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Antitumour Activity and Specificity as a Function of Substitutions in the Lipophilic Sector of Helical Lactoferrin-derived Peptide

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Abstract: A peptide L5 (PAWRKAFRWAWRMLKKAA), derived from the *N*-terminal α-helical region of bovine lactoferrin (LFB 14–31), that is highly active against several tumour cell lines was reported earlier. In this study, a number of L5 analogues were designed in order to investigate how subsequent replacements of the aromatic amino acids in L5 with three amino acids representing different structural parameters influenced antitumour activity and tumour cell specificity relative to normal human cells. The Trp residues were substituted by Lys, Ile or Ala, while the Phe residue was substituted with Ala. The resulting peptides were investigated for their activity against prokaryotic cells, four tumour cell lines, human lung fibroblasts and human erythrocytes. Most of the peptides were highly active against both *E. coli* and *S. aureus*. The peptides were more active against the tumour cell lines than against normal eukaryotic cells but the activity against normal fibroblasts varied more among the peptides than did their antitumour activities. The results revealed that aromatic residues located opposite the cationic sector in L5 were more critical for antitumour activity than were aromatic residues located adjacent to the cationic sector. The biological responses for the peptides against tumour cell lines, fibroblasts, *S. aureus* (but not *E. coli*), were highly correlated with the amino acid descriptors used in our QSAR model. The result obtained from the QSAR study identified specific structural features that were important for lytic activity and membrane specificity. Certain structural properties in positions 3, 9 and 11 were shown to be important for antitumour activity, while additional structural properties in position 7 were found to be important with respect to tumour cell specificity. This information may offer a possibility for *de novo* design of an antitumour peptide with an improved therapeutic index.
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Keywords: antimicrobial peptides; lactoferrin; lytic peptides; tumoricidal peptides; tumour cell specificity; QSAR

INTRODUCTION

Antimicrobial peptides are ubiquitous in nature and are thought to be an important component in innate host defence against infectious agents [1]. Cationic amphipathic peptides, such as the defensins and cecropins, induce cell death in prokaryotic and/or eukaryotic cells by increasing membrane permeability. Increased permeability may lead to cell lysis or, alternatively, may produce subtle changes in the membrane's barrier function that promote cell

Abbreviations: ATCC, American Type Culture Collection; EC_{50}, concentration with 50% red blood cell survival; IC_{50}, concentration with 50% cell survival; MIC, minimal inhibitory concentration; PCA, principal component analysis; PLS, projections to latent structures; QSAR, quantitative structure activity relationship; RBC, red blood cells; TFA, trifluoroacetic acid.

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death. Several antimicrobial peptides display selectivity that is on the borderline between those typical of the venom-like peptides [2–4] and those of the antibacterial peptides [5–8]. These peptides, exemplified by magainins [9,10], lactoferrin derivatives [11,12] and cecropin–magainin hybrids [13–15] appear to be cytotoxic against certain tumour cells at concentrations which are non-toxic toward normal mammalian cells. Structure–activity relationships of helical antimicrobial peptides have been extensively investigated (for a review see [16]). Due to the much greater similarity between tumour cell membranes and normal human cell membranes, the design and refinement of peptides that are able to selectively kill tumour cells is more difficult than is the improvement of antimicrobial peptides.

In a recent study, a group of peptides was designed based on the sequence of the *N*-terminal helical region of bovine lactoferrin (LFB) (residues 14–31) and demonstrated that the angle subtended by the cationic sector is a crucial parameter in modulating antitumour activity and tumour cell specificity relative to normal cells [11]. It was found that helical LFB derived peptides with a narrow angle of cationic sector displayed greatly improved antitumour activity. Peptides with enhanced tumour cell specificity could be constructed in several ways; high lipophilicity and the number of aromatic residues could be counterbalanced by keeping one cationic residue in the lipophilic sector, alternatively the lipophilicity could be lowered by reducing the number of aromatic amino acid residues. The aim of this study was to explore the importance of the number and the position of aromatic residues in the lipophilic sector of a LFB derived peptide with respect to tumour cell specificity in more detail. The starting peptide (PAWRKAFRWAWRMLKKAA, named L5) is highly toxic toward several tumour cell lines but has relatively poor tumour cell specificity. This peptide has all cationic residues concentrated in one sector giving a narrow angle subtended by the cationic sector (Figure 1). One or several of the three Trp and one Phe residues in the lipophilic sector of this peptide were subsequently replaced by each of three amino acids, representing different structural parameters (Ala, Lys or Ile). A quantitative structure–activity relationship (QSAR) was then applied to further explore whether certain structural parameters could be identified as more optimal than others for each position occupied by aromatic residues in the lipophilic sector concerning antitumour activity and tumour cell specificity. Antitumour effects were also compared with antibacterial activities in order to identify structural parameter(s) that distinguish between these activities.

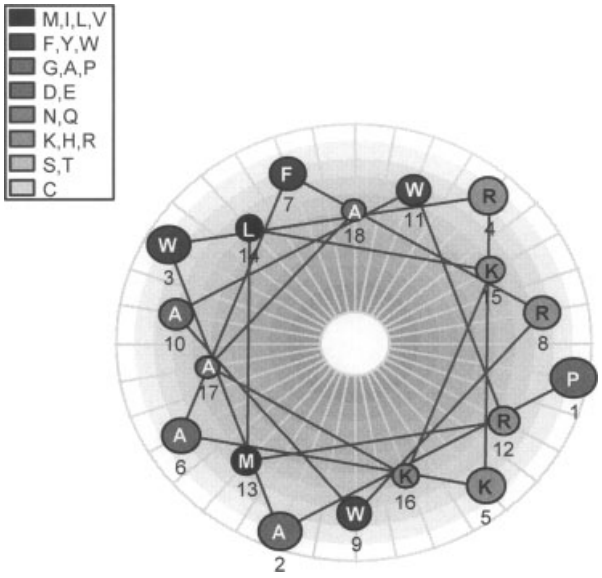

Figure 1 Edmundson helical wheel representation of lactoferrin derivative L5.

