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Original article

Design, synthesis and evaluation of peptide libraries as potential anti-HIV compounds, via inhibition of gp120/cell membrane interactions, using the gp120/cd4/fab17 crystal structure

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Abstract

The crystal structure of a gp120/CD4/Fab17b complex was analysed leading to the design of several peptide libraries in the hope of obtaining novel gp120/cell membrane receptor interaction inhibitors, especially inhibitors of gp120/CD4 and gp120/chemokine receptor interactions. Syntheses of tri- and tetra- and pentapeptides were performed via a solid phase synthesis methodology using a Rink Amide MBHA resin and a Fmoc strategy giving C-terminal amide form peptides. Compounds were assayed against C8166 cells infected by HIV-1 IIIB and screened using a gp120 binding assay and the FIGS reporter gene assay.

Keywords: CD4; gp120; HIV; Peptides; Inhibition; Interaction

1. Introduction

The WHO estimates that 33.6 million people were living with HIV/AIDS by the end of 1999 [1]. The current treatment for HIV/AIDS involves combination of a number of drugs. Although this therapy can suppress viral loads to below detectable levels, suppression has been observed to be reversible, especially once drug therapy is stopped [2]. In addition it still involves side effects, high costs and rigorous adherence to a drug regime [2]. Therefore, there is still an urgent need for new drugs active against novel drug targets. Many steps in the viral life cycle (viral entry, reverse transcription, integration, gene expression, gene assembly, budding and maturation) [3,4] could serve as potential targets for designing new anti-HIV drugs [5–7]. We were particularly interested in developing novel agents aimed at the process of HIV adsorption to the cell.

The adsorption of the virus to the cell is mediated by the strong and specific binding of the virus envelope glycoprotein gp120 to the CD4 receptor in the presence of specific chemokine receptors on the human cell membrane. Initial binding of gp120 to the CD4 leads to conformational changes in gp120 which expose the chemokine receptor binding site. Interaction of the V3 loop with the chemokine receptor [8] then triggers further conformational changes, which enable the fusion peptide gp41 to insert into the target cell lipid bilayer [9,10].

The inhibition of interaction of gp120 with the cells has already been established for many high-molecular weight polyanionic compounds [11–14] including heparin [14], polysulfates [15] and poly-carboxylates [15]. These compounds are thought to bind to positively charged regions of the V3 loop of gp120, preventing gp120/receptor interactions. [16] Similarly, soluble CD4

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and fragments and derivatives of CD4 have been shown to inhibit gp120/receptor interactions.

Following binding to the CD4, gp120 undergoes a conformational change and then binds to chemokine receptors, in particular, the chemokine receptors CXCR4 and CCR5. Therefore, an alternative strategy for treatment is to find inhibitors which bind to the chemokine co-receptor, preventing interaction of the co-receptor with gp120 and hence entry of the virus into the cell. A number of compounds have been discovered which do this [17]. Compounds have been discovered which bind to either CXCR4 co-receptors or CCR5 receptors. Different strains of the virus use different co-receptors for entry. Thus the co-receptor antagonists are specific for particular strains of HIV. Antagonists of the CXCR4 co-receptor include bicyclams, a series of bicyclic peptides and a nona-peptide based on poly-arginine. Whilst a quaternary ammonium derivative, TAK-779 has shown to be an antagonist of the CCR5 co-receptor.

After HIV-gp120 binds to CD4 and the chemokine receptor, the subsequent step in viral fusion is fusion of the cell and virus membranes. This is initiated by HIV-gp41 entering the cell membrane. Various inhibitors of this step have been discovered, including polypeptides [17].

The aim of the work described in this paper is to discover small peptides that bind to gp120 at either the CD4 binding site or the chemokine receptor site. Although these peptides are unlikely to be drug candidates themselves, they could be a valuable lead for developing 'peptoids' [18,19] (a 'peptoid' is a non-peptide agent that has been designed using the chemical structure of natural peptides as the starting point).

