

Cytotoxicity of pilosulin 1, a peptide from the venom of the jumper ant *Myrmecia pilosula*

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Abstract

The synthetic peptide pilosulin 1, corresponding to the largest defined allergenic polypeptide found in the venom of the jumper ant *Myrmecia pilosula*, inhibited the incorporation of [*methyl*-³H]thymidine into proliferating Epstein–Barr transformed (EBV) B-cells. The LD₅₀ was four-fold lower in concentration than melittin, a cytotoxic peptide found in honey bee venom. Loss of cell viability was assessed by flow cytometry by measuring the proportion of cells that fluoresced in the presence of the fluorescent dye 7-aminoactinomycin D. Examination of proliferating EBV B-cells indicated that the cells lost viability within a few minutes exposure to pilosulin 1. Partial peptides of pilosulin 1 were less efficient in causing loss of cell viability and the results suggest that the 22 N-terminal residues are critical to the cytotoxic activity of pilosulin 1. Normal blood white cells were also labile to pilosulin 1. T- and B-lymphocytes, monocytes and natural killer cells, however, were more labile than granulocytes. Analysis of pilosulin 1 using circular dichroism indicated that, in common with melittin and other Hymenoptera venom toxins, it had the potential to adopt an α -helical secondary structure. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The sting of the jumper ant *Myrmecia pilosula* causes severe allergic reactions including anaphylaxis in sensitised individuals. The ant is found in the sandy-soiled high country of south-eastern Australia and Tasmania. The major allergens from the venom

of the jumper ant *M. pilosula* have been cloned and characterised and named according to international convention, Myr p 1 and Myr p 2 [1,2]. The major allergen Myr p 1 (112 amino acid residues) is expressed in the venom sac as a family of immunologically related homologous C-terminal peptides of 56, 48, 45, 42 and 27 residues all of which appear to be derived from a single precursor polypeptide [3] (Fig. 1). Myr p 2, however, is expressed in the venom as a single 27 residue C-terminal polypeptide [3]. The largest expressed polypeptide of the Myr p 1 group

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(56 amino acid residues) has been named pilosulin 1 and has a molecular weight of 6045 Da. This study reports potent cytotoxic activity of pilosulin 1 for a number of different cell types, in particular, proliferating EBV B-cells, normal B-lymphocytes, T-lymphocytes, natural killer cells, monocytes and granulocytes.

2. Materials and methods

2.1. Synthetic peptides

Synthetic peptides were prepared as previously described [4,5] using an Applied Biosystems (Foster City, CA, USA) 430A Peptide Synthesiser or were obtained commercially (Chiron Mimotopes, Melbourne, Australia). Honey bee venom melittin was obtained from Sigma (St. Louis, MO, USA).

2.2. Red blood cell lysis

Whole blood was centrifuged at $600\times g$. Red cells were located at bottom of the cell precipitate and white cells formed a layer at the interface of serum and red cells. Red cells were aspirated from the bottom of the centrifuge tube and resuspended in PBS to give a 0.5% (v/v) suspension of cells. Two hundred μ l of red cells were aliquotted into 96 well round-bottom microtitre plate wells. Venom peptides (pilosulin 1, melittin) and control peptides (Myr p 2 C-terminal 27 residue peptide and ribonuclease A) were added in 20 μ l aliquots to individual wells at final concentrations of 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 μ M. Whole *M. pilosula* venom was added to individual wells at final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 μ g/ml. The plates were incubated for 24 h at room temperature and photographed. Cell lysis was determined by the solubilisation of the cellular precipitate.

2.3. Preparation of Epstein–Barr transformed (EBV) B-cells

Peripheral blood mononuclear cells (PBMC) were prepared from normal heparinised blood by diluting the blood with an equal volume of Hanks' balanced salts solution (modified) (Sigma) supplemented with

10 mM sodium bicarbonate, 60 U/ml penicillin G (sodium salt) (Sigma), 100 mg/l streptomycin sulphate (ICN Biomedicals, Aurora, OH, USA) and 2 units/ml heparin (Calbiochem-Novachem, La Jolla, CA, USA) (HBSS) and performing density gradient separation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The PBMC were washed with HBSS and suspended in RPMI-1640 (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10 mM sodium bicarbonate, 20 mM HEPES buffer (ICN), 3 mM L-glutamine (Sigma), 60 U/ml penicillin G, 100 mg/l streptomycin sulphate and containing 20% (v/v) foetal bovine serum (FBS) (MultiSer, Trace Biosciences, Castle Hill, Australia). One ml of cell suspension (2×10^6 cells/ml) was incubated in a humidified atmosphere for 2 days in the presence of 1 ml of B95-8 marmoset cell line supernatant and 1 μ g/ml cyclosporin at 37°C (5% CO₂). The formation of clumps after 2 days was indicative of EBV immortalisation. Cells were washed and fresh media containing 1 μ g/ml cyclosporin supplied every 7 days for a period of 1 month. Cell lines were expanded by passaging in increasing volumes of culture media.

