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The Effects of Shortening Lactoferrin Derived Peptides against Tumour Cells, Bacteria and Normal Human Cells

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Abstract: A number of shortened derivatives of the lactoferrin model peptide L12, PAWRKAFRWAKRMLKKAA, were designed in order to elucidate the structural basis for antitumour activity of lactoferrin derivatives. Three tumour cell lines were included in the study and toxicity determined by measuring lysis of human red blood cells and fibroblasts. The results demonstrated a strong correlation between antitumour activity and net positive charge, in which a net charge close to +7 was essential for a high antitumour activity. In order to increase the antitumour activity of the shortest peptide with a net charge less than +7, the hydrophobicity had to be increased by adding a bulky Trp residue. None of the peptides were haemolytic, but toxicity against fibroblasts was observed. However, modifications of the peptides had a higher effect on reducing fibroblast toxicity than antitumour activity and thereby resulted in peptides displaying an almost 7-fold selectivity for tumour cells compared with fibroblasts. The antimicrobial activity against the Gram-negative bacteria *Escherichia coli* and the Gram-positive bacteria *Staphylococcus aureus* was also included in order to compare the structural requirements for antitumour activity with those required for a high antimicrobial activity. The results showed that most of the peptides were highly active against both bacterial strains. Less modification by shortening the peptide sequences was tolerated for maintaining a high antitumour activity and selectivity compared with antimicrobial activity. The order of the amino acid residues and thereby the conformation of the peptides was highly essential for antitumour activity, whereas the antimicrobial activity was hardly influenced by changes in this parameter. Thus, in addition to a certain net positive charge and hydrophobicity, the ability to adopt an amphipathic conformation was a more critical structural parameter for antitumour activity than for antimicrobial activity, and implied that a higher flexibility or number of active conformations was tolerated for the peptides to exert a high antimicrobial activity.

Keywords: anticancer peptides; antimicrobial peptides; antitumour peptides; lactoferrin; lytic peptides; tumouricidal peptides

INTRODUCTION

Cationic antimicrobial peptides comprise a structurally diverse group of low molecular mass single gene products exhibiting activity against a range of Gram-positive and Gram-negative bacteria and fungi [1–5]. They have in common the ability to form amphipathic structures with the hydrophobic and hydrophilic faces well separated upon interaction with an anisotropic environment such as the bacterial cell membrane. Several antimicrobial peptides

Abbreviations: ATCC, American Type Culture Collection; EC_{50}, concentration required for 50% haemolysis; IC_{50}, concentration with 50% cell survival; MIC, minimal inhibitory concentration; RBC, red blood cells.

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are also reported to be potent against transformed eukaryotic cells [6–11].

The reason for the antitumour activity of these cationic peptides is thought to be an elevated expression of negatively charged phosphatidyl serine on the outer leaflet of the cell membrane of some tumour cells [12–14]. An electrostatic attraction between the positively charged residues of the peptides favours binding to the lipid membrane of the tumour cells. The packing of the phospholipids is distorted when the peptides penetrate into the membrane, thereby increasing the permeability of the cell membrane that results in death of the tumour cells. It is demonstrated that antitumour peptides are equally active against both sensitive and multidrug-resistant phenotypes [15]. Thus, the development of such antitumour peptides can provide a novel class of broad-spectrum agents that act via a unique mechanism, and that are active against cells with over-expression of p-glycoprotein and with resistance towards existing chemotherapeutic agents [15].

The design and refinement of peptides that are able to selectively kill tumour cells is, however, more challenging than designing peptides for antimicrobial applications due to the high similarity between the cell membrane of tumour and non-transformed cells. Shin *et al.* [11] have shown that an 18 residue cecropin–magainin hybrid peptide, P18, is highly active against tumour cells and displays no haemolytic activity below 100 μM. Shorter *N*- or *C*-terminal deletion and substitution analogues of P18 ranging in size from 14 to 16 residues display even greater improved antitumour activity. However, among these the most potent Leu substituted analogues, N-3L and N-4L, also display measurable toxicity against human RBC.

Johnstone *et al.* [15] have reported that short mammalian indolicidin-derived extended-helical peptides and insect-derived α-helical peptides, are highly active against human and murine tumour cells. In general, the peptides display a low haemolytic activity and are more active against tumour cells than against non-malignant cells. The highest selectivity for tumour cells compared with non-malignant cells is displayed by the indolicidin-derived peptides, in which the OLP-1 and OLP-4 analogues are above 5-fold more active against tumour cells than against normal human umbilical vein endothelial cells (HUVEC).