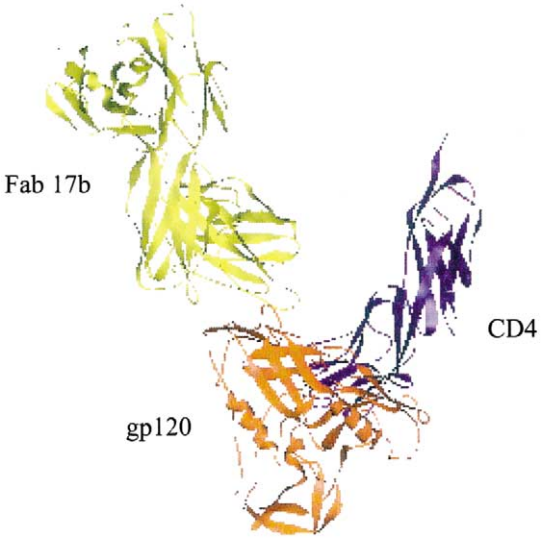

Fig. 1. Ternary complex [21] composed of a truncated form of gp120, the N-terminal two domains of CD4 (black) and a Fab (grey) from the human neutralising monoclonal antibody 17b.

A crystal structure of gp120 complexed with CD4 and Fab 17b from a monoclonal antibody has been published and deposited in the protein database [20,21] (Fig. 1). The Fab is thought to bind to gp120 at an overlapping binding site to the CCR5 chemokine co-receptor binding site. This now provides us with the structural information necessary for rational structure based drug design. By separately studying the interface of gp120 | CD4 and the interface gp120 | Fab, we have identified CD4 and Fab 17b residues which seem to contribute significantly to the binding processes.

By using the results of these studies, we have been able to design peptide libraries to specifically target each of the binding interactions and whilst peptides generally make poor drugs, their relatively easy synthesis and structural and functional diversity make them valuable leads. From peptide leads it should be possible to develop peptoids. We were looking for an initial peptide lead, where the peptide would have a sequence of less than six residues; with longer peptides, it would be harder to convert the peptide lead to a peptoid with a low molecular weight [18].

2. Modelling and peptide library design

2.1. gp120/CD4 interaction

The crystal structure was studied to determine which of the CD4 residues are most important for interaction with gp120. The first criterion was to select CD4 residues within 4 Å of gp120. Then their potential binding interactions were analysed.

The most important residues were found to be Lys35, Gln40, Ser42, Phe43, Leu44, Lys46 and Arg59 (Fig. 2). In particular, we were interested by residues Phe43 and Arg59, which have been reported to give important interactions with gp120 [21]. In particular, Phe43 con-

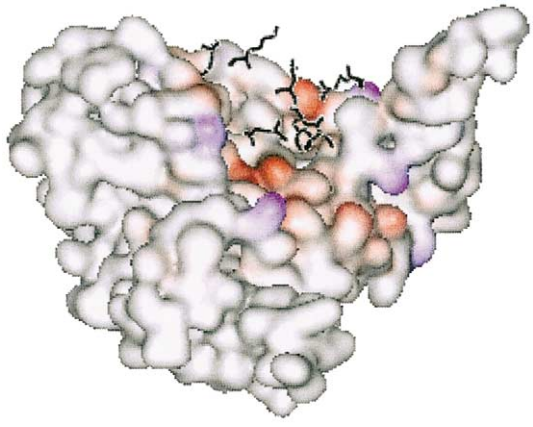

Fig. 2. CD4 studied residues in contact with gp120 surface are represented in black: Lys35, Gln40, Ser42, Phe43, Leu44, Lys46 and Arg59.

tacts a hydrophobic cavity on the gp120 surface accounts for 23% of all interatomic contacts between CD4 and gp120. Arg59 forms an electrostatic interaction with Asp 368 of gp120. Although relatively distant on the primary amino-acid sequence, Phe443 and Arg59 are spatially relatively close. It was, therefore, decided to base the initial library around Phe and Arg, separated by suitable linkers. By measuring the distance between the Cα of each residues and knowing that the span of a glycine unit is 3.8 Å, it was possible to calculate the number of units needed to link any of the discontinuous residues. Calculations suggested that two 'spacer' residues were required between Phe43 and Arg59. Then a study of the distance between the atoms of CD4 residues Phe43 and Arg59 was undertaken in order to determine which residue should take the C-terminal position.

