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| <b>(54) Title:</b> CYTOTOXIC PEPTIDES<br><b>(57) Abstract</b><br><p>This invention relates to cytotoxic, myristylated peptides derived from the N-terminus of the Nef protein. The peptides preferably comprise a domain comprising a net positive charge and an <math>\alpha</math>-helical region. The invention also relates to compounds which inhibit the cytotoxicity of the Nef protein, the methods of preventing Nef toxicity, HIV infection, and to pharmaceutical compositions comprising such compounds.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |

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CYTOTOXIC PEPTIDES

This invention relates to peptides derived from the amino-terminus of the Nef protein and which exhibit  
5 lytic or cytotoxic activities. In particular, it relates to sequences which are myristylated at a site proximal to hydrophobic and/or basic amino acid residues. The invention also relates to a method of screening for inhibitors of cytotoxic activities and to the synthesis of  
10 such inhibitors.

Background of the Invention

The genomes of all primate lentiviruses contain nef, a highly conserved gene that overlaps the 3' LTR.  
15 Recent evidence suggest that the nef gene product, a myristylated protein of 205 amino acids, is involved in AIDS pathogenesis since mutations in HIV-1 nef leading to the loss of Nef correlate with absence of progression to pathogenesis, even though HIV-1 infection persists  
20 (Kirchoff et al., 1995; Deacon et al., 1995). Genetically-engineered point and deletion mutations in molecularly-cloned SIVmac nef also implicate Nef in AIDS pathogenesis in rhesus monkeys (Kestler et al., 1991).

Numerous studies have shown that Nef is  
25 implicated in a diverse range of activities within the cell. However, there has been little consideration of an extracellular mode of action, even though there is some evidence that Nef exists in an extracellular form. Antibodies to Nef appear early after HIV-1 infection  
30 (Culmann et al., 1989, 1991; Reiss et al., 1989, 1991; Bahraoui et al., 1990; Koenig et al., 1990; Gilmour et al., 1990; Wieland et al., 1990; Kienzle et al., 1991; Hadida et al., 1992), and the C-terminus of Nef has been observed on the surface of HIV-1 infected cells (Fujii et al., 1993).  
35 Further, in a vaccinia expression system, Nef was reported to be 'secreted' from cells into the medium (Guy et al., 1990), and in a yeast expression system Nef was released

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from the cells under conditions of stress (Macreadie et al 1995).

The Nef protein of HIV-1 is a 27 kDa cytoplasmic protein targeted to the plasma membrane and to other  
5 cellular membranes by the sequence at the N-terminus which has an N-myristyl group added post-translationally at glycine 2 (Franchini et al, 1986, Guy et al, 1987, Kaminchik et al, 1991, Yu et al, 1992). It is encoded by the nef gene and its mRNA accounts for more than 75% of all  
10 the viral mRNA (Schwartz et al, 1990). Initially, it was thought that nef exerted negative regulatory effects on viral replication by inhibiting transcription of the HIV-1 LTR, thereby possibly contributing to the development or maintenance of viral latency (Ahmad et al 1988, Cheng-Mayer  
15 et al, 1989, Luciw et al 1987, Niederman et al 1989). In contrast, later studies described either neutral or enhancing effects of nef on viral transcription and infection of activated T lymphocytes (Hammes et al 1989 Kim et al 1989). In studies on the simian immunodeficiency  
20 virus (SIV) in macaques, marked reductions in viral burden and in disease pathogenesis were obtained in animals infected with molecularly cloned viruses containing a nef deletion (Kestler et al 1991). Further strong evidence for a positive role for nef in AIDS pathogenesis has come from  
25 studies on the so-called "Sydney" cohort of long-term AIDS-free, HIV-1 positive subjects who had normal CD4 positive cell counts. The HIV-1 strains isolated from all of these subjects had deletions in nef (Deacon et al 1995).

Nef appears to play a number of roles in HIV  
30 infection and pathogenesis. Several of these roles involve cell membrane interactions. Nef downregulates the HIV-1 receptor, CD4 (Guy et al 1987), as well as IL-2 mRNA and the surface expression of the IL-2 receptor (Luria et al 1991). Nef has also been shown to interact with proteins  
35 involved in signal transduction (Saksela et al 1995, Lee et al 1995) and with a cellular kinase (Bodèus et al 1995) where membrane targeting appears to be essential for

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association. Such targeting also appears to be essential for the association of Nef with a range of other membrane-bound proteins (Sawai et al 1995). Residues 2-7 appear to be essential for CD4 downregulation (Salghetti et al 1995), while residues 11-18 appear to furnish a binding site for a serine kinase (Baur et al 1997). There have also been reports that Nef expressed in a variety of eukaryotic cells is cytotoxic, particularly under conditions of stress. Since there is evidence that Nef produced during HIV-1 infection can be exported to the external medium, it has been suggested that such cytotoxicity might be a cause of the killing of bystander cells observed in AIDS pathology.

We have found that myristylated N-terminal peptides of Nef are lytic for cells such as human leucocytes, erythrocytes and yeast. The lytic activity is not merely a result of the presence of the myristyl chain, and appears to be sequence and structure specific.

#### Summary of the Invention

In the first aspect, the invention provides a cytotoxic peptide comprising a myristylated Nef-amino terminal sequence, wherein the site of myristylation is proximal to hydrophobic. Preferably, the myristylation site is proximal to a hydrophobic and basic amino acid residues. In one embodiment, the peptide is haemolytic.

Preferably, the Nef-amino terminal sequence comprises a first flexible domain and a second  $\alpha$ -helical domain. In a preferred embodiment, the myristylation site is in the first flexible domain and the peptide comprises Myr-Nef2-20, Myr-Nef2-22 and Myr-Nef2-26. More preferably, the cytotoxic peptide comprises a flexible domain Nef2-8, which is myristylated at Gly2, and an  $\alpha$ -helical Nef9-21.

In a particularly preferred embodiment, the invention relates to a cytotoxic or lytic peptide comprising a myristylated Nef-amino terminal sequence, wherein the sequence is positively charged. More preferably, the proximal region of the N terminus eg. the

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first seven amino acid residues of sequence, has a net positive charge, and the succeeding residues form an  $\alpha$ -helical region, preferably spanning residues 9 to 21. The flexible site of myristylation is preferably proximal to a hydrophobic amino acid residue, preferably Trp, Ile or Phe, most desirably Trp5. The positive charge can be provided by one or more of Lys and/or Arg residues.

Preferred myristylated peptides of the invention include but are not limited to:

10

GGKWSKSSVIGWPAVRERMRR-OH (Nef 2-22)

GGAWSASSVIGWPAVRGRMRR-OH (Nef 2-22a where a =  
(Ala4,7,Gly18) )

GGRWSRSSVIGWPAVRGRMRR-OH (Nef 2-22b where b =  
(Arg4,7,Gly18) )

15

GGKWSKSRGGGWATVRERMRR-OH (Nef 2-22c where c =  
(Arg9,Gly10-12,Ala14,Thr15) )

GGKWSKSSVIGWPAVRERMRRRAEPAADGV-OH (Nef 2-26)

GKALKVAEVIGWPAVRERMRRRAEPAADGV-OH (Nef 2-26a where a =  
(Lys3,6,Ala4,8,Leu5,Asp9)

20

GGKWSKSSV-OH (Nef 2-10)

VIGWPAVRERMRRRAEPA-OH (Nef 10-26)

The first flexible domain appears to be involved in lytic or cytotoxic activities. Thus, the invention provides cytotoxic or lytic peptides which may be targetted to unwanted cells such as malignant, cancer or pathogenic cells. The peptides of the invention may be administered as selective toxins for treatment of conditions such as cancer or for reducing or ameliorating tumours. Thus, the invention also provides a method of inducing selective cell death.

The inhibition of Nef activity may be attained by targeting one or both of these domains. Compounds which interfere with membrane localisation and perturbation may be used to inhibit lysis of cells and those which form complexes with the second domain to result in non-

35

amphipathic entities may be used to produce non-fusogenic structures.

Thus, in a second aspect, the invention provides a method of screening for inhibitors of cytotoxic, lytic  
5 and/or fusogenic activities, comprising the step of measuring the effect of the presence of one or more putative inhibitors on the activity of the cytotoxic peptide of the invention, or of a domain thereof.

In a third aspect, the invention provides novel  
10 compounds which block the activity of HIV or Nef; these compounds include but are not limited to those listed in Tables 3, 4, 5, and 6. Preferably, the compounds are those listed in Table 6.

Cytotoxic or lytic activities of a compound, or  
15 derivatives or analogues of those given in the Tables can be assayed using cultured cells or artificial membranes. Fusogenic activities can be measured by using artificial membranes or phospholipid vesicles. Observations on the regulation of receptors such as CD4 and IL2-R may also be  
20 made. The person skilled in the art will be able to determine suitable forms of assays to be employed.

Compounds which exhibit inhibitory activities on the first domain preferably contain acidic groups capable of forming salt bridges with the basic amino-acid residues  
25 of the flexible domain to enhance binding and to block binding of Nef to the negatively-charged phospholipids of the cell membrane and also preferably have a specificity-endowing moiety such as an aromatic moiety capable of reacting with the indole ring system of the tryptophan  
30 residue 5. The activity of these compounds may be assayed by inhibition of the cytolytic activity of myristylated Nef2-26 peptide or variants thereof. Compounds which may be used include, but are not limited to lipophilic, aromatic, anionic or zwitterionic or polar moieties. Bis-  
35 inhibitors, made up of mono inhibitors linked by long chain di-acids, or inhibitors comprising multiple peptide sequences are also contemplated.

Non-limiting examples of compounds with the activities described above are one or more of those listed in Tables 3 to 6, preferably the compounds in Table 6.

The fusogenic activity of the second domain may  
5 be targeted with compounds that block the amphiphilic nature of this domain. Such compounds may be amphiphilic themselves and complementary to the hydrophobic phase of the helix. Alternatively, they may be complementary to the hydrophilic face. In both cases, specific interaction  
10 would result in a complex with a second domain, in which the complex is non-amphipathic, and therefore is non-fusogenic. Putative inhibitors of fusogenic activity include, but are not limited to, negatively charged amphipathic helical peptides, coil/coil inhibitors or  
15 anti-sense peptide nucleic acid (PNA) inhibitors.

Preferred compounds which inhibit fusogenic activity as described above include but are not limited to those described in Tables 2 to 6, most preferably those in Table 6.

20 Derivatives or analogues of the peptide and compounds described herein are also contemplated by this invention. A person skilled in the art will be able to obtain such compounds on the basis of the sequence, structure and chemical or biological characteristics of the  
25 peptide and compounds described herein.

In view of the role of the interaction between the Nef protein and cellular membranes in the pathogenesis of HIV infection, the compounds or inhibitors described above are useful for treatment or prevention of HIV  
30 infection by modulating the protein-cell membrane interaction. Thus, in a fourth aspect, the invention provides a method of modulating interaction of Nef protein with a cell membrane, comprising the step of administering a compound as described above to prevent the Nef protein  
35 interacting with membrane bound components. The components are preferably information or energy transducers, and may include proteins, peptides, hormones, ions or the like.

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In a fifth aspect, the invention provides a method of reducing or eliminating the cytotoxicity of Nef or related sequences, comprising the step of administering an inhibitor or compound as described above. In a preferred embodiment, the compound inhibits the down regulation of CD4 receptors and IL-2 receptors by HIV or a part thereof, such as the Nef protein. More preferably, the compound inhibits interaction of Nef with intracellular proteins such as lck and/or serine kinase. Most preferably, the compounds inhibit Nef-induced cytotoxicity in cells of the lymphoid tissue, particularly non-infected lymphoid cells, and/or inhibit Nef-induced killing of bystander cells.

In a sixth aspect, the invention relates to a method of treating HIV infection, comprising the step of administering a compound of the invention to a subject in need of such treatment. The invention also relates to pharmaceutical compositions comprising one or more compounds of the invention, together with a pharmaceutically acceptable carrier. The composition of the invention may be formulated and administered in a manner known to a person skilled in the art. For example, the composition may be in the form of a liquid formulation, aerosols, micronised particles, liposomes, tablets, capsules or powdered form for oral, ip, iv or parenteral administration. It may also be administered via the mucosa using, eg. nasal sprays or nose drops. The dosages and frequency of administration will be easily determined by a person skilled in the art in accordance with the condition to be treated.

Modifications may be made to the peptides or compounds described herein without deleteriously affecting the biologically activity thereof. Such modifications would be within the knowledge and expertise of the person skilled in the art and include, for example, conservative or non-conservative amino-acid substitutions, insertions,

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deletions or derivatisation which do not substantially modify the activity of the molecules.

#### Detailed Description of the Invention

5           The invention will now be described in detail by way of reference only to the following non-limiting examples, and to the drawings, in which:-

          Figure 1 shows the haemolytic effects of Nef peptides on human erythrocytes. The extent of haemolysis  
10       was calculated by dividing the OD540nm at each point by the OD540nm of cells exposed to 2% Tween 80. Cell concentrations were 2% (v/v) and duration of exposure to lytic agent for each point was 5 min. Assays were performed in duplicate and intra-assay variation was within  
15       5%.

t---t Myr-Nef2-22      n---n Myr-Nef2-26    \_---\_ Myr-Nef2-22(Val11)

20    X---X Myr-Nef2-26(Ser13)\*---\* Myr-Nef31-50 1---1 Nef2-22  
\_---\_ Nef2-26            '---' Nef2-22(Val11)   o---o Nef2-26(Ser13)

25           Figure 2 shows the kinetics of lysis of human erythrocytes by Nef peptides. The extent of haemolysis was calculated as in Figure 1. Cell concentrations were 2% and the concentration of each peptide was mg/ml. Assays were performed in duplicate and intra-assay variation was within  
30       5%.

t---t Myr-Nef2-22      n---n Myr-Nef2-26    \_---\_ Myr-Nef2-22(Val11)

35    X---X Myr-Nef2-26(Ser13)\*---\* Myr-Nef31-50 1---1 Nef2-22

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\_---\_ Nef2-26                      "----" Nef2-22(Val11)    o---o Nef2-  
26(Ser13)

Figure 3 comprises photomicrographs of cultured  
5 cells treated with Nef peptides (10X magnification). CEM  
cells were treated with 10mg peptide, or PBS for 30  
minutes. A. PBS B. Myr-Nef31-50 C. Myr-Nef2-22

Figure 4 shows LDH release by Nef-treated CEM  
10 cells. A. Cells were incubated with peptide at a final  
concentration of 12.5 mM for 30 min. Cytotoxicity was  
calculated as a percentage of the maximum LDH release after  
subtracting spontaneous release, as described in the  
methods. Results represent the mean  $\pm$  standard error of the  
15 mean (SEM) for three separate experiments. B. CEM cells  
were incubated with serial dilutions of Myr-Nef2-26 (closed  
squares), Myr-Nef31-50 (open circles), or Nef2-26 (closed  
circles) peptide ranging from 12.5-0.78 mM final  
concentration for 30 min. Cytotoxicity was calculated as a  
20 percentage of the maximum LDH release after subtracting  
spontaneous release, as described in the methods. Results  
represent the mean $\pm$ SEM for three separate experiments.

Figure 4C shows the kinetics of release of LDH  
from CEM cells incubated with Myr-Nef2-26 (squares), Nef2-  
25 26 (triangles), or Myr-Nef31-50 (circles) at a final  
concentration of 6.25  $\mu$ M for up to 4 hr. Values represent  
the mean  $\pm$  SEM for three separate experiments, performed in  
triplicate, and are expressed as the percentage of maximum  
LDH release induced by 1% Triton X-100.

30 Figure 5 shows a fluorescence emission spectra  
for Nef2-26(Ser13) (solid curve) and Myr-Nef2-26(Ser13)  
(dotted curve) in PBS. The excitation wavelength was 290  
nm, temperature-22°C. The concentration of each peptide  
was 5mM.

35 Figure 6 shows Stern-Vollmer plots of  
A) quenching of fluorescence of the Trp 5 residue of  
the non-myristylated peptide Nef2-26(Ser13) by water

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soluble (---- TCC) and lipid soluble (n---n 5NPC, t---t 12NPC, o---o 16NPC) nitroxide quenching agents in the presence of large unilamellar phospholipid vesicles

B) quenching of fluorescence of the Trp 5 residue of the myristylated peptide Myr-Nef2-26(Ser13) by water solution (---- TCC) and lipid soluble (n---n 5NPC, t---t 12NPC, o---o 16NPC) nitroxide quenching agents in the presence of large unilamellar phospholipid vesicles

Figure 7 illustrates the effect of Myr-Nef2-22 on yeast colony formation. The photograph shows the colonies formed after one day of the peptide treatment for *C. albicans* and *C. glabrata*, two days for *K. lactis* and *S. cerevisiae*, and three days for *Sz.* without peptide treatment. With peptide treatment all plates were identical - no colonies formed.

Figure 8 shows the effect of myristylation of Nef peptides on yeast mortality. A. *S. cerevisiae* cells were treated with a 1 mM concentration of the Nef peptides: Myr-Nef31-50, Myr-Nef2-22 and Nef2-22, B Dose responses for treatments of 30 minutes with Myr-Nef2-22 and Nef2-22.

Figure 9 represent cellular propidium iodide (PI) uptake as analysed by flow cytometric analysis. The peptides were Myr-Nef31-50 (Myr control), Myr-Nef2-22 (Myr-Nef), and Nef2-22 (Nef). An arbitrary gate was set such that 5% of untreated cells were classified as stained.

Figure 10 shows the effect of peptides on *E. coli* colony formation. Peptides were added to *E. coli* cells suspended in water. After 30 minutes cells were spread onto solidified 2 x YT and plates were examined after overnight incubating at 37°C.

Figure 11 shows that exposure to myristylated N-terminal Nef peptide reduces the rate of extracellular acidification in CEM CD4+ T cells. (A) Acidification rate responses of CEM cells were perfused with 10<sup>-5</sup> M Myr-Nef2-26 (squares), Nef2-26 (triangles), Myr-Nef31-50 (circles), or medium alone (diamonds). The arrows denote the time of peptide addition and tracings are representative of a

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minimum of three separate experiments. (B) Acidification rate responses of CEM cells perfused with medium alone (diamonds), or with  $5 \times 10^{-5}$  M (squares),  $1 \times 10^{-6}$  M (triangles), or  $5 \times 10^{-6}$  M (circles) Myr-Nef2-26. The  
5 arrows denote the time of peptide addition.

Figure 12 is the region of the 2D  $^1\text{H}$  TOCSY spectrum at 500 MHz of Nef2-26 in 90%  $\text{H}_2\text{O}/10\%$   $2\text{H}_2\text{O}$  at pH 4.5 and 281 K, showing the Ala NH-CbH3 connectivities and the effect of cis-trans isomerism at Pro14. Peaks A and B  
10 were assigned to Ala26, Peaks C and D are due to Ala23, peak E is due to Ala15 when Pro14 is in the trans configuration, peak F arises from Ala15 when Pro14 is in the cis configuration. Assignment of peaks A, B, C or D to a particular conformation was not possible as spectral  
15 overlap prevented an unambiguous assignment of the spectrum. Small cross-peaks were also observed for Ala26 due to the cis-trans isomerism of Pro25, but these peaks are too weak to observe at the contour level of this figure.

Figure 13 shows chemical shift analysis for Nef2-26. All random coil chemical shifts taken from Wishart et al, 1995b. A: Deviation of CaH chemical shift from random coil values for Nef2-26 in 50%  $\text{H}_2\text{O}/50\%$  TFE at pH 5.5 and 281 K. B: Deviation of CaH chemical shift from random coil  
20 values for Nef2-26 in  $\text{C}_2\text{H}_3\text{OH}$  at pH 4.5 and 281 K. C: Deviation of NH chemical shift from random coil values for Nef2-26 in  $\text{C}_2\text{H}_3\text{OH}$  at pH 4.5 and 281 K. D: Deviation of Ca chemical shift from random coil values for Nef2-26 in  $\text{C}_2\text{H}_3\text{O}_2\text{H}$  at pH 4.8 and 281 K. E: Deviation of CdH chemical  
25 shift from random coil values for Nef2-26 in SDS micelles at pH 5.1 and 298 K.

Figure 14 shows regions of the 300-ms mixing time NOESY spectrum of Nef2-26 in  $\text{C}_2\text{H}_3\text{OH}$  at pH 4.5 and 281 K. A: CaH-NH region. Intra-residue NH-CaH cross-peaks are  
35 labelled with a single number. Sequential NOE cross-peaks are not labelled but are indicated by the line joining cross-peak due to consecutive residues (the CaH chemical

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shifts of Ala15 and Pro14 are quite close; as a result the aN connectivity between Pro14 and Ala15 overlapped with the NH-CaH cross-peak of Ala15). Medium-range cross-peaks are indicated by two numbers indicating the residue

5 contributing the CaH and NH protons respectively. B: NH-NH region. The NH-NH cross-peaks are labelled with two numbers identifying the sequence position of the interacting residues; numbers beside the cross-peak identify the contributing residue from the F1 dimension  
10 while the numbers above or below the cross-peak identify the contributing residue from the F2 dimension.

Figure 15 is a summary of the NMR data for Nef2-26 in C<sub>2</sub>H<sub>3</sub>OH at pH 4.5 and 281 K. The intensities of daN, dbN connectivities are represented as strong, medium or  
15 weak by the height of the bars. Shaded lines indicate ddd connectivities to Pro residues. Asterisks indicate that the presence of a NOE could not be confirmed unambiguously due to peak overlap. Values of 3JHN<sub>CaH</sub> < 6 Hz are indicated by -, while those > 8 Hz are indicated by -. Those left  
20 blank could not be measured due to overlap, or were between 6 and 8 Hz. The relative exchange rates of backbone NH protons are indicated in the row labelled NH, based on the strength of cross-peaks in 2H<sub>2</sub>O exchange TOCSY experiments; slowly exchanging amides are indicated by filled circles,  
25 while open circles indicate intermediate exchange. The row labelled aH shows the chemical shift index of the CaH, if the CaH deviation from random coil is > 0.1 ppm higher field than the random coil value it is given a value of -1, if the CaH value is > 0.1 ppm to lower field than the random  
30 coil value it is given a value of 1.

Figure 16 represents parameters characterising the final twenty structures of Nef2-26 in methanol, plotted as function of residue number. A: Upper-bound distance restraints used in the final round of structure refinement;  
35 medium-range (2 < i-j < 5), sequential and intra-residue NOEs are shown in black, hatched and white shading respectively, the one long-range NOE (Trp13 C(4)H to Glu18 NH) is not

shown. NOEs are counted twice, once for each proton involved. B: RMS deviations from the mean structure for the backbone heavy atoms (N, Ca, C) following superposition over the whole molecule. C-F: Angular order parameters (S) (Hyberts et al, 1992; Pallaghy et al, 1993) for the backbone ( $\phi$  and  $\psi$ ) and side-chain ( $\chi_1$  and  $\chi_2$ ) dihedral angles. Gaps in the  $\chi_1$  plot are due to Gly and Ala residues. Gaps in the  $\chi_2$  plot, in addition to Gly and Ala, are due to Ser, Pro, and Val residues.

Figure 17 is a stereo view of the final 20 structures of Nef2-26 in methanol superimposed over the backbone heavy atoms (N, Ca, C) of the well-defined ( $S_{\phi} > 0.9$ ) region of the molecule, encompassing residues 9-22. A: Backbone heavy atoms. B: Orthogonal view showing side chains of residues with well-defined  $\chi_1$  angles ( $S_{\chi_1} > 0.9$ ) are shown.

Figure 18 shows the dose-dependent release of calcein from CEM cells treated with Nef peptides. Calcein-loaded CEM cells were incubated with serial dilutions of Myr-Nef2-26 (squares), Nef2-26 (circles) or Myr-Nef31-50 (triangles) for 30 min and calcein release determined as described in Materials and Methods. Data is representative of at least three separate experiments.

Figure 19 shows the Nef inhibitory activity and cytotoxicity of synthetic compounds in CEM cells. Synthetic compounds were incubated with (solid bars, % Nef inhibition) and without (shaded bars, % cytotoxicity) Nef N-terminal peptide at a molar ratio of 10:1 compound:Nef peptide for 30 min at 37°C prior to incubation with calcein-loaded CEM cells for 1.5 hr. Data represent the mean  $\pm$  SEM for at least two separate experiments.

Figure 20 shows the Nef inhibitory activity of synthetic compounds in PBMC. BRI6209 (closed squares), AP13 (closed triangles), DER-AP3 (closed circles) or DER45 (open squares) were incubated with and without Nef N-terminal peptide at a range of molar ratios for 30 min at 37°C prior

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to incubation with calcein-loaded PBMC cells for 1.5 hr.  
Data is representative of results from two separate donors.

Figure 21 represents the Far-UV circular  
dichroism spectra of Nef peptides in methanol.

5

|   |           |   |            |
|---|-----------|---|------------|
| A | Nef 2-22  | E | Nef 2-26   |
| B | Nef 2-22a | F | Nef 2-26a  |
| C | Nef 2-22b | G | Nef 31-50a |
| D | Nef 2-22c | H | Nef 31-50  |

10

Figure 22 shows the conformational probabilities  
for different Nef N-terminal peptides calculated by  
discrete state-space theoretical analysis (Stultz et al  
1993, White et al, 1994) performed on the Boston University  
Biomolecular Engineering Research Center Protein Sequence  
Analysis server. A Nef2-22, B Nef2-22a, C Nef2-22b, D  
Nef2-22c, E Nef2-26a, F (i) Nef2-9, (ii) Nef10-22, G  
Nef 31-50a, H Nef 31-50

In each frame, the key is:

20

|         |           |
|---------|-----------|
| a-helix | — — — — — |
| strand  | —————     |
| loop    | - - - - - |
| turn    | — . — . — |

25

Figure 23 shows the pressure/surface area  
isotherms for DPPC monolayers on a subphase containing Myr-  
Nef2-22 (————), Myr-Nef 31-50 (— . — .) or Nef2-22  
(- - - -) compared with that of DPPC over HEPES buffer  
(— — — .)

30

Figure 24 represents the dequenching of the  
fluorescence of the fluorochrome octadecyl-rhodamine in the  
presence of non-myristylated Nef2-22 and Nef 9-22,  
indicating the occurrence of membrane-mixing fusion.

35

- 15 -

Key:

Nef2-22 Nef 9-22 

5 Figure 25 represents the dose response curves for the lysis of sheep red blood cells by Nef N-terminal peptides. Peptides were incubated with cells at 20°C for 3 min.

10 Key:

Nef 2-22 Nef 2-22a Nef 2-22b Nef 2-22c 15 Nef 2-26a Nef 2-26 +  +Nef 2-10 +  +Nef 31-50 +  +Nef 31-50a +  +

20

Figure 26 shows the kinetics of lysis of sheep red blood cells by Nef N-terminal peptides at 20°C. The concentration of each peptide was 32 mM.

Key:

25 Nef 2-22 Nef 2-22a Nef 2-22b Nef 2-22c Nef 2-26a 30 Nef 2-26 +  +Nef 2-10 +  +Nef 31-50 +  +Nef 31-50a +  +

Figure 27 represents the dose response curves of peptide cytotoxicity for CD4+ T cells. CEM cells were incubated with peptides at 37°C for 2 hr and LDH release quantitated as described in Materials and Methods. Closed squares, Myr-Nef2-26; closed circles, Myr-Nef2-22; closed triangles, Myr-Nef31-50; open squares, Myr-Nef2-22a; open triangles, Myr-Nef2-22b.; open circles, Myr-Nef2-22c; open diamonds, Myr-Nef2-8; crosses, Myr-Nef31-50a.

Figure 28 shows that the Nef N-terminal peptide induces LDH release in a range of leukocytic cell lines. (A) Cells ( $2 \times 10^4$ /well) were incubated with 10  $\mu$ M Myr-Nef2-26 peptide for 2 hours and LDH release quantified as described in Materials and Methods. (b) Cells ( $2 \times 10^4$ /well) were incubated with 5  $\mu$ M Myr-Nef2-26 peptide for 2 hours and LDH release was quantified. Values represent the mean  $\pm$  SEM for three separate experiments, performed in triplicate, and are expressed as the percentage of maximum LDH release induced by 1% Triton X-100.

Figure 29 shows the Nef N-terminal peptide-induced LDH release uncultured PBMC. Cells ( $1 \times 10^5$ /well) were incubated with 12.5  $\mu$ M Myr-Nef2-26, Nef2-26 or Myr-Nef31-50 for 30 min and LDH release was measured. Values represent the mean  $\pm$  SEM for three separate experiments, performed in triplicate, and are expressed as the percentage of maximum LDH release induced by 1% Triton X-100.

Figure 30 shows the Nef N-terminal peptide-induced LDH release from cells isolated from tonsil lymphoid tissue. Tonsillar cells ( $1 \times 10^5$ /well) were incubated with 10  $\mu$ M Myr-Nef2-26, Nef2-26 or Myr-Nef31-50 for 30 min (solid bars) or 2 hr (shaded bars) and LDH release was measured. Values represent the mean  $\pm$  SEM for three separate experiments, performed in triplicate, and are expressed as the percentage of maximum LDH release induced by 1% Triton X-100.

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## GENERAL METHODS

Peptide Synthesis

Peptides corresponding to the amino-terminal sequence of Nef were synthesised as described previously (Curtain et al 1994) on an Applied Biosystems 430A peptide synthesiser, using the FastMoc® solid-phase technique in which the α-amino groups of the amino acids were protected by base-labile 9-fluorenylmethoxycarbonyl (Fmoc) groups. The sequences from the pLN4.3 strain of HIV-1 were:

10 Gly-Gly-Lys-Trp-Ser-Lys-Ser-Ser-Val-Ile-Gly-Trp-Pro-Ala-Val-Arg-Glu-Arg-Met-Arg-Arg-Ala-Glu--Pro-Ala-OH

2 20  
 <-----Nef2-20----->

15 22  
 <-----Nef2-22----->

26  
 <-----Nef2-26----->

20 Additionally, a 21 residue peptide (Nef2-22(Val11)) was prepared in which Ile6 was replaced with a Val (LAV1a strain) and a second pLN4.3 24 residue N-terminal peptide (Nef2-26(Ser13)) was prepared in which serine was substituted for tryptophan at position 13.

25 A control non-N-terminal 20 residue peptide (Nef31-50) was synthesised according to the following sequence:-

31  
 30 Gly-Ala-Val-Ser-Arg-Asp-Leu-Glu-Lys-His-Gly-Ala-Ile-Thr  
 50  
 -Ser-Ser-Asn-Thr-Ala-Ala-OH.

The non-myristylated peptide GKVSRKLEKHGAITSSNTAA-OH Nef 31-50a where a = (Lys32,36) and non-myristylated Nef2-22 and Nef9-22 were also prepared.

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The peptides were myristylated on the synthesiser by applying myristic acid to the N-terminal amine of the peptide/resin (ie Fmoc group removed) using the protocol normally used for recoupling serine (ie longest dissolving time). The myristylated peptides were coded Myr-Nef2-22, Myr-Nef2-22a, Myr-Nef2-22b, Myr-Nef2-22c, Myr-Nef2-26, Myr-Nef2-26a, Myr-Nef2-22(Val11), Myr Nef2-10, and Myr-Nef10-26. Myr-Nef2-26(Ser13) and Myr-Nef31-50. The peptides were purified using a Vydac C18 column, and their purity and composition confirmed by HPLC, amino acid analysis and electrospray mass spectroscopy.

The sequences of the peptides are as follows:-

|    |                                   |                                                       |
|----|-----------------------------------|-------------------------------------------------------|
|    | GGKWSKSSVIGWPAVRERMRR-OH          | (Nef 2-22)                                            |
| 15 | GGAWSASSVIGWPAVRGRMRR-OH          | (Nef 2-22a where a =<br>(Ala4,7,Gly18) )              |
|    | GGRWSRSSVIGWPAVRGRMRR-OH          | (Nef 2-22b where b =<br>(Arg4,7,Gly18) )              |
|    | GGKWSKSRGGGWATVRERMRR-OH          | (Nef 2-22c where c =<br>(Arg9,Gly10-12,Ala14,Thr15) ) |
| 20 | GGKWSKSSVIGWPAVRERMRRRAEPAADGV-OH | (Nef 2-26)                                            |
|    | GKALKVAEVIGWPAVRERMRRRAEPAADGV-OH | (Nef 2-26a where a =<br>(Lys3,6,Ala4,8,Leu5,Asp9)     |
|    | GGKWSKSSV-OH                      | (Nef 2-10 )                                           |
| 25 | VIGWPAVRERMRRRAEPA-OH             | (Nef 10-26)                                           |

#### Rationale for sequences

The Nef2-22 and Nef2-26 sequences were selected because we had found earlier that Myr-Nef2-22 could provoke the formation of non-lamellar structures in lipid bilayer membranes (Curtain et al 1994). Myr-Nef31-50 was chosen as a potential control sequence to evaluate the effect of myristylation on the membrane activity of the Nef2-22 and Nef2-26 peptides because discrete statespace theoretical analysis (Stultz et al, 1993, White et al 1994) performed on the Boston University Biomolecular Engineering Research Center Protein Sequence Analysis server showed that it

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would form a rigid  $\alpha$ -helix in contrast to Nef2-22 and Nef2-26 in which an  $\alpha$ -helix was only probable from residues 14 on, leaving considerable flexibility at the N-terminus of the peptide. The predicted overall  $\alpha$ -helical content of the peptides was confirmed by circular dichroism measurements made in methanol/water (60/40).

#### Liposome preparation

Large unilamellar vesicles (LUV) were prepared by dispersing dried films of egg yolk phosphatidyl choline (EYPC) in buffer (5 mM Hepes, 100 mM NaCl, pH7.4), freezing and thawing the dispersion 10 times and extruding 10 times through two stacked 0.4 mm pore size polycarbonate filters (Nucleopore, Pleasanton CA) as described by Hope et al (Hope et al 1985). The lipid spin labels were added in the desired molar ratio to the EYPC in chloroform/methanol (70/30) before the preparation of the dried films.

#### Cells

(i) Human erythrocytes and leukocytes.

Buffy coat packs and time-expired, packed erythrocytes were obtained from the Australian Red Cross Blood Bank, Melbourne. Peripheral blood mononuclear cells (PBMC) were purified from buffy coat packs by ficoll-Hypaque density gradient centrifugation.

Tonsil cells were isolated from tonsils surgically removed during therapeutic tonsillectomy. Tonsils were stored on ice in PBS pH7.4 containing 250  $\mu$ g/ml gentamicin and processed within 3-6 hr. The epithelial layer was removed and the tissue mechanically disrupted by sieving through a 200 mesh in PRMI 1640 supplemented with 10% (v/v) heat-inactivated foetal bovine serum (GIBCOBRL). Dead cells, epithelial cells and debris were removed by centrifugation over an isotonic metrizamide gradient at 1600 x g at room temperature for 10 min. Cells with a density of between 1.04 and 1.065 were collected and washed before use.

