. Presence of M1 during infection reduced infection of TZM-bl to 50%.

M7 when added immediately after infection showed 60% reduction in virus replication, however it could not inhibit HIV-1 infection progression when added after 10 hrs of infection. M7 when added during infection showed up to 45% reduction in infection. This data indicated that M1 and M7 act differently to inhibit infection of TZM-bl cells. M1 acts at earlier stages of virus infection and M7 probably inhibits a later step in the lifecycle. It is effective even after infection.
Figure 4.3.1: Anti-HIV-1 activity of phloroglucinols at different time of additions in TZM-bl cells

4.3.2 Inhibition of DNA strand transfer reaction of HIV-1 integrase enzyme by phloroglucinols and buchapine analogues

Integration of proviral DNA in the genomic DNA of the host cell is catalyzed by viral integrase and cellular factors. The integrase binding site in the U3 region of viral promoter contains a purine motif 5′-GGAAGGG-3′ that is specifically targeted by oligonucleotide-intercalator conjugates. These conjugates interact with viral DNA and inhibit catalytic activities of integrase. Rapid generation of drug resistant viruses and low intracellular permeabities of these conjugates created obstacles to develop them into novel therapeutics. The 1-methoxy-3,5-dicaffeoylquinic acid 3,4 dicaffeoylquinic acid binds to catalytic core domain of viral integrase enzyme and irreversibly inhibits its activity. Several antisense RNAs and siRNAs were also used to block integration step of viral life cycle.
We looked for the ability of compounds to inhibit strand transfer reaction of HIV-1 integrase. Except M1, none of the compounds could inhibit in vitro reaction catalyzed by HIV-1 integrase. M1 showed 60% inhibition of integrase enzyme activity at 50 µM. Thus M1 may act by interfering at the integration step of HIV-1 life cycle.

Buchapine and its analogues did not exert any effect on integration reaction.
4.3.3 Mechanism of HIV-1 inhibition by buchapine analogues

Reverse transcription is one of the early steps in viral lifecycle. Most of the currently available drugs like NRTIs and NNRTIs target reverse transcription step of viral life cycle. Other than these drugs several approaches were developed to inhibit reverse transcription. RNA decoys lacking primer binding site (PBS) act as a template and compete with the HIV-1 RNA when RT makes the first jump during the first strand transfer (Lamothe et al., 2000) and are also co-packaged with viral genomic RNA into the virions and can compete with the genomic RNA for RT binding. The tRNALys mutant containing an 11 nucleotide 3′-end complementary region to TAR region primes reverse transcription from TAR region instead of PBS and thereby inhibits viral replication. Strategies that target dimerization of RT, cellular expression of sFvs, antisense RNAs, siRNAs, Ribozymes and RNA aptamers to inhibit RT activity are potent inhibitors of HIV-1 replication (Table-4.1). The aptamers bind to the template-primer binding cleft on HIV-1 RT.

Buchapine is known to act on transcription process by inhibiting Reverse Transcriptase with an EC50 value of 29 µM (McCormick McKee et al., 1996). Hence in order to elucidate the mechanism of action of the two new analogues, we first performed in vitro Reverse Transcriptase assay as described above. All the three compounds inhibited Reverse Transcriptase activity but with different potency, B1 showing least activity. Buchapine (B7) exhibited EC50 at 25 µM, however, B4 inhibited 50% Reverse Transcriptase activity at 100 µM. B1 exhibited significantly weaker activity with an EC50 value of 298 µM. As B1 shows significant anti-HIV-1 activity in cell based assay system in vitro with IC50 values in the range of 2-4 µM, but shows very weak Reverse Transcriptase inhibitory activity, it becomes interesting to identify the mechanism of HIV-1 inhibition in addition to reducing RT activity.
Figure 4.3.2: Concentration of compounds [EC50 (µM)] required to get 50% inhibition of in vitro reverse transcription reaction.
4.4 Inhibition of HIV-1 at different time of addition by quinoline 2,4 diols

In order to get an indication about which part of HIV-1 lifecycle was inhibited by the B4 and B1 in addition to inhibition of reverse transcription, if any, the compounds were added at various stages of HIV-1 infection in single cycle infection experiments with TZM-bl cells. We first pre-treated the HIV-1NL4.3 virus or the TZM-bl cells with the compounds for 1 hour followed by washing of the cells to remove the compounds and then initiating the infection process as described in materials and methods. As shown in Figure 4.3.3A, significant inhibition with all the three compounds was observed in infections where virus was pre-treated with the compounds as compared to very little inhibition in case of pre-treatment with cells. The results demonstrated that these compounds do not interact with HIV receptors or co-receptors on the cells but could be interacting with viral proteins or inhibiting virus during infection process.

To investigate further into the mechanism we did a time course of addition experiment in TZM-bl cells, in which we added the compounds either pre, during and post-infection, or only during the infection or only post-infection at different time points. As shown in Figure 4.3.3B, B1 shows better inhibitory activity than B7 and B4 when added only during infection. It also showed 73% inhibition of infection when added immediately after infection, but lost significant inhibitory activity when added 2 and 6 hours post-infection. This result indicates that B1 plays a role during very early part of virus infection cycle.