In tetrapeptides containing an arginine residue in C-terminal position and a phenylalanine in N-terminal position, the relative distances between the atoms was similar to that of the original CD4 fragment. This contrasts having the Phe in the C-terminal position and the Arg at the N-terminal position, which gave a larger spacing of key functional groups compared to the original CD4 fragment. Therefore, the arginine residue was designated as the C-terminal residue. This led to the design of a library **Phe**–X–X–**Arg**.

Spacers between the key residues were chosen because of the differing representative nature of their side chains and are described in Table 1.

2.2. gp120/Fab 17b interaction

A similar exercise was repeated for the gp120/17b interaction which acts as a model for the gp120–chemokine receptor interaction.

The Fab 17b residues at the interface were isolated from the remainder of Fab 17b using an initial cut off point of 5 Å. The remaining Fab 17b residues were carefully studied in order to determine which residues were interacting with gp120, the distances involved with those interactions, and the type of interactions occurring. The Fab 17b residues studied were Leu55, Asp56, His59, Trp94, Pro95, Glu103, Glu106, Gly107, Glu108, Tyr109 and Asp110 (Fig. 3). In particular, we

Table 1
Designed peptide library for inhibition of gp120/CD4 interaction

Library	Spacers X
Phe–*X*–*X*–**ArgNH$_2$**	***Gly***: very flexible, without reactive functionality ***Ser***: hydroxyl group, hydrophilic ***Val***: aliphatic chain, hydrophobic ***Phe***: aromatic compound, hydrophobic ***Lys***: amine group, hydrophilic

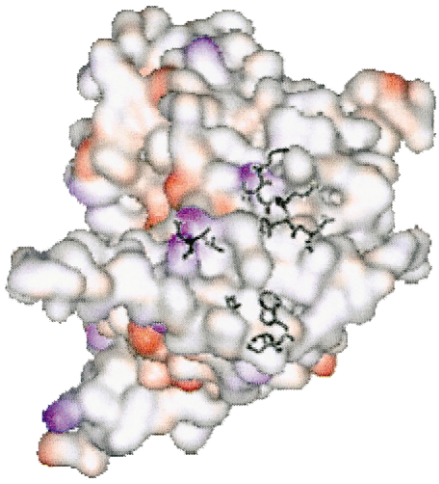

Fig. 3. 17b studied residues in contact with gp120 are in black: Leu55H, Asp56H, His59H, Trp94L, Pro95L, Glu103, Glu106, Gly107, Glu108, Tyr109 and Asp110H.

were interested in residues 106–110 which form a contiguous sequence with good interaction with gp120. In this sequence Glu106–Gly107–Glu108–Tyr109–Asp110, the most significant interactions with gp120 appeared to involve Glu106, Glu108 and Asp110. A salt bridge is thought [21] to be present between Glu106 of 17b and Arg419 of gp120 and H-bonding was determined using MacroModel®. This led to design of a peptide library **Glu**–X_1–**Glu**–X_2–**Asp**. X_1 and X_2 were a series of six amino acids with differing physicochemical properties; this led to a library of 36 peptides (Table 2).

3. Synthesis of peptide libraries (Fig. 4)

The quickest and most efficient way to synthesise peptides is via standard solid phase peptide synthesis methodology. In order to control the coupling of each amino acid, the backbone amine (position N-terminal) was protected by Fmoc (9-fluorenylmethyloxycarbonyl), which is stable to acidic reagents and tertiary amines but can be readily cleaved under basic conditions [22].