METHODS

Peptide Synthesis and Purification

The peptides were synthesized on a MilliGen 9050 Plus PepSynthesizer (Milligen, Milford, MA, USA). Coupling reactions with Fmoc amino acids preactivated as pentafluorophenyl (Pfp)-esters were catalysed by *N*-hydroxybenzotriazole (HOBt, 1.3 equivalents). Coupling reactions with Fmoc amino acids activated *in situ* with O-benzotriazolyl-*N*, *N*, *N*′, *N*′-tetramethyluronium hexafluorophosphate (HBTU) were performed in the presence of *N*,*N*-diisopropylethylamine (DIPEA, 2.4 equivalents). A fourfold excess of amino acids was employed during every coupling step. The *C*-terminal amino acid was preattached to a 4-hydroxymethylphenoxy acetic acid/polyethylene glycol/polystyrene (PAC-PEG-PS) resin, which ensured a free *C*-terminal carboxylic group after the final acid cleavage. All amino acids with reactive side chains were protected with acid labile protecting groups and deprotected during cleavage of the peptide from the solid support upon treatment with reagent K (80% trifluoroacetic acid (TFA), 5% anisol, 5% ethanedithiol (EDT), 5% water and 5% phenol) for no more than 3 h. Crude peptides were purified on a reversed

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phase HPLC C18-column (Delta-Pak™ C18, 100 Å, 15 μm, 25 × 100 mm, Waters Corporation, Milford, MA, USA) using a mixture of water and acetonitrile (containing 0.1% TFA) as the mobile phase and UV detection at 254 nm. All peptides were analysed for purity on an analytical RP-HPLC (Delta-Pak™ C18, 100 Å, 5 μm, 3.9 × 150 mm, Waters Corporation, Milford, MA, USA) with a mixture of water and acetonitrile (containing 0.1% TFA) as the mobile phase and UV detection at 254 nm. The purity of all peptides was found to be above 95%. All purified peptides were characterized by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK). Peptides were lyophilized before biological testing.

Haemolytic Assay

The haemolytic activity of the peptides was determined using freshly isolated human red blood cells (RBC) as recently described [11,17]. The RBC were incubated with peptides dissolved in phosphate buffered salt water (PBS) at concentrations varying from 1 μg/ml to 1 mg/ml for 1 h at 37 °C. The samples were centrifuged at 4000 rpm (corresponding to 2951 × **g**) for 5 min before the absorbance of the supernatant was measured at 540 nm by a microtitre plate reader (Thermomax Molecular Devices, NJ, USA). Zero percent haemolysis and 100% haemolysis were determined in PBS and 1% Triton X-100, respectively. Peptide concentrations causing 50% haemolysis (EC_{50}) were determined from the dose-response curves.

Cell Cultures

The murine fibrosarcoma (MethA) cell line [18], human colorectal adenocarcinoma HT-29 (ATCC-HTB-38) and human mammary carcinoma MT-1 (kindly provided by Dr Fodstad, Department of Tumor Biology, the Norwegian Radium Hospital, Oslo, Norway) were grown in RMPI-1640 medium

Table 1 Amino Acid Sequences of Lactoferrin Derived Peptide L5 and its Analogues

W or F substitution	Peptide	Amino acid sequence																		Calculated Mw	Observed Mw
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
Start peptide	L5	P	A	W	R	K	A	F	R	W	A	W	R	M	L	K	K	A	A	2273.8	2271.8
Alanine																					
W3 → **A3**	B1	—	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2158.6	2157.1
W9 → **A9**	B2	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	—	2158.6	2158.0
W11 → **A11**	B3	—	—	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	2158.6	2157.9
W3,9 → **A3,9**	B4	—	—	A	—	—	—	—	—	A	—	—	—	—	—	—	—	—	—	2043.5	2043.3
W3,11 → **A3,11**	B5	—	—	A	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	2043.5	2043.3
W9,11 → **A9,11**	B6	—	—	—	—	—	—	—	—	A	—	A	—	—	—	—	—	—	—	2043.5	2042.9
Lysine																					
W3 → **K3**	B7	—	—	K	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2215.7	2215.5
W9 → **K9**	B8	—	—	—	—	—	—	—	—	K	—	—	—	—	—	—	—	—	—	2215.7	2214.9
W11 → **K11**	B9	—	—	—	—	—	—	—	—	—	—	K	—	—	—	—	—	—	—	2215.7	2214.6
W9,11 → **K9,11**	B10	—	—	—	—	—	—	—	—	K	—	K	—	—	—	—	—	—	—	2157.7	2157.5
Isoleucine																					
W3 → **I3**	B11	—	—	I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2200.7	2200.5
W9 → **I9**	B12	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—	—	—	2200.7	2200.5
W11 → **I11**	B13	—	—	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—	2200.7	2200.5
W3,9 → **I3,9**	B14	—	—	I	—	—	—	—	—	I	—	—	—	—	—	—	—	—	—	2127.7	2127.5
W3,11 → **I3,11**	B15	—	—	I	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—	2127.7	2127.5
W9,11 → **I9,11**	B16	—	—	—	—	—	—	—	—	I	—	I	—	—	—	—	—	—	—	2127.7	2127.5
W3,9,11 → **I3,9,11**	B17	—	—	I	—	—	—	—	—	I	—	I	—	—	—	—	—	—	—	2054.6	2054.5
Phenylalanine																					
F7 → **A7**	B18	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	—	—	—	2197.7	2197.5
W3F7 → **A3,7**	B19	—	—	A	—	—	—	A	—	—	—	—	—	—	—	—	—	—	—	2252.7	2252.2