Our research group has spent considerable effort to elucidate the structural basis of selectivity of lactoferrin derivatives for the membrane of transformed cells with the goal of optimizing peptide antitumour activity [16–18]. In a recent study, a series of cytolytic peptides was designed based on residues 14–31 of the *N*-terminal α-helical region of bovine lactoferrin [16]. The peptide L12, PAWRKAFRWAKRMLKKAA, is highly active against all the tumour cell lines tested and displays 4-fold selectivity for murine fibrosarcoma MethA cells compared with human lung fibroblast MRC-5 cells.

In the present study a series of truncated L12 derivatives were prepared in order to elucidate the structural basis for antitumour activity of lactoferrin derivatives, and to explore the possibility of designing shorter membrane active antitumour peptides. Three tumour cell lines were included in the study, and the specificity determined by measuring the toxicity against human red blood cells (RBC) and human fibroblasts. The antimicrobial activity against the Gram-negative rod *Escherichia coli* and the Gram-positive cocci *Staphylococcus aureus* was also determined in order to identify any differences between structural parameters affecting antitumour and antimicrobial activity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification

The peptides were synthesized on a MilliGen 9050 Plus PepSynthesizer (MilliGen, Milford, MA, USA), using a solid-phase with Fmoc protection as previously described [16,19]. The crude peptides were analysed and purified by reversed phase HPLC (Waters, Milford, MA, USA). 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).

Haemolytic Activity

The haemolytic activity of the peptides was determined using freshly isolated human red blood cells (RBC) isolated as recently described [16,20]. Briefly, the RBC were incubated with peptides dissolved in 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, and the absorbance of the supernatant 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 [21] and human mammary carcinoma MT-1 (kindly provided by Dr Fodstad, Department of Tumour Biology, the Norwegian Radium Hospital, Oslo, Norway) were grown in RMPI-1640 medium 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 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. 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. Before use, MethA 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 at similar levels of sub-confluency into 96-well plates (Falcon, Becton Dickinson Lab, USA). MethA cells were stimulated immediately, while the fibroblasts, MT-1 and A549 cells were allowed to adhere for approximately 16–24 h, washed with 100 µl corresponding maintenance medium without serum prior to the toxicity assays.

Toxicity Assays

Different cell lines were treated with 100 µl of peptide solution of different concentrations diluted in serum-free culture medium as recently described [16]. After 4 h incubation at 37 °C, the microtetrazolium (MTT)-based colorimetric assay [22] 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 the 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 the wells and the plate was shaken for 1 h on a Termolyne Roto Mix type 50 800 (Dubuque, Iowa, USA). A microtitre plate reader was used to measure the absorbance at 590 nm. 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.

Antimicrobial 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 cfu/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 triplicates. The MIC determinations were performed in accordance with recommendations given in [23].

RESULTS

A series of peptides was prepared based on L12 by successively deleting the *N*-terminal residues (Table 1; peptides S2–S5). The results showed that most of the deletion peptides retained the antitumour activity of the parent peptide L12, and that a similar change in activity was observed against all the tumour cell lines (Table 2). A significant reduction in activity was only observed when all the four *N*-terminal residues of L12 were deleted. The resulting peptide S5 contained one unit of charge less than S2–S4 by the deletion of an Arg residue. It was noteworthy that all the peptides were highly antimicrobial and displayed MIC values below 5.5 µM against *E. coli*, and between <1.1 and 11 µM against *S. aureus*. None of the peptides exhibited haemolytic activity within the concentration range tested, i.e. up to 1000 µg/ml. Some toxicity was observed against human lung fibroblasts for the longest peptides, but fibroblast toxicity rapidly declined through the series of peptides and thereby increased the IC_{50} ratio between fibroblasts and A549 cells for all peptides besides S5.