2.4. Preparation of total blood white cells

Heparinised whole blood was centrifuged at $600\times g$ for 5 min and the cells were suspended in freshly made red cell lysis solution (0.83% w/v ammonium chloride, 0.1% w/v potassium bicarbonate) and allowed to stand at room temperature for 2–5 min to haemolyse red cells. The white cells were then washed with serum-free supplemented RPMI-1640 culture medium and suspended in the same medium.

2.5. Cell proliferation assay

EBV B-cells were cultured to log-phase growth in supplemented RPMI-1640 medium containing 20% foetal calf serum. Two hundred μ l of cells (1×10^6 cells/ml) were aliquotted into 96 well round bottom microtitre plate wells. Peptides dissolved in 10 μ l of culture medium were added to the wells. [*methyl*-³H]Thymidine (1 μ Ci) (ICN) was added to each well in 10 μ l of RPMI-1640 culture medium and the plates cultured as described above for 16 h. The cells were harvested using a Titertek (ICN) cell

(a) Myr p 1

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1      5      10      15      20
Met Lys Leu Ser Cys Leu Leu Leu Thr Leu Ala Ile Ile Phe Val Leu Thr Ile Val His

21     25     30     35     40
Ala Pro Asn Val Glu Ala Lys Asp Leu Ala Asp Pro Glu Ser Glu Ala Val Gly Phe Ala

41     45     50     55     60
Asp Ala Phe Gly Glu Ala Asp Ala Val Gly Glu Ala Asp Pro Asn Ala Gly Leu Gly Ser
                                     1

61     65     70     75     80
Val Phe Gly Arg Leu Ala Arg Ile Leu Gly Arg Val Ile Pro Lys Val Ala Lys Lys Leu
5      10      15      20

81     85     90     95     100
Gly Pro Lys Val Ala Lys Val Leu Pro Lys Val Met Lys Glu Ala Ile Pro Met Ala Val
25     30     35     40

101    105    110
Glu Met Ala Lys Ser Gln Glu Glu Gln Gln Pro Gln
45     50     56

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(b) Melittin

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Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu Ile Ser Trp Ile
1      5      10      15      20

Lys Arg Lys Arg Gln Gln
21     25

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Fig. 1. (a) The derived amino acid sequence of the allergen Myr p 1 (112 amino acid residues). The sequence of pilosulin 1 (56 amino acid residues) is highlighted and its amino acid sequence numbered below the sequence. The arrows indicate the proteolytic processing cleavage points. (b) The amino acid sequence of bee venom melittin.

harvester and counted in 1 ml of EcoLite scintillation fluid (ICN) in a liquid scintillation counter (Packard Instrument, Meriden, CT, USA).

2.6. Cell viability assay using flow cytometry

2.6.1. Cultured cells

Log-phase growth cultured EBV B-cells (2×10^5 cells) were suspended in serum-free supplemented RPMI-1640 culture medium and incubated with peptides and 7-aminoactinomycin D (7-AAD, 4 $\mu\text{g/ml}$, Sigma) for 10 min at room temperature. The cells were then analysed by flow cytometry in a Coulter ELITE flow cytometer (Coulter, Hialeah, FL, USA) equipped with an argon laser operated at 15 mW and emitting light at 488 nm. Cells that fluoresced at 675 nm (i.e. that had taken up 7-AAD) were defined as non-viable [6].