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The human leukocyte cell lines CEM (CD4+T-cell), Jurkat (CD4+T-cell), RPMI 8226 (B-cell), U266 (B-cell), THP-1 (monocyte) and U-937 (monocyte, Sundstrom and Nilsson, 1976) were obtained from American Type Culture Collection, Rockville, Maryland, USA. The RC2a human monocytic cell line was from the Macfarlane Burnet Centre for Medical Research, Melbourne, Australia. Cell lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, 1.5g/l sodium bicarbonate, 10 mM HEPES, 1mM sodium pyruvate, 25 µg/ml gentamicin and 20 (RPMI 8226), or 10% (v/v) heat-inactivated foetal bovine serum (GIBCO-BRL Life Technologies, Auckland, NZ) and passaged twice weekly. Cells were harvested during log-phase growth for use in all experiments.

15 (ii) Yeast

The yeast strains used were *Saccharomyces cerevisiae* strain DY150 (MATa ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11 can1-100), *Candida glabrata* strain L5 (leu), *Candida albicans* clinical isolate JRW#5, *Kluyveromyces lactis* strain MW98-8c (MAT-a uraA arg lys) and *Schizosaccharomyces pombe* (h- ade ura leu1-32). Strains were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). The required number were resuspended in 700-1000 ml modified buffer, mixed with molten agarose and stirred at 39°C until required.

(iii) Fresh red blood cells were obtained from Merino X Border Leicester sheep.

30 Yeast growth assay

Peptides were purified to homogeneity by HPLC and then dissolved in distilled water at a concentration of 2mg/ml and stored at 4°C until required. Aliquots of peptide solutions were added to cells suspended in a final volume of 100ml water, and after 1 hour or other specified time, the mixture was plated onto the appropriate solidified medium. Plates were incubated for one to three

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days, depending on the strain, and the number of viable colonies was determined.

#### Microscopy of yeast

5                   Propidium iodide (PI) uptake of treated cells was examined by microscopy immediately after the addition of PI to 1 mg/ml to cells in suspension. Fluorescence microscopy utilised an Olympus BH2-RFCA microscope equipped with an excitation cube/filter B for PI fluorescence.

10                  Escherichia coli strain MC1061 (F-araD139 D(ara-leu)7696 galE15 galK16 D(lac)X74 rpsL (Str<sup>r</sup>) hsdR2 (rK-MK<sup>+</sup>) mcrA mcrB1) was also employed for toxicity studies and plated onto 2 X YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl).

15

#### Flow cytometry analysis of Yeast

                  Cells were analysed by forward angle and 90° light scatter as well as PI fluorescence using a Coulter EPICS® elite flow cytometer. PI was added to 25 mg/ml and dye penetration was measured by the presence of  
20                   fluorescence emission at 520 nm. Gating was adjusted such that the population defined as "PI stained" comprised 5% of the control cell population.

#### 25   Haemolysis

                  The packed cells were washed three times and then diluted to a 1% or 3% suspension in phosphate buffered saline (PBS). The peptides were dispersed by sonication in PBS to a final concentration of 100 mM. Each was added by  
30                   micropipette to 1 ml of the cell suspension over the desired concentration range (0.05 - 20.0 mM) and the mixture was shaken for 30 seconds, allowed to stand for the desired time with gentle inversion of the tube every 30 seconds and then centrifuged at 1500 x g for 2 minutes.  
35                   The optical density (OD)540nm of the supernatant was determined spectrophotometrically. A blank tube containing 3% cells only was used as a control. 100% haemolysis was

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determined by adding 0.5% Tween 80 to 3% cells and mixing and centrifuging them as described above. The results for each peptide was calculated as a percentage of the OD of the Tween 80 tube after subtracting the value of the control.

#### Microscopic analysis of human cells.

CEM CD4+ T cells were washed once in PBS, pH 7.4 and once in assay buffer (0.96 mM KH<sub>2</sub>PO<sub>4</sub>, 5.28 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.74 mM KCl, 143 mM NaCl, pH 7.4), and resuspended at 1x10<sup>7</sup>/mL in assay buffer. 100 mL cells were added to 10 mL peptide (1 mg/mL in PBS), or PBS and incubated at room temperature for up to 6 hours. At various time intervals, 10 mL of cell suspension was removed, placed on a glass slide and examined by light microscopy.

#### LDH release assay

Nef peptides were assayed for cytotoxic activity against human cells using a commercially available kit (Cytotoxicity Detection Kit (LDH), Boehringer Mannheim, Mannheim, Germany). Cells were prepared as described above and resuspended at 2x10<sup>5</sup>/mL (cell lines) and 1x10<sup>6</sup>/mL (PBMC and tonsil cells) in assay buffer. The cell concentrations were determined in preliminary experiments to give optimal maximum versus spontaneous calcein release for each cell type. The peptides were dissolved in water at a concentration of 1 mM. 100ml cells were mixed with 100 ml peptide diluted to the desired concentration in assay buffer in a microtitre plate and incubated at 37°C in 5% CO<sub>2</sub>, 90% humidity for 30 minutes. At the end of this incubation, the plate was centrifuged at 250xg for 10 minutes and 100 mL supernatant transferred into a EIA plate (Dynatech, Chantilly, Virginia, USA). Lactate dehydrogenase (LDH) activity was determined by adding 100 ml reaction mixture and incubating at room temperature, protected from light, for 30 minutes. The OD<sub>490nm</sub> on an EIA plate reader (Dynatech DIAS; Dynatech, Chantilly,

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Virginia, USA; or Nunc, Roskilde, Denmark), using a reference wave-length of 630 nm. Plates were blanked on wells containing buffer alone. All assays were performed in triplicate and included cells incubated in buffer alone to measure spontaneous LDH release. Spontaneous release was 10% or less of maximum release in all assays. Maximum LDH release was determined by adding 100 ml 2% Triton X-100 to 100 ml cells and used to calculate the percentage cytotoxicity for each peptide as follows:

10                   % cytotoxicity = 100 x (ODpeptide -  
ODspontaneous)/(ODmaximum - ODspontaneous)

Additional controls, consisting of peptide alone and peptide plus LDH (0.05 U/mL; Boehringer Mannheim, Mannheim, Germany), were included to ensure that the peptides themselves did not contain LDH activity and did not inhibit cellular LDH activity.

#### Ultraviolet Fluorescence Studies

Ultraviolet fluorescence spectra of the tryptophan residues in Nef2-26(Ser13) and Myr-Nef2-26(Ser13) were recorded at 22°C with a Perkin Elmer MPF3 fluorescence spectrophotometer using an excitation wavelength of 290 nm. Fluorescence quenching data were obtained by adding the spin labels over the desired range of concentrations to a mixture (1:50 molar ratio). Dynamic (collisional) quenching of a fluorophore in free solution follows the Stern-Vollmer equation (Stern et al 1919)

$$\frac{I_0}{I} - 1 = k_{qr}[Q]t$$

30                   where I and I<sub>0</sub> are the intensities of the tryptophan fluorescence in the presence and absence of the buffer soluble quencher tempo choline chloride, (TCC), k<sub>q</sub> is the bimolecular rate constant, [Q]<sub>t</sub> is the total concentration of TCC and t is the fluorescence lifetime. For the doxyl-labelled phospholipids which partition into the lipid phase

the concentration of [Q] may be taken to be the mole fraction added because electron spin resonance spectroscopy studies on bilayer membrane-associated spin-labelled phospholipids have shown that the spin labels at all  
5 positions are almost entirely confined to the lipid phase (Hubble et al 1971). A plot of  $\frac{I_0}{I} - 1$  against [Q]T will be

linear if collisional quenching occurs. If the quencher forms a non-fluorescent equilibrium complex with the ground state of the fluorophore, static quenching will also occur  
10 and this will be evident from an upward curvature of the Stern-Vollmer plot. Downward curvature of the plot may indicate that the fluorophore is exposed to the quencher in two or more environments.

#### 15 Palmitoyl-stearoyl Probes

For use in ultraviolet fluorescence quenching studies, palmitoyl-stearoyl phosphatidyl choline probes, doxyl spin-labelled at the 5 (5NP), 12 (12NP) and 16 (16NP) carbons of the stearoyl chain, were obtained from Avanti  
20 Polar Lipids Inc, Pelham AL. The water soluble spin probe, tempo choline chloride (TCC) was obtained from Molecular Probes Inc, (Junction City, OR). Spin labels from both sources were checked for purity and to ensure that their number of spins/M were >90% of theory, as described by  
25 Gordon and Curtain (1988). Egg yolk phosphatidyl choline (EYPC, Type XVI\_E) was obtained from Sigma St Louis MO and used without further purification.

#### NMR Spectroscopy

30 NMR samples were prepared by dissolving 2.4 mg of lyophilized Nef2-26 peptide in 0.55 ml of the appropriate solvent (final concentration 1.5 mM); solvents used were C<sub>2</sub>H<sub>3</sub>OH, C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>H, 90% H<sub>2</sub>O/10% 2H<sub>2</sub>O and 2H<sub>2</sub>O. The pH was adjusted with small additions of 0.5 M NaOH or 2HCl. The  
35 peptide was also examined in SDS micelle solution; a 400 mM solution of SDS-2H<sub>2</sub>O was prepared in 1 ml 90% H<sub>2</sub>O/ 10% 2H<sub>2</sub>O

then 2.4 mg of Nef2-26 was dissolved in 0.6 ml of this solution and the pH was adjusted to 5.1. NMR spectra were recorded at 298 K; attempts to accumulate spectra at lower temperatures resulted in the crystallization of SDS from solution. Reported pH values were recorded at room temperature and were not corrected for isotope or solvent effects. The  $^1\text{H}$  chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm, via the chemical shift of residual  $\text{CH}_2\text{OH}$  at  $\text{dCH}_3$  3.35 ppm (Wüthrich, 1976) or the  $\text{H}_2\text{O}$  resonance (Wishart et al, 1995a).

Spectra were recorded on Brüker AMX-500 or AMX-600 spectrometers. Unless stated otherwise, all spectra were recorded at 281 K; probe temperatures were calibrated according to the method of van Geet (1970). All 2D spectra were recorded in phase-sensitive mode using time-proportional phase incrementation (Marion & Wüthrich, 1983). Solvent suppression was achieved by selective, low power irradiation of the water signal during the relaxation delay (typically 2s) and during the mixing time in NOESY experiments. For some spectra obtained in 90%  $\text{H}_2\text{O}$ /10%  $2\text{H}_2\text{O}$ , water suppression was also achieved by pulsed field gradients using the WATERGATE method of Sklenar et al (1993).

2D homonuclear NOESY spectra (Anil-Kumar et al, 1980; Macura et al, 1981) were recorded with mixing times of 50 and 300 ms. TOCSY spectra (Braunschweiler & Ernst, 1983) were recorded using the DIPSI-2 spin-lock sequence (Rucker & Shaka, 1989) with spin-lock times of 70-80 ms. DQF-COSY (Rance et al, 1983) and E-COSY (Griesinger et al) spectra were also recorded. Typically, spectra were acquired with 400-600  $t_1$  increments, 32-128 scans per increment, and 4096 data points.  $^1\text{H}$  sweeps widths were 6024.1 Hz at 500 MHz and 7812.5 Hz at 600 MHz. Spectra were processed using UXNMR-941001.4 (Brüker) and analysed using XEASY01.3.7 (Bartels et al, 1995). Sine-squared window functions, phase shifted by  $60^\circ$ - $90^\circ$ , were applied in

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both dimensions prior to Fourier transformation. A  $^{13}\text{C}$  HMQC spectrum (Bax et al, 1983) was acquired on Nef2-26 (1.5 mM) dissolved in  $\text{C}_2\text{H}_3\text{O}_2\text{H}$  (pH 4.8, 281 K) at 600 MHz. The  $^{13}\text{C}$  sweep width was 11318 Hz and 256 scans and 400 t1  
5 increments were employed.

The  $^3\text{J}_{\text{NHCaH}}$  coupling constants were measured from the DQF-COSY spectra at 500 MHz. The appropriate rows were extracted from the spectrum, inverse Fourier transformed, zero-filled to 32 K, and multiplied by a Gaussian window  
10 function prior to Fourier transformation. The antiphase peak shapes were simulated to take account of the effect of broad line widths on small coupling constants, using an in-house program COUPLING. Slowly-exchanging amide protons were identified by dissolving the lyophilized peptide in  
15 the appropriate deuterated solvent ( $\text{C}_2\text{H}_3\text{O}_2$  or  $2\text{H}_2\text{O}$ ) and recording a series of 1D and TOCSY spectra immediately after dissolution.

#### Structural Constraints:

20 NOESY cross-peak volumes measured from a 300-ms mixing time spectrum in  $\text{C}_2\text{H}_3\text{OH}$  were used to calculate upper bound distance restraints. Peaks from the upper side of the diagonal were used except where peaks from the lower side were better resolved. Only one pair of nondegenerate  
25 C $\beta$ H protons (Pro 14) was free of spectral overlap. As a result, volumes were calibrated using C(6)H/C(7)H cross-peaks from the Trp5 and Trp13 aromatic rings (distance 2.47 Å); distances were calculated using volumes proportional to  $r^{-6}$  in the program CALIBA (Güntert et al, 1991), which  
30 automatically corrects for degenerate and pseudo-atoms. Where cross-peak volume could not be estimated reliably an upper bound of 5 Å was assigned. Corrections of 0.5 and 1.0 Å were added to distance constraints involving only backbone protons and at least one side-chain proton,  
35 respectively, to allow for conformational averaging and errors in volume integration.

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A small number of lower bound restraints  $> 1.8$  Å (Wilcox et al, 1993; Manoleras & Norton, 1994) was also used for NH, CaH and CbH atoms in final structure calculations. These were obtained with an in-house program which checked the initial (DIANA) structures for distances of  $< 3.5$  Å that were not represented in the NOE restraint list. Distances identified in this way were compared with the experimental NOESY spectra to confirm that cross-peaks could have been observed had they been present. Where a cross-peak was clearly absent, a lower bound restraint of 3.5 Å was added to the restraint list; for all other NH, CaH and CbH atoms the lower limit was 1.79 Å.

Backbone dihedral angle constraints were inferred from  $3J_{NHCaH}$  values as follows:

$3J_{NHCaH} \approx 5\text{Hz}$ ,  $f = -60^\circ \pm 30^\circ$ ;  $5\text{ Hz} < 3J_{NHCaH} < 6\text{Hz}$ ,  $f = -60^\circ \pm 40^\circ$ ;  
 $3J_{NHCaH} \approx 8\text{Hz}$ ,  $f = -120^\circ \pm 40^\circ$  (Wüthrich, 1986).

Where  $6\text{Hz} < 3J_{NHCaH} < 8\text{Hz}$ , and the possibility of positive  $f$  angles had been excluded by the NOESY spectrum (Ludvigsen & Poulsen, 1992),  $f$  was constrained to  $-120^\circ \pm 60^\circ$ ; otherwise  $f$  angles were not constrained. Nondegenerate CbH resonances were observed for eleven residues at pH 4.5 and 281 K in  $C_2H_3OH$ . Where possible  $3J_{NHCaH}$  coupling constants were measured from passive couplings as displacements in E-COSY spectra or peaks splittings in DQF-COSY spectra. The relative intensities of intra-residue  $d_{ab}$  and  $d_{Nb}$  NOEs were measured in a 50 ms mixing time NOESY spectrum. None of the patterns fitted those expected for any of the three staggered conformations ( $\phi = -60^\circ$ ,  $60^\circ$ , or  $180^\circ$ ) (Wagner et al, 1987), as a result of which all  $\phi$  angles were left unconstrained.

#### Structure Calculations

Initial structures were generated with the distance geometry program DIANA, version 2.8 (Güntert et al, 1991), using dihedral angle constraints derived from

coupling constant data and distance constraints derived from NOE cross-peaks assigned unambiguously in both chemical shift dimensions. No hydrogen-bonding restraints were used at any stage in the calculations. Ambiguous

5 NOESY cross-peak assignments were resolved where possible using these initial structures. An assignment was accepted if in all structures the appropriate interproton distance was  $<5\text{\AA}$  and the distance between alternative pairs was  $>7\text{\AA}$ . A small number of lower bound restrains  $>1.8\text{\AA}$  was also

10 introduced prior to the final structure calculations.

Once the final set of restraints had been obtained, a new family of distance geometry structures was generated using DIANA, and the 100 structures with the lowest penalty functions were refined by simulated

15 annealing in X-PLOR, version 3.1 (Brünger, 1992). Simulated annealing was performed using 20,000 steps at 1000 K and 10,000 steps as the molecule was gradually cooled to 300 K. A time step of 1 fs was employed throughout. The 100 structures were then subject to

20 further simulated annealing in which they were gradually cooled from 300 to 0 K in 20,000 steps and then energy minimized using 100 steps of Powell conjugate gradient minimization. For each structure this procedure was carried out 10 times and the best of these 10 in terms of

25 total energy and NOE energies was selected. The structures were then energy minimized in the empirical CHARMM force field (Brooks et al, 1983), with all explicit charges neutralized (Monks et al, 1995) and with a distance-dependent dielectric instead of explicit water molecules.

30 The 20 best structures based on their stereochemical energies (ie the sum of all contributions to the calculated energy except the electrostatic term) and NOE energies were chosen for structural analysis.

Example 1: Cytotoxic activity of the amino-terminal region of HIV Nef protein on human erythrocytes and human leukocyte cell lines.

The effects of Nef2-22, Nef2-26, Nef2-22 (Val11),  
5 Nef2-26 (Ser13), the myristylated analogues and of the control peptides Nef31-50 and Myr-Nef31-50 on haemolysis of human erythrocytes and cultured CEM lymphocytes were studied as described above.

#### 10 Dose Response of Haemolysis

A dose-response study showed that significant haemolysis only occurred with the non-myristylated N-terminal peptides at concentrations of 20 mM as shown in Figure 1, whereas the myristylated peptides were active at  
15 concentrations as low as 0.5 mM. The control non N-terminal peptide Myr-Nef31-50 was virtually non-lytic over the concentration range 0.5-20 mM. The four myristylated N-terminal peptides, regardless of sequence or length gave approximately the same dose-response curve.

20

#### Kinetics of haemolysis

The time course of haemolysis for the myristylated and non-myristylated peptides is shown in Figure 2. The control myristylated peptide Myr-Nef31-50  
25 was almost inactive over the period selected (10 min.) and the four active peptides showed very similar kinetics. These kinetics were characterised by a rapid increase in haemolysis over the first four minutes.

#### 30 Microscopy of CD4+ T cells

Microscopic examination of CEM cells treated with myristylated N-terminal Nef peptide Myr-Nef2-22 showed extensive cell aggregation (Figure 3). Aggregation was evident after 10 minutes and on continued incubation over 6  
35 hours, and the cells appeared to be gradually lysed. Uptake of trypan blue and propidium iodide was observed in aggregated cells (data not shown), indicating loss of

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membrane integrity. Cells exposed to the control non-N-terminal peptide Myr-Nef31-50 showed little or no evidence of aggregation over the same time period. Similarly, treatment of cells with non-Myristylated N-terminal peptide Nef2-22 did not cause cell aggregation and neither these cells, nor cells treated with Myr-Nef31-50 showed evidence of trypan blue or propidium iodide uptake (data not shown).

#### Cytotoxicity

CEM cells exposed to 12.5 mM Myr-Nef2-26 showed near-maximal LDH release ( $96.4 \pm 3.2$  % cytotoxicity) after 30 mins (Figure 4A). Toxicity was dose-dependent, with activity evident at concentrations as low as 3 mM (Figure 4B). Little, or no LDH release was detected when cells were treated with non-myristylated Nef2-26 ( $2.7 \pm 2.7$  % cytotoxicity), or the control peptide Myr-Nef31-50 ( $9.7 \pm 1.9$  % cytotoxicity). None of the peptides contained LDH activity, or inhibited the activity of a control preparation of LDH derived from rabbit muscle.

Kinetics studies showed that LDH release from CEM cells exposed to Myristylated N-terminal Nef peptide was evident after only 10 min and increased rapidly over 30 min, before reaching a plateau between 2 and 4 hr (Figure 4C). Non-myristoyled N-terminal peptide caused only low levels of LDH release, which did not change with time. CEM cells treated with the Myristylated, non-N-terminal Nef peptide also showed increasing LDH release, but the kinetics of release were slower than those of the Myristylated N-terminal peptide and only 29% LDH release was attained after 4 hours (compared with 78% release at this time point with the Myristylated N-terminal peptide) as shown in Figure 4C.

#### Fluorescence quenching studies

Fluorescence quenching studies were undertaken to determine the localisation of the tryptophan residue at position 5 in relation to the lipid bilayer in the

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myristylated and unmyristylated peptide. Because the haemolysis results had shown that substitution of the distal position tryptophan 13 with serine did not affect haemolytic activity of the myristylated peptide, Myr-Nef2-26(Ser13) and Nef2-26(Ser13) were used in these experiments. This strategy meant that ultraviolet fluorescence derived only from the proximal tryptophan, which made interpretation of the results simpler.

The fluorescence spectra of the peptides were determined first in PBS (Figure 5). A slight blue shift and increased intensity of the emission spectrum of Myr-Nef2-26(Ser13) was observed relative to Nef2-26(Ser13). These effects suggest that myristylation had increased the hydrophobicity of the environment of the indole ring of tryptophan residue. It was noted that, when the peptides were added to the LUV, Myr-Nef2-26(Ser13) caused a noticeable reduction in the slight turbidity of the liposome suspension. This was in accord with our earlier observations (Curtain et al, 1994) that the myristylated N-terminal 21 residue peptide (NPC) corresponding to Myr-Nef2-22 caused a marked reduction in light-scattering of SUV due to the formation of smaller peptide/lipid adducts.

Stern-Vollmer plots of the emission at 340 nm were determined with all four quenching agents for Myr-Nef2-26(Ser13) and Nef2-26(Ser13) (Figures 6A & B). With peptide Nef2-26(Ser13) (Figure 6A) significant quenching was only observed with the buffer-soluble TCC, the slopes of the lines with the lipophilic quenchers being quite shallow. The plots are linear, suggesting that simple collisional quenching occurred in a single environment. On the other hand, in the case of Myr-Nef2-26(Ser13) the steepest slope was found with 5NPC and the plot curved downwards slightly (Figure 6B). The water soluble TCC gave a similar plot but with a much less steep slope. These results indicate that myristylation results in the indole ring of the proximal tryptophan being pulled into the upper regions of the lipid bilayer where it is accessible to the

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phospholipid quenchers, although it still spends a fraction of its time in the headgroup-buffer interface, as shown by the quenching with TCC.

These results show that myristylated N-terminal peptides of Nef are lytic for human erythrocytes and toxic to cultured lymphocytic cells, and that these activities are not merely a result of the presence of the myristyl chain but are related to the structure of the peptide. These findings may be significant in two ways: in relation to the membrane targeting and interactions of Nef and in relation to the possible involvement of Nef and Nef fragments in AIDS pathogenesis.

Example 2: Cytotoxic effect of the amino-terminal regions of HIV Nef protein on yeast.

Myr-Nef2-22 (10mM) and control Nef31-50 (10mM) were added to the five yeast strains as described above. All yeast species responded similarly to Myr-Nef2-22. Figure 7 shows that treatment with Myr-Nef2-22 caused the loss of colony formation of the entire cell population. Although further experiments were performed on the above strains and numerous other strains, results below are restricted to those involving the *S. cerevisiae* strain, DY150, since cell killing was observed as a general phenomenon in all yeast strains tested. Addition of 1 mM Myr-Nef31-50 peptide to cells caused no loss of colony formation (Figure 8A), showing that myristylation per se is not responsible for the peptide toxicity.

However, treatment with a 1 mM amount of Nef2-22 resulted in an 80% loss of colony formation, while the same concentration of Myr-Nef2-22 caused total loss of colony formation. To compare the Myr-Nef2-22 and Nef2-22 in more quantitative terms, dose response studies were performed. In a 30 minute interval the lowest Myr-Nef2-22 concentration for complete cell killing was about 1 mM (Figure 8B) while the Nef2-22 peptide required about three

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times that level to cause a similar effect. Concentrations of Myr-Nef2-22 down to 0.05 mM were partially effective but below this concentration there was no effect. Thus the myristylated end group either adds stability or enhances the activity of the Nef2-22 peptide.

#### Cell permeabilization by Nef peptides

The loss of colony formation was investigated to see whether permeabilization was a likely cause of cell death: yeast provides an advantage for this kind of examination because the cell wall remains intact even when the membranes are permeabilized. Following peptide treatment cells were examined for staining with propidium iodide (PI). By fluorescence microscopy, cells treated with Myr-Nef2-22 were stained with PI within 30 minutes of the peptide addition while untreated cells did not stain. PI staining is useful to distinguish unstained viable cells or cells with an intact membrane from those that are dead or have a compromised membrane (Shapiro, 1994).

Quantitation of the peptide-induced permeabilization was investigated by flow cytometry (Figure 9). Essentially all of the cells treated with Myr-Nef2-22 were stained with PI after a 30 minutes treatment. In contrast Nef2-22 caused about a third of cells to be permeabilized, while the treatment with the control peptide, Myr-Nef31-50 produced about 5% PI-staining, similar to the untreated cells. These data indicate that the Nef N-terminal peptides disrupt the plasma membrane and other cell membranes leading to cell death.

In order to determine the cellular localization of Myr-Nef2-22 the peptide was labelled with FITC. The resulting labelled peptide did not associate with cells (as judged by flow cytometry and fluorescence microscopy) and had no cell killing activity. This suggests that the Lys residue(s) are important for the killing activity. Further studies to examine the cellular localization will require alternate labelling strategies.

Nef peptides kill E. coli cells

Nef peptides were added to a suspension of E. coli cells in water to determine whether they would be capable of killing prokaryotic microbial cells. The Nef peptides killed E. coli cells in a manner comparable to yeast (Figure 10). Peptide cytotoxicity could be delayed by suspending the cells in buffer (50 mM HEPES/2% glucose) so that complete killing was not observed until 20 hours incubation at 30°C. Taken together with the yeast data, these results suggest that extracellular Nef-induced toxicity for eukaryotic and prokaryotic cells is exerted through a common effect on cell membranes.

15 Example 3:      Effect of Extracellular Nef Peptides in the Cytosensor Microphysiometer.

The Cytosensor Microphysiometer system is an extremely sensitive assay system and was used to examine the effects of extracellular Nef peptides on human CD4+ cells. This system is also used to develop assays to quantitatively assess and compare the effect of inhibitors of these Nef peptides.

The Cytosensor Microphysiometer (Molecular Devices Inc., CA) is a light addressable potentiometric sensor-based device that can be used to indirectly measure the metabolic rate of cells in vitro (Parce et al., 1989; McConnell et al., 1992). Metabolism is determined by measuring the rate of acid metabolite production from cells immobilised inside a microvolume flow chamber.

Human CEM cells were centrifuged and resuspended in low-buffered serum-free/bicarbonate-free RPMI 1640 medium (Molecular Devices; hereafter referred to as modified medium). The cells were seeded at a density of 30,000 - 63,000 cells/capsule on to the polycarbonate membrane (3µm porosity) of cell capsule cups (Molecular Devices). Cells were immobilised using an agarose entrapment medium (Molecular Devices). The seeded capsule

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cups were transferred to sensor chambers containing the silicon sensor which detects changes in pH (and thus cellular metabolism). The Cytosensor system used for this set of experiments contained four separate chambers for the measurement of acidification rates. Modified medium was pumped across the cells at a rate of 100 - 120  $\mu$ l/min. Each cell chamber was served by fluid from either of two reservoirs, which could be alternated using a software command.

To measure the acidification rate, flow of the modified media was periodically interrupted, allowing the accumulation of excreted acid metabolites (lactic acid and CO<sub>2</sub>). In this set of experiments, flow was stopped for 30 s, during which time, a least squares fit slope to the change in voltage signal over time, the acidification rate (measured as  $\mu$ V/s), was calculated. This rate data was normalised (using the 4-5 rate points prior to addition of compound) to allow direct comparison of the signals from the four chambers. Measurements of the acidification rate were made every 2 min. The chamber was held at 37°C.

Basal acidification rates were monitored (in the absence of any treatment) for at least 20 min. After this time, the peptides were exposed to the cells for periods of no less than 1 hour. In all experiments, at least one chamber was not exposed to any of the compounds, providing a negative control. The results are shown in Figure 11.

#### Response of CEM cells to Nef peptides.

Continuous exposure of the cells to 105M N-terminal myristylated peptide Myr-Nef2-26 caused a 33-75% reduction of the basal metabolic rate (n=4) after a 1 hour period (Figure 11A). After the same time period, the control cells had a 0-5% reduction in metabolic rate. When Myr-Nef2-26 was initially introduced to the cells, a transient increase (5-10%) in metabolic rate was observed (n=4). This increase in metabolic rate lasted for 6 - 10 minutes before the rapid decrease in metabolic rate

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occurred. This initial increase in metabolic rate was not observed in the control cells. After the addition of Myr-Nef2-26 was ceased and flow of untreated media was resumed, the metabolic rate of the cells did not recover and in most cases continued to decline. These results are consistent with rapid, irreversible cell toxicity (cell lysis) induced by Myr-Nef2-26 at 10-5M. The initial increase in cell metabolic rate induced by Myr-Nef2-26 may be due to a membrane activity of the peptide.

10           The observed effect of Myristylated Nef N-terminal peptide was dose-dependent and still evident at a peptide concentration of 1  $\mu$ M, as shown in Figure 11B, but not at 0.1  $\mu$ M.

15           10-5M non-myristylated peptide Nef2-26 did not have any significant effect on the basal metabolic rate of the cells after continual exposure for 1hr (n=3) (Figure 11A). However, as observed for Myr-Nef2-26, Nef2-26 did cause an initial, transient increase in metabolic rate (6-12%) when first introduced to the cells (n=3). This suggests that the peptide Nef2-26 also has membrane activity but is without any lytic activity.

20           10-5M myristylated peptide Myr-Nef31-50 did not demonstrate a consistent effect on cell metabolic rate after continual exposure for 1hr (n=3) (Figure 11A). The metabolic rate varied from -5- +5% of the basal rate throughout exposure and no initial increase in rate was consistently observed.

25           These results suggest that myristylation at the N-terminal of the peptide Nef2-26 is necessary for the lytic toxicity of peptide. These results also indicate that a region within the Nef2-26 amino acid sequence is responsible for the transitory membrane effect observed in these experiments.

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Example 4:      Solution structure of the amino-terminal  
                         region of HIV Nef protein.

                 The solution structure of a 25-residue  
polypeptide (Nef2-26) was investigated by 1H-NMR  
5    spectroscopy as described above.

                 1D and 2D 1H NMR spectra of Nef2-26 in 90%  
H<sub>2</sub>O/10%<sup>2</sup>H<sub>2</sub>O and <sup>2</sup>H<sub>2</sub>O indicated that the peptide had no  
well-defined structure in aqueous solution. This was  
evident from the limited dispersion of the backbone NH (Dd  
10    0.6 PPM) and CaH (Dd 0.9 ppm) chemical shifts and the lack  
of cross-peaks other than intra-residue and sequential in  
the NOESY spectrum. When the peptide was dissolved in <sup>2</sup>H<sub>2</sub>O  
the amide protons underwent rapid deuteration (complete  
within 10 minutes at 281 K and pH 4.8), indicating an  
15    absence of stable hydrogen bonds.

                 In aqueous solution, the peptide bond preceding  
Pro14 co-existed in both cis and trans conformations. The  
conformation of the Trp-Pro peptide bond, as determined  
from sequential NOEs between the Trp13 CaH and the Pro CdH  
20    (trans conformation) or CaH (cis conformation) resonances  
(Wüthrich, 1986), was approximately 55% trans and 45% cis,  
and the interconversion rate between the two forms was  
sufficiently slow for separate sets of peaks to be observed  
for a number of residues. An example of this is shown in  
25    Figure 12, where six Ala NH-CbH3 cross-peaks were observed  
in the 2D TOCSY spectrum even though there are only three  
Ala residues in the molecule. Peaks A and B were due to  
Ala26, and C and D to Ala23, while peaks E and F were due  
to Ala15 with Pro14 in the trans and cis conformations,  
30    respectively. Peaks A, B, C and D were not assigned to  
particular conformers as the limited dispersion in 1H  
chemical shifts resulted in highly overlapped spectra,  
preventing unambiguous assignments for the two conformers.  
Only one set of signals was observed for the residues 2-7,  
35    indicating that conformational differences associated with  
cis-trans isomerism at Pro14 had negligible effects on the  
chemical shifts of these residues. From Ser8 to Ala26 two

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sets of signals were observed for most residues and it was possible to follow the sequential assignment of the two forms from Ser8 to Glu18, although spectral overlap prevented the assignment of peaks from Arg19-Ala26 to individual conformers. For some residues the chemical shift difference between the two conformations was > 0.1 ppm. The largest difference was for Pro14 CaH which resonated at 3.30 ppm in the cis configuration, 1.12 ppm upfield of its random coil value (Wishart et al, 1995b) and 0.93 ppm upfield of the corresponding shift in the trans conformer. This large shift to higher field may have been the result of ring-current effects from the neighbouring Trp13 (Wüthrich, 1986).

Only three medium-range NOE cross-peaks were observed in the NOESY spectrum of Nef2-26 in aqueous solution, as follows: Ile11 dNN(i,i+2), Trp13 daN(i,i+2) and dNN(i,i+2). These cross-peaks were all associated with the conformer having Pro14 in the cis configuration, suggesting that this conformer may have a slightly more stable structure than the trans conformer.

Cis-trans isomerism was also observed for Pro25, but only a small proportion (<5%) was present in the cis form. The presence of the cis isomer was confirmed by observation of a weak NOESY cross-peak from Glu24 CaH to Pro25 CaH. Only the flanking residues Glu24 and Ala26 gave rise to separate NMR cross-peaks due to this conformation, indicating that the structural differences between the two forms were limited, in contrast to those due to Pro14 isomerism.

2,2,2-trifluoroethanol (TFE) has been used extensively to stabilize helical structures in aqueous solutions (Nelson & Kallenbach, 1986; Dyson et al, 1992, Sönnochsen et al, 1992; Morton et al, 1994). When TFE was titrated (up to 50%) into an aqueous solution of Nef2-26 (the pH increased from 4.8 to 5.5), there was a small increase in the dispersion of the backbone NH chemical shifts (to 0.7 ppm) and a larger increase (to 1.4 ppm) in

the CaH chemical shift dispersion. The effect of cis-trans isomerism at Pro14 became less pronounced at a TFE concentration around 30% and disappeared once the TFE concentration reached 50%. The CaH resonances not only had a greater chemical shift dispersion in 50% TFE but several also shifted to higher field (Figure 13A), consistent with formation of a helical structure. In fact, the profile of the CaH chemical shift index is very similar to that of Nef2-26 in methanol (Figure 13B), the structure of which is discussed below. These results are in broad agreement with those of Sabatier et al (1990), who found using circular dichroism spectroscopy that a peptide corresponding to residues 2-32 of Nef was partially helical in TFE but a mixture of b-sheet and random structures in aqueous solution, whereas a shorter peptide (residues 1-17) was not helical in either solvent and actually formed a b-sheet in TFE.