The next series of peptides was based on S3 in order to design shorter deletion peptides by successively deleting the *C*-terminal residues (Table 1; peptides S6–S11). S3 was chosen as the parent peptide since it retained most of the antitumour activity of L12, and in order to keep the Trp residues and a high net positive charge, which are reported to be essential for the antimicrobial and antitumour activity of bovine lactoferrin derivatives

Table 1 *N*- and *C*-terminal Deletion Analogues

Peptide	Calc. Mw	Obs. Mw	N[a]	C[b]	Sequence
L12[c]	2215.7	2214.6	18	+7	P A W R K A F R W A K R M L K K A A
S2	2144.6	2143.3	17	+7	A W R K A F R W A K R M L K K A A
S3	2073.6	2072.8	16	+7	W R K A F R W A K R M L K K A A
S4	1945.4	1943.8	15	+7	R K A F R W A K R M L K K A A
S5	1817.5	1816.0	14	+6	K A F R W A K R M L K K A A
S6	1976.5	1975.8	15	+7	W R K A F R W A K R M L K K A
S7	1905.4	1904.5	14	+7	W R K A F R W A K R M L K K
S8	1719.2	1718.6	13	+6	W R K A F R W A K R M L K
S9	1563.0	1562.9	12	+5	W R K A F R W A K R M L
S10	1434.8	1433.1	11	+5	W R K A F R W A K R M
S11	1363.7	1363.3	10	+5	W R K A F R W A K R

[a] N, number of amino acids in the peptides.
[b] C, net positive charge.
[c] Ref. [16].

Table 2 Biological Activities (μM) of *N*- and *C*-terminal Deletion Analogues

Peptide	IC_{50}				EC_{50}	MIC		IC_{50}
	MethA[a]	MT-1[b]	A549[b]	Fibroblast[b]	RBC[b]	*E. coli*[c]	*S. aureus*[c]	Ratio[d]
L12[e]	7.9	10	14	31	—[f]	2.3	<1.1	2.2
S2	12.3	14	15	41	—	4.7	2.3	2.7
S3	14.5	16	22	60	—	4.8	2.4	2.7
S4	33.4	27	31	135	—	5.1	3.9	4.4
S5	157	68	156	367	—	5.5	11	2.4
S6	28	40	24	163	—	3.8	3.8	6.8
S7	29	50	34	232	—	3.9	3.9	6.8
S8	—[f]	—	—	—	—	20	44	
S9	—	—	—	—	—	48	>96	
S10	—	—	—	—	—	52	>105	
S11	—	—	—	—	—	>110	>110	

[a] The maximum concentration of the peptides tested was 500 μg/ml and [b] 1000 μg/ml.
[c] The minimum concentration of the peptides tested was 2.5 μg/ml.
[d] IC_{50} ratio fibroblasts/A549.
[e] The data were cited from ref. [16] except for A549.
[f] — , no activity within the concentration range tested.

[5,18,19,24,25]. Compared with S3, the peptides S6 and S7 displayed a 2-fold reduced antitumour activity against the MethA and MT-1 cell lines, whereas the activity against A549 cell line and the bacterial strains was mainly unchanged (Table 2). As observed for the previous peptides, S6 and S7 were also non-haemolytic. It is noteworthy that the toxicity of S6 and S7 against human lung fibroblasts was greatly reduced compared with S3 and resulted in a 7-fold IC_{50} ratio between fibroblasts and A549 cells. A total loss of antitumour activity was observed when the *C*-terminal residues of S7 were further deleted, resulting in peptides S8–S11, which contained less than 14 residues and a net positive charge of no more than +6. A reduction in antimicrobial activity was also observed for these peptides and with the most severe effect observed against *S. aureus*.

The results above implied that a sequence length of 14 residues and a net positive charge of +7 were

required for a high antitumour activity. Seemingly, any deviations from these requirements resulted in peptides displaying low or no antitumour activity. In order to test this hypothesis, a third set of peptides was designed based on S7 by varying the charge and number of residues of the peptides in a systematic manner while maintaining the hydrophobic residues (Table 3; peptides M1–M6). The peptide M1 was two residues shorter than S7 due to deletion of the internal Ala residues, but had the same net charge. Surprisingly, M1 displayed no antitumour activity and also showed reduced antimicrobial activity (Table 4). The peptide M2 exhibited low, but measurable antitumour activity and increased antimicrobial activity compared with M1. Further reduction in net positive charge by deletion of a Lys residue gave M3, which only showed antitumour activity against the MT-1 cell line. A further reduction in hydrophobicity by deletion of a Leu and Met residue in M3 gave M4 and M5, respectively, and resulted in a total loss of antitumour activity. It was noteworthy that this reduction in hydrophobicity did not affect the antimicrobial activity, which remained high and with MIC values below 10 μM against both bacterial strains. None of these peptides displayed toxicity against RBC or fibroblasts within the concentration range tested. In an attempt to increase the antitumour activity, the *C*-terminal Lys residue of M5 was replaced by Trp, thereby increasing the hydrophobicity and lytic potential of the resulting peptide M6. As shown in Table 4, M6 displayed measurable antitumour activity and 2-fold selectivity for tumour cells despite its