Table 2
Designed peptide library for inhibition of gp120/17b interaction

Library	Spacers X
Glu–X_1–Glu–X_2–AspNH$_2$	X_1: Gly, Phe, Ala, Ser, Asp, Asn X_2: Tyr, Asp, Leu, Gly, Lys, Ser ***Gly***: very flexible, without reactive functionality ***Ser***, ***Lys***, ***Asp***, ***Asn***, ***Tyr***: hydrophilic ***Ala***, ***Val***: aliphatic chain, hydrophobic ***Phe***: aromatic compound, hydrophobic

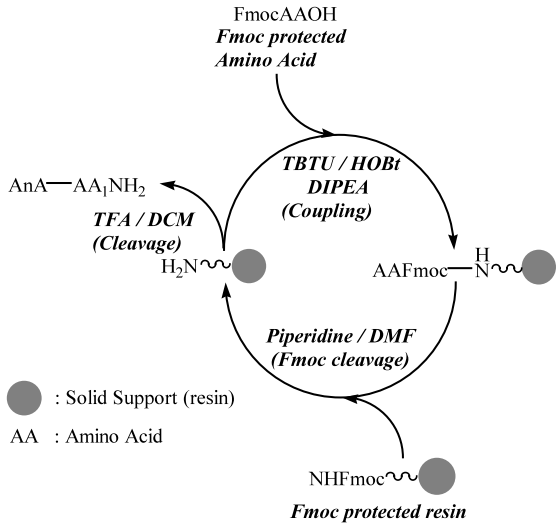

Fig. 4. General peptide synthesis scheme.

Where necessary, the amino acid side chains were protected by base stable and acid labile groups [22,23] such as *t*-Boc (*tert*-butoxycarbonyl), Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl), Trt (trityl) and Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl). For increased stability, the peptides were prepared as C-terminal protected amides using a Rink Amide MBHA resin. The libraries were prepared via both large batch and split parallel synthesis techniques.

Standard Fmoc deprotection of the pre-swollen Rink Amide resin using 20% piperidine in DMF was followed by coupling of the next amino acid of the sequence using standard TBTU/HOBT coupling methodology. The sequences were constructed in reverse using successive Fmoc deprotection/amino acid coupling steps. Deprotection of the final Fmoc group, followed by TFA mediated cleavage of the peptide from the resin and concomitant cleavage of the acid labile side chain protecting groups, gave the desired peptides as C-terminal protected amides.

Three libraries of discrete peptides were obtained, the structures of each compound were confirmed by mass spectroscopy and homogeneity was checked by HPLC analysis. However, in the case of the library directed at gp120/17b interaction, there was some evidence in the mass spectrometry data for the presence of $[M+15]^+$, thus suggesting that methyl esterification of the free glutamic/aspartic acid side chains may have occurred to some degree. This most probably occurred during the isolation step, which involved trituration of the peptide from methanol with cold ether and was probably catalysed by the presence of residual TFA from the cleavage step.

High resolution mass spectrometry (HRMS) of a random sample of each library was also obtained (Table 3).

Table 3
Accurate mass of representative peptides from the three libraries, by positive ES mass spectrometry

Library Peptide	Accurate mass	
	Calculated mass	Measured mass
HPhe–X_1–X_2–**ArgNH$_2$**		
HPhe–Gly–Phe–ArgNH$_2$	525.2938	525.2941
HPhe–Ser–Val–ArgNH$_2$	507.3043	507.3041
HPhe–Phe–Phe–ArgNH$_2$	615.3407	615.3414
HPhe–Lys–Ser–ArgNH$_2$	536.3309	536.3312
HPhe–X–**ArgNH$_2$**		
HPhe–Arg–ArgNH$_2$	477.3052	477.3050
HPhe–Gln–ArgNH$_2$	449.2625	449.2625
HPhe–Phe–ArgNH$_2$	468.2723	468.2722
HPhe–Trp–ArgNH$_2$	507.2832	507.2832
HGlu–X_1–Glu–X_2–**AspNH$_2$**		
HGlu–Ser–Glu–Tyr–AspNH$_2$	640.2340	641.2420
HGlu–*Gly*–Glu–Leu–AspNH$_2$	562.2122	561.2515
HGlu–Asn–Glu–Gly–AspNH$_2$	563.1711	562.2109
HGlu–Ala–Glu–Asp–AspNH$_2$	578.1708	577.2114