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Table 2 Biological Data of Lactoferrin Derivative L5 and its Analogues

Peptide	Mic *E. coli* (μM)[b]	Mic *S. aureus* (μM)[b]	RBC EC_{50} (μM)[c]	MethA IC_{50} (μM)[a]	MT-1 IC_{50} (μM)[c]	HT IC_{50} (μM)[c]	A549 IC_{50} (μM)[c]	Fibroblast IC_{50} (μM)[c]	Ratio of IC_{50} Fibroblast/ A549
L5[d]	2	2	33	6.6	8	14	4.8	17	3.5
B1[d]	6.9	4.6	>463	24	20	35	25	190	7.6
B2[d]	4.6	2.4	382	16	11	16	15	46	3
B3[d]	4.6	<1.2	278	11	12	15	15	46	3
B4	7.3	25.5	>489	>245	224	>489	410	>489	>1.2
B5	7.3	25.5	>489	>245	247	489	358	>489	>1.4
B6[d]	14.7	14.7	>489	110	87	180	137	>489	>3.6
B7	4.5	1.7	>451	57.3	31	101	42	230	5.5
B8[d]	13.5	4.5	>451	13.5	12	29	14	59	4.2
B9[d]	2.3	<1.1	>451	7.9	10	25	14	31	2.2
B10	35	19	>463	199	214	328	158	>463	>2.9
B11	2	2	323	9	10	11	7.7	20	2.6
B12	5	<1	155	12	12	15	10.2	26	2.5
B13	2	<1	64	6	9.3	15	6.8	15	2.2
B14	5	5	>470	36	18	35	19	108	5.7
B15	2.5	5	413	16	9.4	19	11.3	45	4
B16	4.6	1.7	284	22	17	30	12.7	26	2
B17	2.5	10	>487	47	36	136	65	280	4.3
B18[d]	15	10	>455	34.6	32	52	34	289	8.5
B19[d]	7.2	14.4	>480	116.4	75	248	130	>480	>3.7

[a] The maximum concentration of the peptides tested was 500 μg/ml.
[b] The minimum concentration of the peptides tested was 2.5 μg/ml.
[c] The maximum concentration of the peptides tested was 1000 μg/ml.
[d] All biological data are cited from Yang *et al.* [11] except IC_{50} values for A549.

supplemented with 10% heat inactivated fetal bovine serum (Biochrom, KG, Berlin Germany) and 1% L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C. The human embryonic fibroblast cell line MRC-5 (ATCC-CCL-171) was maintained in MEM medium, supplemented with heat inactivated 10% fetal bovine serum and 1% L-glutamine. The human lung carcinoma A549 (ATCC-CCL-185) was maintained in Ham's F12K medium with 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, supplemented with heat inactivated 10% fetal bovine serum. Before use, MethA cells (4×10^4 cells/well), HT-29 cells (4×10^4 cells/well), MT-1 cells (1.5×10^4 cells/well), A549 cells (1.5×10^4 cells/well) or fibroblasts (1×10^4 cells/well) were seeded into 96-well plates (Falcon, Becton Dickinson Lab, USA). MethA cells were stimulated immediately, while the fibroblasts, MT-1, A549 and HT-29 cells were allowed to adhere for approximately 16–24 h, washed with 100 μl corresponding maintenance medium without serum prior to cytotoxicity assays.

Cytotoxicity Assays

The different cell lines were treated with 100 μl of peptide solutions of different concentrations diluted in serum-free culture medium. After 4 h incubation at 37°C, the microtetrazolium (MTT)-based colorimetric assay [19] was performed. Briefly, 20 μl MTT solution (5 mg/ml MTT in PBS, Sigma Germany) was added to each well and the plate was incubated for 2 h. An aliquot of 120 μl of supernatant was removed from MethA cells while 70 μl of medium was removed from each well of adherent cells. A solubilization solution (100 μl of 0.33% concentrated HCl in 2-propanol) was added to each well and the plate was shaken on a Termolyne Roto Mix type 50800 (Dubuque, Iowa, USA) for 1 h. The absorbance at 590 nm was measured by a microtitre plate reader. Zero percent cell lysis and 100% cell lysis were determined in serum-free medium and 1% Triton X-100, respectively. The IC_{50} values were determined from the dose response curves. The final results were recorded by averaging at least three repeated experiments.

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Determination of Antibacterial Activity

The bacterial strains, *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC-25923, were grown in 2% Bacto Peptone water (Difco 1807-17-4). A standard microdilution technique with an inoculum of 2×10^6 colony-forming units per ml was used. The minimal inhibitory concentration (MIC) of the peptides was determined in 1% Bacto Peptone water after incubation overnight at 37 °C. All assays were performed in triplicate. MIC determinations were performed in accordance with recommendations given by Amsterdam [20].

QSAR

The program package Simca-P 9.0 from Umetrics, Umeå, Sweden was used for all calculations. As descriptors for the peptides the *z*-scales derived by Hellberg *et al.* [21,22] were employed, since the results from the analyses can later be used for interpretation of which macroscopic properties of the individual amino acids are responsible for the biological response. In all calculations the *z*-variables were centred and scaled to unit variance since the variation in substitutions was not the same for all amino acid positions. Toxicity against red blood cells was not included in any of the calculations since most responses were outside the measured range. The other biological responses were used as the logarithm of the MIC or IC_{50} value, with responses outside of the range practical for measurements omitted.