B4 exhibited weak activity when added during infection but showed significant inhibition of infection when added immediately after infection. However, B4 also failed to show significant inhibition when added 6 hours post-infection as compared to buchapine which showed nearly 50% inhibitions when added 6 h post-infection. AZT, which was used as a positive control, inhibited HIV-1 very significantly at all time points tested except at 10 hours post-infection, where its activity got reduced to around 50% inhibition.
Figure 4.3.3: Time of addition experiment. Pre with virus: Virus was treated with compounds for 1h and then added to infect cells. Pre with cells: Cells were treated with compounds for 1h and washed to remove unbound compounds. Cells were then infected with HIV-1NL4-3. Activity of compounds B7 (20.2µM), B4 (43.3µM), B1 (8.4µM) and AZT (5µM) at different timepoints. Compounds were added during infection, and after 0h, 2h, 6h and 10h post infection. PDP denotes Pre-during and post treatment with compounds.
4.4.1 B1 prevents binding of gp120 to CD4 receptors

Our experiments in TZM-blcells above indicated that B1 is active at very early steps of HIV-1 infection and its activity is independent of co-receptor use by the virus. In order to see if it acts as an entryinhibitor, we then performed a cell-cell fusion assay. For this, we used 293T cells transientlytransfected to express gp120 and Tat proteins of the virus in a co-culture experiment with TZM-blcells in presence or absence of the compounds.

After 12 hours of co-culture, cellswere stained for β-galactosidase activity. Buchapine and B4 did not show any reduction in β-galactosidase activity whereas B1 showed more than 70% reduction in number of blue colored cells, clearly indicating that it inhibits fusion of gp120 expressing 293T cells with CD4 expressing TZM-blcells

![Figure 4.3.4: Inhibition of CD4 - HIV-1 gp120 fusion in cell – cell fusion by compounds](image_url)

Figure 4.3.4: Inhibition of CD4 - HIV-1 gp120 fusion in cell – cell fusion by compounds
4.5 Evaluation of M7 as microbicide candidate

4.5.1 Effect of M7 on vaginal epithelial cells

Cytotoxicity to vaginal mucosa by the microbicide leads to increased viral permeation and infection. To begin with, CC50 of compounds was determined using ME180 cells. Tenofovir and M7 were added at different concentrations. The Cytotoxicity data indicated that CC50 of M7 and tenofovir were 27 µM and 272 µM respectively.

The effect on secretion of pro-inflammatory cytokines by ME180 cells was assessed upon treatment with the compounds. ME 180 cells were treated with 5.7 µM of M7 and 8 µM of Tenofovir. Triton X-100 was used as cytotoxic control. As shown in Figure 4.4.1, addition of M7 compound did not exert significant effect on secretion of inflammatory cytokines like IL1b, IL6, IL8, IL10RANTES and TNFα after 24h. Triton X 100 treatment induced secretion of inflammatory cytokines. M7 was found to be nontoxic and safe for evaluation as microbicide.
4.5.2 Assessment of Safety of quinolone2, 4 diols on epithelial cells

Latently infected cells may get activated and start producing mature virus particles upon activation by inflammatory cytokines (Fichorova2004; Trifonova, Bajpai et al., 2007). In order to see the cytotoxicity of these compounds on cervicalepithelial cell line, ME180, we first analyzed the CC50 of these compounds using MTT assay. As shown in Figure-4.4.2A, the CC50 values for compound 1, 2 and 3 were found to be 89.9, 150 and 55.2 µM for these compounds. Tenofovir exhibited 50% toxicity at 272 µM.
We then evaluated the effect of these compounds on secretion of pro-inflammatory cytokines by ME180 cells. Stimulatory or inhibitory effect on secretion of cytokines was assessed by bead based Luminex assay system. For this, ME180 cells in 24-well plates were incubated with compounds for 24 h. Culture supernatants from treated and untreated wells were collected and assayed for levels of pro-inflammatory cytokines. We assessed 7 cytokines like IL1b, IL6, IL8, IL10, IL12, TNFα and RANTES. Addition of these compounds to ME180 culture did not exert significant effect on secretion of pro-inflammatory cytokines like IL1b, IL6, IL8 and IL10, however, marked reduction in levels of cytokines like RANTES and TNFα was observed compared to untreated ME180 cells (Figure-4.4.2B).