The polypeptides melittin (Bazzo et al, 1988; Brown & Wüthrich, 1981; Brown et al, 1982; Inagaki et al, 1989; Ikura et al, 1991) and d-haemolysin (Lee et al, 1987, Tappin et al, 1988) have very similar structures in methanol and lipid micelles, implying that methanol is an appropriate solvent for mimicking structures of peptides in membrane-like environments. When Nef2-26 was dissolved in methanol, 1D and 2D spectra were consistent with an  $\alpha$ -helical structure (the CaH CSI is shown in Figure 13B) and there was a good correlation between spectra in methanol and those in 50% H<sub>2</sub>O/TFE (ie similar chemical shifts and NOESY cross-peaks). As the overall quality of the spectra recorded in methanol was superior to those in 50% H<sub>2</sub>O/TFE (better signal-to-noise and resolution), the methanol spectra were used to calculate the structure of Nef2-26.

#### NMR Resonance Assignments in Methanol.

Spin systems of the peptide dissolved in c<sub>2</sub>H<sub>3</sub>OH were assigned using a combination of DQF-COSY and TOCSY spectra at 281 K, while sequence-specific assignments were

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made using NOESY spectra (Wüthrich, 1986). Figure 14 shows the fingerprint (Figure 14A) and dNN regions (Figure 14B) of the NOESY spectrum, and the assignments are listed in Table 1 below and shown in Figure 14.

Table 1: Proton Chemical Shifts of Nef<sub>2-26</sub> in C<sup>2</sup>H<sub>3</sub>OH at pH 4.5 and 281 K.<sup>a</sup>

| Residue | NH   | C <sup>α</sup> H | C <sup>β</sup> H | Others                                                                                                                                                     |
|---------|------|------------------|------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Gly1    | 8.60 | 3.91, 3.91       |                  |                                                                                                                                                            |
| Gly2    | 8.71 | 3.85, 3.85       |                  |                                                                                                                                                            |
| Lys3    | 8.51 | 4.05             | 1.61, 1.61       | C <sup>γ</sup> H <sub>2</sub> 1.12; C <sup>δ</sup> H <sub>2</sub> 1.47; C <sup>ε</sup> H <sub>2</sub> 2.69 N <sup>ε</sup> H <sub>3</sub> <sup>+</sup> 7.78 |
| Trp4    | 8.03 | 4.62             | 3.41, 3.25       | N(1)H 10.45; C(2)H 7.21; C(4)H 7.60;<br>C(5)H 7.01; C(6)H 7.09, C(7)H 7.33                                                                                 |
| Ser5    | 7.99 | 4.31             | 3.97, 3.87       |                                                                                                                                                            |
| Lys6    | 8.35 | 4.20             | 1.85, 1.85       | C <sup>γ</sup> H <sub>2</sub> 1.47; C <sup>δ</sup> H <sub>2</sub> 1.65; C <sup>ε</sup> H <sub>2</sub> 2.90 N <sup>ε</sup> H <sub>3</sub> <sup>+</sup> 7.82 |
| Ser7    | 8.11 | 4.28             | 3.92, 3.85       |                                                                                                                                                            |
| Ser8    | 8.11 | 4.26             | 3.91, 3.83       |                                                                                                                                                            |
| Val9    | 7.87 | 3.94             | 2.17             | C <sup>γ</sup> H <sub>3</sub> 1.00, 0.94                                                                                                                   |
| Ile10   | 7.76 | 4.16             | 1.95             | C <sup>γ</sup> H <sub>2</sub> 1.56, 1.27; C <sup>γ</sup> H <sub>3</sub> 0.94; C <sup>δ</sup> H <sub>3</sub> 0.88                                           |
| Gly11   | 8.23 | 4.01, 3.93       |                  |                                                                                                                                                            |
| Trp12   | 8.33 | 4.70             | 3.49, 3.42       | N(1)H 10.49; C(2)H 7.32; C(4)H 7.60;<br>C(5)H 7.01; C(6)H 7.09, C(7)H 7.33                                                                                 |
| Pro13   | -    | 4.06             | 2.22, 1.80       | C <sup>γ</sup> H <sub>2</sub> 2.04, 1.52; C <sup>δ</sup> H <sub>2</sub> 3.70, 3.64                                                                         |
| Ala14   | 7.59 | 4.08             | 1.48             |                                                                                                                                                            |
| Val15   | 7.78 | 3.52             | 2.31             | C <sup>γ</sup> H <sub>3</sub> 1.03, 0.97                                                                                                                   |
| Arg16   | 8.35 | 3.91             | 1.84, 1.75       | C <sup>γ</sup> H <sub>2</sub> 1.54; C <sup>δ</sup> H <sub>2</sub> 3.17; N <sup>ε</sup> H 7.61                                                              |
| Glu17   | 8.27 | 4.22             | 2.18, 2.04       | C <sup>γ</sup> H <sub>2</sub> 2.45, 2.33                                                                                                                   |
| Arg18   | 8.10 | 3.96             | 2.02, 1.95       | C <sup>γ</sup> H <sub>2</sub> 1.65; C <sup>δ</sup> H <sub>2</sub> 3.18; N <sup>ε</sup> H 7.54                                                              |
| Met19   | 8.26 | 4.14             | 2.23, 2.12       | C <sup>γ</sup> H <sub>2</sub> 2.79, 2.53; C <sup>ε</sup> H <sub>3</sub> 2.14                                                                               |
| Arg20   | 8.22 | 4.01             | 2.00, 1.90       | C <sup>γ</sup> H <sub>2</sub> 1.71; C <sup>δ</sup> H <sub>2</sub> 3.11; N <sup>ε</sup> H 7.79                                                              |
| Arg21   | 7.79 | 4.08             | 1.90, 1.86       | C <sup>γ</sup> H <sub>2</sub> 1.62; C <sup>δ</sup> H <sub>2</sub> 3.14; N <sup>ε</sup> H 7.95                                                              |
| Ala22   | 7.82 | 4.25             | 1.48             |                                                                                                                                                            |
| Glu23   | 7.84 | 4.72             | 2.10, 2.00       | C <sup>γ</sup> H <sub>2</sub> 2.49, 2.43                                                                                                                   |
| Pro24   | -    | 4.46             | 2.30, 1.99       | C <sup>γ</sup> H <sub>2</sub> 2.17, 1.87; C <sup>δ</sup> H <sub>2</sub> 3.70, 3.66                                                                         |
| Ala25   | 8.27 | 4.31             | 1.39             |                                                                                                                                                            |

<sup>a</sup>All shifts are relative to DSS at 0 ppm, via δ<sub>CH<sub>3</sub></sub> of C<sup>2</sup>H<sub>3</sub>OH at 3.35 ppm, (Wüthrich, 1976).

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The deviations of CaH and backbone NH from random coil values (Wishart et al, 1995b) are shown in Figure 13. The upfield shifts of the alpha protons were compatible with the formation of a helical structure and the gradual upfield shift of the NH resonances when going from the N- to C-terminus was consistent with the shielding effect of a helical macrodipole (Wishart et al, 1991). The variation in the NH chemical shift from random coil values (Figure 13C) for residues 7-24 was consistent with a 3-4 repeat pattern observed for  $\alpha$ -helices (Zhou et al, 1992) and the Ca chemical shifts shown in Figure 3 D were consistent with a helical conformation for residues 13-21. Increasing the temperature from 281 K to 298 K had no significant effect on the spectra (the chemical shift range for the CaH resonances of residues - decreased only slightly, from 1.20 ppm at 281 K to 1.17 ppm at 298K), implying that the helical structure was retained over this temperature range.

#### Amide Exchange

When Nef2-26 was dissolved in C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>H at pH 4.5 and 281 K, the amides of residues near the N- and C-termini exchanged more rapidly, with the NH signals of Gly3, Lys4, Lys7, Ser8, Ser9, Ala26 and the C-terminal amide disappearing within 3 hours and those Gly2, Trp5 and Ser6, disappearing shortly thereafter. The other amides were still present after 24 hours, and those from Ile11, Val16, Arg17, Glu18, Arg19, Met20 and Arg21 were observable in a TOCSY spectrum after 48 hours (Figure 15). These slowly exchanging amide protons were from the centre of the helix, while the amides with intermediate exchange rates (Val10, Arg22, Ala23, and Glu24) were from the ends of the helix. The amides of Gly12, Trp13 and Ala15, although not at the ends of the helix, were not among the most slowly exchanging group, presumably because of their proximity to Pro14, which is the site of a kink in the helix (see below).

Structure Determination

Figure 15 summarises the sequential and medium-range NOE connectivities,  $^3J_{NH\alpha H}$  coupling constants, and slowly exchanging amide protons observed in methanol. The presence of helical structure encompassing residues 7 to 23 is indicated by the low  $^3J_{NH\alpha H}$  coupling constants ( $< 6$  Hz), numerous  $d_{\alpha N}(i, i+3)$ ,  $d_{\alpha N}(i, i+4)$  and  $d_{\alpha \beta}(i, i+3)$  NOE connectivities and the presence of slowly exchanging backbone amide protons in this region of the molecule. The low coupling constants indicate that under these conditions the peptide is not undergoing significant conformational averaging.

Structures were calculated using 392 upper bound distance constraints inferred from NOEs, made up of 239 intra-residue, 81 sequential and 73 medium-range ( $1 < |i-j| < 5$ ) NOEs (Figure 16A); only one long-range NOE was observed, between C(4)H of Trp13 and NH of Glu18. Six lower bound constraints based on the absence of NOEs from the NOESY spectrum were also used. Fourteen backbone dihedral angle constraints based on spin-spin coupling constants were used, but no side chain restraints were employed. Initial structures were calculated using DIANA, then refined by simulated annealing in X-PLOR and finally energy minimized in X-PLOR with the CHARMM force field. A summary of geometric and energetic parameters for these structures is given in Table 2 below.

Table 2: Structural Statistics for the 20 Energy-Minimized Structures of Nef<sub>2.26</sub> from X-PLOR.<sup>a</sup>

|                                                                              |                 |
|------------------------------------------------------------------------------|-----------------|
| RMS deviations from experimental distance restraints Å (394) <sup>o</sup>    | 0.0278 ± 0.0009 |
| RMS deviations from experimental dihedral restraints (deg) (14) <sup>b</sup> | 0.283 ± 0.162   |
| RMS deviations from idealised geometry                                       |                 |
| bonds (Å)                                                                    | 0.0115 ± 0.0014 |
| angles (deg)                                                                 | 2.615 ± 0.046   |
| impropers (deg)                                                              | 0.343 ± 0.022   |
| energies (kcal mol <sup>-1</sup> )                                           |                 |
| E <sub>NOE</sub>                                                             | 15.36 ± 0.97    |
| E <sub>cdih</sub>                                                            | 0.08 ± 0.07     |
| E <sub>L-J</sub>                                                             | -64.1 ± 4.8     |
| E <sub>bond</sub> + E <sub>angle</sub> + E <sub>improper</sub>               | 79.1 ± 2.8      |
| E <sub>elec</sub>                                                            | -310.7 ± 13.9   |

<sup>a</sup> The best 20 structures after energy minimization in the distance geometry force-field were subsequently energy-minimized in the CHARMM force-field, using a distance-dependent dielectric and neutralized side chains, as described under Materials and Methods.

<sup>b</sup> The numbers of restraints are shown in parentheses. None of the structures had distance violations > 0.5 Å or dihedral angle violations > 5°.

Structural Analysis and Description.

Analysis of the angular order parameters ( $S$ ) (Hyberts et al, 1992; Pallaghy et al, 1993) of the final 20 structures indicates that residues 10-23 are well defined, with  $S > 0.9$  for both  $f$  and  $y$  angles as shown in Figures 16B and C. The RMSD from the mean structure is plotted as a function of residue number in Figure 16D, which shows that the structure is well defined over the central part of the molecule. For this well-defined region the mean pairwise RMSD is  $0.40 \pm 0.11$  Å for the backbone heavy atoms C, Ca and N, and  $1.43 \pm 0.20$  Å for all heavy atoms. Corresponding values for the whole molecule are  $3.11 \pm 0.80$  and  $3.96 \pm 0.84$  Å, respectively. Although no  $c$  angles were restrained, several of the residues have well-defined  $c1$  angles and some also have well-defined  $c2$  angles (Figures 16E, F). These residues with well-defined  $c1$  and  $c2$  angles are located in the helical region of the molecule. Although several of the  $c$  angles are well defined, stereospecific assignments were not made because the CaH-CbH cross-peaks due to Arg17, 19, 21 and 22 were overlapped in the E-COSY spectrum, while for the cross-peaks that could be resolved the coupling-constants and intensities from the 50 ms NOESY spectrum did not fit the expected patterns (Wagner et al, 1987). Trp13 has a well-defined  $c1$  angle, but in this case the conformation about the ca-Cb bond ( $c1 = -d126^\circ$ ) is eclipsed.

The overall conformation of Nef2-26 in methanol is shown in Figure 17A, where the backbone heavy atoms of the 20 best structures (those with the lowest overall energies, excluding the electrostatic term) have been superimposed over residues 10-23 (where the molecule is  $\alpha$ -helical). An orthogonal view of the structures, which also shows the well-defined side chains, is presented in Figure 17B.

35

Structure in Detergent Micelles.

The structure of Nef2-26 in methanol has been described in detail because the good quality of the spectra in this solvent permitted an extensive set of NMR  
5 restraints to be obtained. We have also investigated the structure in micelles of the detergent SDS. The cis-trans isomerism about the Trp13-Pro14 amide bond, which was evident in aqueous solution, was not present in SDS micelles. It is assumed that the trans conformer is now  
10 dominant, but this could not be confirmed from NOEs to the CaH resonance of Trp13 as the peak was coincident with the water resonance.

The deviation of CaH chemical shifts from random coil (Wishart et al, 1995b) (Figure 13E) indicated that  
15 residue 13-23 were predominantly  $\alpha$ -helical, and several dNN (i,i+2) and daN (i,i+3) NOESY cross-peak associated with these residues supported this. While there was no evidence that the first ten residues were also helical, the observation of several medium-range NOEs, including dNN  
20 (i,i+2) for residues 7/9 and 8/10, daN (i,i+2) for residues 2/4 and 5/7, and daN(i,i+4) for residues 1/5, suggest that the N-terminal end of Nef2-26 may be more structured in SDS micelles than in aqueous solution.

25 Altered N- and C- Termini.

The effects of replacing the N-acetyl group of Nef2-26 with a myristyl group were examined in C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>H at 281 K and pH 4.5. Only small changes were observed in the chemical shifts of the three N-terminal residues compared  
30 with those of Nef2-26, the largest difference in NH and CaH chemical shifts being observed for Gly2 (dNH 8.48, representing a shift of 0.12 ppm to higher field, and dCaH 3.92, a shift of 0.01 ppm to lower field). Otherwise the spectra were essentially identical. The same medium-range  
35 NOESY cross-peak were also observed. It appeared, therefore, that the myristyl group had no effect on the  $\alpha$ -helical structure in methanol.

- 47 -

When the N-terminal acetyl group was replaced by NH<sub>3</sub><sup>+</sup> and the C-terminal amide by a carboxylate, several changes were observed in the spectra, mostly from protons near the N-terminus. The NH to CaH cross-peaks for residues 2-8 were quite weak compared with those for other residues, and the NH signals were quite broad (<sup>3</sup> 18 Hz) in the 1D spectrum, suggesting that some intermediate exchange process was occurring. Consistent with this, the linewidths of resolved amide resonances from this region decreased as the temperature was raised to 298 K. The amide chemical shifts of these residues were also shifted to lower field, by 0.03 (Ser6) up to 0.14 ppm (Lys4), presumably due to the effect of the positive charge at the N-terminus. The effect on the CaH shifts was smaller (0.03-0.05 ppm) and not consistently in one direction. The effect at the C-terminus was less dramatic, with only the NH peak of Ala26 shifting significantly, to lower field by 0.18 ppm. Other residues were largely unaffected by the changes at the N- and C-termini. The NOESY spectrum showed that the daN (i,i+3), daN (i,i+4), and dab (i,i+3) NOEs characteristic of the  $\alpha$ -helix were preserved (compared with the NOESY spectrum of Nef2-26 in methanol), suggesting that the  $\alpha$ -helical region of the structure in methanol was essentially unaffected by changes at the N- and C-termini.

Spectra of Nef2-26 with free N- and C-termini in water at pH 4.5 and 281 K were very similar to those of Nef2-26. When the N-acetyl group was replaced by myristylate, spectra recorded in water at pH 4.5 showed broad lines (for example the width at half-height of the indole NH of Trp5 was 5 Hz for Nef2-26 but 15 Hz for Myr-Nef2-26), and the 2D NOESY and TOCSY spectra showed very few clear cross-peaks. Thus, it appeared that myristylated Nef2-26 was aggregating in aqueous solution.

35

Example 5:     The design of peptides which will have potential coiled-coil interactions with the N-terminal peptides of Nef.

The hydrophobic face of the helical region Nef2-26 consists of residues Trp13, Val15, Met20 and Ala23. This allows a peptide to form potential coiled-coil interactions over two turns of the helix, with Trp13 and Val15 in the a and d positions of the first turn, Val10 and Gly12 occupy the interface border positions e and g. For the second turn Met20 and Ala23 are in the a and d positions while the e and g positions are occupied by Arg17 and Arg19. A coiled-coil with the peptides in an antiparallel arrangement would give a favourable alignment of the helical dipoles. A suitable coiled-coil interaction would be similar to that shown below, with amino acid residues denoted by standard single letter codes.

|                |    |
|----------------|----|
| LXAAXXAEXELXXA |    |
| 1              | 14 |

The peptide AXXLEXEAXXAAXL represents a starting position from which to design the peptide with the most efficient coiled-coil interactions. The residues labelled X in this peptide, could be occupied by any helix-enhancing residues such as alanine, although for solubility and spectroscopic reasons, some of these residues should be more hydrophilic in nature, such as Gln, Asn, and Ser at position f. The ability to form Glu to Lys lactam bridges i to i+4 residues apart could also be used in these positions to increase the helical propensity of the peptide (Houston et al., 1996).

Variations of this peptide include replacing some or all alanine residues (1, 11, 12) with other hydrophobic residues such as valine, leucine or isoleucine, to maximise hydrophobic interactions. The residue at position 8 will interact with Trp13 on Nef2-26 and it is possible that an aromatic residue such as Phe or Tyr may give a stronger

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interaction, although steric restrictions may rule this possibility out. Other variations include extending the peptide to allow it to interact with Lys4 and 7 of Nef2-26. This could be achieved by adding one or more Glu residues to the C-terminus of the peptide, a variable length spacer, made of Gly or Ser residues may be required to achieve the best interaction.

Example 6: Inhibitors that interact with the N-terminal region of the Nef protein

Markush Structure embodying compounds made:

Lip-Ar-Pol-AA

15

"Lip" is a lipophilic moiety necessary for complexing with the hydrophobic regions of the Nef terminus. This is exemplified by a number of different structures. These include cholic acid, the hydrophobic tripeptide Ile-Val-Ile, peptoids and polyglycols.

20

"Ar" is a link which contains an aromatic ring and is a point at which conformational constraint may be used to effect. Encompassed moieties range from phenylalanine derivatives to heterocyclic compounds.

25

"Pol" is a link with a polar sidechain, typically aspartic acid or asparagine.

"AA" is typically a neutral amino acid residue with a small sidechain, such as 2-aminobutyric acid, cysteine, proline or 3-chloroalanine. However, attachment of bulky groups, such as 4-nitrobenzyl was compatible with good inhibitory potency. Also, inspection of inhibitor models docked onto the Nef structure indicate the possibility of extending the sidechain such as in Lysine to interact with a glutamate residue on the Nef structure.

35

Design Methodology:

Effort was put into improving the solubility of initial lead inhibitors, some of which were completely intractable solids. A number of modifications were found to improve solubility:

- Modifications of "Lip" (lead structure based on IVI)
- Attachment of an ether or polyglycol chain to the N-terminal isoleucine residue
  - 10 -Removal of the N-terminal acetamide
  - Replacement of the N-terminal isoleucine residue with an N-alkylated amino acid
  - Replacement of the N-terminal tripeptide sequence IVI with a hydrophobic but more soluble moiety such as cholic acid
  - 15 or a polyglycol or a tripeptoid (N-alkylation).

- Modifications of "Ar" (lead structure based on Phenylalanine)
- Use of an indoline or isoquinoline instead of phenylalanine.
  - 20

- Modifications of "Pol-AA" (lead structure based on Asn-Cys-NH<sub>2</sub>)
- Primary amides frequently lead to insoluble compounds, and it was found that Asp-Cys, the diacid equivalent of Asn-Cys-NH<sub>2</sub>, resulted in water solubility.
  - 25
  - Cysteine is undesirable in therapeutics. 2-Aminobutyric acid (Abu) can be isosteric with cysteine replacement of cysteine with Abu was found to also improve solubility.

30

Examples

- Examples of suitable inhibitors include combinations of:-
- a) Lipophilic moieties are steroids; ethylene oxide fatty acids; multi-fatty acid chains eg phosphatidylcholine esters; branched fatty acids; branched polymers with acid terminal group.
  - 35

- 51 -

b) Aromatic moieties: Different aromatic groups may be used to increase the interaction with Trp groups eg naphthalene or indane groups. There are also a number of Phe replacements that fix the position and orientation of the aromatic ring (eg (Z)-dehydrophenylalanine, 2-aminoindan-2-carboxylate, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylate, and indoline-2-carboxylate) and which may be used to locate increased binding. It is also possible to include cyclodextrins, which have a strong affinity for inclusion of aromatic residues.

c) Anionic/Zwitterionic Moieties: Anionic-carboxylate, phosphate, phosphonate, sulfonate. Zwitterionic groups may also be used to reduce lytic effects of inhibitors and also for possible additional ionic interactions with the protein. Long chain di-acid-inhibitors may also be used, as such compounds have little detergent activity and have the possibility of reducing lytic effects.

Multi-presentation of inhibitors or peptide sequences may also be utilised.

d) Complementary, negatively charged amphiphatic helical peptides or coil/coil inhibitors.

Small synthetic cationic helical peptides made up of repeating hexamer units as anti-microbial agents may be used, in particular, negatively charged amphiphathic helical peptides as Nef inhibitors.

e) Anti-sense Peptide Nucleic Acid (PNA) Inhibitors: Anti-sense PNAs directed to essential regions of the Nef gene are synthesised and the anti-HIV effects of such compounds determined.

#### Best Inhibitors.

Certain combinations of the above modifications led to superior inhibitory activity over other combinations. Thus Abu as "AA" was effective when used in conjunction with IVI as "Lip", but not when "Lip" was cholic acid.

Thus the most potent inhibitors with best solubility may be exemplified as the following:

R1-Ile-Val-Ile-R2-Asp-R3

Cholyl-R2-R4-R5

5 where R1 may be an acetamide cap or a solubilizing group as stated above, R2 is Phe or its 2-naphthyl counterpart, R3 is Cys (free acid) or Abu (free acid), R4 is Asp or Asn, R5 is Cys or (S-4-nitrobenzyl)Cys and may be terminally amidated or not.

10

Example 7: Synthesis and Characterisation of Putative Inhibitors of the Cytotoxic Activity of the Amino-Terminus of HIV-1 Nef and Screening of these Compounds in a Rapid In Vitro Assay.

15 A number of compounds were synthesised and tested for their ability to block Nef peptide-induced cell membrane damage, using a short-term assay based on the release of the fluorescent probe, calcein, from CD4+ T-cells.

20 General synthetic methodology

All compounds designed as Nef inhibitors were assembled by peptide or amide bond formation using conventional peptide chemistry coupling techniques. This involved activation of a carboxylic acid group, through  
25 generation of an acid chloride, an anhydride or an active ester, with subsequent reaction with an amine. Constituent units were natural and unnatural amino acids, N-alkylated amino acids ("peptoids") and lipophilic carboxylic acids. Some units were synthesised because they were not  
30 commercially available. Where required, amino acids were fully protected. In all cases, incremental assembly was diagnostically monitored by <sup>1</sup>H NMR. As a final step, full deprotection yielded the target molecules. The HBTU coupling method was predominantly used with solution-phase  
35 t-BOC chemistry, but any peptide coupling method and strategy may be used. Carboxylic acids were protected as

esters and removed by saponification, acidolysis or hydrogenolysis, but any other sensible protecting groups could also be used. Dissolution of carboxylic esters prior to hydrogenolysis required solvents such as

5 hexafluoropropanol, trifluoroethanol, trifluoroacetic acid or DMF and were often unprecedentedly slow, requiring many days reaction for completion. This was a function of the substrate and not the solvent. The thiol of cysteine was protected as a nitrobenzyl or disulfide (removal by

10 reduction), an acetamidomethyl (removal by oxidation), or a trityl (removal by acidolysis), but any other suitable protecting groups could also be used. Amines were protected as t-butyloxycarbamates (BOC; removal by acidolysis or heat), fluorenylmethoxycarbonyl (Fmoc;

15 removal by secondary amines) or benzyloxycarbamates (CBZ; removal by acidolysis or hydrogenolysis), but any other suitable protecting groups may be used.

#### General HBTU coupling method

The carboxylic acid or N-protected amino acid and

20 O-benzotriazole-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU; 1 equivalent) were dissolved in dry DMF with stirring and treated with a tertiary base such as diisopropylethylamine (DIPEA; 2 equivalents) followed by the amine (1 equivalent). After 90 minutes, the reaction

25 mixture was dumped into water. If the precipitated product was solid, this was filtered off and sucked to dryness, with final trituration with ether. If the precipitated product was a gum, this was taken up in ethyl acetate and the organic layer was subjected to the standard washing

30 protocol, which will be outlined once as follows:

The organic later was washed twice with a saturated aqueous solution of sodium hydrogen carbonate and then twice washed with aqueous citric acid (10%w/v), then shaken with a saturated aqueous solution of sodium

35 chloride, then dried and evaporated under vacuum to furnish the product.

Chromatographic purification was performed if necessary.

Exemplary syntheses

Ac-IVIFD-Abu

5 (a) TFA.FD(Bn)Abu(Bn)

BOC-Asp( $\beta$ -Bn) (10.3 g, 32 mmol) and HBTU (12.1 g, 32 mmol) were dissolved in dry DMF (120 ml) and treated with DIPEA (5.57 ml; 64 mmol) followed by 2-aminobutyric acid benzyl ester (Abu(Bn); 32 mmol). After stirring for  
10 90 min, the reaction mixture was worked up according to the above standard protocol to give the product, BOC-D(Bn)Abu(Bn) (12.8 g, 95% yield), as a pale yellow oil. This was dissolved in 1:1 TFA:DCM, stirred for 30 mins, and concentrated rigorously under vacuum to give the  
15 trifluoroacetate salt, TFA.D(Bn)Abu(Bn) as a gum. After neutralization with DIPEA, this dipeptide was added to an HBTU-activated (as above) solution of BOC-Phe (32 mmol) in DMF (120ml) and treated in the usual way to furnish BOC-FD(Bn)Abu(Bn) (91 % overall yield) as a gum which  
20 solidified on standing. This was treated with TFA as above to give the title compound as an oil.

(b) Ac-IVI

Starting from BOC-Val and Ile(Bn) and subsequently BOC-Ile, BOC-IVI(Bn) was made by the above  
25 HBTU coupling/TFA deprotection protocol. At this point, AcIVI could be readily made by two methods. In one method, the BOC group was removed and the resulting TFA.IVI(Bn) was acylated with excess acetyl chloride or acetic anhydride in DMF in the presence of excess triethylamine to give Ac-  
30 IVI(Bn) as a solid after precipitation with water, filtering, and drying. Hydrogenolysis with hydrogen gas and 10%Pd/C in trifluoroethanol for 6 hours, followed by filtering, evaporating, dissolving in aqueous ethanol and triethylamine, gave the triethylamine salt of Ac-IVI as a  
35 colourless solid after concentrating to dryness and

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tritulating with ether. In the second method, BOC-IVI(Bn) was debenzylated by catalytic hydrogenation transfer using ammonium formate in methanol to give, after concentrating and precipitating with water, BOC-IVI as a gum which  
5 hardened to a solid on standing. After removal of the BOC group, the resulting TFA.IVI could be acylated and treated by the above protocol to give Ac-IVI as the triethylamine salt.

(c) AcIVIFDAbu

10 AcIVI-.Et<sub>3</sub>NH<sup>+</sup> was coupled to TFA.FD(Bn)Abu(Bn) as per the usual HBTU protocol to give AcIVIFD(Bn)Abu(Bn) as a colourless solid. This was subjected to hydrogenolysis in hexafluoropropanol for five days to give the title compound as a colourless solid.

15

MPEG(5000)Ile-Val-Ile-Phe-Asn-Abu-NH<sub>2</sub> (BRI6211)

A solution of succinimidyl carboxymethyl polyethyleneglycol monomethyl ether (MW 5000) [MPEG(5000)SCM] (17.5mg) in dry DMF (2ml) was added to a  
20 solution of Ile-Val-Ile-Phe-Asn-Abu-NH<sub>2</sub> (2.3mg) in DMF (2ml). The solution was stirred for 30 minutes, then triethylamine (2 drops) added and the mixture stirred overnight at room temperature. Diethyl ether (30ml) was then added and the precipitated white solid collected by  
25 filtration, washed with diethyl ether and dried to give a white powder (15mg). <sup>1</sup>H nmr (D<sub>2</sub>O) δ: 0.7-1.0, br m, 21H; 1.1, vbr s; 1.42, vbr s; 1.77, vbr s; 2.67, vbr s; 3.02, vbr s; 3.2-4.2, m, ca.500H; 7.1-7.4, 5ArH.

30 Glycol conjugates:

n-BuO(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>C(=O)-FDBu

In order to incorporate an amine-reactive group, the hydroxyl group of monoether-protected polyglycols was conventionally functionalized to a carboxylic acid or  
35 activated carbonate. For the former, the hydroxyl proton

was removed with strong base and alkylated with an alkyl bromoacetate followed by saponification to give the oxyacetate. For the latter, the hydroxyl group was reacted with succinimidyl carbonate to give the active carbonate.

5           Thus, hexa(1,2-butylene glycol) monobutyl ether in dry acetonitrile over molecular sieves was treated with disuccinimidyl carbonate (1.5 equivalents) and triethylamine (1.5 equivalents) and stirred overnight under dry nitrogen. The solvent was evaporated under vacuum and  
10 the reaction mixture partitioned between dichloromethane and a saturated aqueous solution of sodium hydrogen carbonate. The organic layer was again washed with a saturated aqueous solution of sodium hydrogen carbonate and then twice washed with aqueous citric acid (10%w/v), then  
15 shaken with a saturated aqueous solution of sodium chloride, then dried and evaporated under vacuum to give the active carbonate as a treacle-like material (95% yield). This was treated with an equivalent of TFA.FD(Bn)Abu(Bn) and triethylamine in DMF and the product  
20 subjected to purification on a silica gel column with dichloromethane as eluent to give n-BuO(CH<sub>2</sub>CH<sub>2</sub>O)<sub>6</sub>C(=O)-FD(Bn)Abu(Bn) (40% yield) as a gummy solid. Standard hydrogenolysis yielded the title compound.

25 Cholic acid/peptide conjugates:

CHOLYL-FDC via CHOLYL-FD(Bn)C(OEt) disulfide dimer

Cysteine ethyl ester hydrochloride (20g) in water (50ml) was treated with 1.1eq. H<sub>2</sub>O<sub>2</sub> (13ml of 30% aq. solution) slowly and external cooling was applied during a  
- 30 30 min period. The reaction mixture was stirred for another hour, basified (HCO<sub>3</sub><sup>-</sup>) and extracted with ethyl acetate (4 X 100ml). The organic layer was dried and evaporated and the residue dissolved in dioxan (200 ml) and acidified with conc. HCl (10 ml). After cooling, the product, cystine  
35 diethyl ester, was filtered off as colourless crystals in the form of the dihydrochloride salt. BOC-D(Bn) and BOC-F

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were then sequentially used in the standard HBTU coupling/TFA deprotection procedure to finally give TFA.FD(Bn)C(OEt) as the disulfide dimer which could be recrystallized from acetonitrile. HBTU coupling with  
5 cholic acid gave the title compound. BRI6209 was obtained after ester saponification and disulfide cleavage with 2-mercaptoethanol. A useful system for column chromatography with silica gel used n-butanol:28%aq. NH<sub>3</sub>: water: ethanol in a ratio of 28:8:4:3 and product visualisation was aided  
10 by vanillin spray.

CHOLYL-FNC(S-4-Nitrobenzyl)-NH<sub>2</sub> and CHOLYL-FDC(S-4NBn)

(a) CholyPhe- $\beta$ -Benzyl-Asp-S-4-Nitrobenzyl-Cys Ethyl Ester (BRI6197))

To cysteine ethyl ester hydrochloride (18.6 g,  
15 100 mmol) and triethylamine (200 mmol) in chloroform (200 ml) was added p-nitrobenzyl chloride (17.1 g, 100 mol) and the reaction mixture was stirred overnight. The reaction mixture was neutralized with 10M HCl and washed thrice with sat. aq. NaCl, dried and concentrated. The product  
20 crystallized from the concentrate as needles in 83% yield. The succinimidyl active ester of BOC-D( $\beta$ -Bn), (i.e. BOC-D( $\alpha$ -Succ) (4.20g, 10 mmol), was treated with Cys(S-4-Nbn)OEt.HCl (3.21g, 10 mmol) and triethylamine (20 mmol) in DCM (50 ml). After 2 h, the reaction mixture was subjected  
25 to the standard washing protocol. The resulting dipeptide was de-BOCed and coupled with BOC-F( $\alpha$ -Succ) and deBOCed by the same procedure to yield the tripeptide TFA.FD(Bn)C(NBn)(OEt) as the TFA salt.

A solution of diisopropylethylamine (82mg;  
30 0.63mmol) in dry DMF (0.5ml) was added to a solution of cholic acid (130mg; 0.32mmol) and HBTU (120mg; 0.32mmol) in dry DMF (2ml). The solution was stirred for 5 minutes at room temperature when a solution of Phe- $\beta$ -Benzyl-Asp-S-4-Nitrobenzyl-Cys Ethyl Ester Hydrochloride (211mg; 0.31mmol)  
35 and diisopropylethylamine (41mg; 0.32mmol) in dry DMF (1ml) added. The mixture was stirred for 4 hours, then diluted

with water (50ml) and stirred vigorously for an additional 30 minutes. The white solid that separated was collected by filtration, washed with water and dried to give a white powder (253mg; 79%). <sup>1</sup>H nmr (CDCl<sub>3</sub>) δ: 0.63, s, 3H; 1.23, t, 3H; 2.5-3.2, 12H; 3.42, br m, 1H; 3.75, s, 2H; 3.82, br s, 1H; 3.92, br s, 1H; 4.17, q, 2H; 6.55, br d, 1H; 7.1-7.4, m, 10ArH + 2NH; 7.49, d, 2H; 8.14, d, 2H. The product was used without further purification, but was spectroscopically identical to material purified by reverse phase HPLC (C18; 55% CH<sub>3</sub>CN/H<sub>2</sub>O).