Principal component analysis. A PCA was performed in order to check the homogeneity of the data, in which the biological responses that were included in the calculations were scaled to unit variance and centred to have a comparable influence of all variables. This resulted in a model with five significant components explaining 86% of the variation in data with peptide B10 being just outside the confidence limit (Figure 2). It was still deemed that this should be included in later calculations. The peptides were evenly distributed along the two first components with the peptides having the highest activity being situated to the left of the plot. This was also evident from a closer inspection of the loading plot (Figure 3) which revealed that the largest influence on the model came from the biological activities. The even distribution of the peptides in the PCA made a good starting point for the QSAR using projection to latent structures (PLS) analysis [23, 24].

Projections to latent structures. In order to gain more information about the role of structural parameters in different positions a PLS analysis was performed, in which the *z*-values were centred and scaled and biological data were used as the centred logarithm of the MIC/IC_{50} value. This resulted in a model with four significant components explaining 81% of the variation in the *y*-variables using 70% of the variation in *x*. The importance of the variables on the model can be visualized in plots of w^*c, in which w^* is the weight that combines the original *x*-variables to form the scores *t* and *c* is the weight of the *y*-variables that form the scores *u*, with large values indicating high correlation (Figure 4). Another way of presenting the results is to compare

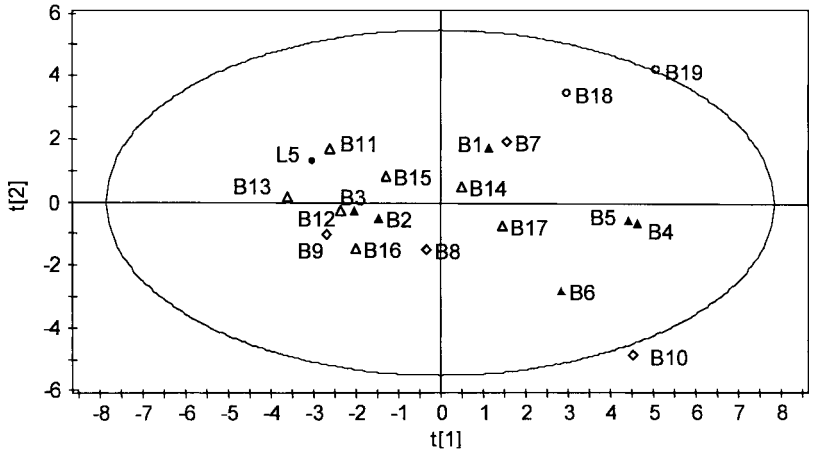

Figure 2 t[1] vs. t[2] from PCA of complete dataset. ● Starting peptide, ▲ alanine substituted peptides, ◊ lysine substituted peptides, Δ isoleucine substituted peptides, ○ fenyl alanine substituted peptides.

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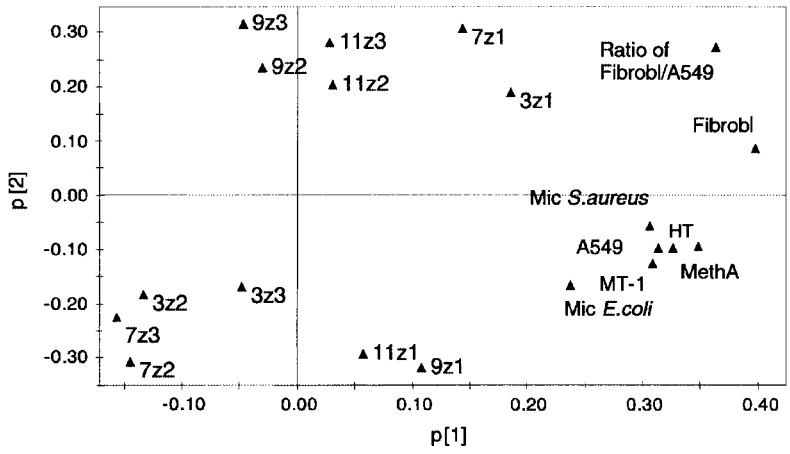

Figure 3 First two loadings (p[1] vs. p[2]) from PCA of complete dataset.

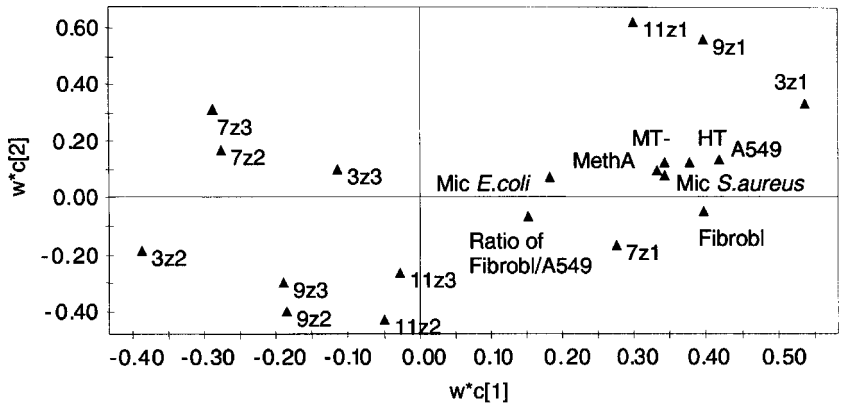

Figure 4 First two loadings ($w^*c[1]$ vs $w^*c[2]$) from PLS analysis of dataset.

the observed vs predicted responses (Figures 5 and 6).