Inflammatory cytokine IL12 could not be detected in supernatants of untreated and compound-treated cells however in Triton treated sample, 0.2 pg/ml of IL12 was observed. Overall, this data indicate that these compounds are nontoxic and safe for evaluation as vaginal microbicide candidate. ME180 cells treated with 2% Triton-X100 solution showed increase in levels of pro-inflammatory and anti-inflammatory cytokines (Figure-4.4.2B).
Figure 4.4.2: Effect of quinoline 2,4 diols on secretion of proinflammatory cytokines from ME180 cells

4.5.3 HIV-1 microbicidal activity of compounds using dual chamber trans-well assay system

Compounds were evaluated for microbicidal activity at non toxic concentrations. HIV-1 is thought to be sexually transmitted by genital epithelium transmigration followed by DC- and/or LC mediated capture and transmission to CD4+T cells. Compounds that act at early steps of life cycle like attachment, entry and reverse transcription of HIV-1, are presently being evaluated as HIV-1 microbicide candidates. Since M1 is an Integrase Inhibitor we did not consider it for evaluation as microbicide candidate. Buchapine, its two analogues were evaluated
for their ability to inhibit transmission of HIV-1 across epithelial barrier using ME180 cells in a dual chamber model.

For this purpose ME180 cells were cultured in apical chamber of trans-well system for 9 days and integrity of monolayer was assessed by measuring transepithelial electrical resistance (Figure 4.4.3). To confirm that there were no gaps left in between the cells and monolayer formed was tight and dense fluorospheres were used. Fluorescence was not detected in the samples collected from basal chamber (Table 4.5).

![Figure 4.4.3: Measurement of Confluence of ME180 epithelial layer in trans-well system using transepithelial Electrical Resistance](image-url)
Table 4.5: Assessment of Confluence of ME180 epithelial layer in trans-wells using fluoresphere

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<tr>
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<tr>
<td></td>
<td>Empty trans-well</td>
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<td>Apical chamber</td>
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<tr>
<td>Basal chamber</td>
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4.5.4 Prevention of transmission of HIV-1 across epithelial barrier by M7

It is now well known that HIV-1 transmigrate through genital epithelium followed by dendritic cell and/or langerhans cell mediated capture and transmission to CD4+ T-cells (Ayehunie Groves et al., 1995; Miller & Shattock, 2003). In order to see effect of M7 on transepithelial cell migration and infection of monocytes and T-cells, a dual chamber model comprising an apical chamber having ME180 epithelial cell layer and hPBMCs in basal chamber was used. ME180 cells form confluent monolayer when grown on insert chambers.

They are characterized by several desmosomes connecting adjacent cells, and prominent epidermal filaments. These morphological characteristics are typical of stratified squamous epithelia such as those which line the cervix, and urethra.

Completely confluent ME180 cells in apical chamber were incubated with activated human PBMCs in basal chamber HIV-1 NL4-3 virus (0.1 MOI) was added to apical chamber in presence or absence of compounds. After 6 h of infection, apical chambers were removed and culture supernatants collected on day 5 from hPBMCs...
were assayed for presence of p24 antigen. We found that M7 showed 30% inhibition of infection of hPBMCs while tenofovir inhibited infection up to 86.49% (Figure 4.4.4). As evident from our results, M7 moderately inhibited HIV-1 transmission across epithelial cells.

Figure 4.4.4: Inhibition of transmission and infection of HIV-1 across epithelial layer by M7. In dual-chamber model system, ME180 cells in apical chamber were grown to confluence and then treated with compounds M7 (5µM) and Tenofovir 8 µM and HIV-1NL4-3. Infection of hPBMCs by transmitted HIV-1NL4-3, in basal chamber was assessed using p24 antigen ELISA according to manufacturer’s protocol. % inhibition was calculated by comparing p24 values of compound treated cells to untreated control cells.
4.5.5 HIV-1 Microbicidal activity of quinoline 2,4 diols

Completely confluent ME180 cells in apical chamber were incubated with activated human PBMCs in basal chamber. HIV-1 NL4-3 virus (equivalent to 0.1 MOI) was added to apical chamber in presence or absence of compounds. After 6 h of infection, apical chambers were removed and hPBMC culture was continued till day 5. Culture supernatants collected on day 5 from hPBMCs were assayed for presence of p24 antigen. Compound B1 significantly inhibited virus transmission to hPBMCs (Figure 4.4.5A). Compounds B7 and B4 were not able to prevent infection of hPBMCs significantly. Tenofovir used as standard showed 85% reduction in p24 levels of hPBMCs (Figure 4.4.5 A).

Finally, we evaluated ability of compounds to inhibit infection from cell associated HIV-1 in a co-culture of ME180 and HIV-1 infected human PBMCs. Compounds were added to ME180 cells in a co-culture with HIV-1 NL4-3 infected hPBMCs for 4 hours followed by washing and removal of PBMCs. On day 5, supernatants from the ME180 cells were analyzed for p24 antigen. Buchapine and compound 2 showed around 20% reduction in infection of ME180 cells whereas Compound 3 exhibited 82% inhibition of infection. Tenofovir used as a positive control blocked infection of epithelial cells up to 90% (Figure-4.4.5B). These results clearly indicate that compound B1 significantly inhibits transmission of cell free and cell associated virus as compared to other two compounds.
Figure 4.4.5: Activity of compounds against cell associated and cell free virus. A, Anti-HIV-1 activity of compounds against cells associated virus transfer assay. B, Inhibition of cell free virus from ME180 cells to hPBMCs in dual chamber trans-well assay (Concentration of compounds used 8µM each).