4. Results and discussion

4.1. gp120/CD4 interaction

4.1.1. Results

Peptides were screened in two assays, firstly for inhibition of gp120/CD4 interaction using a cell-free modified ELISA assay and secondly, for anti-viral effect activity against HIV-infected C8166 cells. ELISA assays gave some interesting structure–activity relationship data.

For the first peptide library **HPhe**–X_1–X_2–**ArgNH$_2$** (Table 4), the following can be deduced:

- IC_{50} of tetrapeptides containing a phenylalanine residue in position X_1 and/or X_2 were far more active than those without one. **HPhe**–Phe–Phe–**ArgNH$_2$**, was very effective at inhibit of binding between gp120 and CD4.
- The presence of glycine residue in peptide structure was appeared to decrease the inhibitory effect, possibly because of the high flexibility of the corresponding peptides. Phenylalanine containing peptides were probably more constrained.
- Other residues, like serine or valine, were noticed to have an influence on the fusion.

Unfortunately in cellular assays, the compounds were not active ($EC_{50} > 500$ μM). This may be due to hydrolysis of the compounds in the presence of bovine serum.

These results were used to design a new shorter library **HPhe**–X–**ArgNH$_2$**. This new library was made to see if a shorter peptide based on this sequence would be more

Table 4
Residue effect on IC_{50} (mM) in the modified ELISA assay for the first library **HPhe**–X_1–X_2–**ArgNH$_2$**

Library **HPhe**–X_1–X_2–**ArgNH$_2$**					
Position 1 (X_1)	Position 2 (X_2)				
Effect on inhibition	H (*Gly*)	CH_2OH (*Ser*)	$CH(CH_3)_2$ (*Val*)	$(CH_2)_4NH_2$ (*Lys*)	$CH_2C_6H_6$ (*Phe*)
H (*Gly*)	25	$\gg$ 12.25	10	$\approx$ 25	**7.70**
CH_2OH (*Ser*)	10.46	10	8.74	$>$ 25	**2.99**
$CH(CH_3)_2$ (*Val*)	$\approx$ 10.4	6.74	13	14.25	**2.63**
$(CH_2)_4NH_2$ (*Lys*)	$\gg$ 25	$>$ 12.5	$\gg$ 25	12.5	**1.78**
$CH_2C_6H_6$ (*Phe*)	**9.75**	**2.75**	**0.92**	**2.91**	**0.72**

efficient or not, and to confirm the need of constrained and hydrophobic residues like phenylalanine. This library was synthesised using the same general procedures used for the first library described below and used a wider range of residues such as ***Arg***, ***Asp***, ***Gln***, ***Gly***, ***Lys***, ***Phe***, ***Pro***, ***Ser***, ***Trp***, ***Val***, ***None***.

This second tripeptide library **HPhe**–X–**ArgNH$_2$** was more active than the tetrapeptide library (Table 5). But no clear correlation between activity and the nature of the X substituent could be found.

Unfortunately, as for the first library, cellular assays against HIV-infected cells gave no significant activity (with EC_{5O}'s between 200 and 2000 μM in comparison with 0.016 μM for AZT).

