Chapter 6

Design and Evaluation of peptides with enhanced activity
Introduction

Further molecular analysis of all the 4 peptides with gp120 revealed that all the major possible interactions lie in the first 15 residues. The docking was performed by taking the first 10-15 residues of all the 4 peptides with gp120. First peptide did not yield any significant interaction, while other 3 peptides P-2.1, P-3.1, P-4.1 showed 10, 7 and 1 interactions respectively. To experimentally confirm these interactions the above 3 peptides were synthesized commercially and analyzed for their anti-HIV activity and molecular action on gp120 mediated cell fusion.

Results

Molecular interactions of the peptides

Modeling was done for all the four initial peptides by talking the first fifteen residues where the maximum number of interactions was found. The first peptide which acquired a new structure could not bind to gp120. The second peptide increased the interactions to ten with gp120. And the third peptide has shown seven interactions with gp120. Fourth peptide was also omitted since it exhibited only one interaction with gp120. All the residues of the three peptides fall in the allowed regions of the Ramachandran’s plot (Fig 6.1, 6.2 and 6.3).

Analysis of cytotoxicity and anti-HIV activity of designed peptides

Cytotoxicity of all the 3 peptides was analyzed in Sup-T1 cells at 50, 100 and 150 µg/ml concentrations by using MTT cell based proliferation assay. All the three peptides were considerably less toxic (<5%) (Fig 6.4). Analysis of anti-HIV
activity of the peptides on HIV-1 93IN101 (Fig 6.5) and HIV-1 93RW024 (Fig 6.7) strains in Sup-T1 cells revealed that P2.1 and P3.1 could exhibit 80% inhibition whereas these peptides exhibited 60% activity on HIV-1 94 UG103 (Fig 6.8) strain. P-4.1 was inactive.

**Synergistic action of peptides**

The synergistic action of both peptides P 2.1 and P3.1 in combination were tested for their HIV inhibition that resulted in 80% activity at 25ug concentration each (ie., total of 50ug) (Fig 6.6). This confirmed that the peptides P2 and P3 have affinity to gp120 and anti-HIV active. Further analysis was taken up to analyze its molecular action.

**Interaction of peptides with virus surface gp120.**

The virus was captured using monoclonal antibodies spanning gp120 and gp41 regions in the presence of mixture of P-2.1 and P-3.1. The exposure of various epitopes of gp120 in the presence of peptides was monitored in terms of the amount of virus binding to corresponding antibody. The amount of bound virus was estimated by quantifying in terms of viral p24. The results of these experiments show that peptides binding to gp120 can block specifically at V3-F425 and 257DIV epitopic regions (Fig 6.9).

**Action of P-2.1 and P-3.1 on Reporter based TZM-BL cells**

Anti-HIV-1 activities of P-2.1 and P-3.1 were reconfirmed by reporter based assay. TZM-BL cells were challenged with HIV-1 in the presence and absence of
peptides. Expression of β-gal occurred during HIV infection to the untreated TZM-BL cells and the reduction of X-gal took place, whereas the presence of T20, P-2.1 and P-3.1 inhibited the viral infection and the subsequent expression of β-gal (Fig 6.10, Panel A). The results of FACS analysis presented in Fig 6.10 Panel B, confirm that Peptides possess affinity to gp120 and prevented HIV-1 infection.

Discussion

Modeling and docking of Epap-1 with trimeric gp120 showed the presence of specific interactions in stabilization of their binding. These molecular interactions with gp120 showed high affinity to V3 loop region. Along with this V3 loop there exists a potential interaction with other aminoacids in C2 (Glu268, Ile285 in P3.1), C3 (Asn340, Gln344, Ser347 in P3.1; Ser347, Ile339, Ile345, Ala346, Lys348, Arg350, Asn356 in P2.1) and C4 regions (Arg456 in P3.1). Indeed the insilico mutations of interacting aminoacids in P2.1 (Ser2, Ala5, Thr7, Ala8) and P3.1 (Val1, His14, Asp5, Cys6, Leu4, Ser10) abolishes the affinity of peptides to trimeric gp120. This suggests the significant role played by these amino acids in interacting with other residues spanning neighboring regions around V3 loop.