2.6.2. White cells

Whole white cells or PBMC (200 μl , 10^6 cells/ml) suspended in serum-free supplemented RPMI-1640

culture medium were incubated on ice for 30 min with fluorescent labelled monoclonal antibodies recognising cell specific markers: CD3-FITC (T-cells); NKH1-RD1 (CD56; natural killer cells and a T-cell subset); B4-ECD (CD19; B-cells); Mo2-RD1 (CD14; monocytes); My7-RD1 (CD13; granulocytes and monocytes). Antibodies were from Coulter (Miami, FL, USA). The cells were washed in ice cold RPMI by centrifugation ($500 \times g$) and re-suspension. The cells were then incubated with the peptides and 7-AAD at room temperature in the dark and then analysed by flow cytometry. The individual cell subpopulations were defined with the appropriate mix of antibodies. Thus, for example, PBMC were labelled with CD3-FITC, NKH1-RD1, and B4-ECD to define the T-cells (CD3^+), natural killer cells (CD3^- , CD56^+), and B-cells (CD19^+); after incubation with peptide and 7-AAD, viable cells were 7-AAD-negative and dead cells were 7-AAD-positive. In this example, four colour flow cytometric analysis made it possible to simultaneously monitor the killing of T-, B-, and natural killer cells.

2.7. Analysis of pilosulin 1 peptides by circular dichroism

Measurements were made using either an Aviv Model 62DS spectrometer equipped with an electronic temperature controlling device and calibrated with 7-camphorsulphonic acid or with a Jasco JX710 CD spectrometer.

Measurements using the Aviv Model 62DS spectrometer were made at 25°C in a 0.5 mm path length cuvette, at a protein concentration of 0.12 mg/ml in 20 mM phosphate buffer pH 7.0. Data were collected from 195 to 250 nm at 0.5 nm intervals and were corrected for the buffer baseline. The fraction of α -helix (f_h) was calculated according to the following equation [7].

$$F_h = ([\theta]_{222} - [\theta]_{222}^0) / ([\theta]_{222}^{100}) \quad (1)$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm and $[\theta]_{222}^0$ and $[\theta]_{222}^{100}$, the ellipticities corresponding to 0 and 100% α -helix, are -2000 and $-30\,000$ deg cm² dmol⁻¹, respectively [8,9].

Data using the Jasco JX710 CD spectrometer were recorded at 21°C from 195 to 265 nm with a 0.01 cm path-length cell at 10 nm/min with a resolution of 1 nm in 0.02 M phosphate buffer pH 7.14 containing up to 50% (v/v) trifluoroethanol. Secondary structure was estimated using the programme of Yang et al. [10] supplied with the instrument.

2.8. Preparation of unilamellar vesicles

Large unilamellar vesicles composed of egg yolk phosphatidylcholine were prepared by pressure extrusion through polycarbonate membranes of 1 nm pore size [11].

3. Results

3.1. Red blood cell lysis

Red cell lyses (Fig. 2) were carried out in the presence of Ribonuclease A control (row A), whole venom (row B), pilosulin 1 (row C), Myr p 2 C-terminal 27 residue control peptide (row D), melittin (row E). After 24 h incubation, complete cell lysis was obtained with melittin at a concentration of 10 μ M with partial lysis visible down to 0.625 μ M. Complete lysis in the presence of pilosulin 1 was obtained at a concentration of 40 μ M with evidence of partial lysis visible down to 1.25 μ M. Whole venom caused complete cell lysis at the highest concentration used (200 μ g/ml) with partial lysis visible to 25 μ g/ml. The controls were without effect.