RESULTS

Peptide Design

All the LFB-derived peptides presented in an earlier study, including peptide L5 itself, possess a considerable degree of α-helicity in the CD investigation in both sodium dodecyl sulfate (SDS) and hexafluoroisopropanol (HFIP) solutions [11]. In the peptide L5, Trp are located at either side of the cationic sector (Trp^9 and Trp^{11}) and one Trp is located opposite to the cationic sector (Trp^3) (Figure 1). The L5 analogues were designed by replacing the Trp residues by Lys, Ile or Ala, or by replacing the Phe^7 residue with Ala (Table 1). The amino acid Ala was chosen because it is small relative to Trp and Phe. Since an earlier study [11] has shown that enhanced tumour cell specificity was obtained by retaining one cationic residue in the lipophilic sector [11], the effect of subsequent replacement of the Trp residues with Lys was also investigated. The effect of substituting the Trp residues by Ile was included since Ile has similar hydrophobicity to Trp but is much less bulky [22]. Since a significant loss of tumoricidal activity was obtained when two out of the three Trp residues were replaced with either Ala or Lys (Table 2), three Trp residues were simultaneously replaced with Ile.

Antibacterial Activity

The antibacterial activities of the peptides were included in the study in order to check the correlation between antibacterial and antitumour activities with respect to structural changes in the peptides. The L5 derivatives were tested against the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. aureus* (Table 2). For most

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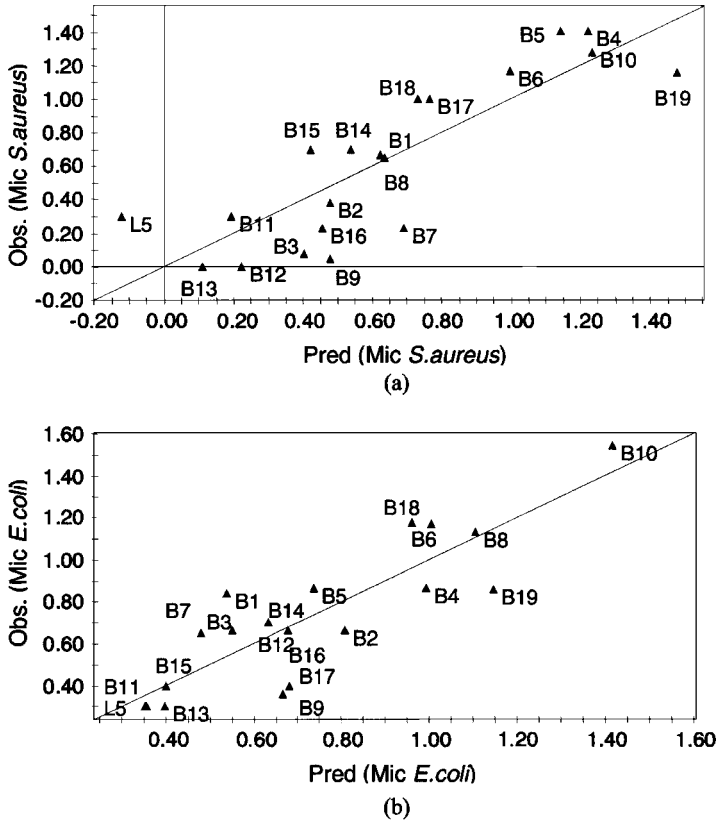

Figure 5 Observed vs predicted activity against (a) *S. aureus* and (b) *E. coli*.

of the peptides the antibacterial activities varied more against *S. aureus* than against *E. coli*. Four of the peptides (B3, B9, B12 and B13) displayed MIC values against *S. aureus* of less than 1.2 μM. This group of peptides has either an Ala, Lys or Ile substitution at Trp^{11} or an Ile substitution at Trp^{9}. A severe loss (3.7 to 17 fold) of antibacterial activity was observed when two out of three Trp residues were replaced with either Ala or Lys, whilst all combinations involving two substitutions with Ile only gave rise to a slight reduction in activity (1.3–2.5 fold). The replacement of Phe at position 7 with Ala resulted in a 5–7 fold reduced activity. As can be seen from the QSAR results (Figure 5), the correlation between observed and predicted values for *S. aureus* was high, while the correlation for *E. coli* was poor.

Haemolytic Activity

The effect of the L5 derivatives against RBC was also investigated (Table 2). In general, the peptides exerted low haemolytic activity. The substitution of one Trp at any position in L5 led to decreased haemolytic activity. Replacement of Trp with one Lys resulted in the complete loss of haemolytic activity, while replacement with one Ala caused a severe reduction but still with detectable haemolytic activity. The substitution of Trp with one Ile resulted in smaller changes in haemolytic activity than the replacements with Ala and Lys. Substitution of any two or three Trp caused a loss of haemolytic activity except the peptides B15 with $I^{3,11}$ and B16 with $I^{9,11}$. The replacement of Phe with Ala (B18 and B19) resulted in a loss of haemolytic activity.