Even though the V3 region is immunodominant due to its extended nature and antibody accessibility (Huang et al., 2005) it is often masked by folding of the V2 loop along the V1/V2 stem (Wyatt et al., 1998). And the conformational shifts that occur in other variable regions make the binding of antibodies difficult in theses cryptic residues. On the other hand the recessed nature of other receptor binding sites contributes to their poor immunogenicity (Wyatt et al., 1998). But
the involvement of constant regions in virus neutralization need further attention
as these regions may be unique and conserved among strains and thus may be
involved in host recognition and pathogenicity. One of the highlighting
achievements in the present study is that the peptide not only binds to the V3 loop
but also encompasses the adjacent constant regions. This is supported by the
synergistic action of the two peptides. This kind of cooperative interdomain
interactions offered by the peptides may provide some important clues in the
development of efficient small molecule inhibitors.
Panel A shows the structure of peptide, P-2.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120. Here peptide P-2.1 exhibited ten interactions with gp120 that were shown in the below table.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Interacting Molecules</th>
<th>Bond Lengths in A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GLN352{NE2}-(O)ALA9</td>
<td>2.79</td>
</tr>
<tr>
<td>2</td>
<td>LYS348(O)-(O)THR7</td>
<td>3.24</td>
</tr>
<tr>
<td>3</td>
<td>ARG350(N)-(O)ALA8</td>
<td>3.14</td>
</tr>
<tr>
<td>4</td>
<td>ILE345(O)-(N)ALA8</td>
<td>2.50</td>
</tr>
<tr>
<td>5</td>
<td>ALA346(O)-(OG)SER2</td>
<td>3.32</td>
</tr>
<tr>
<td>6</td>
<td>SER347(N)-(O)ALA5</td>
<td>2.53</td>
</tr>
<tr>
<td>7</td>
<td>LYS357(O)-(O)ARG1</td>
<td>2.43</td>
</tr>
<tr>
<td>8</td>
<td>ASN356(OD1)-(N)THR7</td>
<td>2.35</td>
</tr>
<tr>
<td>9</td>
<td>LYS357(O)-(OG1)THR7</td>
<td>2.36</td>
</tr>
<tr>
<td>10</td>
<td>ILE339(N)-(OG1)THR7</td>
<td>3.49</td>
</tr>
</tbody>
</table>
Panel A shows the structure of peptide, P-3.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120. Here peptide P-3.1 exhibited seven interactions with gp120 that were shown in the below table.

<table>
<thead>
<tr>
<th>Interacting Residues of gp120 with epitope</th>
<th>Bond Distances in A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU268(O) – (N)VAL1</td>
<td>3.24</td>
</tr>
<tr>
<td>SER347(OG) – (O)LEU4</td>
<td>2.94</td>
</tr>
<tr>
<td>ASN340(O) – (N)ASP5</td>
<td>3.47</td>
</tr>
<tr>
<td>GLN344 (O) – (N)CYS6</td>
<td>3.21</td>
</tr>
<tr>
<td>PHE396(OXT) – (O)LEU7</td>
<td>3.37</td>
</tr>
<tr>
<td>ARG456(NE) – (OG)SER10</td>
<td>3.35</td>
</tr>
<tr>
<td>ILE285(O) – (O)HIS14</td>
<td>2.13</td>
</tr>
</tbody>
</table>
Fig 6.3