(b) Preparation of CholylPhe-Asn-S-4-Nitrobenzyl-Cys-NH<sub>2</sub> (BRI6199)

CholylPhe-β-Benzyl-Asp-S-4-Nitrobenzyl-Cys Ethyl Ester (205mg) was dissolved in a solution of dry methanol saturated with ammonia gas (6ml) and the yellowish solution left to stand at room temperature overnight. The solution was then concentrated to give a gel-like material. The crude product was then dissolved in methanol with gentle heating and then diethyl ether added to precipitate a white powder, which was collected by filtration and dried (145mg). <sup>1</sup>H nmr (D<sub>6</sub>-DMSO) δ: 0.59, s, 3H; 0.8-2.3, 30H; 2.5-3.5; 3.64, br s, 1H; 3.79, br s, 1H; 3.91, s, 2H; 4.04, 1H; 4.12, 1H; 4.3-4.6, 3H; 7.15-7.4, 5ArH; 7.64, d, 2H; 8.21, d, 2H.

(c) Preparation of CholylPhe-Asp-S-4-Nitrobenzyl-Cys-OH (BRI6198)

1M aqueous sodium hydroxide solution (0.04ml; 0.04mmol) was added to a solution of CholylPhe-β-Benzyl-Asp-S-4-Nitrobenzyl-Cys Ethyl Ester (20mg; 0.02mmol) in methanol (2ml) and the solution left to stand at room temperature for 4 hours and then concentrated. <sup>1</sup>H nmr (D<sub>2</sub>O) showed the disappearance of the ethyl and benzyl ester groups of the starting material.

CHOLYL-FDP and CHOLYL-FNP-NH<sub>2</sub>

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(a) Choly1Phe- $\beta$ -Benzyl-Asp-Pro Benzyl Ester

A solution of diisopropylethylamine (26mg; 0.2mmol) in dry DMF (1ml) was added to a solution of Choly1Phe- $\beta$ -Benzyl-Asp-OH (76mg; 0.1mmol) and HBTU (38mg; 0.1mmol) in DMF (1ml). The solution was stirred for 5 minutes and then a solution of Pro Benzyl Ester Hydrochloride (24mg; 0.1mmol) and diisopropylethylamine (13mg; 0.1mmol) in DMF (1ml) added. The mixture was stirred at room temperature for 3 hours, diluted with water and the milky mixture extracted with ethyl acetate. The ethyl acetate extract was washed with 10% sodium chloride solution (2X), water (3X), and finally 10% sodium chloride solution before being dried (MgSO<sub>4</sub>) and concentrated to give a waxy solid (92mg). <sup>1</sup>H nmr (CDCl<sub>3</sub>)  $\delta$ : 0.66, s, 3H; 0.8-2.4, 37H; 3.01, 2H; 3.44, vbr s, 1H; 3.63, vbr s, 1H; 3.84, br s, 1H; 3.95, br s, 1H; 4.47, br s, 1H; 4.71, br s, 1H; 4.95-5.30, m, 4H; 6.10, br d; 6.25, br d; 7.05-7.50, 15ArH.

## (b) Preparation of Choly1Phe-Asp-Pro-OH

10% Palladium on carbon (50mg) was added to a solution of Choly1Phe- $\beta$ -Benzyl-Asp-Pro Benzyl Ester (55mg) in methanol (4ml) and the mixture stirred under an atmosphere of hydrogen for 16 hours. The mixture was then filtered and the filtrate concentrated to give a white solid (27mg). <sup>1</sup>H nmr (CD<sub>3</sub>OD)  $\delta$ : 0.71, s, 3H; 0.9-2.4, 37H; 2.5-3.2, 2H; 3.4-3.9, br m; 3.81, br s, 1H; 3.95, br s, 1H; 4.38, br m, 1H; 4.62, br m, 1H; 7.1-7.4, 5ArH.

(c) Preparation of Choly1Phe-Asn-Pro-NH<sub>2</sub>

Choly1Phe- $\beta$ -Benzyl-Asp-Pro Benzyl Ester (23mg) was dissolved in a solution of dry methanol saturated with ammonia gas (4ml) and the solution left to stand at room temperature overnight. The solution was then concentrated to give the crude product as a glassy residue. <sup>1</sup>H nmr (CD<sub>3</sub>OD)  $\delta$ : 0.69, s, 3H; 0.85-2.4, 37H; 2.5-3.2, 2H; 3.80, br s, 1H; 3.94, br s, 1H; 4.39, br m, 1H; 4.65, br m, 1H; 7.1-7.4, 5ArH.

Conformational constraints

## 1-Aminoindane-1-carboxylic acid

This compound was made according to the procedure in Biochemical Pharmacology, 11, p. 847. Briefly, indan-2-one (23g) was heated at 55-60 deg. with NaCN (25g) and (NH<sub>4</sub>)<sub>2</sub>O<sub>3</sub>C (210g) 840 ml of 1:1 EtOH:H<sub>2</sub>O. After standing and filtering, the washed filter cake was recrystallized from ethanol to give the hydantoin as pale needles (75%). This was hydrolyzed in 10M HCl (300 ml) for 2h at 100 deg. to yield the title compound, which could be Fmoc or BOC N-protected by the usual means for amino acids (for example, 20 mmol in iPrOH (20 ml)/water (20 ml) with triethylamine (120 mmol) and (BOC)<sub>2</sub>O (1.1 eq) for 4h). Another convenient procedure for the acid hydrolysis step involved gradual dissolution of the hydantoin (10g) in sulfuric acid (70 ml) and water (60 ml) at 140 deg. for 24 h, followed by cooling and filtration on standing and washing with THF.

The commercially available indoline and tetrahydroisoquinoline constraints could be N-protected analogously.

Example of Fmoc chemistry

## Fmoc-DC(S-Trityl)(Piperonyl ester)

Fmoc-Cys(S-Trt) (2 mmol) and piperonyl alcohol (2 mmol) and DCC (2.1 mmol) in EtOAc (20 ml) was treated with DMAP (30 mg) and stirred overnight, filtered, concentrated, deprotected in 1:1 DCM:diethylamine (4 ml) for 5 h and purified through a silica plug, eluting with 5%MeOH/CHCl<sub>3</sub> to give Cys(S-Trt)(Pip) in 63% yield. This was HBTU-coupled with Fmoc-D(β-t-Bu) as per usual to give the title compound as a gum after chromatographic purification.

Peptoids

Peptoids can be conveniently made by a number of methods, two of the most useful of which are exemplified below.

N-Isobutyryl-N-isobutyl glycine compounds:

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(Me)<sub>2</sub>CHC(=O)[N(CHCH(Me)<sub>2</sub>)CH<sub>2</sub>C(=O)]<sub>3</sub>FDAbu

Ethyl bromoacetate (100 mmol, 11 ml) in THF (20 ml) was added slowly to isobutylamine (2.5 eq) in THF (50 ml), with cooling. After 30 min., the product was  
5 partitioned between ether and aq. HCO<sub>3</sub><sup>-</sup>, the organic layer washed, dried and evaporated to give N-isobutyl glycine ethyl ester as an oil in quantitative yield. Of this oil, 10 mmol was taken into THF (10 ml) and triethylamine (10 ml) and treated slowly with isobutyryl chloride (10 mmol)  
10 in THF (10 ml). After 30 min, the concentrated reaction mixture was subjected to the standard washing protocol, and the resulting product oil saponified with excess aq. NaOH in MeOH overnight to give (Me)<sub>2</sub>CHC(=O)N(CHCH(Me)<sub>2</sub>)CH<sub>2</sub>CO<sub>2</sub>H in 95% overall yield. This carboxylic acid was coupled  
15 with 1 equivalent of the N-isobutyl glycine ethyl ester stock in the standard HBTU method, the resulting dipeptoid ester saponified, and these two last steps repeated once more to furnish (Me)<sub>2</sub>CHC(=O)[N(CHCH(Me)<sub>2</sub>)CH<sub>2</sub>C(=O)]<sub>3</sub>OH as gum. HBTU coupling with TFA.FD(Bn)Abu(Bn) and  
20 hydrogenolysis in the usual way yielded the title compound. N-Isobutyryl-N-isobutyl leucine.

To HCl.Leu(OMe) (2.9 g, 20 mmol) and isobutyraldehyde (1.44 g, 20 mmol) in 1,2-dichloroethane (60 ml) after 15 min was added AcOH (1.2 g, 20 mmol) and  
25 NaBH(OAc)<sub>3</sub> (30 mmol). After stirring overnight, the solvent was evaporated and the product partitioned between ether and 1M HCl, the aqueous layer separated and basified, extracted with ether and the ether layer separated, dried and evaporated to give N-isobutyl leucine methyl ester as  
30 an oil. This methyl ester was acylated with isobutyryl chloride and saponified as above to give the title compound as a gum.

#### Compound testing in the calcein release assay

35 A fluorogenic assay system for the measurement of Nef peptide-induced membrane damage in human cells was developed for the screening of putative Nef inhibitory

compounds. Based on the release of the fluorochrome calcein from pre-loaded cells, the method was developed from a cell-mediated cytotoxicity assay described by Lichtenfels et al (1994). Unlike the LDH assay, this assay does not  
5 rely on the measurement of an enzymic activity, and so is not susceptible to the presence of similar activity, or inhibitors of activity, in the samples to be tested.

CEM cells or PBMC were washed twice in PBS containing 5% FBS and resuspended at a concentration of  
10  $2 \times 10^6$ /ml in PBS/5% FBS. Calcein AM (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 20 mM and the cells were incubated in the dark at 37°C for 30 min. Unincorporated dye was removed by washing once in PBS/5% FBS and once in PBS and the cells were resuspended  
15 at  $5 \times 10^5$ /ml (CEM) or  $5 \times 10^6$ /ml (PBMC) in Nef assay buffer. These concentrations of cells were determined in preliminary experiments to give optimal maximum versus spontaneous calcein release for each cell type. 100 ml cells were mixed with 100 ml Nef peptide, diluted to the  
20 desired concentration in Nef assay buffer, and incubated at 37°C in 5% CO<sub>2</sub>, 90% humidity for 1.5-2 hours. At the end of this incubation, the plate was centrifuged at 250xg for 10 min and 100 ml supernatant transferred to a white polystyrene 96-well plate (Packard, Meriden, Connecticut, USA). Fluorescence intensity (FI) was measured on an LS50B  
25 luminescence spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK), using an attenuating filter and excitation and emission wavelengths of 485 and 538 nm respectively. All assays were performed in triplicate and  
30 plates were blanked on wells containing assay buffer alone. Spontaneous calcein release was measured using cells incubated with buffer alone (100 ml) and maximum release was determined by adding 2% Triton X-100 (100 ml) to cells. The percentage cytotoxicity was calculated using the  
35 following formula:

$$\% \text{ cytotoxicity} = 100 \times (\text{FI}_{\text{peptide}} - \text{FI}_{\text{spontaneous}}) / (\text{FI}_{\text{maximum}} - \text{FI}_{\text{spontaneous}})$$

Putative inhibitors of Nef peptide cytotoxicity were tested by mixing equal volumes (50  $\mu$ l) of Myr-Nef2-22 peptide and synthetic compound, diluted in Nef assay buffer as required, in a U-bottom microplate (Disoposable Products, Adelaide, Australia) and incubating for 30 min at 37°C in 5% CO<sub>2</sub>, 90% humidity prior to addition of cells. Results were expressed as the percent inhibition of the cytotoxicity in the presence of the Nef N-terminal peptide alone. Compounds were also tested in the absence of Nef peptide to assess cytotoxicity

#### Results

Tables 3 and 4 list the compounds synthesised and the diagnostic protein NMR signals for some representative compounds respectively.

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Table 3

LISTING OF PUTATIVE NEF INHIBITORS

All amino acids are "L" unless specified otherwise.

- 5    Comments Code
- (A) Water-soluble
- (B) Slightly water-soluble
- (C) Note: N-terminus not amidated
- (D) Note: C-terminus free carboxylic acid
- 10   (E) Belongs to the set of the most potent Nef-Inhibitors
- (G) TFA=Trifluoroacetic acid salt
- (H) BOC=t-butyloxycarbonyl
- (I) Mpr=3-methoxypropionyl
- (J) PEG5k=polyethylene glycol; average m.w. 5000;
- 15   PEG4=tetraethylene glycol, monomethyl ether; PBG6=poly(1,2-butylene glycol (n=6), monobutyl ether
- (K) Benzyl and ethyl ester, monomer or disulfide dimer as indicated
- (L) ClAla=3-chloroalanine
- 20   (M) Ch=cholic acid
- (N) Nb=para-Nitrobenzyl (on S atom)
- (O) iBu=isobutyryl; RLeu=N-(isobutyl)Leucine; RGly=N-(isobutyl)Glycine
- (P) Tic="L"(S)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Indo="L"-(S)-indoline-2-carboxylic acid; Inda=2-amino-indane-2-carboxylic acid; Cl2F=3,4-dichlorophenylalanine; 1-npAla=(1-naphthyl)-alanine; 2-npAla=(2-naphthyl)-alanine
- 25
- 30   Section 1.
- Hexapeptides containing a variant C-terminus residue, such as L-2-aminobutyric acid (Abu; dlAbu for the racemate) and/or modified end-caps to enhance solubility/activity
- Truncated sequences

35

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| Compound                          | Code       | Comments |
|-----------------------------------|------------|----------|
| Ac-IVIFN-Abu-NH <sub>2</sub>      | DER-AP9    | BE       |
| IVIFNC-NH <sub>2</sub>            | DER-AP10   | BCE      |
| (IVIFN-Abu-NH <sub>2</sub>        | DER-AP11   | BCE)     |
| Ac-IVIFDC                         | DER-AP13   | BED      |
| IVIFNC                            | DER-AP15   | CD       |
| Ac-IVIFDS-NH <sub>2</sub>         | DER-JB-AP2 |          |
| Ac-IVIFD-Abu                      |            | BD       |
| (TFA.IVIFD(Bn)C(Et)) <sub>2</sub> | BRI6202    | GK       |
| TFA.IVIFD-dlAbu                   | BRI6233    | CDG      |
| TFA.IVIFN-dlAbu-NH <sub>2</sub>   | BRI6239    | CG       |
| TFA.IVIFN-Abu-NH <sub>2</sub>     | BRI6245    | G        |
| BOC-FN-Abu-NH <sub>2</sub>        | BRI6242    | H        |
| TFA-FN-Abu-NH <sub>2</sub>        | BRI6243    | AG       |
| TFA.IVIN-Abu-NH <sub>2</sub>      | BRI6244    | G        |
| MPr-IVIFD-dlAbu                   | BRI6230    | BDEI     |
| MPr-IVIFN-dlAbu-NH <sub>2</sub>   | BRI6236    | I        |
| PEG5k-IVIFN-Abu-NH <sub>2</sub>   | BRI6211    | AJE      |
| PEG4-IVIFD-Abu                    |            | DJ       |
| Ac-IVIFDP                         |            | D        |
| Ac-IVIFD-ClAla                    |            | DL       |
| BOC-IVIFN-Abu-NH <sub>2</sub>     | BRI6223    |          |
| BOC-IVIN-Abu-NH <sub>2</sub>      | BRI6224    |          |
| MPr-IVIFD-Abu(OMe)                | BRI6269    | I        |
| Ac-IVIFD-Abu(OMe)                 | BRI6270    |          |
| Ac-IVIFDK                         | BRI6274    | AD       |

Section 2.

-N-terminus tripeptide modifications/replacements as  
solubility/activity enhancers

- 5        -Cholic acid conjugates  
          -Peptoids  
          -Glycols

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| Compound                                             | Code    | Comments |
|------------------------------------------------------|---------|----------|
| Ch-FNC (Nb) -NH <sub>2</sub>                         | BRI6199 | BENM     |
| (Ch-FDC) 2                                           | BRI6200 | ADEM     |
| (Ch-FD (NMe <sub>2</sub> ) C (NMe <sub>2</sub> ) ) 2 | BRI6201 | M        |
| (Ch-FD (Bn) C (Et) 2                                 | BRI6205 | KM       |
| Ch-FDC                                               | BRI6209 | ADEM     |
| Ch-FN-Abu-NH <sub>2</sub>                            | BRI6221 | M        |
| Ch-N-Abu-NH <sub>2</sub>                             | BRI6222 | M        |
| Ch-FD (Bn) C (Et)                                    | BRI6225 | KM       |
| Ch-F                                                 | BRI6273 | BDM      |
| iBu (RLeu) FN-Abu-NH <sub>2</sub>                    | BRI6214 | O        |
| iBu (RGly) 3FD-dlAbu                                 | BRI6235 | ADO      |
| iBu (RGly) 3FN-dlAbu-NH <sub>2</sub>                 | BRI6241 | O        |
| iBu (RGly) 3N-Abu-NH <sub>2</sub>                    | BRI6216 | O        |
| iBu (RGly) 5FN-Abu-NH <sub>2</sub>                   | BRI6217 | O        |
| iBu (RGly) 5N-Abu-NH <sub>2</sub>                    | BRI6218 | O        |
| iBu (RGly) VIFD-dlAbu                                | BRI6231 | OD       |
| iBu (RGly) VIFN-dlAbu-NH <sub>2</sub>                | BRI6237 | O        |
| (R,R-Gly) VIFD-dlAbu                                 | BRI6232 | CDO      |
| (R,R-Gly) VIFN-dlAbu-NH <sub>2</sub>                 | BRI6238 | CO       |
| PBG6-FD-dlAbu                                        | BRI6271 | J        |
| PBG6-FN-dlAbu-NH <sub>2</sub>                        | BRI6272 | J        |

Section 3.

- Conformationally constrained inhibitors through phenylalanine modification in particular, to enhance solubility/activity.
- 5

| Compound                                                           | Code    | Comments |
|--------------------------------------------------------------------|---------|----------|
| AcIVI-Indo-D-Abu                                                   |         | P        |
| AcIVI-Tic-D-Abu                                                    | BRI6268 | BDP      |
| AcIVI-Inda-D-Abu                                                   |         | P        |
| AcIVI- (Cl <sub>2</sub> F) -D-dlAbu                                | BRI6266 | BDP      |
| AcIVI- (Cl <sub>2</sub> F) -NH <sub>2</sub> -dlAbu-NH <sub>2</sub> |         | P        |
| AcIVI- (1-npAla) -D-dlAbu                                          | BRI6264 | PD       |
| AcIVI- (2-npAla) -D-dlAbu                                          | BRI6265 | PDE      |

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Table 4

Diagnostic proton NMR signals for some exemplary compounds

| Compound <sup>1</sup>                             | Diagnostic Signals <sup>2</sup>                                                                                                                                                                                                                                                                                                                                                                                            |
|---------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| AcIVIFD(Bn)Abu(Bn)                                | Amide NH protons (6; 7.6-8.5)<br>Aromatic protons (15; 7.1-7.3)<br>BnCH <sub>2</sub> protons (4; 5.2)<br>$\alpha$ -CH protons (6; 4.1-4.8)<br>Ac protons (3; 1.85; singlet)<br>Methyls (21; 0.6-0.9)                                                                                                                                                                                                                       |
| AcIVIFDAbu                                        | Loss of Benzylic protons                                                                                                                                                                                                                                                                                                                                                                                                   |
| AcIVI-Ar-DAbu<br>(Ar=Indo, Inda,<br>Tic or npAla) | Appropriate Integration of Aromatic<br>Protons                                                                                                                                                                                                                                                                                                                                                                             |
| PEG4-IVIFD-Abu                                    | Glycol CH <sub>2</sub> s (12; 3.4-3.7)<br>Methoxy (3; 3.3; singlet)                                                                                                                                                                                                                                                                                                                                                        |
| PBG6-FD-dlAbu                                     | Glycol protons (17; 3.3-3.7)                                                                                                                                                                                                                                                                                                                                                                                               |
| (Ch-FD(Bn)C(Et)) <sub>2</sub>                     | NH amide (2; 8.4)<br>NH amide (1; 8)<br>ArH (10; 7.4 and 7.3)<br>BnCH <sub>2</sub> (2; 5.15)<br>$\alpha$ -CHs (3; 4.6-4.8)<br>Ester CH <sub>2</sub> (2; 4.1)<br>Cholyl (1; 4.35)<br>Cholyl (1; 4)<br>Cholyl (2; 3.6 and 3.8)<br>Cholyl and $\beta$ -CH <sub>2</sub> s (numerous<br>overlapping multiplets; 2.5-3.3)<br>Cholyl and ester CH <sub>3</sub> (numerous<br>overlapping multiplets and ester<br>triplet; 0.6-2.3) |
| Ch-FNC(Nb)-NH <sub>2</sub>                        |                                                                                                                                                                                                                                                                                                                                                                                                                            |

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|                                            |                                                                                                                                                                                                                                                                                                                                                              |
|--------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Ch-FD(t-Bu)C(Trt)(Pip)                     | Phe and Tft ArH (20; 7.2-7.5)<br>Pip ArH (3; 6.7-6.9)<br>OCH <sub>2</sub> O (2; 5.95)<br>OCH <sub>2</sub> (2; 5.0)<br>t-Bu (9; 1.35; singlet)                                                                                                                                                                                                                |
| Ch-FDC                                     | Loss of protecting group signals<br>[from, for example, Ch-FD(Bn)C(Et)) <sub>2</sub><br>or Ch-FD(Bn)C(Nb)(OEt) or Ch-FD(t-Bu)C(Trt)(Pip)]                                                                                                                                                                                                                    |
| iBu(RLeu)FN-Abu-NH <sub>2</sub><br>(CDC13) | $\alpha$ -CH (1; 3.95; triplet)<br>"N- $\beta$ -CH <sub>2</sub> " (2; 3.0-3.4; pair of doublets of doublets)<br>isobutyryl CH (1; 2.8-2.9; multiplet)<br>isobutyl CHs (2; 1.9-2.1; multiplet)<br>$\beta$ -CH <sub>2</sub> (2; 1.5-1.8; multiplet)<br>isobutyryl CH <sub>3</sub> s (6; 1.15; doublet)<br>isobutyl CH <sub>3</sub> s (12; 0.9-1.0; multiplets) |
| iBu(RGly)3FD-Abu<br>CDC13                  | $\alpha$ -CH <sub>2</sub> s (6; 3.9-4.3)<br>"N- $\beta$ -CH <sub>2</sub> s" (6; 3.1-3.3)<br>isobutyryl CH (1; 2.8-3.0)<br>isobutyl CHs (3; 1.8-2.0)<br>isobutyryl CH <sub>3</sub> s (6; 1.1-1.2)<br>isobutyl CH <sub>3</sub> s (18; 0.8-1.1)                                                                                                                 |

1 In deuterated DMSO unless stated otherwise. See previous tables for explanation of abbreviations.

2 Diagnostic signals are not generally re-described on recurrence of the same structural feature. Given in parentheses are: integration; chemical shift in ppm relative to TMS

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#### Activity of Nef peptides in the calcein release assay

The neutral, non-fluorescent calcein acetoxymethyl ester, calcein AM, passively enters cells and is hydrolysed by cellular esterases to give a polar, fluorescent compound which is retained by viable cells with intact membranes. Addition of a myristylated 21-residue N-terminal Nef peptide to calcein-loaded CEM CD4+ T cells caused dose-dependent release of calcein as shown in Figure 18. Non-myristylated N-terminal Nef peptide and a myristylated, non-N-terminal Nef peptide caused little or no calcein release under the same conditions. These responses are similar to those observed in the LDH cytotoxicity assay, using non-calcein-loaded CEM cells (see Example 1).

#### Inhibitors of Nef cytotoxicity

Approximately 120 synthetic compounds were tested in the calcein assay for their ability to abrogate calcein release from CEM cells induced by myristylated N-terminal Nef peptide. Initial screening at a 10-20-fold molar excess of synthetic compound revealed of the order of 24 compounds which gave 50% or more inhibition of Nef peptide cytotoxicity in this system as shown in Table 5. Of these compounds, 15 were selected for further study. At a compound: Nef peptide ratio of 10:1, AP9, BRI6199, BRI6203, BRI6209, BRI6211 and DER-AP3 showed greater than 50% Nef inhibition and were not cytotoxic as shown in Figure 19. AP13, DER45 and DER-AP2 also showed at least 50% Nef inhibition and gave less than 25% cytotoxicity. Titration of these compounds revealed one, BRI6199, with which caused 50% inhibition of Nef peptide-induced calcein release at a molar ratio of approximately 1:1 (compound: Nef peptide) and 90% inhibition at a molar ratio of 2:1 (compound: Nef peptide) as shown in Table 6. The next best compounds, DER-AP2, DER-AP3, AP13, DER45, BRI6209, showed 50% inhibition of Nef peptide cytotoxicity at 1 to 5:1 compound:Nef peptide molar ratios and 90% inhibition at 4-14:1 compound:Nef peptide molar ratios. Testing of representative compounds using

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calcein-loaded PBMC showed that they could also block the cytotoxic activity of myristylated Nef N-terminal peptide against primary human cells, as shown in Figure 20. Two compounds, DER31 and DER45 were also tested in the

5 microphysiometer, using a 10-fold molar excess over Nef peptide, and were found to be able to block the decline in metabolic activity of CEM CD4+ T cells observed following exposure to Myristylated Nef N-terminal peptide (data not shown) .

Table 5  
Nef inhibitory activity and cytotoxicity of synthetic compounds

| Compound     | Structure                  | Compound:<br>Nef peptide<br>molar ratio | Nef<br>inhibition | Cytotoxicity |
|--------------|----------------------------|-----------------------------------------|-------------------|--------------|
| None         | -                          |                                         | nd1               | ++++2        |
| AP1          | Ac-GGAANALESEANNAASLAG-NH2 | 6                                       | +2                | -            |
| AP1 (water)  | Ac-GGAANALESEANNAASLAG-NH2 | 6                                       | +                 | -            |
| AP8          | Ac-IVIFDV-NH2              | 14                                      | +++               | -            |
| AP8 (water)  | Ac-IVIFDV-NH2              | 10                                      | -                 | ++           |
| AP9          | Ac-IVIFN-Abu-NH2           | 15                                      | ++++              | -            |
| AP9 (water)  | Ac-IVIFN-Abu-NH2           | 10                                      | ++                | -            |
| AP10         | IVIFNC-NH2                 | 15                                      | +++               | ++           |
| AP10 (water) | IVIFNC-NH2                 | 10                                      | +++               | ++           |
| AP11         | IVIFN-Abu-NH2              | 15                                      | +++               | +            |
| AP11 (water) | IVIFN-Abu-NH2              | 10                                      | ++                | ++           |
| AP12         | Ac-IVIFNC-NH2              | 10                                      | +                 | +            |
| AP13         | Ac-IVIFDC-OH               | 10                                      | ++++              | +            |
| AP17S        | H-LKLLKI-OH                | 10                                      | +                 | -            |
| AP17SA       | Ac-LKLLKI-OH               | 10                                      | +                 | nd           |
| AP18S        | H-LTLLTI-OH                | 10                                      | -                 | ++++         |

|                 |                                         |    |      |      |
|-----------------|-----------------------------------------|----|------|------|
| AP18SA          | Ac-LTLLTI-OH                            | 10 | -    | +    |
| AP19S           | Ac-LKLLLEI-NH2                          | 10 | -    | +    |
| AP19S(heated)   | Ac-LKLLLEI-NH2 (L-E amide<br>link)      | 10 | -    | +    |
| AP19SA          | Ac-LK(Ac) LLEI-NH2                      | 10 | +    | nd   |
| BM01            | Ac-ELAEELAEELAE-NH2                     | 10 | +    | -    |
| BM01 (water)    | Ac-ELAEELAEELAE-NH2                     | 10 | +    | +    |
| BRI6197         | Choly1-FD(OBn)C(SBnNO2)OEt              | 10 | -    | +++  |
| BRI6198(water)  | Choly1-FDC(Nb)-OH                       | 10 | +    | ++++ |
| BRI6199         | Choly1-FNC(Nb)-NH2                      | 10 | ++++ | -    |
| BRI6199(water)  | Choly1-FNC(Nb)-NH2                      | 10 | ++++ | -    |
| BRI6200(water)  | (Choly1-FDC)2 disulphide                | 10 | -    | ++++ |
| BRI6200P(water) | (Choly1-FDC)2 thioether                 | 10 | ++   | ++   |
| BRI6201         | (Choly1-FD(NMe2)C(NMe2))2<br>disulphide | 10 | -    | ++++ |
| BRI6202         | (TFA.IVIFD(Bn)C(Et))2                   | 10 | +++  | ++   |
| BRI6203         | (Ac-IVIFDC-OEt)2                        | 10 | +++  | +    |
| BRI6204         | Ac-IVIFDC-Oet                           | 10 | ++   | +    |
| BRI6205         | (Choly1-FDC(Bn)C(Et))2                  | 10 | +    | +    |
| BRI6206         | (C9-FD(OB21)C(OEt))2                    | 10 | +    | +    |
| BRI6209(water)  | Choly1-FDC                              | 10 | ++++ | -    |
| BRI6210         | Choly1-FD(OB21)C                        | 10 | -    | ++++ |

|                 |                           |    |     |     |
|-----------------|---------------------------|----|-----|-----|
| BRI6211 (water) | PEG (5000) IVIFN-Abu-NH2  | 10 | +++ | nd  |
| BRI6214         | iBu (RLeu) FN-Abu-NH2     | 10 | +   | -   |
| BRI6215         | iBu (RGly) 3FN-Abu-NH2    | 10 | -   | nd  |
| BRI6216         | iBu (RGly) 3N-Abu-NH2     | 10 | -   | +   |
| BRI6217         | iBu (RGly) 5FN-Abu-NH2    | 10 | -   | -   |
| BRI6218         | iBu (RGly) 5N-Abu-NH2     | 10 | -   | -   |
| BRI6219         | Choly1-FN-Abu-NH2         | 10 | -   | +++ |
| BRI6220         | Choly1-N-Abu-NH2          | 10 | +   | -   |
| BRI6221         | Choly1-FN-Abu-NH2         | 10 | -   | -   |
| BRI6222         | Choly1-N-Abu-NH2          | 10 | -   | -   |
| BRI6223         | BOC-IVIFN-Abu-NH2         | 10 | +   | ++  |
| BRI6224         | BOC-IVIN-Abu-NH2          | 10 | +   | +   |
| BRI6225         | Choly1-FD (Bn) C (Et)     | 10 | -   | ++  |
| BRI6226         | FN-Abu-NH2                | 10 | +   | -   |
| BRI6230 (water) | MPr-IVIFD-dlAbu           | 10 | ++  | -   |
| BRI6231 (water) | iBu (RGly) VIFD-dlAbu     | 10 | +   | nd  |
| BRI6232 (water) | (R,R-Gly) VIFD-dlAbu      | 10 | ++  | nd  |
| BRI6233 (water) | TFA. IVIFD-dlAbu          | 10 | +   | nd  |
| BRI6234 (water) | Choly1-FD-dlAbu-OH        | 10 | +   | nd  |
| BRI6235 (water) | iBu (RGly) 3FD-dlAbu      | 10 | ++  | -   |
| BRI6236         | MPr-IVIFN-dlAbu-NH2       | 10 | +   | nd  |
| BRI6237         | iBu (RGly) VIFN-dlAbu-NH2 | 10 | ++  | nd  |

|                 |                                              |    |      |     |
|-----------------|----------------------------------------------|----|------|-----|
| BRI6238         | (R,R-Gly) VIFN-dlAbu-NH <sub>2</sub>         | 10 | ++   | +++ |
| BRI6239         | TFA. IVIFN-dlAbu-NH <sub>2</sub>             | 10 | ++   | +++ |
| BRI6240         | CHOLYL-FN-dlAbu-NH <sub>2</sub>              | 10 | +    | +   |
| BRI6241         | iBu(RGly) 3FN-dlAbu-NH <sub>2</sub>          | 10 | -    | +   |
| BRI6242         | BOC-FN-Abu-NH <sub>2</sub>                   | 10 | +    | +   |
| BRI6243 (water) | TFA. FN-Abu-NH <sub>2</sub>                  | 10 | +    | -   |
| BRI6244         | TFA. IVIN-Abu-NH <sub>2</sub>                | 10 | +    | +   |
| BRI6245         | TFA. IVIFN-Abu-NH <sub>2</sub>               | 10 | ++   | nd  |
| BRI6264         | Ac-IVI (1-npAla) D-dlAbu                     | 10 | ++   | nd  |
| BRI6265         | Ac-IVI- (2-npAla) D-dlAbu                    | 10 | ++++ | nd  |
| BRI6265L        | Ac-IVI- (Ala-2-naphthyl) -D-Abu              | 10 | -    | nd  |
| BRI6266         | Ac-IVIF (C12F) -D-dlAbu                      | 10 | +    | nd  |
| BRI6267         | Ac-IVIF (3-Cl, 4-Cl) N-dlAbu-NH <sub>2</sub> | 10 | +    | nd  |
| BRI6268         | AcIVI-Tic-D-Abu                              | 10 | -    | nd  |
| BRI6269         | MPr-IVIFD-Abu (OMe)                          | 10 | +    | nd  |
| BRI6270         | Ac-IVIFD-Abu (OMe)                           | 10 | +    | nd  |
| BRI6271         | PBG6-FD-dlAbu                                | 10 | -    | nd  |
| BRI6272         | PBG6-FN-dlAbu-NH <sub>2</sub>                | 10 | -    | nd  |
| BRI6273         | Cholyl-F                                     | 10 | -    | nd  |
| BRI6274         | Ac-IVIFDK                                    | 10 | -    | nd  |
| BRI6274 (Na2)   | Ac-IVIFDK                                    | 10 | +    | nd  |

|               |                                                                  |    |      |      |
|---------------|------------------------------------------------------------------|----|------|------|
| BRI6274 (TFA) | Ac-IVIFDK                                                        | 10 | +    | nd   |
| BRI6281       | MeO(CH <sub>2</sub> CH <sub>2</sub> O)4CO-IVI-(Ala-2-naph)-D-Abu | 10 | ++++ | nd   |
| Cholic acid   |                                                                  | 10 | -    | nd   |
| CCC12         | Ac-LLLHNM-NH <sub>2</sub>                                        | 5  | -    | +    |
| DER31         | Ac-IVIFDC-NH <sub>2</sub>                                        | 10 | ++++ | ++   |
| DER31b        | Ac-IVIFDC-Acm                                                    | 13 | +++  | -    |
| DER32         | Ac-LISWIC-NH <sub>2</sub>                                        | 14 | -    | -    |
| DER35         | Ac-IVIWDC-NH <sub>2</sub>                                        | 10 | ++++ | +++  |
| DER36         | Ac-IVSWDC-NH <sub>2</sub>                                        | 14 | +    | -    |
| DER38         | IVILDC                                                           | 15 | +++  | -    |
| DER39         | (IVIFD)2K                                                        | 8  | +++  | -    |
| DER40         | Ac-(NorLeu)VI(Phg)DC-NH <sub>2</sub>                             | 14 | +    | -    |
| DER45         | Ac-IVIFNC-NH <sub>2</sub>                                        | 10 | ++++ | +    |
| DER47         | Ac-IVIFDM-NH <sub>2</sub>                                        | 14 | +++  | -    |
| DER60         | C14-FDC-NH <sub>2</sub>                                          | 18 | ++++ | ++   |
| DER62         | C3-FDC-NH <sub>2</sub>                                           | 21 | ++   | ++   |
| DER65         | C11-FDC-NH <sub>2</sub>                                          | 19 | -    | ++++ |
| DER69         | Ac-                                                              | 14 | ++   | +    |
|               | (NorLeu)2(pg)N(penicillamine)-Acm-NH <sub>2</sub>                |    |      |      |
| DER7-10b      | (OH)2-C14-cystamine                                              | 27 | -    | +++  |

|                         |                           |    |      |      |
|-------------------------|---------------------------|----|------|------|
| DER73                   | C14-NC-NH <sub>2</sub>    | 24 | -    | nd   |
| DER74                   | C14-FNC-NH <sub>2</sub>   | 18 | +    | nd   |
| DER76                   | Ac-ITTFDC-NH <sub>2</sub> | 14 | -    | +    |
| DER77                   | Ac-IVTFDC-NH <sub>2</sub> | 14 | +    | -    |
| DER84                   | H-LLLFNI-OH               | 10 | ++   | +    |
| DER84 (water)           | H-LLLFNI-OH               | 10 | +    | +    |
| DER84A (DMSO)           | H-LLLFNI-OH               | 10 | +    | -    |
| DER-AP1-NH <sub>2</sub> | C6-FDC                    | 22 | +    | -    |
| DER-AP2-NH <sub>2</sub> | C9-FDC                    | 10 | +++  | +    |
| DER-AP3-NH <sub>2</sub> | C10-FDC                   | 10 | ++++ | -    |
| DER-AP4-NH <sub>2</sub> | C12-FDC                   | 19 | +    | +++  |
| DER-AP5-NH <sub>2</sub> | C16-FDC                   | 17 | ++++ | +++  |
| DER-AP6-NH <sub>2</sub> | C13-FDC                   | 18 | -    | ++++ |
| DER-AP7                 | C9-WNC-NH <sub>2</sub>    | 19 | -    | +    |
| DER-AP14                | C16-FN-Abu                | 10 | ++   | ++   |
| DER-AP15                | Chau-FN-Abu-OH            | 10 | -    | ++++ |
| DER-AP16                | Cin-FN-Abu-OH             | 10 | +    | +    |
| IGM07                   | H-Phg-DIGC-OH             | 21 | +    | -    |
| JB-AP1                  | H-C9-FDS-NH <sub>2</sub>  | 21 | +    | +    |
| JB-AP2                  | Ac-IVIFDS-NH <sub>2</sub> | 14 | +    | +    |

1. nd - not done

2. -, 0%; +, 1-25 %; ++, 26-50%; +++, 51-75 %; +++++, 75-100 %.