4.1.2. Discussion

A phenylalanine residue in the X positions of **HPhe**–X_1–X_2–**ArgNH$_2$** seemed to increase activity more than other substituents, leading to IC_{50}'s from 0.72 mM for HPhe–Phe–Phe–$ArgNH_2$ to 9.75 mM for HPhe–Gly–Phe–$ArgNH_2$. This may have been due to either increased hydrophobicity of Phe derivatives and/or less conformational flexibility. To try and understand this observed trend, the log P values were calculated using ACDLabs program [24] and this compared with their activity in the ELISA assay (Fig. 5). The more lipophilic compounds seem to have the greater activity in the gp120/CD4 binding assay. Whilst this data is theoretical and does not take account of charge, the trend observed is what would be expected. However, no correlation could be found between the inhibition of gp120/CD4 interaction and activity of compounds against HIV infected cells. For the **HPhe**–X–**ArgNH$_2$** library, there seems to be no correlation between the nature of X and the inhibition of gp120/CD4 interaction. A plot of calculated log P against inhibition confirms this (Fig. 6). However, the tripeptides were more active than the tetrapeptides. This could be due to entropic factors favouring the tripeptide.

Table 5
Residue effect on IC_{50} (mM) in the modified ELISA assay for the second library HPhe–X–$ArgNH_2$

Library **HPhe**–X–**ArgNH$_2$**	
Position X	IC_{50}
Arg	0.69
Asp	0.17
Gln	0.61
Gly	0.28
Lys	0.39
Phe	0.55
Pro	0.34
Ser	0.82
Trp	0.31
Val	0.27
None	0.24

4.2. gp120/17b Interaction

The library **HGlu**–X_1–**Glu**–X_2–**AspNH$_2$** assayed against the gp120/17b interaction was screened in two assays. The first assay was a reporter cell assay (FIGS assay) [25] which detects inhibition of fusion events. Essentially, in the FIGS assay, there are two cell lines. One of these is chronically infected HIV cell. The other is transfected with a plasmid coding a β-galactosidase. Expression of the β-galactosidase is linked to Tat, a viral transcription factor. Therefore, expression of β-galactosidase is dependent on fusion with the chronically infected cell.

The second assay was a cellular assay to detect inhibition of HIV infection in C8166 cells. None of the peptides showed significant activity in the FIGS assay at concentrations of 100 μM or with the cellular assay at concentrations of $>$250 μM. This could be due to instability of the peptides in the medium or insufficient binding to the 17b binding site. The lack of activity may alternatively be due to lack of exposure of the17b binding site except during binding to CD4.

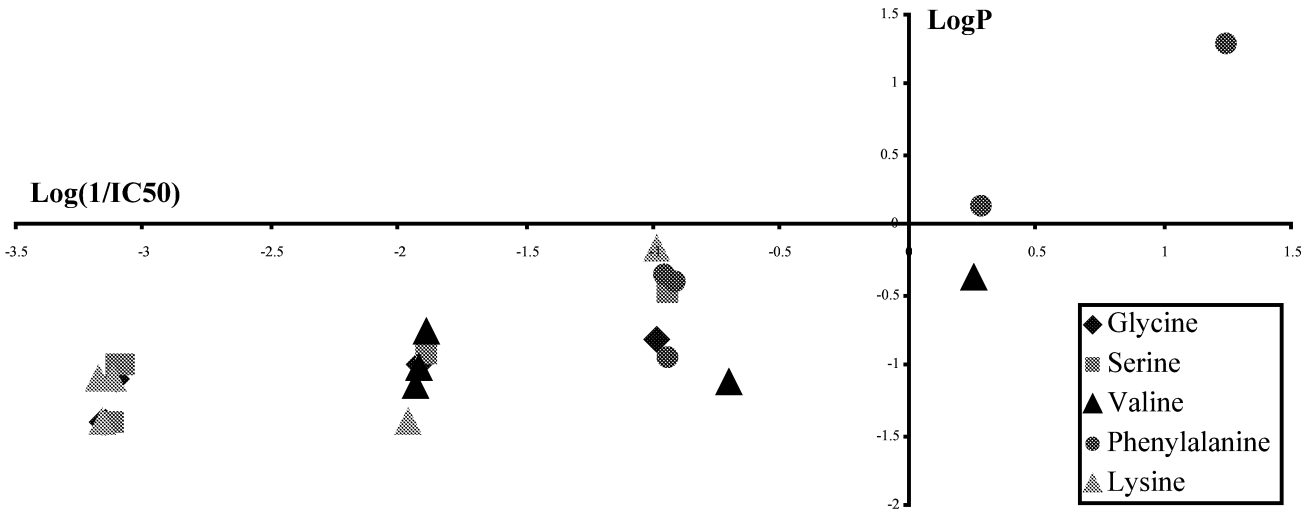

Fig. 5. Theoretical log P of peptides from the first library HPhe–X_1–X_2–ArgNH$_2$ (indicated residue is in X_1 position), calculated using ACDLabs program.