Structure and Molecular interactions of P-4.1

Panel A shows the structure of peptide, P-4.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120. Peptide P-4.1 exhibited only one interaction at phe353 of gp120.
Cytotoxicity of all the three peptides was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple color which indicates the percentage of live cells. All the three peptides were not cytotoxic and exhibited negligible cytotoxicity of 4% at 150µg concentration.
Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Three concentrations 50, 100, 150 µg/ml of all three peptides were used. 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2.1 and P-3.1 have shown 80% antiviral activities.
Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Two concentrations 50, 100µg/ml of the two peptides were used. 1 µg/ml of T-20 was used as control. To test the synergistic anti-viral action of peptides mixture of 25 µg/ml of each P-2.1 and P-3.1 were added. Here each peptide exhibited 40% activity at 50µg concentration individually whereas the mixture of both peptides of 50µg concentration exhibited 80% activity.
Sup T-1 cells were challenged with HIV-1 93RW024 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. All peptides were of 50 µg/ml concentration. 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2 and P-3.1 have shown 80% antiviral activities along with the combination of P-2.1 and P-3.1.
Sup T-1 cells were challenged with HIV-1 94UG103 in the presence and absence of peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. All peptides were of 50 µg/ml concentration. 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2 and the mixture of P-2.1 and P-3.1 have shown 60% antiviral activities.
Virus was incubated with increasing concentrations of P-2.1 and P-3.1 (in equal amounts). Virus was captured with gp120 monoclonal antibody coated wells as indicated on X-axis. The bound virus was lysed and p24 was estimated and plotted on Y-axis. Decrease in p24 value in the antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region. Binding of the two peptides in combination to the same regions of gp120, 257D and F425 shows the synergistic effect of these peptides.
HIV-1 mediated β-gal expression in TZM-BL cells: TZM-BL cells were infected with HIV-1. HIV-1 entry allows tat mediated LTR based expression of β-gal and the fluorescent cells (due to azo dye) were counted by confocal microscopy and flow cytometry. Panel A indicates the confocal image of the TZM-BL cells which shows the expression of β-gal upon HIV-1 infection in control cells and the inhibition of expression by T-20(control), P-2.1 and P-3.1. The Panel B shows the FACS analysis of the same assay to count the number of cells fluoresced.
Conclusions

- Tryptic digestion of both native and rec Epap-1 showed identical peptide pattern in MALDI analysis
- Trypsin Digest of rec Epap-1 showed significant anti-HIV-1 activity
- Trypsin Digest was separated by HPLC and the two Fractions could be isolated, in which Fraction-1 showed ~40% anti-HIV-1 activity, affinity to HL-2/3cells and inhibited the fusion reaction
- Epap-1 model was generated by threading technique using WURST server and docking was performed between Epap-1 and gp120 by HEX 5.6
- Out of four peptides obtained from docking studies, peptides 2 and 3 showed considerable interactions (8 and 7) with gp120
- All the 4 peptides showed binding to HL 2/3 cells and did not bind to Sup-T1 cells and showed considerably less cytotoxicity at 100µg concentration
- Peptide 2 and 3, which are having 8 and 7 interacting residues have shown anti-HIV activity, but peptide 2 (IC-50 - 59µg) possess higher activity compared to Peptide 3 in both Sup-T1 and PBMC’s
- In studies conducted using soluble gp120 and virus, peptide 2 has shown a conserved interaction at V3 loop regions recognized by 257D and F425 antibodies.
P-2 inhibited cell fusion mediated dye transfer between HL2/3 and Sup T1 cells suggesting that it blocks viral entry, which is further confirmed by its action on HIV infection mediated by Tat activated beta gal expression. These results together suggest that peptide 2 inhibits viral entry through interaction at V3 loop region.

Peptides (15mers) P-2.1 and P-3.1 have shown 10 and 7 interactions respectively with gp120 and were significantly anti-HIV active. They exhibited synergistic action when used in combination which is confirmed by anti-HIV assay and epitopic analysis using ELISA. They also inhibit the replication of subtype A viruses namely, HIV-1 93RW024 and HIV-1 94UG103 in Sup-T1 cells.

Thus P- 2.1 and P-3.1 synergistically interacts with gp120 in V3 loop leading to the blockage of viral entry. The observation that P-2.1 and P-3.1 acting synergistically with 100 fold increase in activity suggests that binding sites of P-2.1 and P-3.1 in V3 region are distinctly different and cooperative. This suggests that the present study identified two new regions in the Epap-1 involved in the gp120 interaction, one is from Ile 339 to Asn 356 and the other is from Gln 268 to arg 456. Further these results indicate that Epap-1 interaction with gp120 is multidimensional in nature.