3.2. Effect of venom peptides on proliferation of cultured EBV B-cells

Log-phase EBV B-cells were cultured with pilosu-

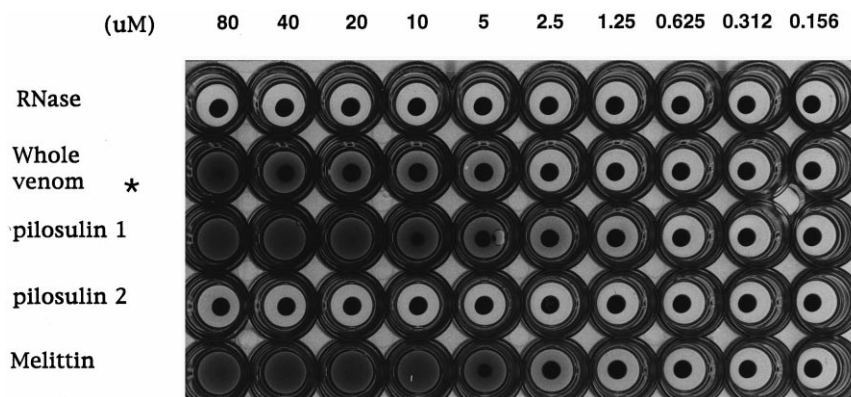


Fig. 2. The effect of whole *M. pilosula* venom, pilosulin 1, melittin, Myr p 2 C-terminal 27 residue peptide and ribonuclease A on red blood cells. Red blood cell suspensions (0.5% (v/v) 200 μ l) were incubated with different concentrations (in 20 μ l) of whole venom* (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 μ g/ml) and the peptides (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 μ M) in a 96 well round bottom microtitre plate. After 24 h incubation at room temperature, the plate was photographed. Cell lysis is indicated by the visual solubilisation of the cellular precipitate as determined by eye.

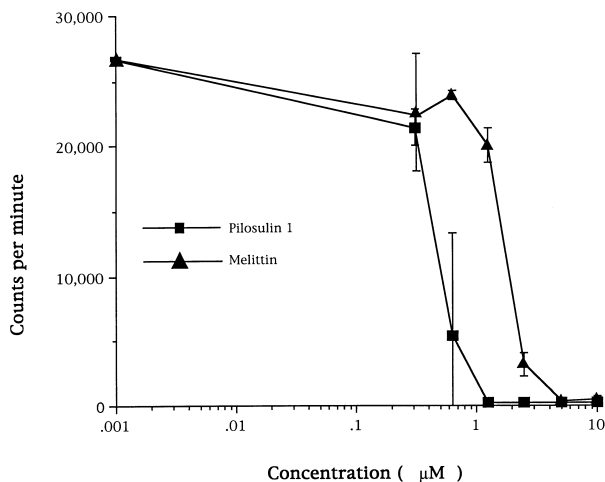


Fig. 3. The effect of pilosulin 1 and melittin on the proliferation of EBV B-cells. Log-phase cells (see Section 2) were incubated for 16 h in the presence of different concentrations of peptides and [*methyl*-³H]thymidine and then harvested and liquid scintillation counted. Each data point is shown as mean $\pm$ S.E. of the mean of three measurements.

lin 1 and bee venom melittin at concentrations ranging from 0.3125 to 10 μ M (Fig. 3). An LD₅₀ of 0.4 μ M was obtained for pilosulin 1 and this was approximately four times lower than for the honey bee toxin melittin.

3.3. The effect of pilosulin 1 and partial peptides of pilosulin 1 on EBV B-cell viability measured by flow cytometry

Pilosulin 1 and peptides corresponding to partial sequences of pilosulin 1 were tested at concentrations up to 8 μ M for their effects on viability of log-phase proliferating EBV-transformed B-cells (Fig. 4). Pilosulin 1 was most potent whereas the partial peptides 1–22 and 11–56 were less effective: the molar concentration of the 1–22 peptide required to kill 40% of the cells was 70 times greater than that of the full peptide, and the required concentration of the 11–56 peptide was 200 times greater. The other partial peptides, 23–56, 37–56 and 47–56 (data not shown) were without effect.

3.4. Effect of pilosulin 1 on the cell sub-populations of white cells and PBMC

Whole white cells differ from PBMC predomi-

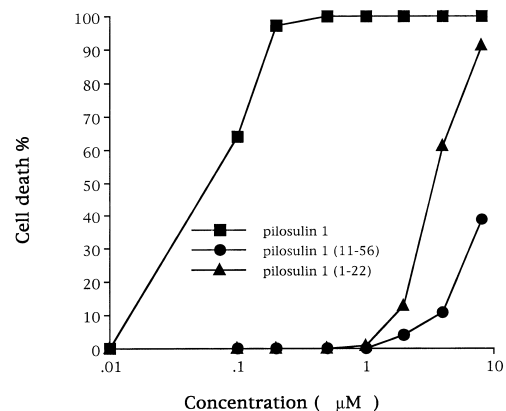


Fig. 4. The effect of pilosulin 1 and partial pilosulin 1 peptide sequences on the viability of EBV B-cells. Log-phase EBV B-cells (2×10^5 cells) were incubated for 10 min in the presence of the peptides and 7-AAD (4 μ g/ml). Uptake of 7-AAD was measured by flow cytometry.

nantly because of the granulocyte sub-population which accounts for approximately 70% of the white cell population. Sub-populations of whole white cells and PBMC were identified using fluorescent-labelled monoclonal antibodies as described in Section 2. Lysis caused by pilosulin 1 was rapid and usually complete by 5 min at room temperature (data not shown). Cells from different normal volunteers dif-