5. Conclusion

The first peptide library (HPhe–X_1–X_2–ArgNH$_2$) showed a relatively good binding inhibition but unfortunately no potential anti-viral activity. Hydrophobic and constrained residues enhanced the inhibitory properties of these peptides. Shorter peptides (tripeptides) showed higher activity than tetrapeptides. This data shows that peptides containing a Phe and Arg inhibit gp120/CD4 interaction and may represent a lead for the design of inhibitors of gp120/CD4 interaction.

For the gp120/17b interaction no significantly active peptide was discovered. This may be due to degredation of the peptides in the assay conditions, which were all cellular based. This contrasts to the case for the assays used for gp120/CD4 interaction which are based on recombinant protein.

6. Experimental

6.1. Modelling

The interfaces of gp120 with the CD4 receptor and the 17b Mab have been studied using the crystal structure of a ternary complex [21] composed of a truncated form of gp120, the N-terminal two domains of CD4 and a Fab from the human neutralising monoclonal antibody 17b using a molecular modelling software MacroModel® (Interactive Molecular Modelling System Version 6.0), running on a Silicon Graphics workstation [26].

6.2. Chemistry

The reagents and solvents were purchased from Aldrich or Sigma. The resins were purchased from

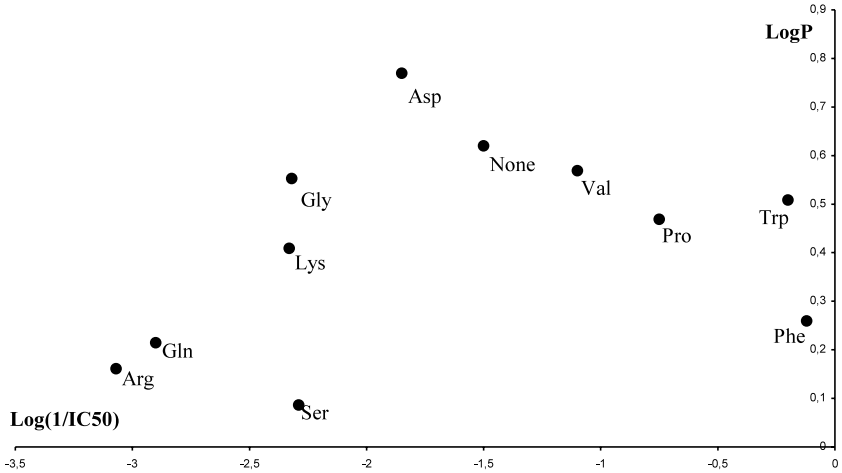

Fig. 6. Theoretical log P of peptides from the second library HPhe–X–ArgNH$_2$, calculated using ACDLabs programme.

Novabiochem. The reactions were monitored by TLC using silica gel 60F254 plates from Merck with visualisation via UV light and/or Ninhydrin solution (by spraying then heating). The mass spectra were recorded on a Fison VG Platform II spectrometer using the electrospray (ES) or atmospheric pressure chemical ionisation (APCI) techniques. All the new compounds were fully characterised by mass spectroscopy. Accurate mass measurements (HRMS) were performed at the EPSRC National Mass Spectrometry Service Centre Chemistry Department of University of Wales Swansea. The RP-HPLC were performed on TSP (Thermo Separation Product, System AS3000, P4000, UV3000) with a Waters column (Spherisorb ODS2, 250×4.6 mm^2, particle size, 5 μ) at $\mu = 254$ and 220 nm. RP-HPLC analytical runs were performed using the following standard conditions: gradient of MeCN in water from 0 to 100% over 50 min.