Antitumour Activity

All the L5 derivatives were less effective against tumour cells than against prokaryotic cells (Table 2). Most of the L5 derivatives exhibited lower activity against the tested human tumour cell lines (MT-1, HT-29 and A549 cell lines) than that of L5, while B13 and B11 displayed a slightly higher activity for MethA cells and HT-29 cells, respectively. Replacement of Trp^{3} with either Ala or Lys caused a large reduction in antitumour activity compared with the replacements of Trp at position 9 or 11 with

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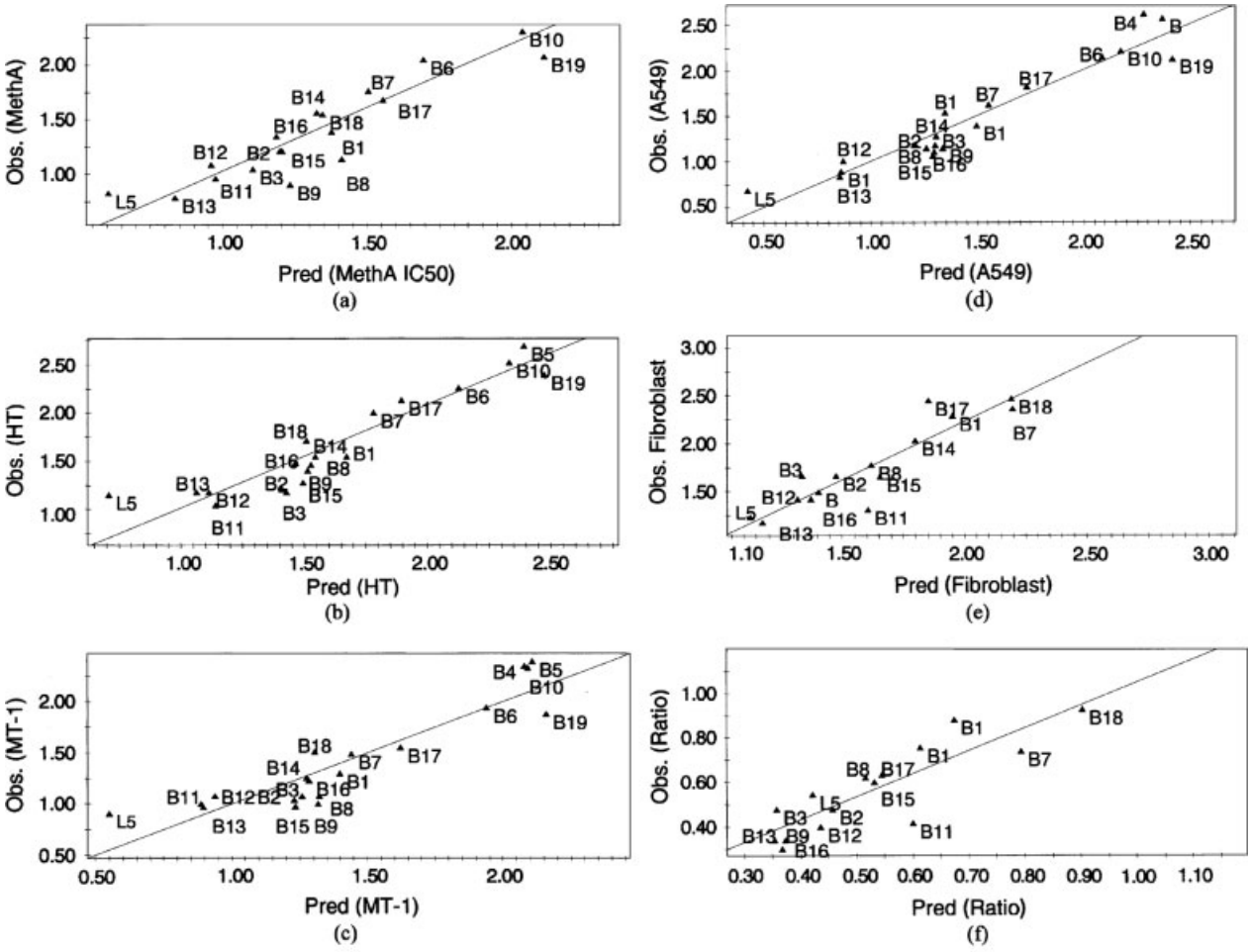

Figure 6 Predicted vs observed activity against (a) MethA, (b) HT, (c) MT-1, (d) A549, (e) Fibroblasts, and (f) IC_{50} ratio between fibroblast/A549.

the same amino acids. When two Trp were replaced with two Ala or two Lys, a substantial reduction in tumoricidal activity was obtained against all tumour cell lines (>11 fold). When one Trp was replaced with Ile at any position, almost no change in antitumour activity was observed, and when two Trp were substituted with two Ile relatively small reductions in antitumour activities were observed (MethA; 2.4–5.5 fold, MT-1; 1.2–2.3 fold, HT-29; 1.4–2.5 fold, A549; 2.4–4 fold). When all three Trp were replaced with Ile, a moderate decrease in tumoricidal activity was observed (MethA; 7 fold, MT-1; 4 fold, HT-29; 10 fold, A549; 13 fold). The replacement of Phe^{7} by Ala (B18) reduced antitumour activity more than the Trp^{3} substitution with Ala, and when the Trp residue in position 3, and the Phe residue in position 7 were simultaneously replaced by Ala (B19), a large drop in activity was observed. When these data were treated in our QSAR model, good correlations between observed and predicted values for all four tumour cell lines were observed (Figure 6a–d).