Table 3 Short Derivatives Based on Peptide S7

Peptide	Calc. Mw	Obs. Mw	N[a]	C[b]	Sequence[c]											
M1	1763.2	1762.8	12	+7	W	R	K	F	R	W	K	R	M	L	K	K
M2	1505.9	1505.3	10	+6	W	R		F	R	W	K	R	M	L	K-NH_2	
M3	1378.7	1377.0	9	+5	W	R		F	R	W		R	M	L	K-NH_2	
M4	1264.6	1264.3	8	+5	W	R		F	R	W		R	M		K-NH_2	
M5	1133.4	1133.8	7	+5	W	R		F	R	W		R			K-NH_2	
M6	1191.4	1191.5	7	+4	W	R		F	R	W		R			W-NH_2	
O1	1763.2	1762.8	12	+7	W	F	K	R	R	W	K	R	M	L	K	K
O2	1763.2	1762.5	12	+7	R	F	K	R	W	W	K	R	M	L	K	K

[a] N, number of amino acids in the peptides.
[b] C, net positive charge.
[c] The $-NH_2$ group denotes peptides with an amidated *C*-terminal carboxylic acid group.

Table 4 Biological Activities (μM) of Derivatives Based on Peptide S7

Peptide	IC_{50}				EC_{50}	MIC		IC_{50}
	MethA[a]	MT-1[b]	A549[b]	Fibroblast[b]	RBC[b]	*E. coli*[c]	*S. aureus*[c]	Ratio[d]
M1	—[e]	—	—	—	—	28	11	
M2	155	319	275	—	—	6.6	6.6	>2.4
M3	—	580	—	—	—	7.2	7.2	
M4	—	—	—	—	—	7.9	7.9	
M5	—	—	—	—	—	8.8	8.8	
M6	215	327	338	592	765	4.2	<2.1	1.8
O1	—	—	—	—	—	28	14	
O2	110	152	190	—	—	11	11	>3

[a] The maximum concentration of the peptides tested was 500 μg/ml and [b] 1000 μg/ml.
[c] The minimum concentration of the peptides tested was 2.5 μg/ml.
[d] IC_{50} ratio fibroblast/A549.
[e] — , no activity within the concentration range tested.

short size of only seven residues. M6 was also highly active against both *E. coli* and *S. aureus* displaying MIC values of 4.2 μM and <2.1 μM, respectively.

The loss of activity observed by the Ala deletions from peptide S7, as well as the antitumour activity of M6 indicated that limitations in sequence length and net positive charge were not the only parameters influencing antitumour activity. The order of the amino acid residues was suspected to play a more critical role in affecting antitumour activity compared with antimicrobial activity. Such a correlation was recently demonstrated in a series of longer bovine lactoferrin derivatives by varying the positions of the hydrophobic and cationic residues based on an α-helical conformation [16]. The peptides O1 and O2 were therefore designed in order to investigate the effects of peptide conformation on antitumour activity of short peptides (Table 3). Based on the peptide M1, which displayed no activity against tumour cells, O1 was designed by interchanging the Arg and Phe residues in positions 2 and 4, respectively, in order to optimize an amphipathic α-helical conformation (Table 3 and Figure 1).

To further optimize the amphipathicity, O2 was designed by interchanging the Trp and Arg residues in positions 1 and 5 of O1, respectively. Compared with M1, the simple modification resulting in peptide O1 did not improve the antitumour or the antimicrobial activity (Table 4). However, peptide O2 exhibited significantly improved antitumour activity and also displayed a 2.5-fold improved antimicrobial activity against *E. coli*. Neither O1 nor O2 displayed measurable haemolytic activity. It was also noteworthy that the fibroblast toxicity was unaffected by these substitutions and resulted in an above 3-fold IC_{50} ratio between fibroblasts and A549 cells.