6.2.1. General coupling procedure

Fmoc-AA loaded resin or Rink Amide MBHA resin was placed in flask. A piperidine–DMF solution (20/80, 10 mL g^{-1} of resin) was added and the mixture was stirred for half an hour. The resin was drained by applying vacuum, then washed three times with DMF and checked using a Ninhydrin test (if not positive, deprotection was repeated). Then, 2 equiv. of Fmoc-AA, TBTU, HOBt were dissolved in DMF (5 mL g^{-1} of resin) and 4 equiv. of DIPEA were added to the mixture. This mixture was added to the resin and stirred for 1 h 30 until a negative Ninhydrin test on a small sample of resin was obtained. These two steps were repeated until required peptide was assembled. After the final coupling, the resin was washed three times with DMF, three times with DCM and three times with MeOH then dried under high vacuum over P_2O_5 or KOH.

6.2.2. General cleavage procedure

The resin was slurried in 10% TFA in DCM. The solvent was allowed to percolate slowly through the resin beads then the resin was washed with 5% TFA, allowing it to pass through the resin bed slowly. The detachment is an acid-catalysed equilibrium, so it was important to continually remove the detached peptide by using this flow method. The excess of TFA–DCM was removed under reduced pressure and the deprotection was completed with 95% TFA. The resin was washed three times with DCM and the fractions of washing were collected.

6.2.3. Ninhydrin test for amino groups

A solution of EtOH–water (2/1) was prepared and a sample of a few resin beads was transferred to a small glass tube with 2–3 mL of EtOH–water solution. Three to four drops of Ninhydrin were added and the mixture was heated to 80–100 °C for 5–15 min. A positive test was indicated by blue resin beads.

6.3. Biology

6.3.1. gp120/CD4 binding assay, modified ELISA

Microtitre plate wells were coated with 50 uL of recombinant sCD4 at 0.5 μg mL^{-1} in PBS overnight at room temperature (r.t.). Plates were washed with PBS and unbound sites were blocked with 100 μL of RPMI 10% calf serum or 1 mg mL^{-1} BSA for 60 min at r.t.

After washing plates with PBS, dilutions of gp120 in RPMI containing 5% calf serum (final) were added and incubated at 37 °C for 3.5 h. Three dilutions containing 0.1, 0.05 and 0.025 μg mL^{-1} of gp120 were used for the standard curve.

To test inhibition of gp120 binding, 25 μL of peptides were added just before adding 25 μL of gp120 (0.1 μg mL^{-1}) in 10% calf serum RPMI. Peptides were added: (a) either directly to the CD4 coated wells in PBS; (b) or pre-diluted in PBS containing 5% calf serum.

Plates were washed with PBS containing 0.1% detergent Tween 20.

CD4 bound gp120 or its inhibition was detected with human anti-HIV antibodies (1/500 and incubation at 4 °C overnight) and *anti*-human conjugated with Horse Radish Peroxidase (1/1000 and incubation at 37 °C for 90 min).

After washing with PBS Tween 20, colour was developed with a substrate OPD and quantitated, using Pharmacia programme and the standard curve [27].

6.3.2. CD4-mediated cell fusion using the vaccinia expression system

The FIGS assay was preformed in 24-well format as described [25]. Colorimetric assays of β-galactosidase activity were performed to quantitate fusion-dependent gene activation. A population of cells was co-infected with vaccinia recombinants encoding RNA polymerase and HIV-1 env, meanwhile a second population was transfected with plasmid containing a gene linked to a promoter then infected with vaccinia recombinant encoding CD4. Both populations were mixed in presence of tested compound and at indicated times, detergent lysates were prepared and analysed by the colorimetric assay.

6.3.3. Anti-viral activity

Compounds were evaluated for in vitro anti-viral activity, against C8166 cells infected by HIV-1 IIIB and the decreased in viral load was determined by measuring the levels of gp120 using antibodies [27].

Acknowledgements

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