Fibroblast Cytotoxicity

The peptides that were most effective against the tumour cell lines were also the most toxic against fibroblasts. Fibroblasts were relatively less sensitive to the peptides than tumour cells (Table 2) and the cytotoxic activities against the fibroblasts varied more relative to the antitumour activities. Replacement of Trp^{3} with Ala (B1) or Lys (B7) resulted in an 11-fold and a 13.5-fold decreased activity respectively, whereas Trp^{9} or Trp^{11} replacement with Ala (B2 and B3), or Trp^{9} or Trp^{11} replacement with Lys (B8 and B9) resulted in a 2–4 fold decreased activity relative to L5. A 17-fold reduction in toxic activity

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against the fibroblasts was observed for the Phe^{7} replacement with Ala (B18). Furthermore, substitution of two $Trp^{3,9}$ with Ile (B14) led to a 6.4-fold decrease in activity, whereas 1.5–2.5 fold reduced activity was observed when $Trp^{3,11}$ or $Trp^{9,11}$ were replaced with Ile. The predicted and observed IC_{50} values correlated well for the fibroblasts (Figure 6e). Moreover, Figure 6f shows that QSAR could model the IC_{50} ratio fibroblast/A549 cells well.

DISCUSSION

In a recent study, the relationship was investigated between cationic properties and antitumour activities in peptides derived from the *N*-terminal α-helical sequence of bovine lactoferrin (LFB). One of the peptides (L5), which has all cationic residues concentrated in one sector, displayed high antitumour activity but low tumour cell specificity (i.e. low IC_{50} normal cells/tumour cell ratio) [11]. It was, however, found that increased tumour cell specificity could either be obtained by placing one of the cationic residues in the lipophilic sector, or by substituting one of the aromatic amino acid residues in the lipophilic sector with Ala. In the present study, the role of several structural parameters in the lipophilic sector in LFB-derived peptides was investigated by replacing one or several of the aromatic amino acid residues present in the L5 sequence, with Ala, Lys or Ile. The peptides were tested against normal and transformed eukaryotic cells as well as prokaryotic cells. QSAR analysis was also employed to further explore whether certain structural parameters could be identified as more optimal than others for each position possessed by the aromatic amino acid residues concerning antitumour activity and tumour cell specificity. In addition to using chemical knowledge of amino acid properties for study of new peptides, different multivariate methods have successfully been employed in earlier studies. These methods can either be based on the use of macroscopic properties for the peptides [25] or of the amino acid properties, e.g. the *z*-scales developed by Hellberg *et al.* [21,22]. Since the effect of modulating the size of the cationic sector in helical LFB-derived peptides was the main focus in the recent study by Yang *et al.* [11], a number of experimentally derived parameters and parameters describing the charge distribution were used in these QSAR studies. The aim of the current study was to investigate the effect of replacing aromatic residues with amino acids representing different structural properties. Hence a model using theoretically derived descriptors which contain not only qualitative data but also information about macroscopic properties such as lipophilicity-hydrophilicity and size was chosen. This method has proven to be useful in structure–activity studies of antibiotic peptides and a brief introduction to this model can be found in the literature [26].

The antibacterial activity of the peptides was investigated in order to determine whether structural parameters critical for antitumour activity differ from those critical for antibacterial activity. All the peptides were active against *E. coli* and *S. aureus*, and their antibacterial activities varied much less than their activities against the different eukaryotic cells tested. This is consistent with our recent report [11]. Interestingly, some of the peptides with one Trp replaced with Ile, Ala or Lys exerted higher activity against *S. aureus* than the L5 peptide (B3, B9, B12, B13, B16), indicating that reduction of the number of aromatic residues resulted in enhanced activity against *S. aureus*. The replacement of two aromatic amino acids with Ala or Lys, however, led to a significant reduction in antibacterial activity. This is in agreement with earlier results showing that the number of Trp in shorter LFB peptides is critical for antibacterial activity [27]. It is noteworthy, however, that even two or three Trp could be replaced with Ile residues without losing antibacterial activity (*E. coli*: B15, B17; and *S. aureus*: B16). Earlier studies have pointed out that the bulkiness of Trp is important for antibacterial activity in shorter LFB peptides [28]. Moreover, it has been demonstrated that increased antibacterial activity is obtained [28] by replacing aromatic amino acid residues with larger non-coded amino acids. Along the scale of hydrophobicity of an amino acid residue in a protein, Trp and Ile are among the most hydrophobic, Ala is in the middle, and Lys is the least hydrophobic [29]. Our results suggest that even though bulkiness may improve antibacterial activity, antibacterial activity can be maintained when aromatic amino acids are removed as long as the hydrophobicity and hydrophobic moment are not significantly changed. The peptide activity against *S. aureus* was more correlated to the three human tumour cell lines than *E. coli* (Figure 4) indicating that the mode of peptide action toward *S. aureus* was more similar to that toward tumour cell lines than *E. coli*. This correlation may be due to the more complex cell envelope of the Gram-negative bacterium *E. coli* than that of the Gram-positive *S. aureus* and than the cytoplasmic membrane of

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eukaryotic cells since *E. coli* has an outer membrane exterior to the cytoplasmic membrane [30].