Table 6  
Fifty and ninety percent inhibitory doses of selected synthetic compounds

| Inhibitor               | Structure                                       | ID501          | ID902          |
|-------------------------|-------------------------------------------------|----------------|----------------|
| AP9                     | Ac-IVIFN-Abu-NH <sub>2</sub>                    | 12.9±2.9 (n=2) | nd             |
| AP10                    | IVIFNC-NH <sub>2</sub>                          | 7.7±3.5 (n=4)  | 18.8 (n=1)     |
| AP13                    | Ac-IVIFDC-OH                                    | 2.4±0.3 (n=5)  | 6.7±1.9 (n=4)  |
| BRI6199                 | Cholyl-FNC (SBnNO <sub>2</sub> )NH <sub>2</sub> | 1.4±0.6 (n=3)  | 2.0±0.8 (n=4)  |
| BRI6200P                | (Cholyl-FDC) 2                                  | 0.9±0.1 (n=4)  | nd             |
| BRI6203                 | (Ac-IVIFDC-OEt) 2                               | 8.2±1.2 (n=2)  | 20 (n=1)       |
| BRI6209                 | Cholyl-FNC                                      | 1.0±0.1 (n=4)  | 4.2±2.0 (n=4)  |
| DER31                   | Ac-IVIFDC-NH <sub>2</sub>                       | 5.5±1.0 (n=5)  | 11.4 (n=1)     |
| DER35                   | Ac-IVIWDC-NH <sub>2</sub>                       | 3.8±0.8 (n=5)  | 5.8 (n=1)      |
| DER45                   | Ac-IVIFNC-NH <sub>2</sub>                       | 4.9±0.9 (n=5)  | 13.5±3.3 (n=3) |
| DER-AP2-NH <sub>2</sub> | C9-FDC                                          | 3.5±1.1 (n=5)  | 4.5 (n=1)      |
| DER-AP3-NH <sub>2</sub> | C10-FDC                                         | 3.8±0.8 (n=5)  | 5.6±2.0 (n=3)  |

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- 1 ID50 - 50% inhibitory dose. Calculated from titration  
of the synthetic compound.
- 2 ID90 - 90% inhibitory dose. Calculated from titration  
of the synthetic compound.
- 5 3 nd - not determined

Example 8: Structural Requirements for the Cytotoxicity  
of the Nef N-Terminal.

The effects of modifications to the sequence and  
10 secondary structure of the Nef N-terminus on the  
cytotoxicity of the peptide were investigated.

Fresh red blood cells and the CD4+ T-cell line,  
CEM, were obtained as described above (see "General  
Methods"). Measurement of haemolysis and CEM cell LDH  
15 release was performed according to the methods described  
above (see "General Methods").

Circular dichroism spectra

Far ultraviolet circular dichroism spectra of the  
20 peptides in methanol were measured over the range 250 nm to  
200 nm using an AVIV spectropolarimeter at 309K using 0.01  
or 0.05 cm pathlength rectangular quartz cells. Peptide  
concentration in the sample solution was measured by  
quantitative amino acid analysis. Thirty six spectra were  
25 averaged, baseline-corrected and analysed for %  $\alpha$ -helix,  $\beta$ -  
strand and disordered structures using the K2d Kohonen  
neural network program (Andrade et al. 1993; Merelo et al.  
1994) obtainable by ftp anonymous to swift.embl-  
heidelberg.de directory /group/andrade. Estimates of  
30 distribution of secondary structure along the sequences  
were obtained by discrete state-space theoretical analysis  
(Stultz et al, 1993, White et al, 1994) performed on the  
Boston University Biomolecular Engineering Research Centre  
Protein Sequence Analysis server  
35 (<http://bmerc-www.bu.edu/psa/about.htm#about>).

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## Membrane fusion studies

Membrane-mixing fusion was measured using concentration-dependent fluorescence dequenching (Hoekstra et al, 1984). SUV made from dipalmitoyl phosphatidyl choline (DPPC, Sigma, St Louis MO) were labelled with octadecyl-rhodamine (R18, obtained from Molecular Probes Inc, Junction City OR) in a molar ratio of 1 fluorochrome to 100 lipid molecules by adding the dye in a 100 mM ethanolic solution. Ethanol and excess dye were removed by gel filtration through Sephadex G50 in 50 mM Tris-HCL buffer (pH 7.0). The fusion assay was carried out in a darkened room by adding 100 mg of the labelled vesicles to 1 mg of unlabelled SUV in 2 ml of 50 mM Tris-HCl buffer (pH 7.0) and adding the fusogenic agent at the desired molar ratio to this mixture. The fluorescence of the mixture was followed at 43°C using excitation and emission wavelengths of 460 and 680 nm, respectively in a Perkin Elmer-Hitachi M35 spectrofluorimeter. The fluorescence intensity of the mixture in the absence of fusogen was taken as baseline.

20

Interfacial behaviour of peptides and monolayers of dipalmitoyl phosphatidyl choline (DPPC)

5.3 mg of DPPC (Sigma, St Louis Mo) was dissolved in 5 ml of hexane:propanol (9:1 v/v). The DPPC solution was added dropwise to the surface of a subphase (170 ml) contained in a Teflon® Langmuir trough. The trough was mounted in a thermostatted cabinet with high humidity and the temperature was controlled to 30.2° C. The film was compressed at a rate of 38 mm<sup>2</sup> s<sup>-1</sup>. Isotherms of DPPC were measured on subphases of both Milli-Q® (Millipore) water (pH 5.9) and 25 mM HEPES (Merck) adjusted to a pH 7.2 with sodium hydroxide (AR).

Injection of Peptides below DPPC Monolayers: A DPPC monolayer was spread on the HEPES subphase. The peptides were dispersed in 10 ml of 25 mM HEPES (pH 7.2). 10 ml of subphase was removed and replaced by the 10 ml of peptide dispersion ensuring a uniform distribution of peptide in

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the subphase. After 10 minutes equilibration the DPPC monolayer film was compressed and the resulting pressure/area isotherms plotted.

## 5 Results

Effect of sequence modification on secondary structure of peptides

In order to determine whether sequence modification had any effect on secondary structure the latter was determined for each of the peptides by CD spectroscopy and by computation from its sequence. The CD spectra of the peptides in methanol are compared in Figure 21 and the calculated proportions of  $\alpha$ -helix,  $\beta$ -strand and disordered structures are given in Table 7.

Table 7

Structural data on Nef peptides calculated from CD spectra

| Peptide    | $\alpha$ -helix % | $\beta$ -structure % | random % |
|------------|-------------------|----------------------|----------|
| Nef 2-22   | 65                | 10                   | 25       |
| Nef 2-26   | 67                | 8                    | 25       |
| Nef 2-22a  | 74                | 11                   | 15       |
| Nef 2-22b  | 65                | 88                   | 23       |
| Nef 2-22c  | 48                | 19                   | 33       |
| Nef 2-26a  | 78                | 6                    | 16       |
| Nef 31-50a | 79                | 6                    | 15       |
| Nef 31-50  | 76                | 4                    | 20       |

Small differences were in structure observed between peptides Nef 2-22a and Nef 2-22c. On the other hand Nef 2-22b, Nef 2-22e and Nef 31-50 showed a marked increase in  $\alpha$ -helix and a corresponding reduction in disorder. Increasing the chain length from 21 to 25 residues (Nef 2-26) had little effect on the CD spectrum.

Theoretical calculation of the secondary structures showed that most of the increase in  $\alpha$ -helix in Nef 2-22b,c & e occurred over residues 2-10 while Nef 31-50, in contrast to its CD spectrum showed a equal probability of  $\alpha$ -helix and loop over its entire length, as

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shown in Figure 22. Increasing the positive charge of this sequence (Nef 31-50a) resulted in a modest increase in  $\alpha$ -helicity.

5 The overall CD-derived value for  $\alpha$ -helix for Nef 2-22 and Nef 2-26 and the distribution of the probability for  $\alpha$ -helicity for Nef 2-22 and Nef 2-26 agreed well with the structure obtained from NMR3 for Nef 2-26.

10 Interaction of Nef 2-22a and Nef 31-50 with monolayers of DPPC.

The study of the interaction of peptides with phospholipid monolayers often give clues to the mechanisms of their behaviour with cell membranes. It was found that there was a slight difference between the Milli-Q® and  
15 HEPES isotherms for DPPC monolayers. DPPC monolayers are relatively pH insensitive, so the difference may be attributed to the HEPES and it was decided, therefore, to use the latter in the subphase for the experiments with the peptides. The isotherms of DPPC on the subphases containing  
20 respectively unMyristylated Nef 2-22, Nef 2-22 and Nef 31-50 illustrate a strong interaction between the lipid monolayer in the cases of Nef 2-22 and Nef 31-50, as shown in Figure 23. These two peptides had expanded the monolayer film substantially. Their collapse point (50-52  
25 mN m<sup>-1</sup>) showed that the isotherms observed remain a function of the DPPC molecules. The result also indicated that with both peptides, a significant amount of the amino acid side chains were interacting with the monolayer.

### 30 Membrane fusion studies

Fluorescence fusion assays were carried out on the non-Myristylated Nef 2-22 and Nef 9-22 peptides to find out whether the fusion of SUV by non-Myristylated Nef N-terminal peptides that we had observed previously required  
35 the presence of the relatively unstructured seven N-terminal residues. These assays rely on the fact that the fluorochrome R18 is heavily self quenched when inserted in

a lipid bilayer at high lipid/fluorochrome ( $< 1/100$ ). In the absence of lipid mixing the dye only very slowly transfers from the bilayers of the labelled to those of the unlabelled vesicles. When lipid-mixing fusion occurs the dye is rapidly diluted throughout the system and relief of self-quenching results in an increase in fluorescence. When added to SUV at a molar ratio of 1/150, both peptides caused a rapid increase in R18 fluorescence as shown in Figure 24, indicating that lipid mixing had taken place.

10

Lysis of sheep red blood cells

Figure 25 shows dose-response curves for the lysis of sheep red blood cells by all the Nef peptides. Broadly, reduction of the positive charge in the 2-7 region of the peptide (Nef 2-22b) reduced the lytic activity of the peptide as did truncation of the peptide at the C-terminal end (Nef 2-10). An increase in positive charge in the C-terminal region (Nef 2-22c) led to a slight increase in lytic activity. Secondary structure at the N-terminus seemed to take second place to positive charge as a determining lytic activity. Nef 2-26 had a much higher  $\alpha$ -helical content than Nef 2-22c or Nef 2-22, yet had the same order of haemolytic activity. As we have shown previously, Nef 31-50 which has a very high  $\alpha$ -helical content is poorly lytic. On the other hand, the addition of two positively-charged lysine residues to its N-terminal region increased its lytic activity fourfold. The kinetics of haemolysis as shown in Figure 26 show a similar trend with the peptides with positive charges at the N-terminal end reaching almost 100% haemolysis in the first five minutes after addition. It should be noted that the other peptides, with the exception of Nef 31-50, attained more than 50% haemolysis after 160 minutes.

35 Cytotoxicity in CEM CD4+ T cells

The ability of various wild-type and mutant Nef peptides to cause membrane perturbation and subsequent LDH

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release in CD4+ T cells was examined to determine the sequence and/or structural requirements for Nef N-terminal cytotoxicity. C-terminal truncation (Myr-Nef2-10) greatly reduced the ability of Nef N-terminal peptide to cause  
5 membrane damage in CEM cells, as did reduction of the positive charge in the 2-7 region of the peptide by substitution of the lysines at residues 4 and 7 with alanines (Myr-Nef2-22b) as shown in Figure 27. Increasing the positive charge of this region, conversely, had little  
10 effect on peptide activity. The secondary structure at the N-terminus appeared to be of lesser importance than the positive charge in determining Nef peptide cytotoxic activity. Myristylated Nef31-50, which had a high alpha-helical content, caused only moderate LDH release from CEM  
15 cells, but this is increased to the level of the N-terminal peptide (Myr-Nef2-22) following the addition of two lysine residues (positively charged) at positions 32 and 36.

Removal of the the first 8 amino acids of the Nef N-terminus (Myr-Nef10-26) caused a four-fold reduction in  
20 activity in the LDH assay, confirming the importance of this region for cytotoxicity (Table 8). Increasing the alpha-helical content of this region (Myr-Nef2-26a) resulted in a moderate increase in LDH release from CEM cells, suggesting that the observed lack of secondary  
25 structure is not crucial for its activity. As previously observed, myristoylation is important for the membrane targeting of peptides, and little or no LDH release was observed in the presence of non-Myristylated Nef peptides (Nef10-26, Nef2-26a).

30

Table 8

Nef peptide cytotoxicity for CD4+ T cells.

| Peptide      | Sequence                                      | TD50 ( $\mu$ M) # |
|--------------|-----------------------------------------------|-------------------|
| Myr-Nef2-26  | Myr-GGKWSKSSVIGWPAVRERMRRAEPA-NH <sub>2</sub> | 4.8 $\pm$ 1.0*    |
| Myr-Nef10-26 | Myr-VIGWPAVRERMRRAEPA-NH <sub>2</sub>         | 19.1 $\pm$ 1.8    |
| Myr-Nef2-26a | Myr-GKALKVAEVIGWPAVRERMRRAEPA-NH <sub>2</sub> | 0.85 $\pm$ 0.04   |
| Nef10-26     | Ac-VIGWPAVRERMRRAEPA-NH <sub>2</sub>          | >20               |
| Nef2-26a     | Ac-GKALKVAEVIGWPAVRERMRRAEPA-NH <sub>2</sub>  | >20               |

# TD50 - 50% toxic dose.

- 5 \* Data represents the mean  $\pm$  SEM from three separate experiments performed in triplicate.

These data show that the myristylated Nef N-terminus possesses haemolytic and cytotoxic activities and a net positive charge in the proximate N-terminal region of the sequence as well as an  $\alpha$ -helical region extending from residues 9-21. Since this region seems to be the fusogenic part of the non-myristylated peptide, it may be that the degree of membrane perturbation associated with fusogenicity is important to the overall haemolytic activity. Indeed, the NMR data given in this application show that the sequence similarity of Nef2-26 to the haemolytic peptide melittin extends to a structural similarity in the 9-26 region. Unlike melittin, however, the Nef N-terminal 7 residue sequence lacks hydrophobic residues with the exception of Trp4. Adding the myristyl chain would increase the hydrophobicity of this region, possibly making Nef 2-2n peptides behave more like

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melittin. On the other hand, Nef 2-22d is still quite haemolytic although the  $\alpha$ -helix apparently does not commence until around residue 14. It should be noted, however, that sequence changes that even slightly reduce the  $\alpha$ -helicity of melittin render it non-toxic, although simply shortening the peptide at the C-terminal end by 3-5 residues has little effect (cf the very slight activity differences between Nef 2-22 and Nef 2-26)

The kinetic data suggest that a major difference brought about by sequence changes that reduce haemolytic activity is in the kinetics of haemolysis by the modified peptides. All are fairly lytic if left in contact with red cells for 160 min. but the full-length peptides with the more positively-charged 2-7 regions act on red cells much more rapidly. These observations may explain, in part, why the modified peptides were more cytotoxic towards CEM cells. Here, exposure to the peptides was of the order of 2-4 hr. Additionally, it is possible that the 9-22 residue helical region may be internally toxic to the cells after endocytosis, i.e. acting not only on the plasma membrane which in the case of a more complex cell like the lymphocyte is less likely to be irreversibly damaged by the peptide at the low concentrations used. Supporting an internal toxicity role for Nef 9-22 are the data showing that Nef 31-50 is significantly haemolytic after 160 min., whereas it is of low toxicity towards CEM cells.

The film balance data show that the ability to interact with membrane lipid is not a sufficient condition for lytic activity. If anything, the poorly-lytic Myr-Nef 31-50 reacts more strongly with DPPC monolayers than the lytic Myr-Nef 2-22. On the other hand, it should be noted that non-Myristylated Nef 2-22 does not penetrate the monolayer to any extent showing that myristoylation is essential for lipid interaction, although it should be noted that sufficient interaction does occur with the SUV membranes to bring about fusion.

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There is a remarkable correspondence between the proximate N-terminus of Nef and that of a number of Myristylated proteins of the src family (Silverman and Resch, 1992) (Table 9). All have a net positive charge due to the presence of lysine residues, and all have one or more serine residues. The lysine residues have been shown to be essential for the membrane targeting of these Myristylated proteins. As in the case of Nef, it is assumed that the presence of the positive charge which can interact with the negatively charged lipids of the membrane is essential to reinforce the weak membrane targeting properties of the myristyl chain. It has been suggested, moreover, in the case of the src proteins that serine phosphorylation with its consequent neutralisation of the N-terminal positive charge could provide a "switch" mechanism to mediate the membrane interactions of these proteins (McLaughlin and Aderem 1995). Since it is known that Nef interacts with a number of kinases (Sawai et al 1995), and phosphorylated serine residues have been found at the N-terminus (Bodèus et al 1995), it is possible that a similar mechanism operates to control the interaction of Nef with cell membranes and so modulate its interactions with other membrane-associated systems. Interference with such a switch mechanism could be an attractive therapeutic objective.

Table 9

Comparison of Nef proximal N-terminal sequences with those of Myristylated src proteins

|    |     |          |
|----|-----|----------|
|    | Nef | GGKWSKSS |
|    | src | GSSKSKPK |
|    | hck | GCMKSKFL |
| 35 | lyn | GCIKSKGK |

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Example 9: Further Studies of the Cytotoxicity of Nef N-terminus in Leukocytes and Lymphoid Tissue.

The effects of the Nef N-terminus on membrane integrity were investigated in a range of cultured eukaryotic cell lines and in freshly-isolated PBMC and lymphoid tissue cells. The human cell lines CEM, Jurkat, RPM1 8226, U266, THP-1 and U-937, PBMC and tonsil lymphoid cells used in these studies were obtained as described above under "General Methods". The peptides used and their cytotoxicity were respectively synthesised and measured in the LDH release assay as described above under "General Methods".

Results

Nef cytotoxicity in various leukocyte cell lines

A range of leukocytic cell lines were examined for susceptibility to peptide-induced cytotoxicity, using the LDH release assay, to determine whether the cytotoxic activity of the Nef N-terminus is restricted to CD4+ T cells. At a concentration of 10  $\mu$ M, Myristylated N-terminal Nef peptide was highly cytotoxic for both the Jurkat and CEM T cell lines, the U266 B cell line and the RC2a monocyte cell line, as shown in Figure 28A. After 2 hr, near-maximal LDH release was observed in all four cell lines. At a lower concentration of peptide (5  $\mu$ M), although all the cell lines tested were susceptible to Nef N-terminal cytotoxicity, some differences in the percentage of LDH release were apparent, as shown in Figure 28B.

30

Nef cytotoxicity in PBMC

Since cultured cells, particularly immortalised cell lines, may only superficially resemble cells in situ, freshly-isolated, uncultured PBMC were examined for their susceptibility to Nef N-terminal peptide-induced cytotoxicity using the LDH release assay. Marked cytotoxicity was observed in PBMC treated with Myristylated

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N-terminal Nef peptide (Myr-Nef2-26) after only 30 min (Figure 29). In contrast, non-Myristylated N-terminal Nef peptide (Nef2-26) and the control, Myristylated non-N-terminal Nef peptide (Myr-Nef31-50) caused little or no cytotoxicity. These results are similar to those for the CD4+ T cell line, CEM, shown in Figure 4A and suggest that primary human leukocytes (particularly lymphocytes and/or monocyte/macrophages) are also susceptible to Nef-mediated cytotoxicity.

Nef cytotoxicity in tonsil lymphoid tissue cells

Lymphoid tissues are the major site of viral replication and are progressively destroyed in HIV-infected individuals. The effect of Nef peptides on lymphoid tissue cells was assessed using cells freshly isolated from human tonsils. Myristylated N-terminal Nef peptide was cytotoxic for uncultured tonsil lymphoid cells, causing near-maximal LDH release after 30 min. While some LDH release occurred following exposure of cells to the Myristylated non-N-terminal control peptide, this was only apparent after 2 hr and levels were less than 25% of maximum. The non-Myristylated N-terminal peptide caused virtually no LDH release, even after 2 hrs. Results are shown in Figure 30.

The results of this study show that:-

- the structure of the Nef N-terminus comprises an alpha helix from residues 6 to 22, and less well-ordered structures in C- and N-terminal regions.

Other studies have shown that:-

- the N-terminus of Nef is highly conserved (residues 2-7) and important for a number of functions such as CD4 and IL-2R downregulation, and interaction with intracellular proteins,
- the deletion of residues 16-22 (amphipathic helix) abolished serine kinase binding and reduced infectivity,
- the deletion of residues 4-7 reduced infectivity,

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- the lymphoid tissue site is the site of a majority of viral replication and that the tissue progressively destroyed,

- the extracellular Nef detected in culture and serum  
5 has N-terminal protease sites and is present in virions mainly as a 20kD cleaved molecule (cleaved by the HIV PR).

The results of the studies described herein demonstrate that myristylation of the amino-terminal of the HIV Nef peptide renders the sequence cytotoxic to a number  
10 of cell types. Without wishing to be bound by any proposed mechanisms for the observed advantages, ultra-violet fluorescence studies indicate that the presence of a bulky hydrophobic residue such as tryptophan which is proximal to the myristylation site along with one or more polar  
15 residues at the Nef N-terminus on a flexible peptide segment could be sufficient to perturb the phospholipid head group and upper acyl chain regions of the phospholipid bilayer and lead to disorganization of the membrane. The importance of the N-terminus of Nef is highlighted by the  
20 fact that the artificially myristylated sequence comprising amino acid residues 31-50 of Nef does not have cytotoxic activities comparable to that of the N-terminus myristylated peptide.

Finally, Nef belongs to a suite of membrane  
25 active proteins associated with HIV. Others include the accessory protein Vpr which has regions capable of creating pores in membranes (Macreadie et al 1996, Piller et al 1996), and the gp41 transmembrane protein which has a number of membrane-active regions associated with lysis and  
30 fusion (Gallaher et al 19887, Gawrisch et al 1993). For example, the N-terminal gp41 peptide (519-541) is also lytic for red cells and toxic to cultured lymphocytes (Mobley et al 1992). It seems possible that during  
infection there may be a synergism between these membrane  
35 active proteins which would enhance cell membrane disruption and death.

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When Myr-Nef2-22 was labelled with FITC the killing activity of the peptide was totally abrogated. Such treatment attaches the FITC on to the exposed amino groups, which in the case of Myr-Nef2-22 would be the lysine residues. This suggests that these residues are involved in cytolytic activity of the Nef peptide. The observation that the lysine residues of Myr-Nef2-22 were also critical for function is of interest when compared to melittin.

10           The biological relevance of the current findings to HIV-1 pathogenesis are still unclear. Cell death is a feature noted in HIV-1 infection (Levy, 1993; Wei et al, 1995; Ho et al, 1995; Finkel et al, 1996) and such death could be mediated by a transmitted factor. The possibility  
15   that such a factor could be Nef makes this study all the more relevant. The appropriate presentation of Nef in vivo still needs to be discovered. For example, certain breakdown products of Nef would be expected to have the cell killing activity described here. Several other  
20   peptides, including Myr-Nef2-20 and Myr-Nef2-26, produce effects similar to Myr-Nef2-22 in yeast and human cells. In addition, presentation of the N-terminus of Nef on a cell surface may be a mechanism of killing cells by contact. Alternatively, these studies may have relevance  
25   to the release of Nef and to the killing of cells producing high Nef levels.

          If cell killing by Nef is biologically relevant in AIDS pathogenesis, it is an advantage to reproduce the effect in a microbial system. Apart from allowing the  
30   mechanism of cell killing to be investigated, it enables methods for the convenient, rapid screening of inhibitors of such an activity to be developed.

          It is clear from the NMR data for this system that the helical structure is present in the C-terminal  
35   part of the N-terminal peptide molecule, consistent with the structure in methanol. It appears, however, that the N-terminal region is more structured (although not helical)

- 91 -

in the micelles, possibly due to interactions of the lysine residues at positions 3 and 6 with the negatively-charged detergent head groups. Similar interactions might also be possible with negatively-charged lipid head groups in  
5 biological membranes.

In aqueous solution no stable structure was observed for the Nef2-26 peptide. Addition of up to 300 mM phosphate buffer at pH 5.3 produced no change in the 1D spectrum. The Nef peptide, seems less prone to aggregation  
10 than melittin, presumably as a reflection of its smaller hydrophobic surface compared with melittin.

In conclusion, Nef is a multifunctional protein, and it has been shown that the N-terminus is of vital importance to some of these functions. In aqueous solution  
15 the N-terminus is unstructured, but is likely that the functions of this region of the protein involve interactions with cell membranes, in which environment the structure is probably significantly different from that in aqueous solution. The structure of Nef2-26 in methanol may  
20 be expected to resemble more closely the structure adopted by the N-terminus of Nef when it interacts with cell membranes.

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It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, there  
5 are modifications and alterations to the embodiments and methods described herein which may be made without departing from the scope of the inventive concept disclosed in the specification.

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10 following pages, and are incorporated herein by this reference.

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CLAIMS

1. A cytotoxic peptide comprising a myristylated Nef-amino terminal sequence, wherein the site of myristylation is proximal to a hydrophobic amino acid residue, derivatives or analogues thereof.
2. A cytotoxic peptide according to claim 1, wherein the site of myristylation is proximal to a hydrophobic and basic amino acid residue, derivatives or analogues thereof.
3. A peptide according to claim 1 or claim 2 comprising a first flexible domain with a net positive charge and a second,  $\alpha$ -helical domain.
4. A peptide according to any one of claims 1 to 3, selected from the group consisting of Myr-Nef2-20, Myr-Nef2-22a, Myr-Nef2-22b, Myr-Nef2-22c, Nef2-22d, Myr-Nef2-26, Myr-Nef2-26a, Myr-Nef2-10, Myr-Nef10-26 as herein described.
5. A peptide according to claim 4, wherein the first flexible domain comprises amino acid residues 2 to 8 having a net positive charge, and wherein residues 9 to 21 comprise the  $\alpha$ -helical domain.
6. A peptide according to any one of claims 1 to 5, further having lytic activity.
7. A peptide according to any one of claims 1 to 5, further having fusogenic activity with cellular membranes.
8. A method of screening putative inhibitors of cytotoxic, lytic, and/or fusogenic activities, comprising the step of measuring the effect on the activity of the peptide according to any one of claims 1 to 7 of the presence of one or more putative inhibitors.
9. A method according to claim 8, wherein the effect on the activity of a domain of the peptide according to any one of claims 1 to 6 of the presence of one or more putative inhibitors is measured.
10. A compound as listed in any one of Tables 3 to 6, which inhibits the activity of HIV, Nef or of a peptide according to any one of claims 1 to 7.

11. A compound according to claim 10, which is listed in Table 6.
12. A method of modulating interaction of Nef protein with a cell membrane, comprising the step of administering  
5 a compound according to claim 10 or claim 11 thereby to prevent the Nef protein from interacting with membrane bound components.
13. A method of reducing or eliminating the cytotoxicity of Nef comprising the step of administering a  
10 compound according to claim 10 or claim 11.
14. A method of treating HIV infection, comprising the step of administering a compound according to claim 9 or claim 11.
15. A pharmaceutical composition comprising a  
15 compound according to claim 10 or claim 11, together with a pharmaceutically acceptable carrier.
16. A method of inhibiting Nef-induced cytotoxicity in lymphoid tissue, comprising the step of administering a compound according to claim 10 or claim 11.
- 20 17. A method according to claim 16, wherein cytotoxicity in non-infected lymphoid cells is inhibited.
18. A method of inhibiting Nef-induced killing of bystander cells, comprising the step of administering a compound according to claim 10 or claim 11.
- 25 19. A method of inducing selective cell death, comprising the step of administering a peptide according to any one of claims 1 to 7.
20. A pharmaceutical composition comprising a peptide according to any one of claims 1 to 7, together with a  
30 pharmaceutically acceptable carrier.