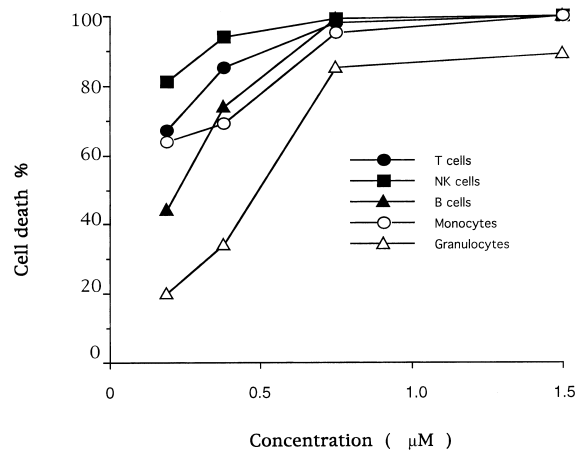


Fig. 5. The effect of pilosulin 1 on the viability of normal white blood cells. Cells were first labelled with fluorescent-labelled monoclonal antibodies recognising cell-specific markers and sub-populations were defined according to the pattern of antibody binding. Pilosulin 1 and 7-AAD were added to the cells for 10 min at room temperature prior to analysis by flow cytometry. Cells which took up 7-AAD were defined as non-viable. Four colour flow cytometric analysis enabled three cell sub-populations to be monitored simultaneously.

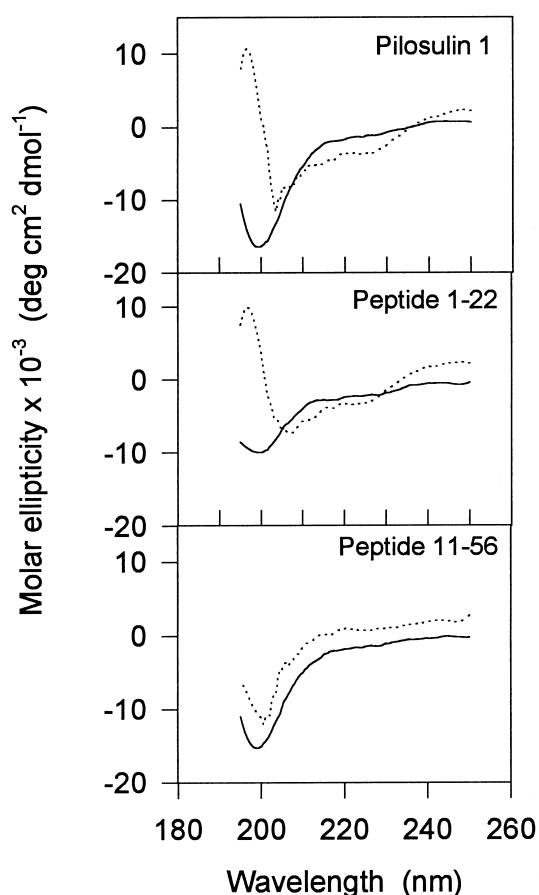


Fig. 6. Circular dichroism spectra of pilosulin 1 and its partial peptides in solution (—) and in the presence of phospholipid vesicles composed of egg phosphatidylcholine (---). The molar ratio of lipid to peptide was in the range of 35–85.

ferred as much as five-fold in their lability to pilosulin 1; however, in all cases, the granulocyte population was more resistant to cell lysis than the mononuclear cells (Fig. 5).

3.5. Circular dichroism

The circular dichroism spectra of pilosulin 1 peptides 1–56, 1–22 and 11–56 in phosphate buffer at pH 7.0 are presented in Fig. 6. Each peptide displays a minimum at 199–200 nm characteristic of the random coil form. The percentage of α -helix calculated according to Eq. 1 is 11, 14 and 12% for peptides 1–56, 1–22 and 11–56, respectively. In the presence of unilamellar vesicles the spectral form for the peptide 11–56 remains much the same. However, the spectra for peptides 1–56 and 1–22 show a maximum at 196–

197 nm and a stronger ellipticity at 222 nm, indicative of an increase in the content of α -helix. Indeed the helix content calculated according to Eq. 1 increased by 7% for pilosulin 1 (1–56) and 4% for peptide 1–22. The results suggest that under the conditions of the experiment peptides 1–56 and 1–22 interact with a phospholipid surface, whereas peptide 11–56 does not. These experiments were designed to determine if binding to the lipid surface induced secondary structure in the peptides. Detection of differences in the affinity of the peptides 1–56 and 1–22 for the lipid surface will have to await a full binding analysis of the interaction.