The most active peptides were almost equally active against all four tumour cell lines tested, while the peptides with low or moderate activity were generally less active against the HT-29 cells than against the MethA, MT-1 and A549 cells. The effect of replacing one Trp with Ala or Lys resulted in relatively small decreases in tumoricidal activity. However, a larger drop in activity was observed for the replacement of Trp^3 with Ala or Lys compared with Ala replacements of Trp^9 or Trp^{11}. An even larger drop in activity was obtained when Phe^7 was replaced with Ala, suggesting that aromatic residues located away from the cationic sector (Figure 1), are more important for the activity of L5 than the Trp residues located adjacent to the cationic sector (Trp^9 and Trp^{11}). When two aromatic residues were replaced by Ala or Lys, the antitumour activity was almost lost. This observation is consistent with our earlier results [11]. The decrease in antitumour activity by the replacements of $Trp^{9,11}$ with Ala was less than the decrease obtained by the replacement of Trp^3 together with Trp^9, Trp^{11} or Phe^7 with two Ala, supporting the findings discussed above that the aromatic residue located opposite the cationic sector had a stronger impact on antitumour activity than those located adjacent to the cationic sector. However, two Trp residues could be replaced by Ile without decreasing the antitumour activity significantly, whereas the replacement of all three Trp with Ile led to a large reduction in antitumour activity, especially against the HT-29 cell line. Similar to the effects against the prokaryotic cells by the peptides, it seems that until a certain threshold in hydrophobicity is reached, bulkiness is more important than hydrophobicity, whereas above that, the hydrophobicity content seems to be a more critical parameter. This was also clear from the plot of $[w^*cl]$ vs $[w^*c2]$ (Figure 4) that z_1 (which contains information about hydrophilicity) for amino acid positions 3, 9 and 11 were positively correlated with the anti-tumour activity, while the other *z*-values (which contain information on bulk and hydrophobicity/hydrophilicity) were inversely correlated. It is noteworthy that the descriptors chosen for the QSAR study contained most of the information needed for a successful modelling of the antitumour activities by the peptides since good correlations between observed and predicted log IC_{50} values were obtained for all four tumour cell lines tested (Figure 6a–d).

In order to construct antitumour peptides that selectively target transformed eukaryotic cells, structural parameters critical for tumour cell specificity have to be identified. A number of tumour cell lines have been shown to contain an elevated level of negatively charged phosphatidylserine in the outer membrane leaflet compared with their normal counterpart cells [31–33]. Other attributes, such as lipoprotein content [34] and fluidity [35] of the cell membranes may also explain the ability of lytic peptides to distinguish between tumour cells and normal cells. Erythrocytes are commonly used to check the toxicity of antibacterial peptides [25,28,36,37]. The cell membrane of erythrocytes is more neutral and dissimilar to the membranes of most body cells including tumour cells [38], and thus represents a poor model for probing tumour cell specificity. Indeed, the aromatic amino acid residues present in the L5 peptide could not be replaced without significantly reducing the haemolytic activity. The smallest reduction in haemolytic activity was obtained when one Trp was replaced with Ile. A higher number of aromatic residues seemed to be needed for the haemolytic activity than that needed for cytotoxic activities against the tumour cell lines. The fibroblast cell membrane is, however, more representative and sensitive than the membrane of erythrocytes, and the cytotoxic effect of the lytic peptides on normal fibroblasts was therefore investigated. The biological data revealed that the peptides that were highly active against tumour cells were also highly toxic against fibroblasts. Several of the peptides with a slightly reduced antitumour activity, however, showed an enhanced tumour cell specificity. This is consistent with earlier findings [11]. The variation in tumour cell specificity among the peptides, seemed to be dependent on which aromatic amino acid was replaced. For example, substitution of Trp^3 or Phe^7 with Ala and substitution of $Trp^{3,9}$ with Ile slightly diminished the antitumour activity, but the selectivity increased significantly (e.g. B1, B14 and B18). On the other hand, replacement of Trp^9 or Trp^{11} with Ala or Lys led to a slight decrease in cytotoxic activity against fibroblast without any significant improvement in tumour cell specificity (B2, B3, B8 and B9), indicating that the improvement of tumour cell specificity was dependent on which aromatic amino acid was replaced and the structural properties of amino acids that were used to replace the aromatic amino acid residue(s). These findings prompted us to examine the relationship between structural changes and tumour cell specificity by QSAR. The data compiling the cytotoxic

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effects on fibroblasts could be treated successfully in QSAR analysis since a good correlation between observed and predicted $\log IC_{50}$ values for fibroblast toxicity was obtained (Figure 6d). Since the tumour cell lines were almost equally sensitive to the peptides, one tumour cell line was selected for QSAR analysis of tumour cell specificity. The fact that the ratio between the tumour cell line A549 and fibroblasts could be modelled quite accurately (Figure 6f) is very interesting in the view of the aim of the current study — to explore whether anti-cancer agents with high tumour cell specificity can be constructed. With regard to tumour specificity it seems that position 7 is of most interest among the four positions, since z_1 is positively correlated to the ratio, which is in contrast to negatively correlated z_1-values for the other positions. Due to the limited amount of data for the substitution in this position, more studies are needed before further details regarding critical structural parameters in this position can be revealed.

It is clear from the Figure 4 that z_1 for amino acid residues at positions 3, 9 and 11 together with z_2 for amino acid at position 3 contains the most information regarding the first component. As a consequence, choosing amino acids, in positions 3, 9, and 11 with high z_1 values will increase the activity of the peptides. Similarly, an increase in z_2 for the amino acid in position 11 should increase the tumour cell specificity without affecting activity to a large extent. In addition, size related z-values for positions 3, 9 and 11 seem to influence the tumour cell specificity more than the activity.

CONCLUSION

Our results have demonstrated that Trp^3 and Phe^7 located away from the cationic sector in the helical peptide L5 was more critical for activity against eukaryotic cells than Trp^9 and Trp^{11} located on either side of the cationic sector. Our QSAR models successfully predicted antitumour activities and tumour cell specificity and demonstrated that structural properties affecting biological responses for the tumour cell lines, fibroblasts and *S. aureus*, but not *E. coli* are highly correlated. The QSAR study also revealed that among the positions in which the aromatic amino acids are located, modulations critical for antitumour activity can be performed in positions 3, 9 and 11 and for tumour cell specificity in position 7. The design and synthesis of new peptides including the use of non-coded amino acids in order to verify the present results and to further improve the predictive ability of the model is in progress.

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