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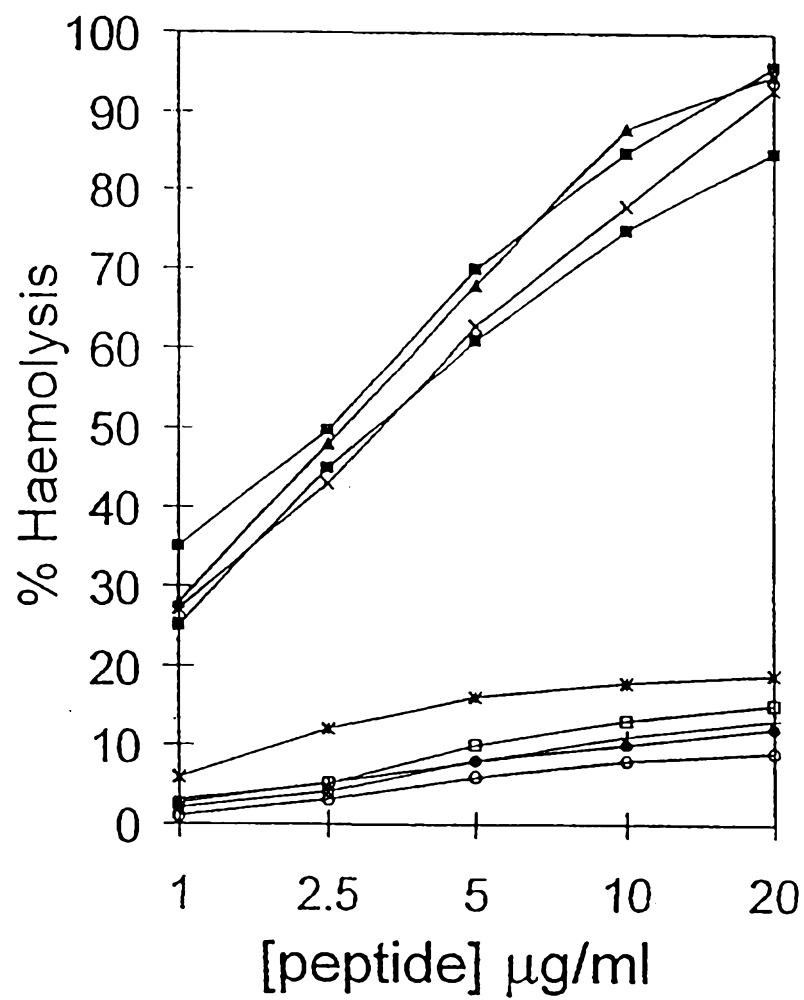


FIGURE 1

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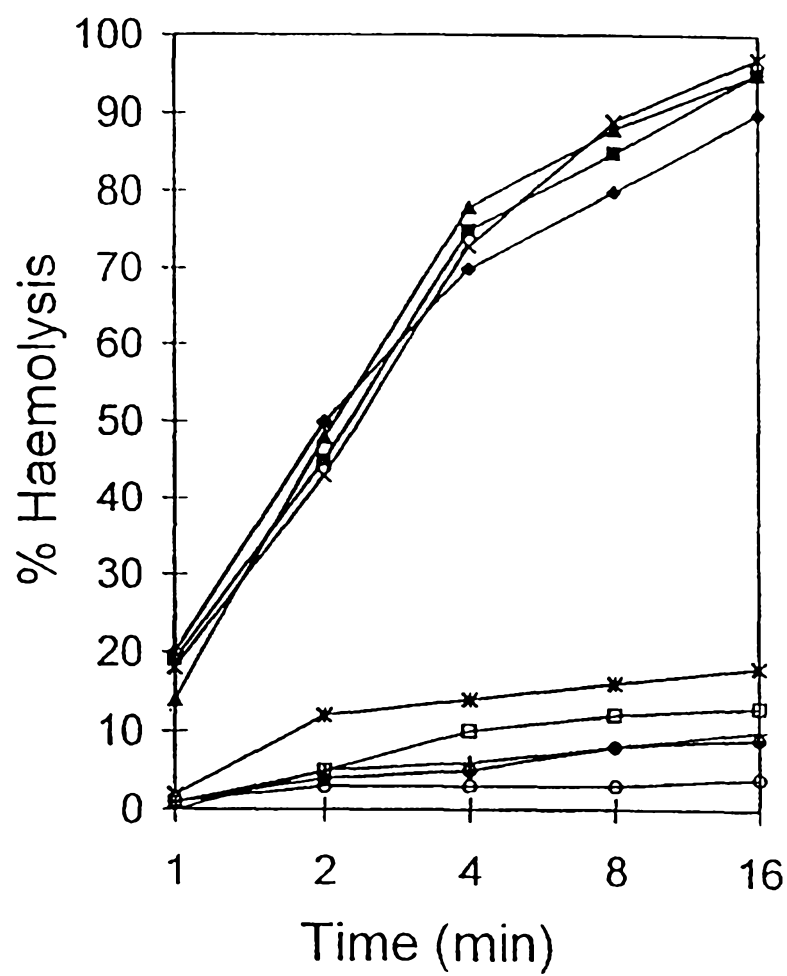


FIGURE 2

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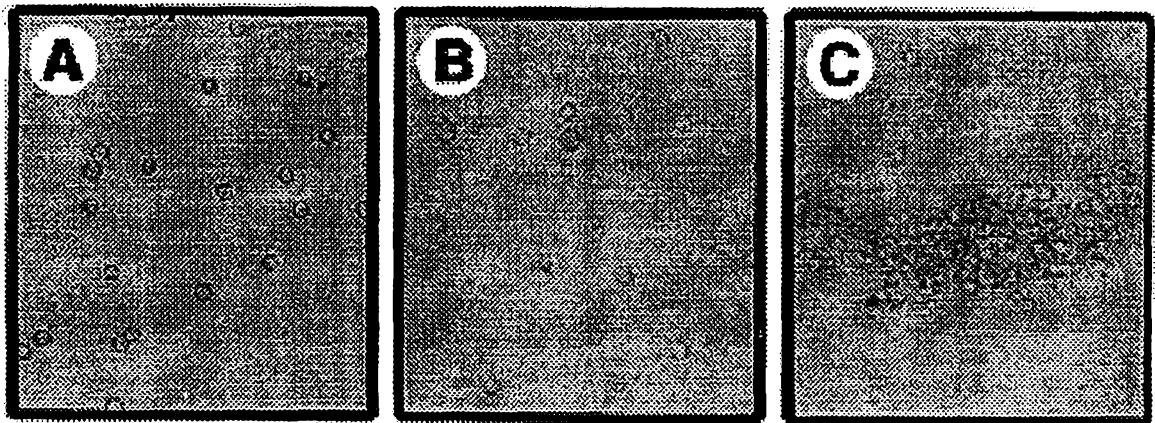


FIGURE 3

SUBSTITUTE SHEET (RULE 26)

4 / 34

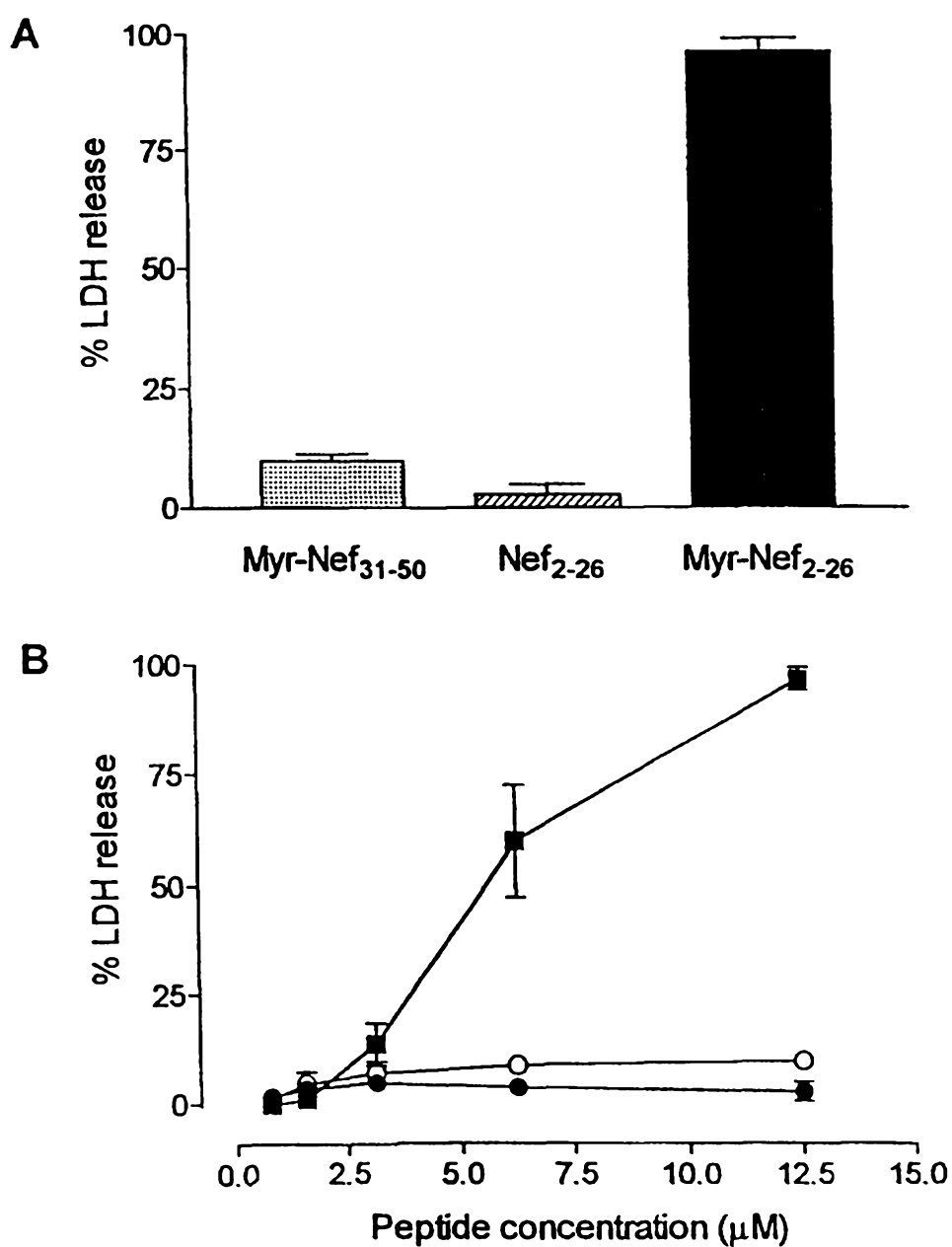


FIGURE 4

SUBSTITUTE SHEET (RULE 26)

5/34

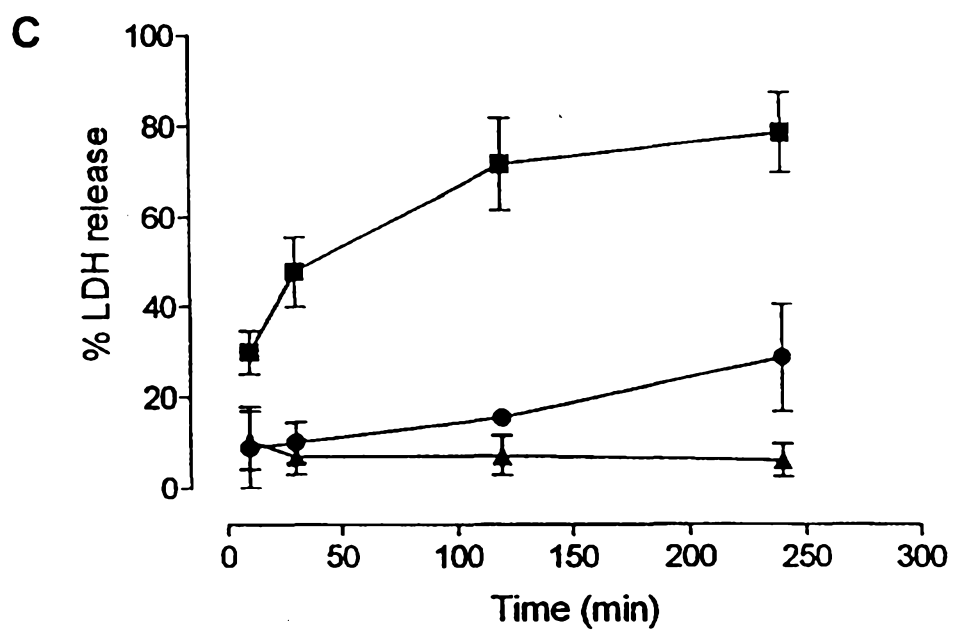


FIGURE 4 (cont.)

SUBSTITUTE SHEET (RULE 26)

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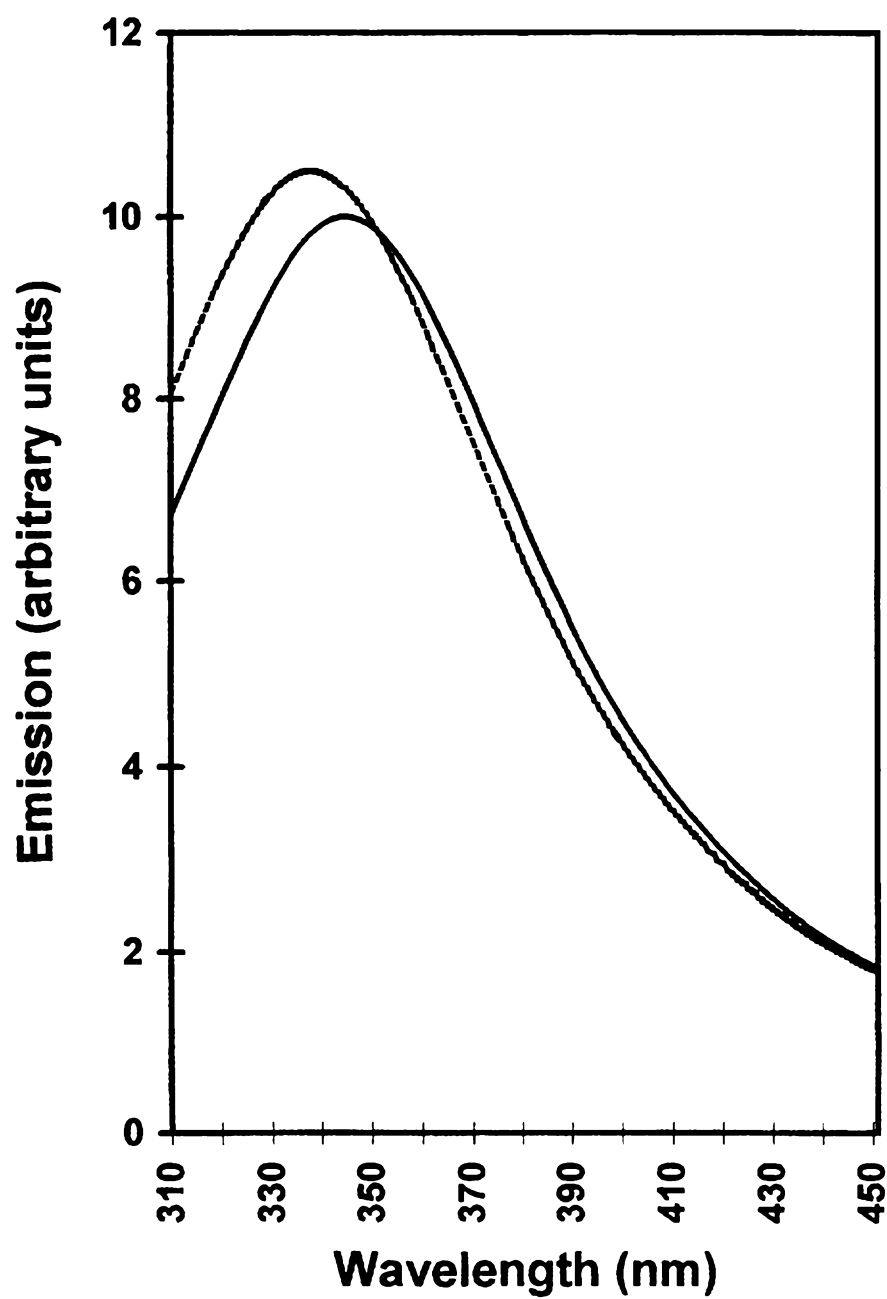


FIGURE 5

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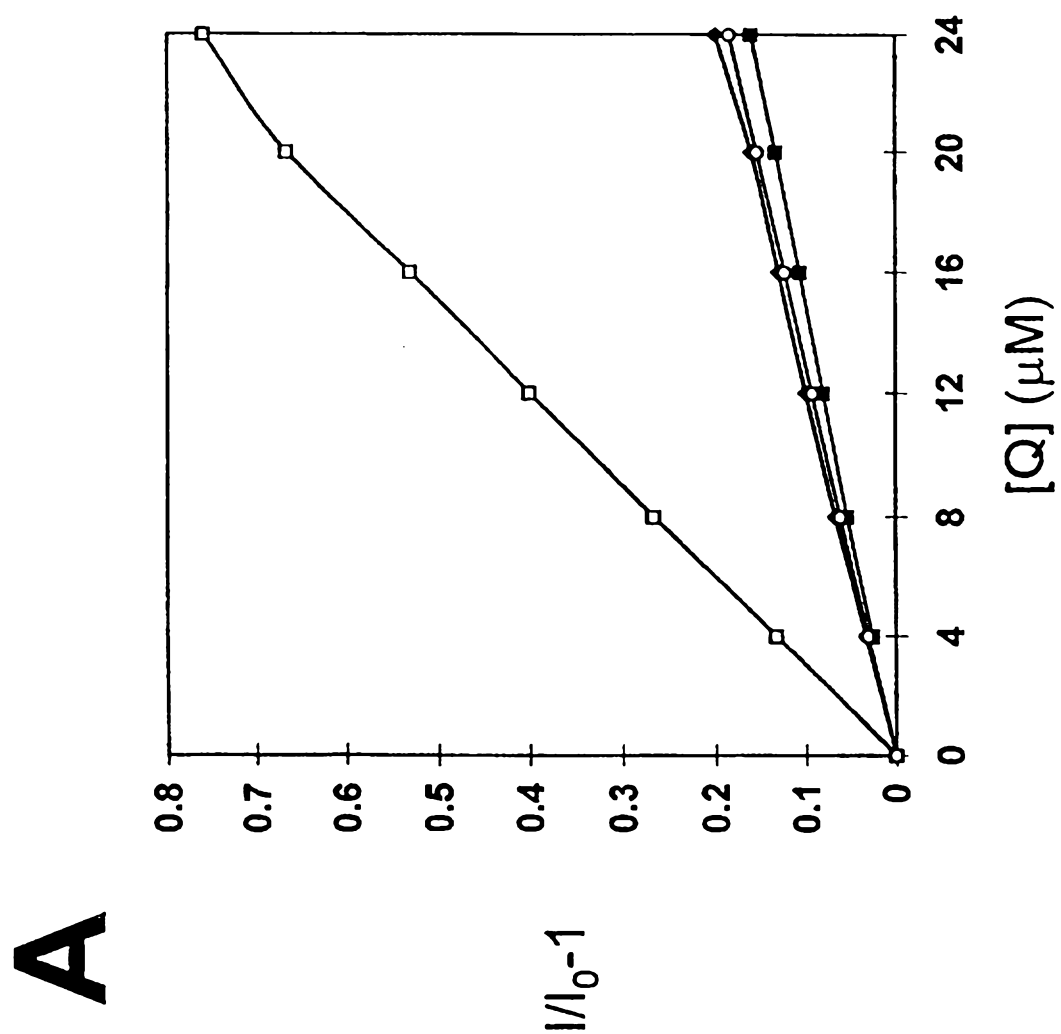


FIGURE 6

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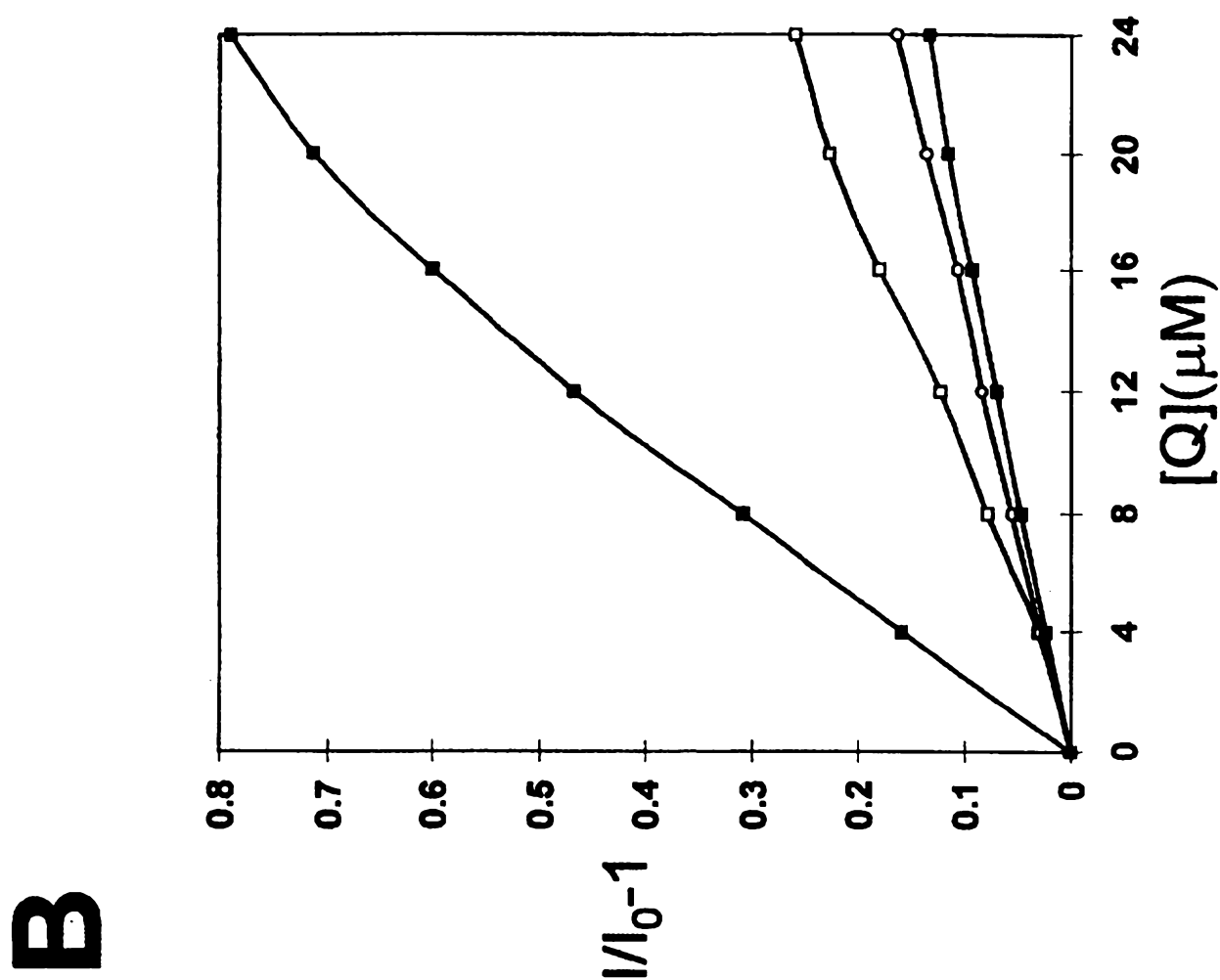


FIGURE 6 (cont.)

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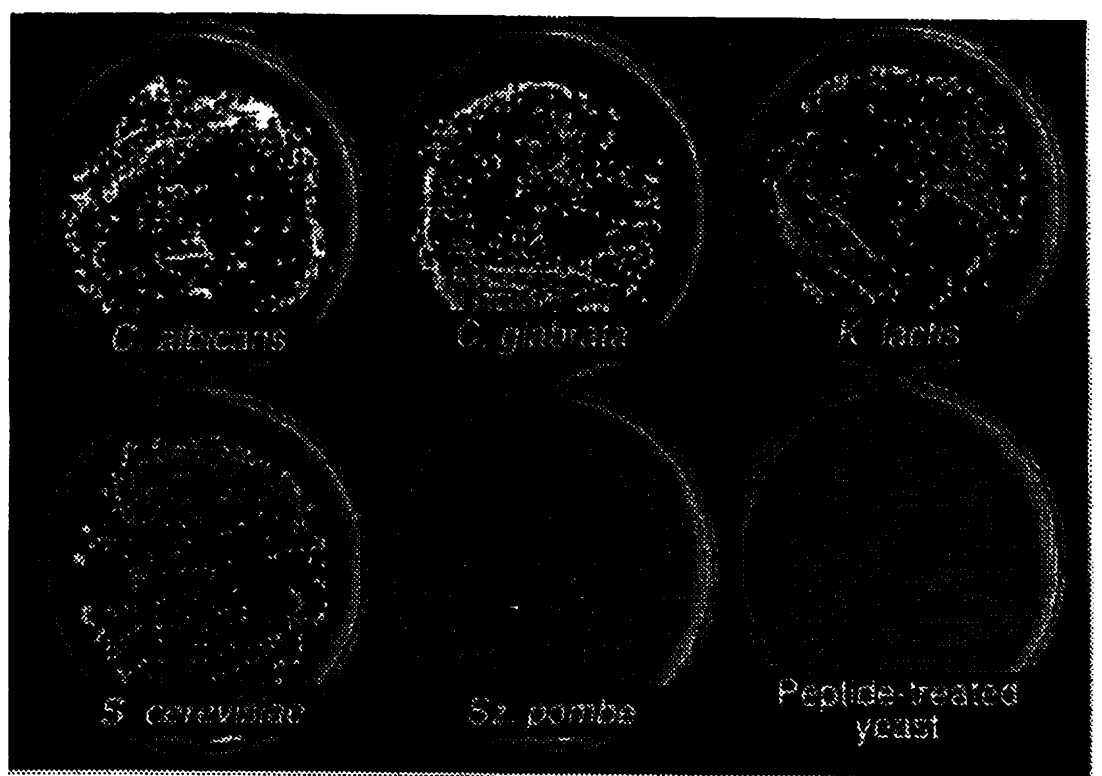


FIGURE 7

10/34

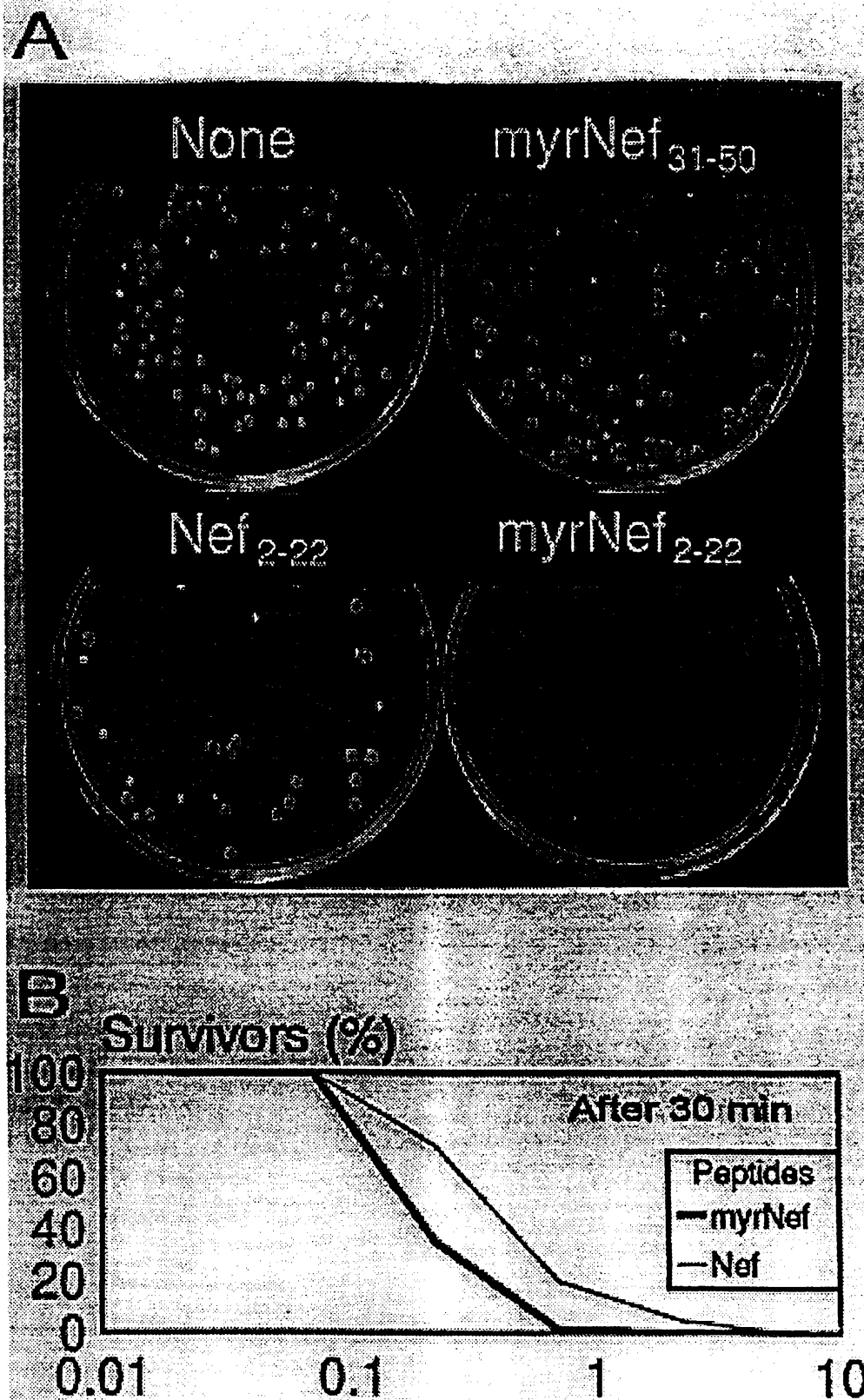


FIGURE 8

11/34

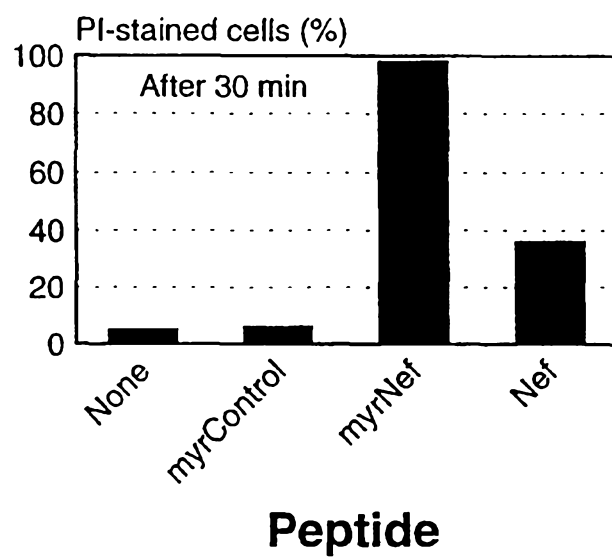


FIGURE 9

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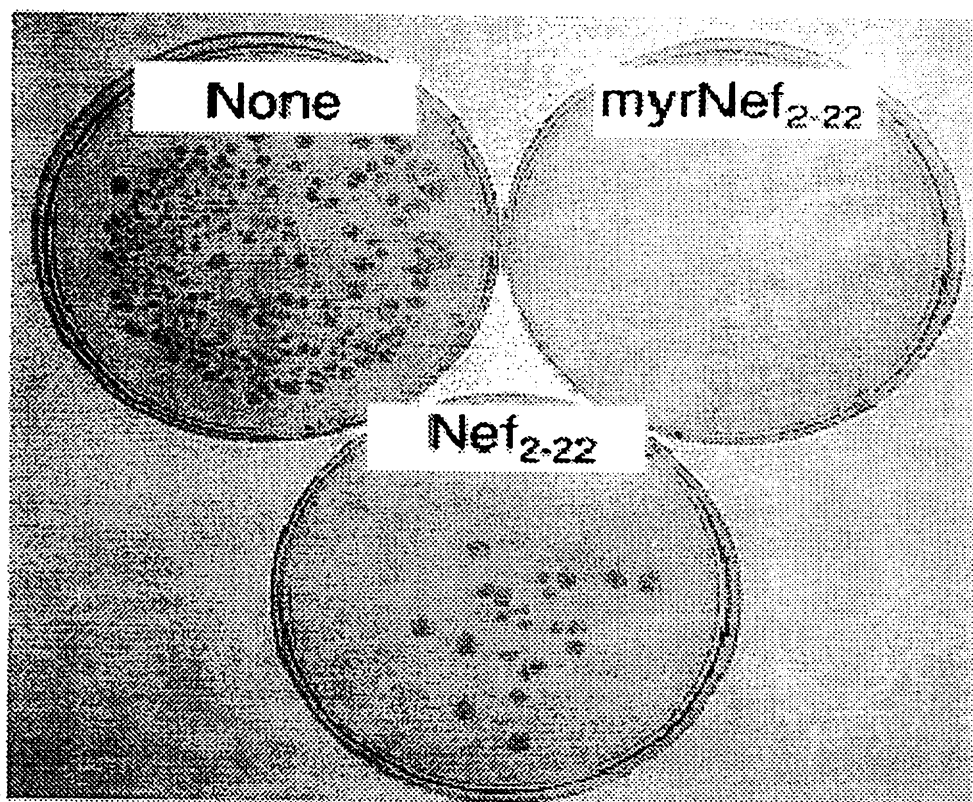


FIGURE 10

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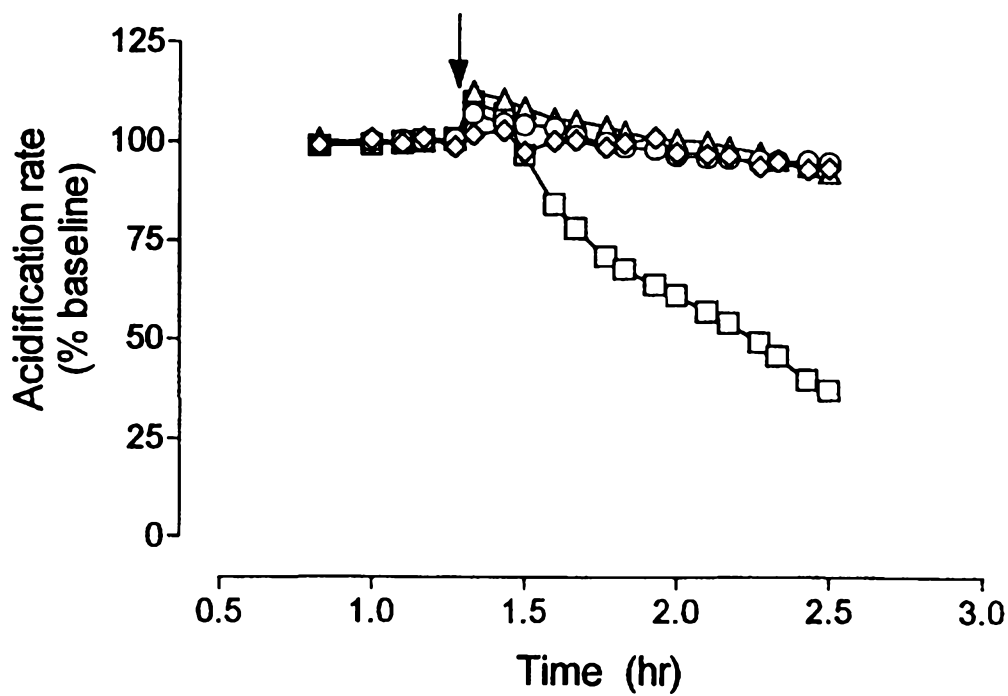
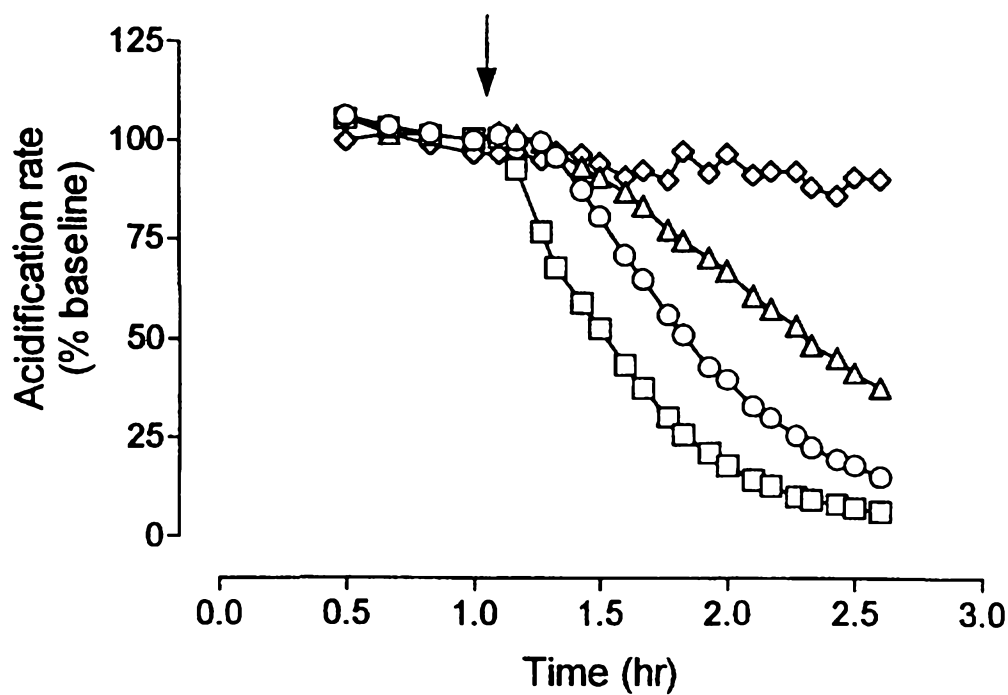
**A****B**

FIGURE 11

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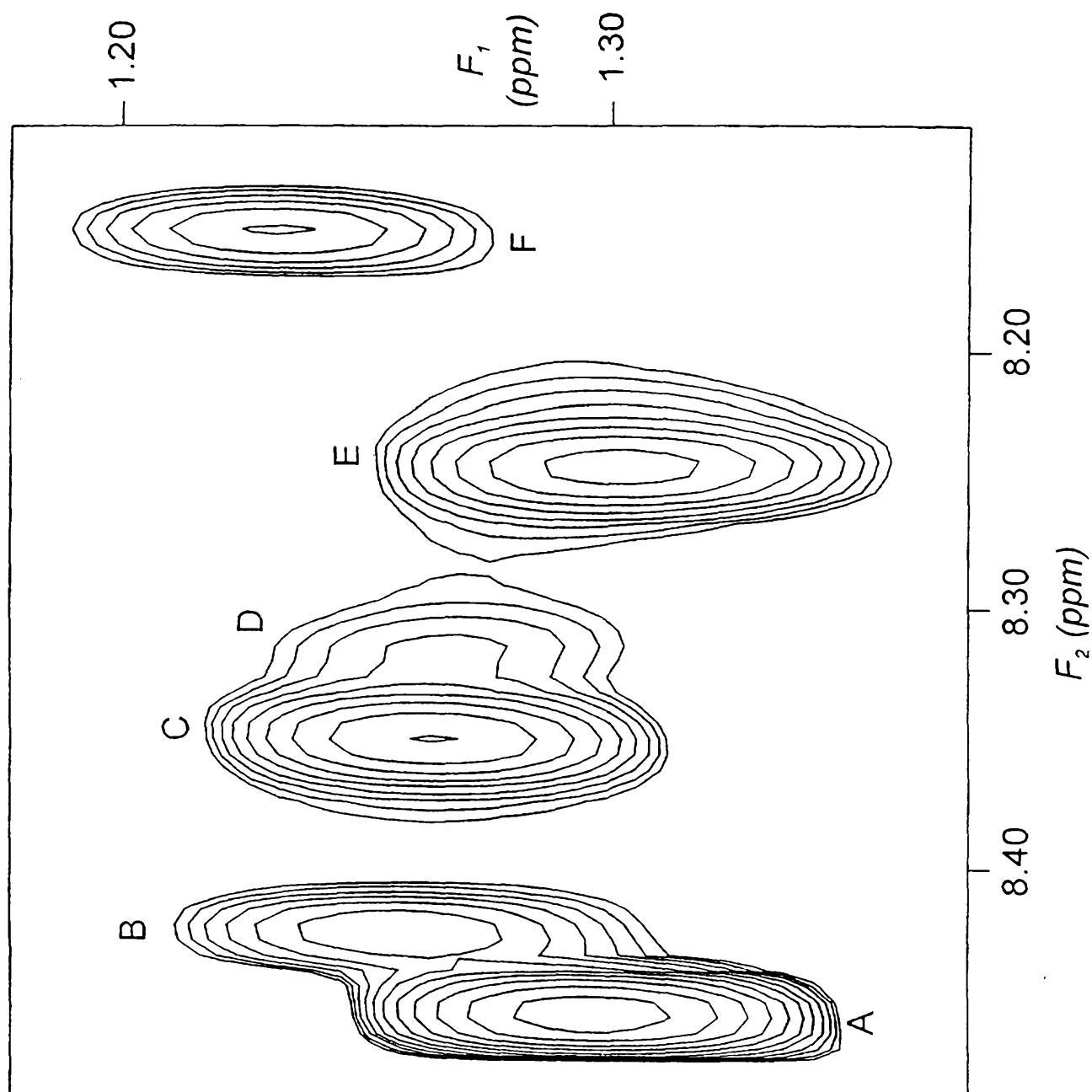