Circular dichroism spectral analysis of the peptides in the presence of 50% trifluorethanol increased the percentage of α -helix in pilosulin 1 (1–56) from 11 to 32% and from 14 to 31% for peptide 1–22.

4. Discussion

The results convincingly show that pilosulin 1, like bee venom melittin, is a potentially cytotoxic molecule. Cell death, as evidenced by the uptake of the fluorescent marker dye 7-AAD, was caused by a loss of membrane integrity [6]. Different cell types were not equally affected by pilosulin 1 and melittin. Pilosulin 1 and bee venom melittin both caused the lysis of red blood cells though melittin was more potent, causing complete haemolysis at a lower molar concentration than pilosulin 1. In the EBV B-cell proliferation experiments, however, pilosulin 1 was more inhibitory on a molar basis than melittin. The flow cytometry results confirmed that pilosulin 1 was more cytotoxic for EBV B-cells than melittin on a molar basis.

The effect of pilosulin 1 on normal white cells and the PBMC indicates that the peptide has cytotoxicity not only for immortalised dividing EBV B-cells but also for normal non-dividing cells. There is evidence of a differential cytotoxic effect with the white cells, with the granulocytes being more resistant to loss of membrane integrity. This relative resistance by granulocytes may be due to differences in membrane structure of these cells. The differences observed between red blood cell and EBV B-cell lysis caused by melittin and pilosulin 1, respectively, indicates that the cell membranes of these two cell types are sufficiently different to give sharply different responses to

the two peptides. It is therefore not surprising that the different cell types that make up a white cell preparation may also have different sensitivities to a cytotoxin that acts by affecting membrane integrity.

The mechanism by which the toxin melittin from the venom of the honey bee causes cell death is thought to be due to a membranolytic effect that appears to involve the C-terminal basic hexapeptide since the remaining segment between residues 1–20 is not lytic, although it will still bind to the erythrocyte membrane [12]. In dilute solution melittin exists as a random coil, but assumes a largely α -helical conformation in lipid bilayers and other hydrophobic environments [13,14]. One hypothesis is that oligomerisation of the melittin monomer to a tetrameric form may form bilayer-spanning polar pores that allow ionic permeability [13,15]. Pilosulin 1 has no sequence homology with melittin (see Fig. 1), however, analysis of pilosulin 1 by circular dichroism in dilute neutral buffer indicated that it exists largely as random coil with little secondary structure, but under increasingly hydrophobic conditions (50% TFE), 32% of pilosulin 1 and 31% of peptide 1–22 had adopted α -helical conformation. Both of these latter peptides also interact with unilamellar vesicles with induction of increased secondary structure. The potential to form an α -helix is probably crucial for any interaction of the molecule with membranes. In this context, it is very interesting that basic amino acid residues (lysine and arginine) are found at regular intervals along the polypeptide chain of pilosulin 1, amidst a larger number of hydrophobic residues (Fig. 1). This is a feature common to numerous polypeptides that are able to form the amphiphilic secondary structures which determines their ability to lyse cells [16,17].

The experiments using partial pilosulin 1 peptides strongly suggested that the 22 N-terminal residues contains a moiety that is critical for its cytotoxicity. It is not clear, however, whether the N-terminal 22 residues contain sequences that are more important for membrane association rather than for membrane lysis. The partial 46 residue peptide of pilosulin 1 (11–56) was only weakly cytotoxic and no cytotoxic activity was observed with the partial 34 residue pep-

tide of pilosulin 1 (23–56). This decline in cytotoxicity could be due either to a loss of membrane association or to the loss of a unique lytic moiety. Experiments using unilamellar vesicles suggested that the 22 residue partial pilosulin 1 peptide (1–22) interacts with the vesicles, but that the 46 residue partial pilosulin 1 peptide (23–56) did not. This, together with the cytotoxicity data, suggests that the moiety responsible for membrane association lies in the N-terminal sequence with the lytic moiety probably extending beyond residue 22 of the molecule, since this partial peptide was less cytotoxic than the full-length pilosulin 1.

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