FIGURE 12

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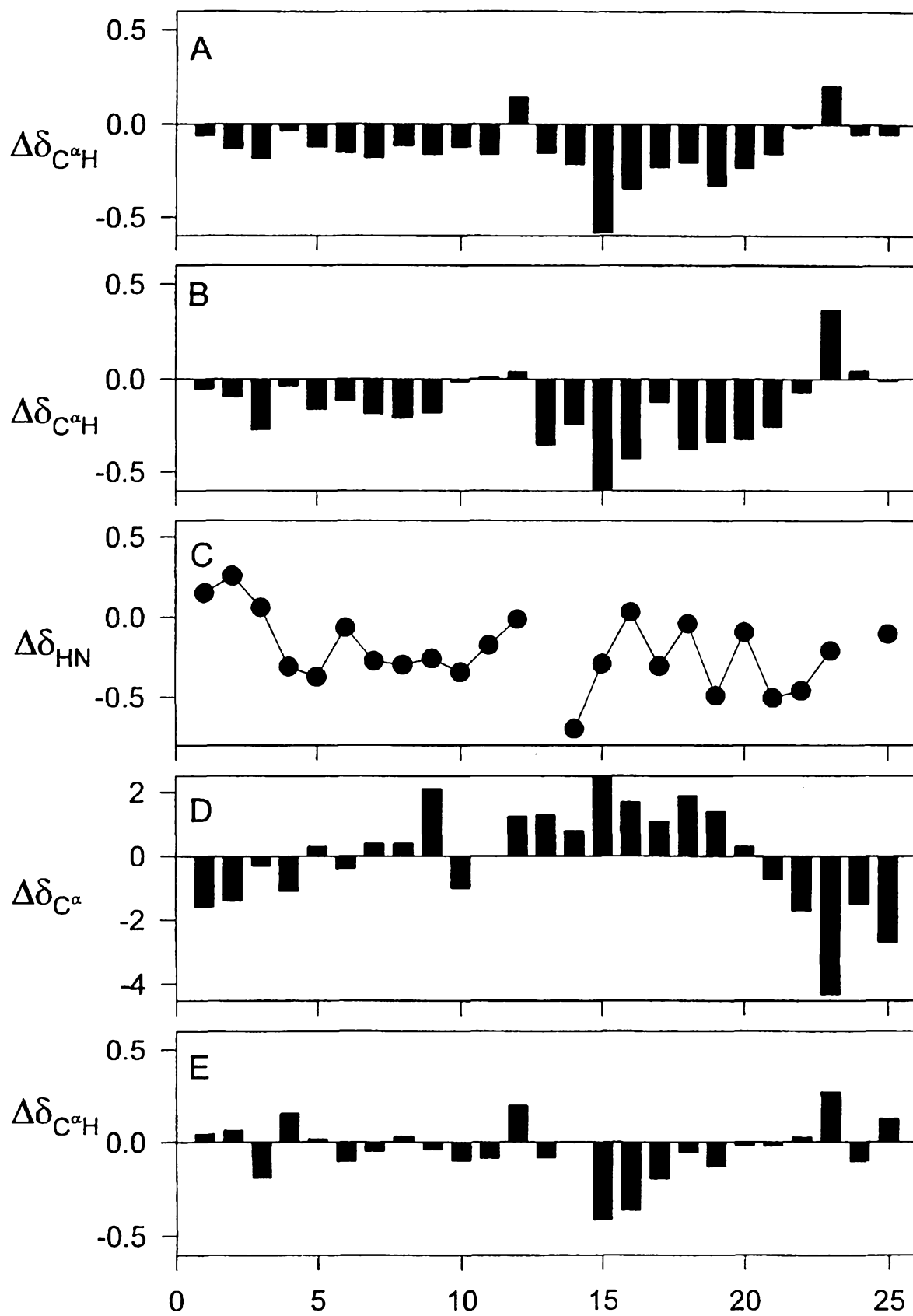


FIGURE 13

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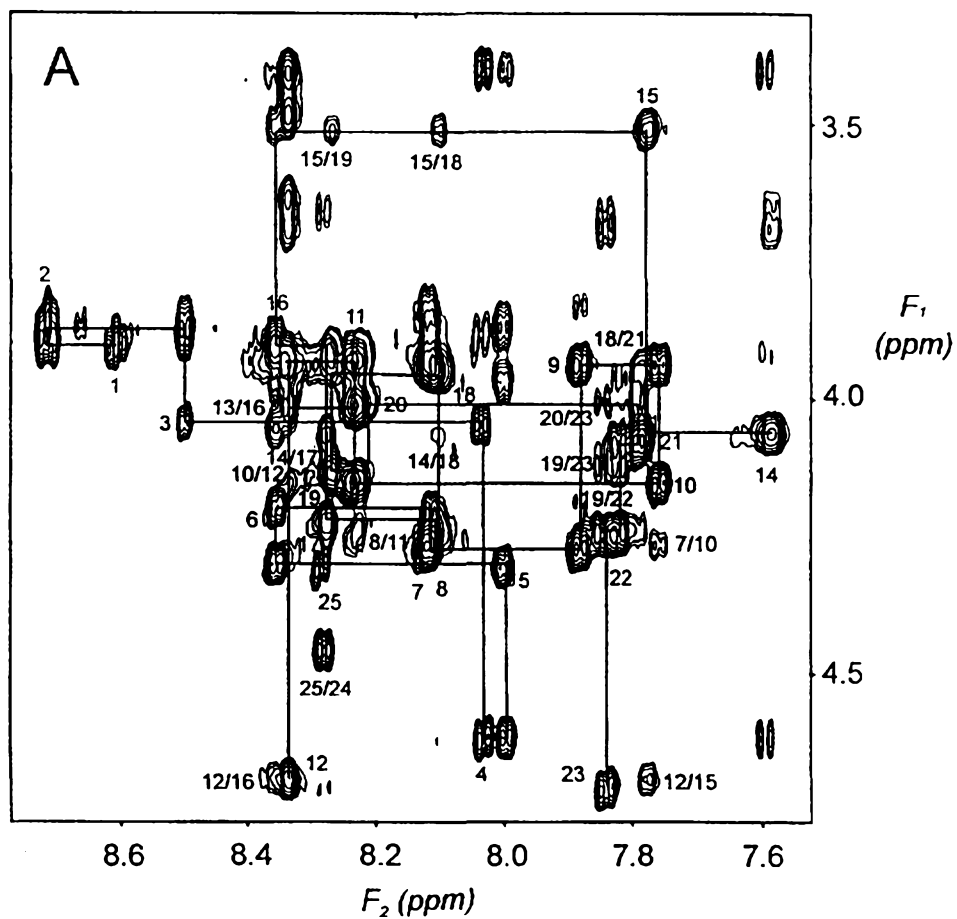


FIGURE 14

SUBSTITUTE SHEET (RULE 26)

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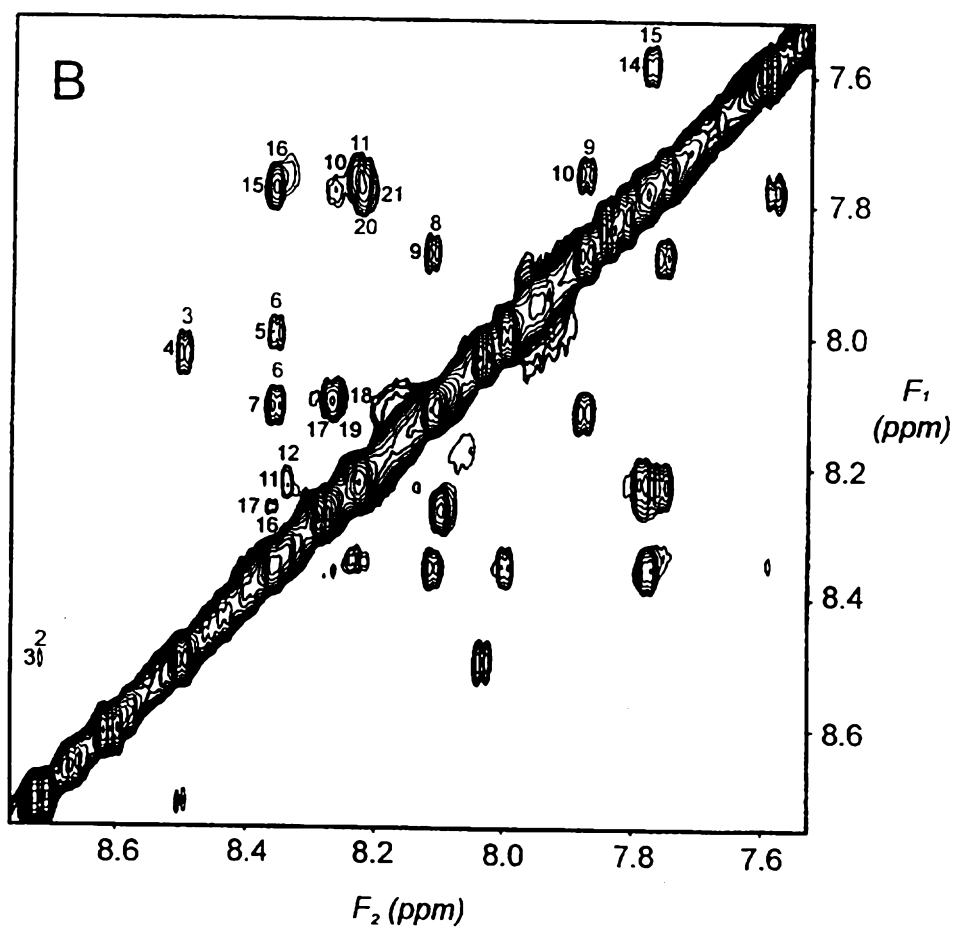


FIGURE 14 (cont.)

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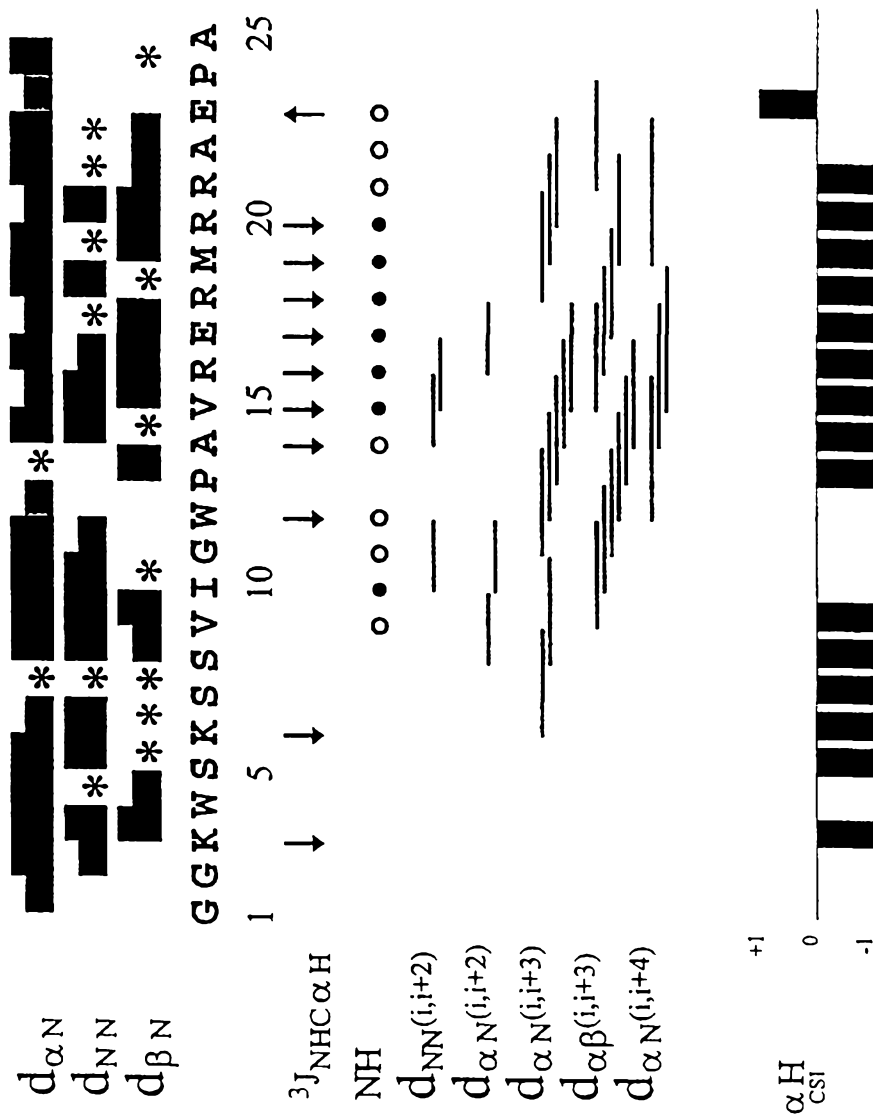


FIGURE 15

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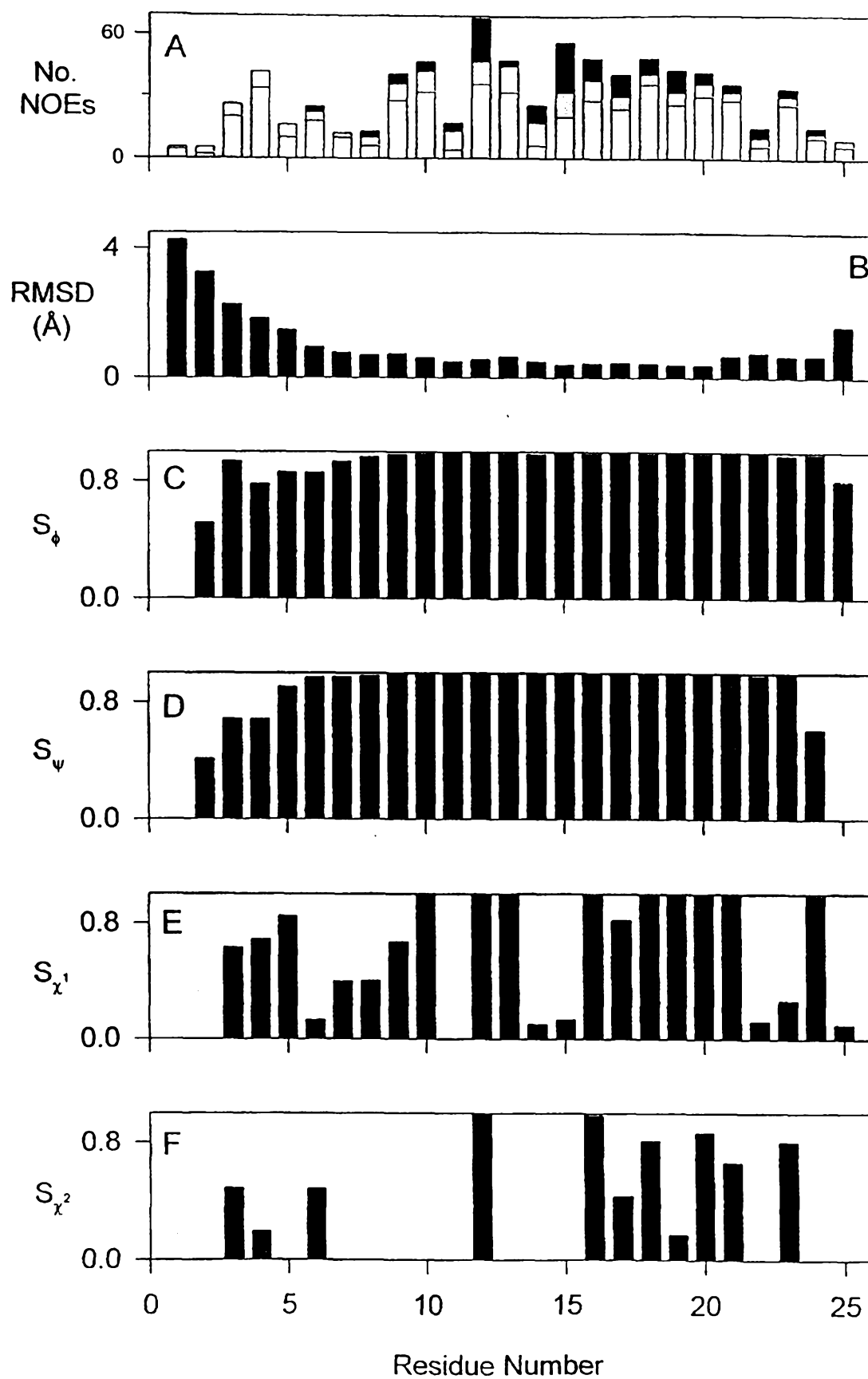
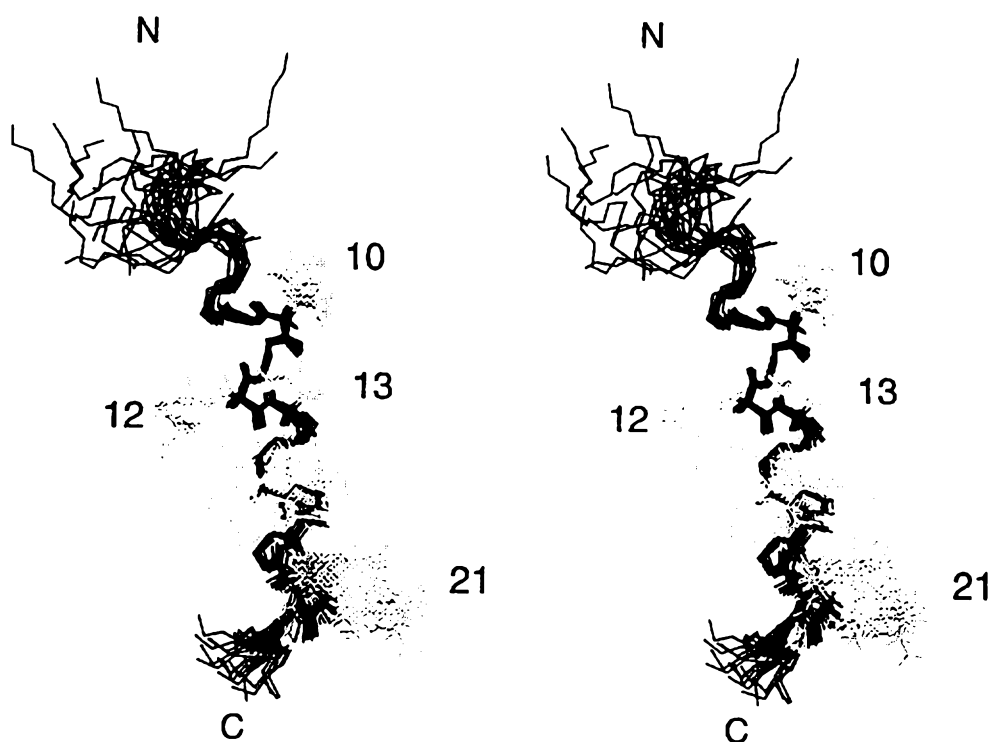


FIGURE 16

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A



B

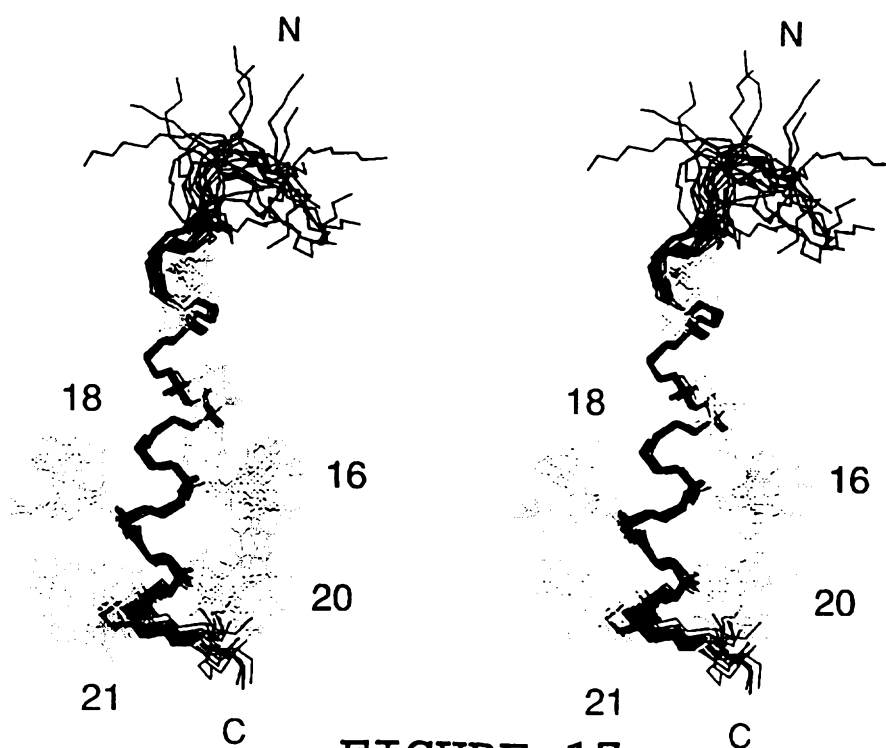


FIGURE 17

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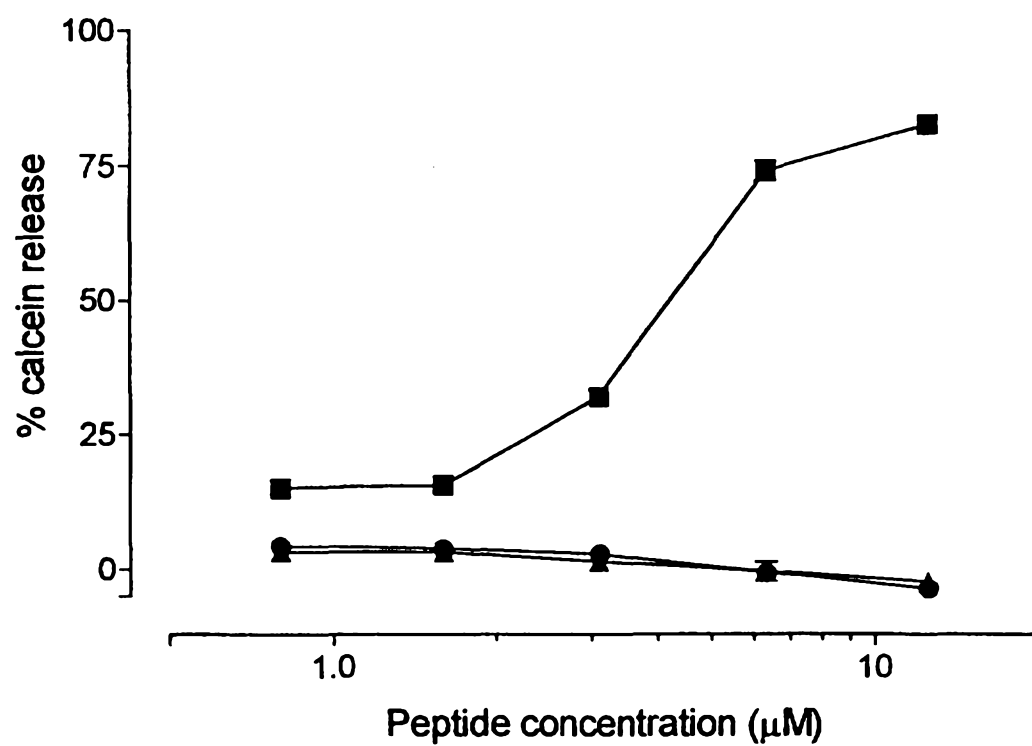


FIGURE 18

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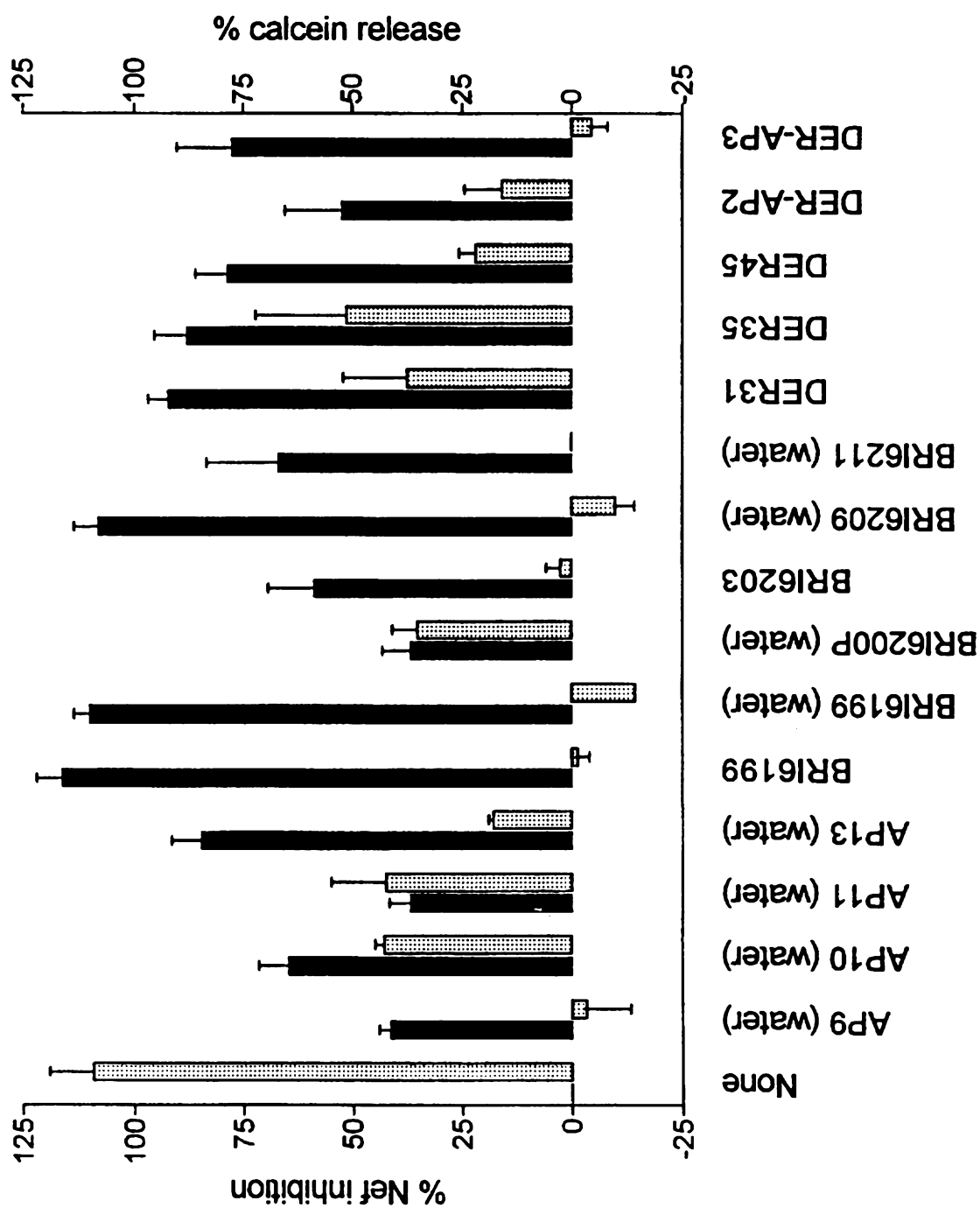


FIGURE 19

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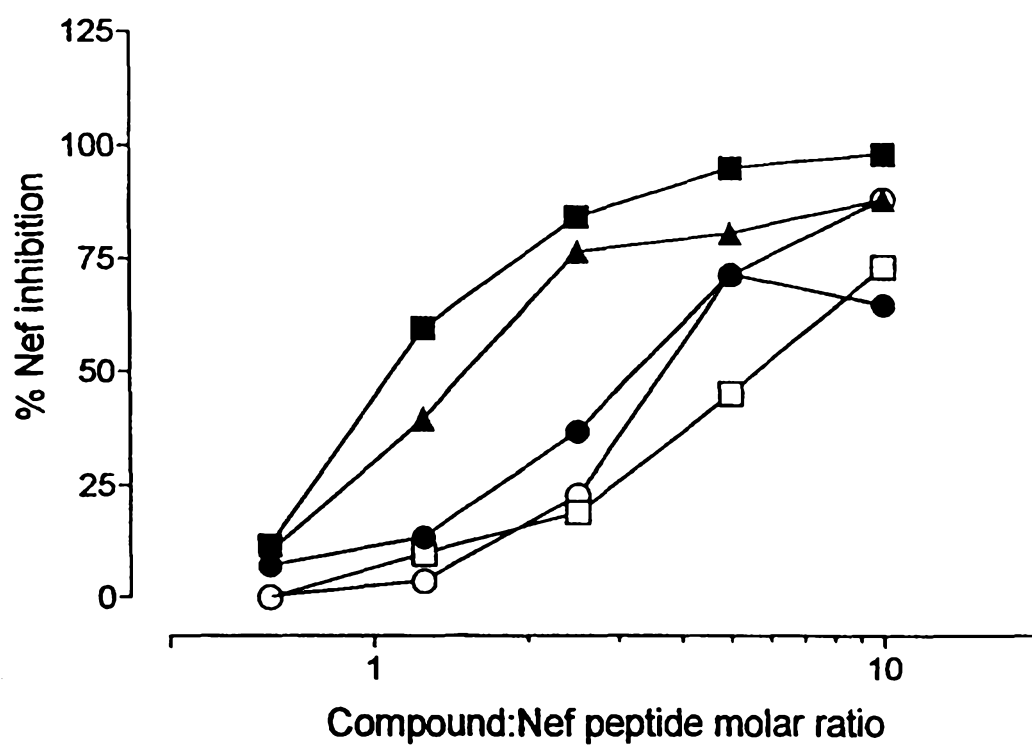


FIGURE 20

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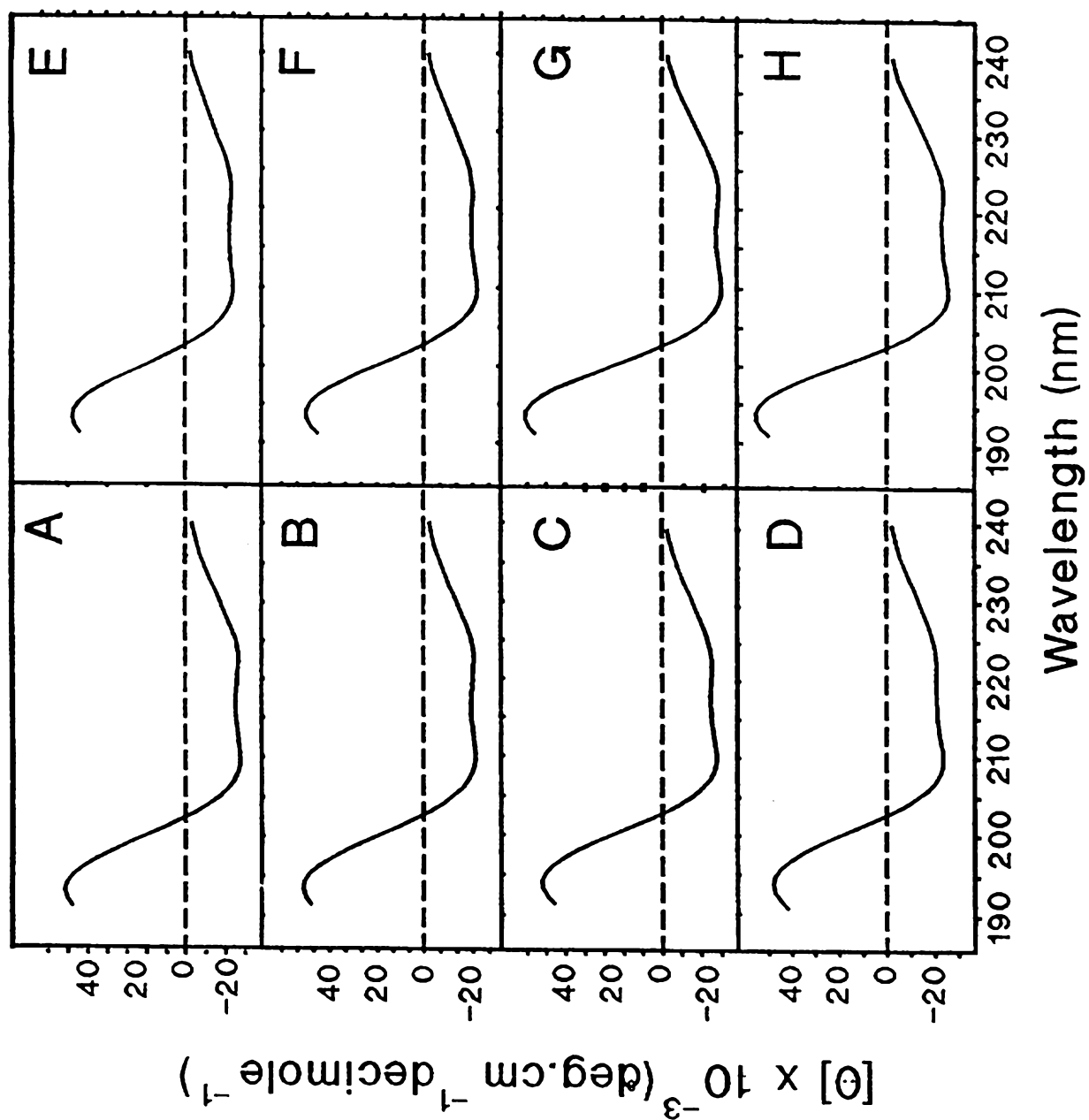


FIGURE 21

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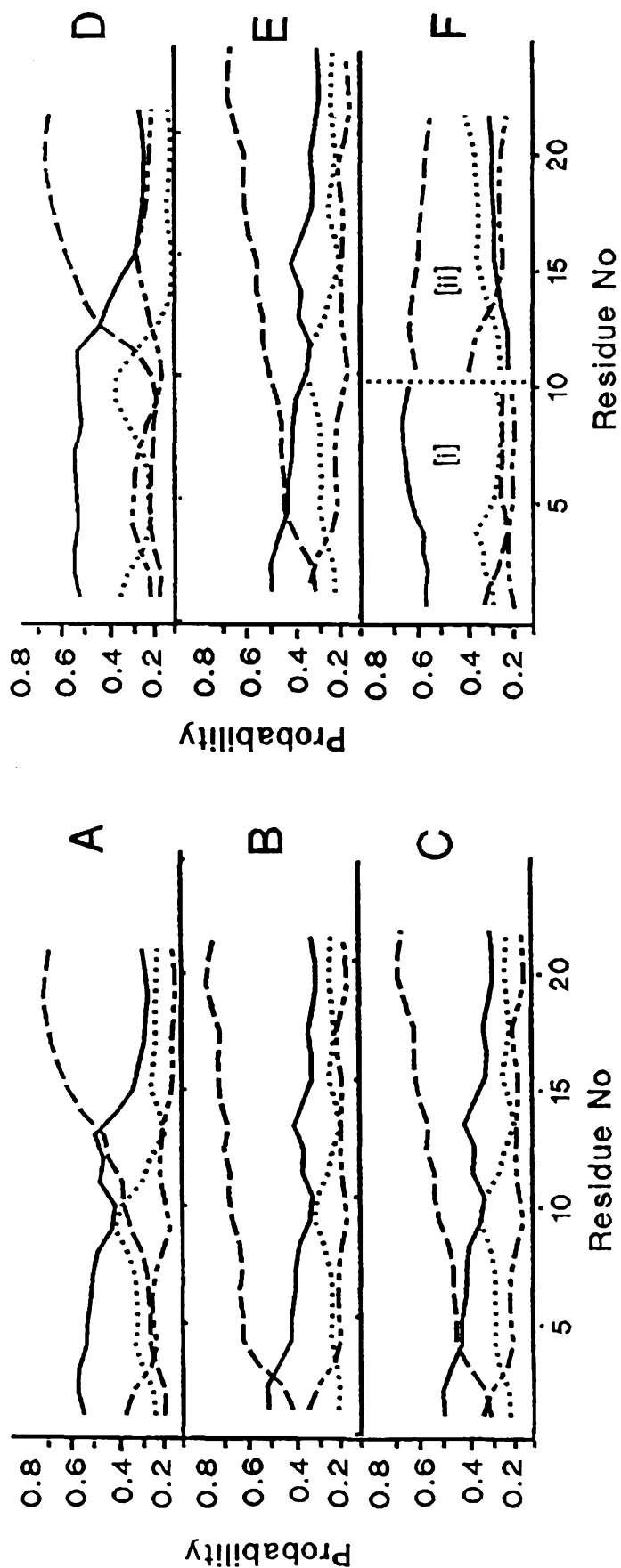


FIGURE 22

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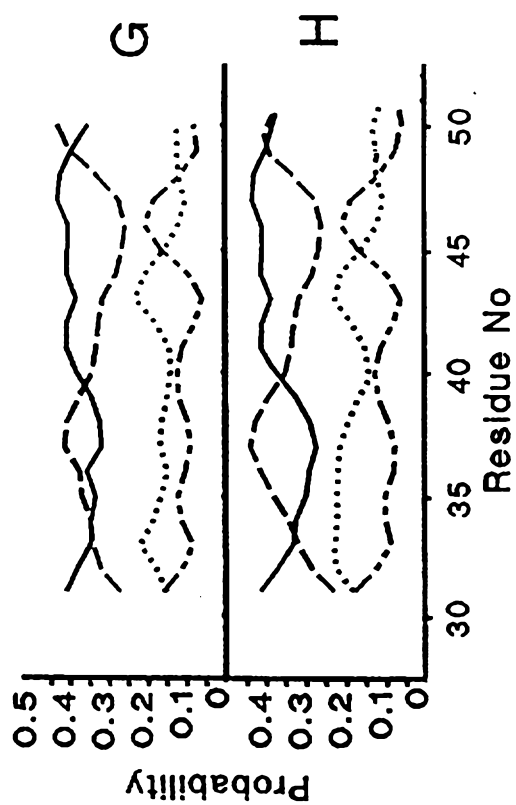


FIGURE 22 (cont.)

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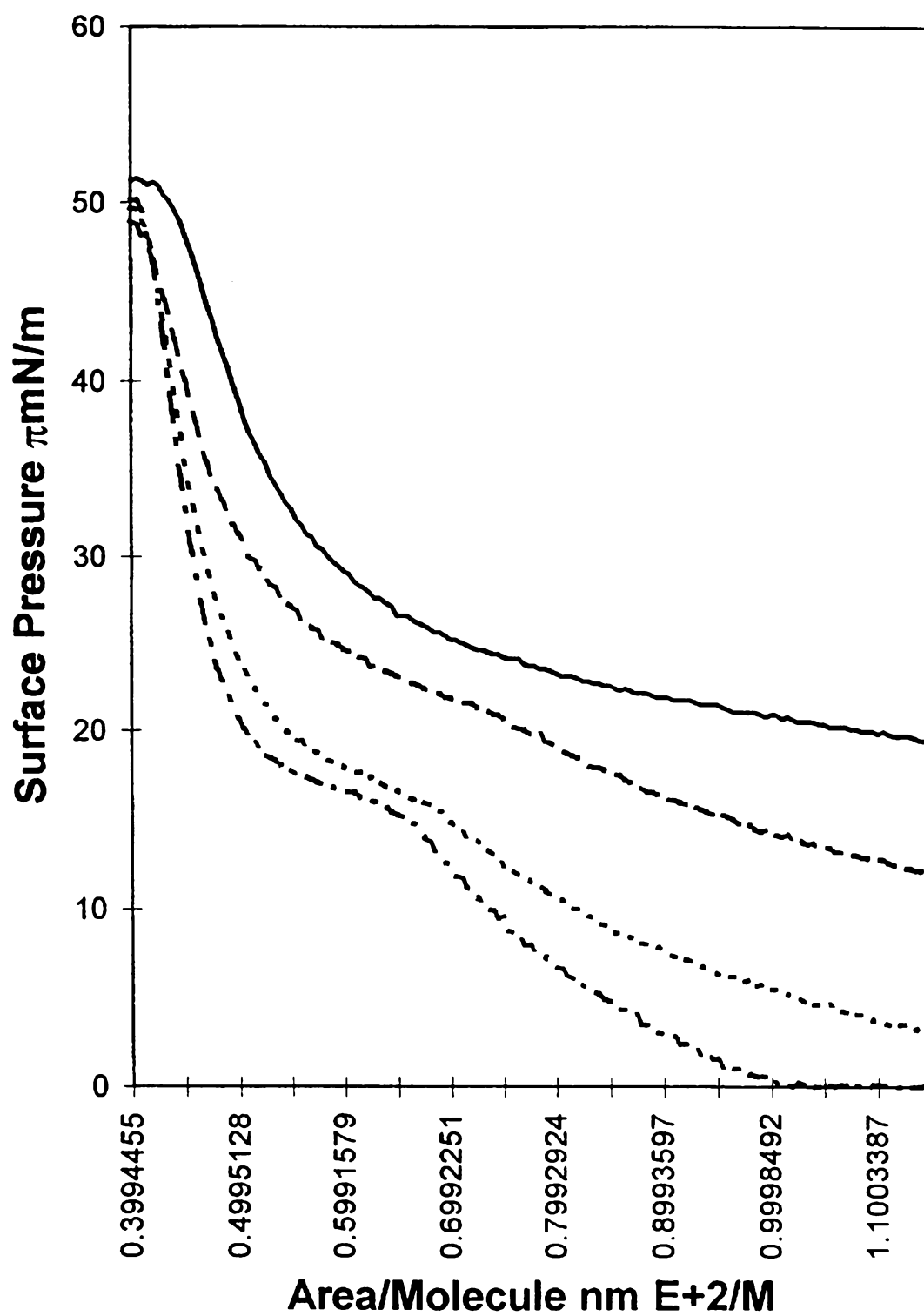


FIGURE 23

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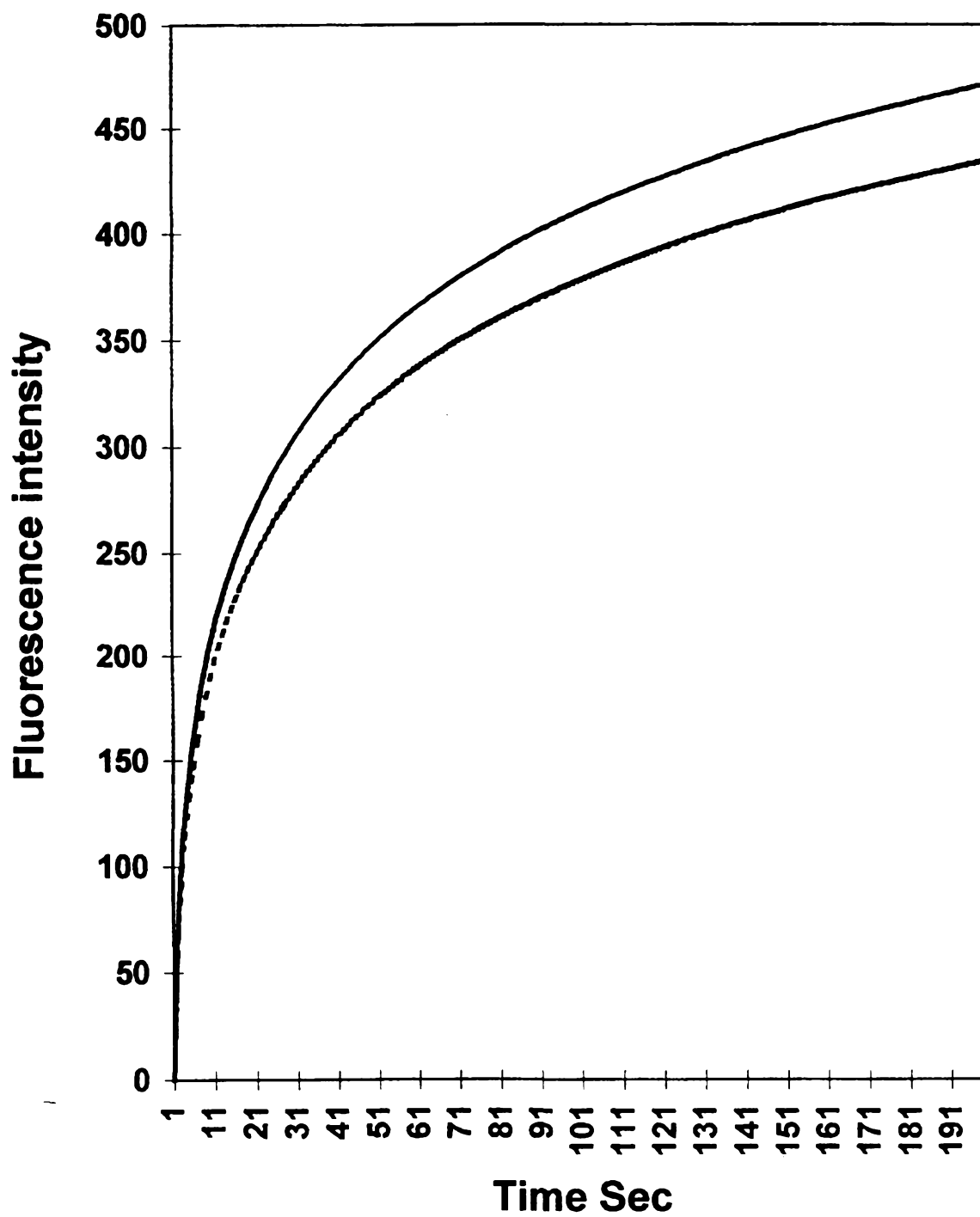


FIGURE 24

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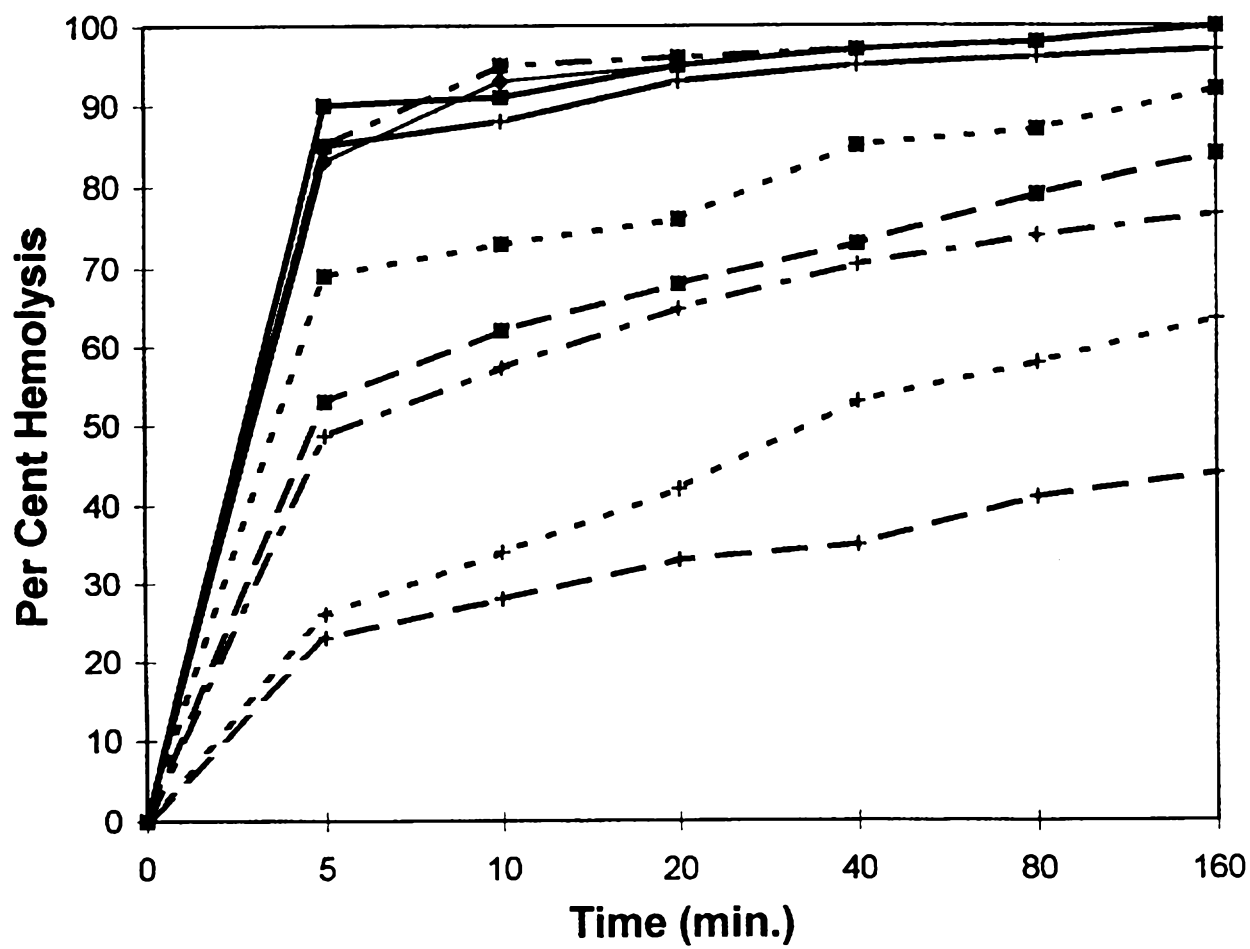


FIGURE 25

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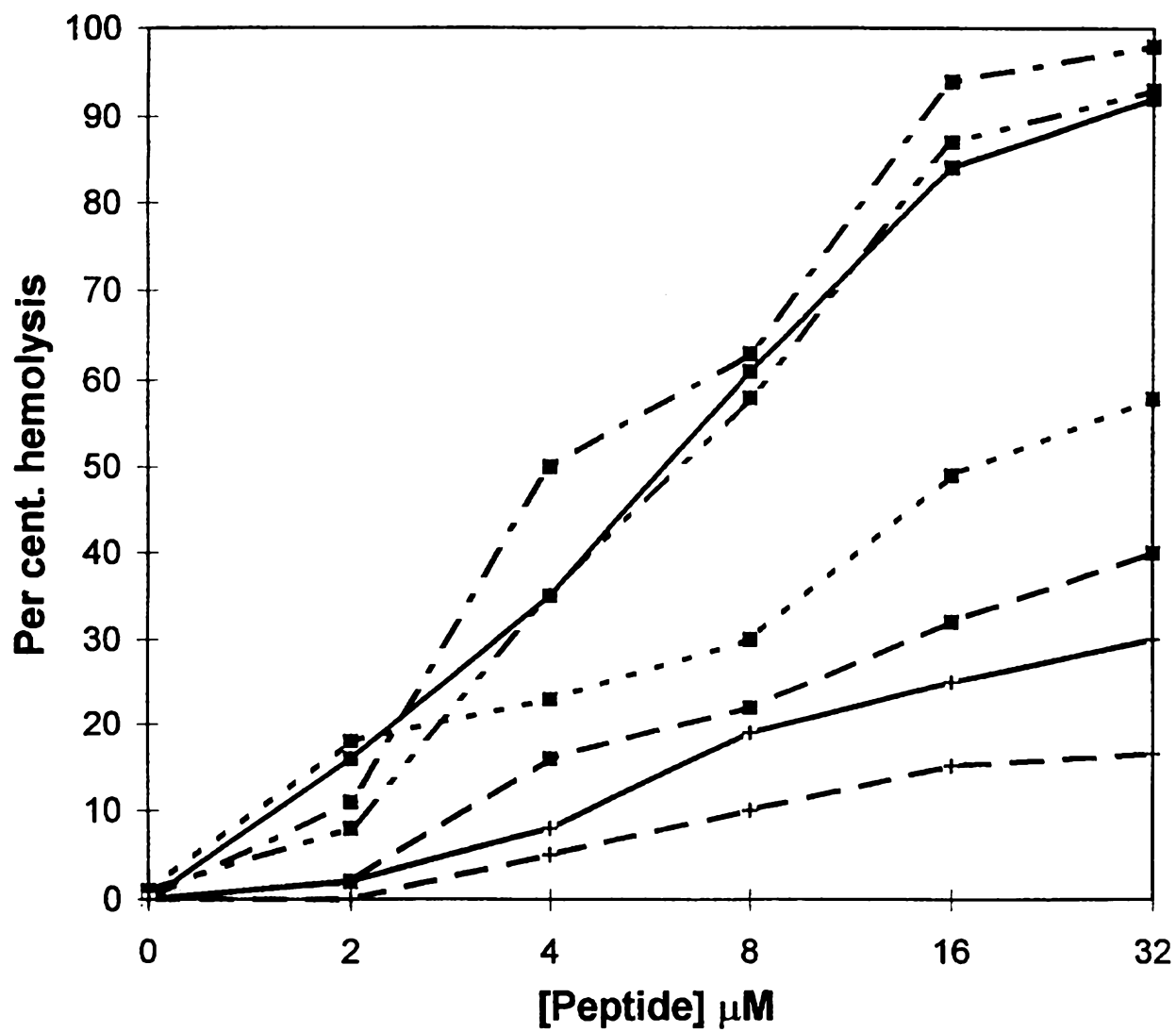


FIGURE 26

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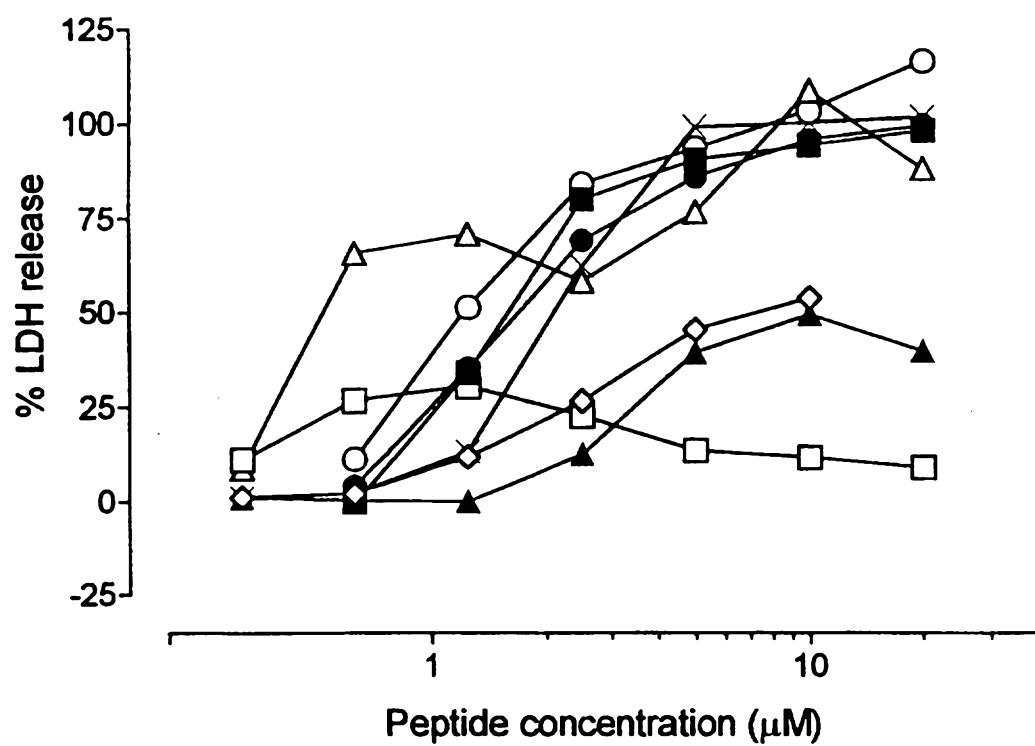


FIGURE 27

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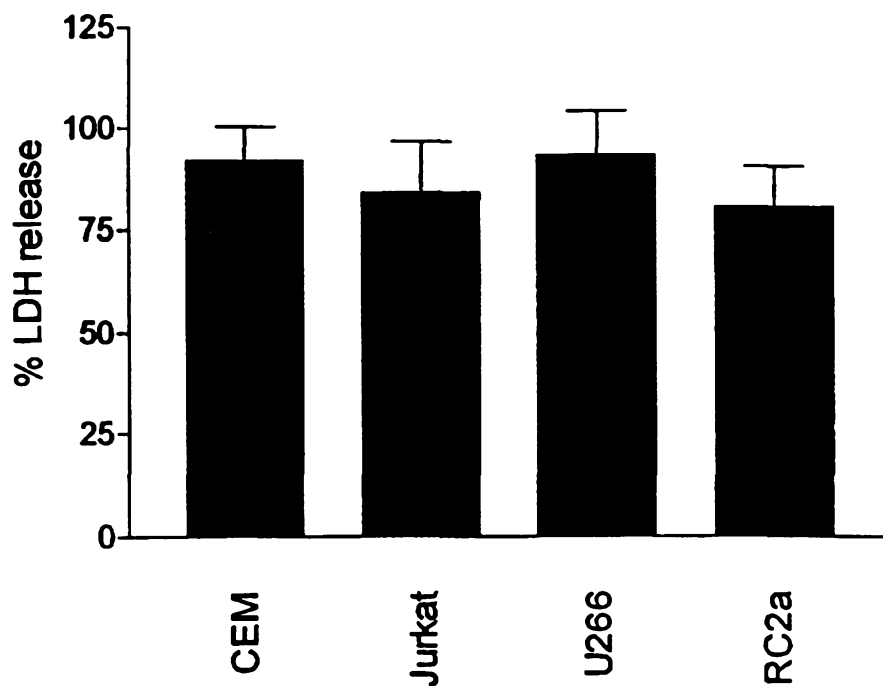
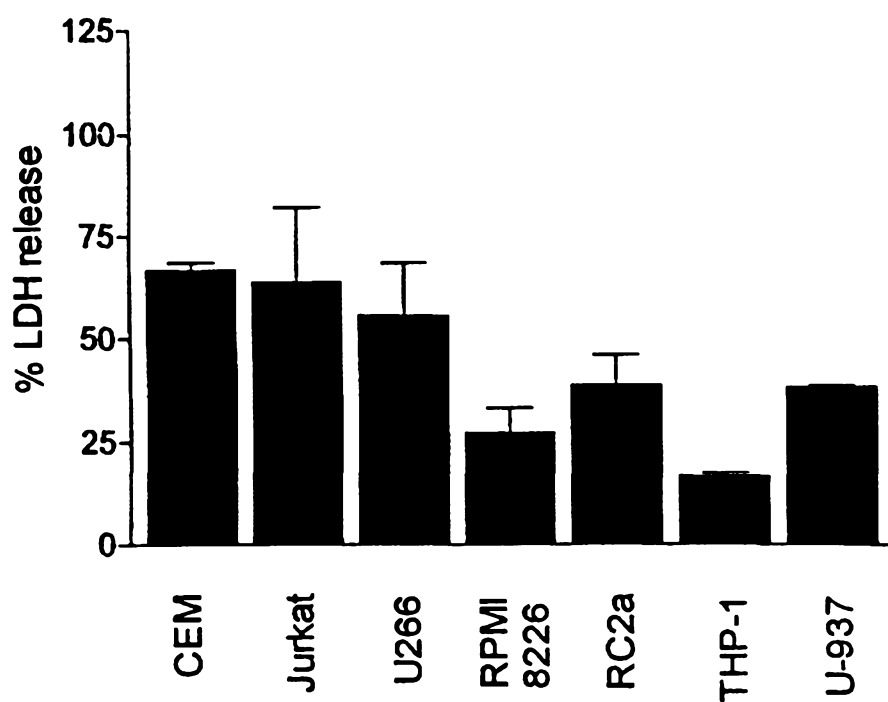
**A****B**

FIGURE 28

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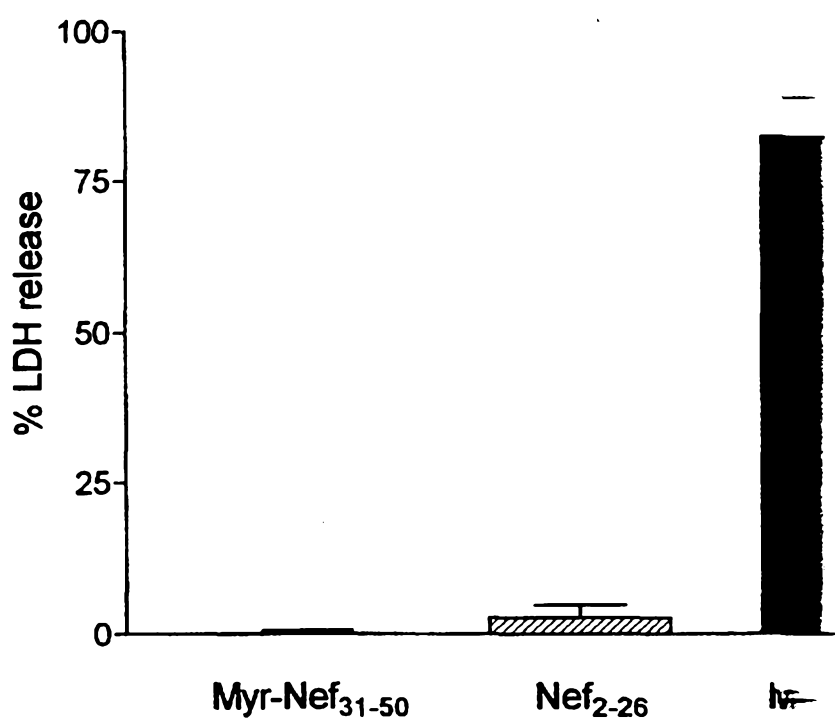


FIGURE 29

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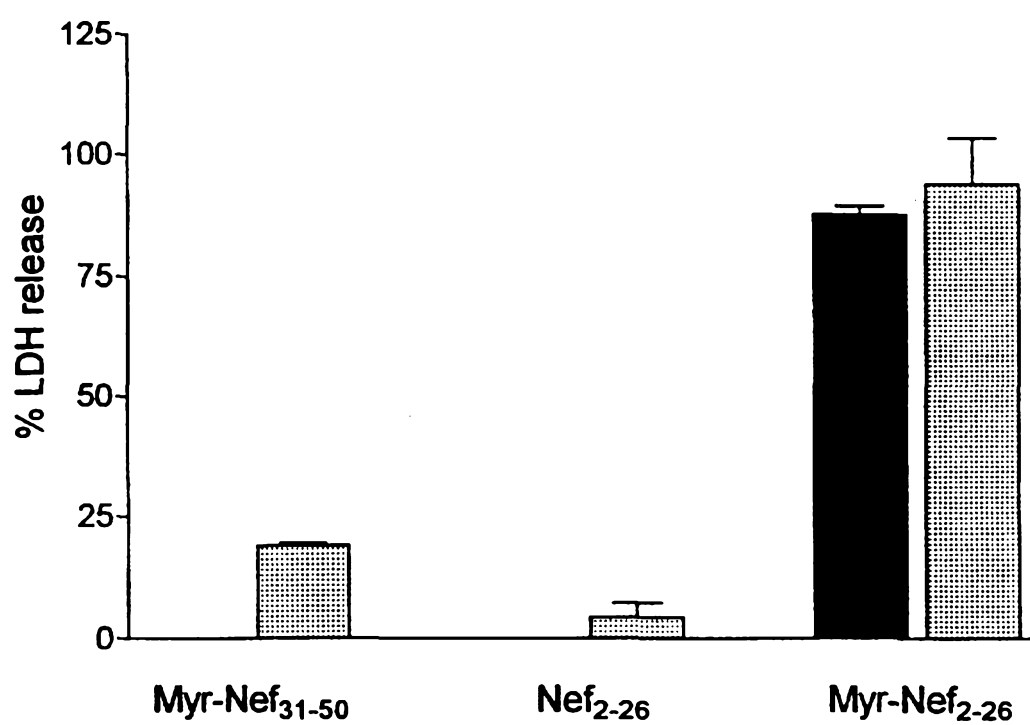
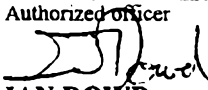


FIGURE 30

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00640

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|--------------------------------------------------------------------------|-----|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|--------------------------------------------------------------------------|-----|-------------------------------------------|-----|----------------------------------------------------------------------------------------------------|--|--|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Int Cl <sup>6</sup> : C07K 14/16, C07K 7/06, C07K 5/087, C07K 5/072, C07K 5/103, C12Q 1/02.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| <b>B. FIELDS SEARCHED</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Minimum documentation searched (classification system followed by classification symbols)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>See Supplemental Box.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                 | Relevant to claim No.                                                                                                                                          |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | US A 5556762 (Pinilla et al) 17 September 1996.<br>Col. 42, Lines 5-37, Col. 55, Seq. ID 105, 107, Col. 56, Lines 29-36.                                                                           | 1 - 20                                                                                                                                                         |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | JOURNAL OF PROTEIN CHEMISTRY, Vol. 15, No. 4, 29 February 1996, pp 395-403, D. Hewish et al: "Peptide Inhibitors of Melittin Action".                                                              | 1 - 20                                                                                                                                                         |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | JOURNAL OF VIROLOGY, Aug. 1993, Vol. 67, No. 8 p 4639-4650. D.C. Shugars et al: "Analysis of Human Immuno deficiency Virus Type 1 Nef Gene Sequences Present In Vivo", p4642, Col. 1, Lines 60-63. | 1 - 20                                                                                                                                                         |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                    |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| <p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier document but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&amp;"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table> |                                                                                                                                                                                                    |                                                                                                                                                                | "A"                                                                                                                                                                                                                                          | document defining the general state of the art which is not considered to be of particular relevance | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention | "E" | earlier document but published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone | "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art | "O" | document referring to an oral disclosure, use, exhibition or other means | "&" | document member of the same patent family | "P" | document published prior to the international filing date but later than the priority date claimed |  |  |
| "A"                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | document defining the general state of the art which is not considered to be of particular relevance                                                                                               | "T"                                                                                                                                                            | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| "E"                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | earlier document but published on or after the international filing date                                                                                                                           | "X"                                                                                                                                                            | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| "L"                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)                                | "Y"                                                                                                                                                            | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| "O"                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | document referring to an oral disclosure, use, exhibition or other means                                                                                                                           | "&"                                                                                                                                                            | document member of the same patent family                                                                                                                                                                                                    |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| "P"                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | document published prior to the international filing date but later than the priority date claimed                                                                                                 |                                                                                                                                                                |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Date of the actual completion of the international search<br>24 December 1997                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                    | Date of mailing of the international search report<br><b>13 JAN 1998</b>                                                                                       |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION<br>PO BOX 200<br>WODEN ACT 2606<br>AUSTRALIA Facsimile No.: (02) 6285 3929                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                    | Authorized officer<br><br><b>IAN DOWD</b><br>Telephone No.: (02) 6283 2273 |                                                                                                                                                                                                                                              |                                                                                                      |     |                                                                                                                                                                                                 |     |                                                                          |     |                                                                                                                                                                          |     |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |     |                                                                          |     |                                           |     |                                                                                                    |  |  |

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00640

| C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                          |                       |
|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                       | Relevant to claim No. |
| X                                                    | YEAST, Vol. 9: p 565-573 (1993) I.G. MacReadie et al: "Expression of HIV - 1 nef in Yeast: The 27KDa Nef Protein is Myristylated and Fractionates with the Nucleus."                                                     | 1 - 20                |
| T                                                    | BIOCHEMICAL & BIOPHYSICAL RESEARCH COMMUNICATIONS 232, (1997), p707-711 Article No. RC976349 I.G. MacReadie, et al: "Cytotoxicity Resulting from Addition of HIV-1 Nef N-Terminal Peptides to Yeast an Bacterial Cells." | 1 - 20                |
| A                                                    | AIDS RESEARCH AND HUMAN RETROVIRUSES Vol. 8, No. 5, 1992, M. Harris, et al: "In Vivo Desired HIV-1 nef Gene Products are Heterogeneous and Lack Detectable Nucleotide Binding Activity".                                 | 1 - 20                |
| A                                                    | GENE. 162 (1995) p239-243, I.G. MacReadie, et al: "Stress -and sequence- dependant release into the culture medium of HIV-1 Nef produced in Saccharomyces cerevisiae".                                                   | 1 - 20                |
| A                                                    | RESEARCH IN VIROLOGY, Vol. 143, No. 1, p59-62, A.G. Laurent et al.: "Characterization of the Nef protein in HIV-1-infected CEM cells."                                                                                   | 1 - 20                |

## INTERNATIONAL SEARCH REPORT

International Application No.

**PCT/AU 97/00640**

## Supplemental Box

Continuation of Box B, Electronic data base consulted during the International Search

- (1) STN, Sequence search [ig][vt][ist][fwlnx][dnix][lkt][flh][nkte][im]|elaelaelaela|ggaanaleseannaaslag|liswig/sqsp, sql<20
- (2) STN File CA, Keywords (NEF and Myristyl?)
- (3) Substructure for sequence <4 amino acids combined with keywords (NEF or HIV)

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International Application No.  
**PCT/AU 97/00640**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report |               | Patent Family Member |
|----------------------------------------|---------------|----------------------|
| US A 5556762                           | AU A 91418/91 | 25/06/92             |
|                                        | CA A 2090860  | 22/05/92             |
|                                        | EP A 558671   | 08/09/93             |
|                                        | JP A 6507378  | 25/08/94             |
|                                        | WO A 9209300  | 11/06/92             |
|                                        | US A 5504190  | 02/04/96             |
| END OF ANNEX                           |               |                      |