DISCUSSION

Recently antitumour structure–activity relationship (SAR) studies were performed on α-helical amphipathic lactoferrin-derived 18-mer peptides [16,18]. Macroscopic properties such as the angle of the cationic sector, the number and positions of aromatic residues, and other properties at the amino

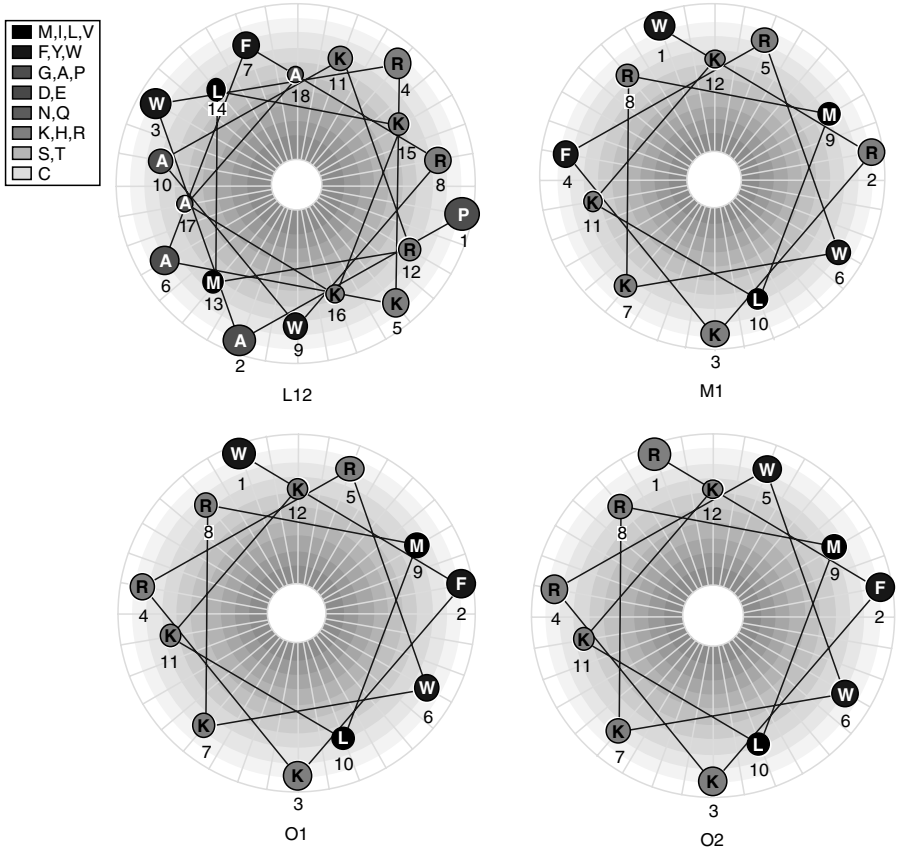

Figure 1 Edmundson helical wheel presentations of L12, M1, O1 and O2.

acid level (amino acid descriptors) have also been investigated by employing quantitative-SAR (QSAR) models [16,18]. However, certain structural parameters are difficult to include in multivariate data analysis, such as peptide length and the order of the amino acids. In the present study the 18-mer model peptide L12 was examined for antitumour activity by successively deleting residues from the *N*- and *C*-terminal ends, as well as by deleting certain residues within the sequence of the shorter derivative S7. It was also of interest in the present study to explore the possibility of designing short peptides with antitumour activity, and to compare the effects of peptide length and the order of the amino acids for antitumour and antimicrobial activity.

Most of the L12-derived peptides exerted similar variations in antitumour activity against all the tumour cell lines tested. None of the peptides were haemolytic and the peptides also showed a higher affinity for tumour cells relative to normal human fibroblasts, as observed by the higher IC_{50} values against the latter cells. In general, modifications of the peptides had a higher impact on reducing fibroblast toxicity than antitumour activity, resulting in an increased IC_{50} ratio between fibroblasts and the A549 carcinoma cells for several of the deletion peptides.

By successively deleting the *N*-terminal residues of L12 the results revealed that the *N*-terminal Trp and Arg residues were essential for antitumour activity, whereas the Pro and Ala residues could be deleted without any major effects on the antitumour activity. The importance of aromatic and cationic amino acid residues for antimicrobial activity of short cationic peptides, as well as antitumour activity of larger lactoferrin derivatives, is emphasized in several reports [5,16,18,19,24–27]. It was noteworthy that a 2-fold reduction in fibroblast toxicity was obtained by deletion of the *N*-terminal Trp-residue of S3, and that the resulting peptide S4 displayed above 4-fold selectivity for tumour cells, which was the highest among the *N*-terminal deletion peptides. Both antitumour activity and fibroblast toxicity were reduced by the following deletion of an Arg residue, whereas the antimicrobial activity of the resulting peptide S5 was unaffected. This implied a difference in the structural requirement for antitumour activity and selectivity compared with antimicrobial activity, and may eventually define a limit to how short membrane active antitumour peptides that can be designed.

Deletion of one or both of the Ala residues from the *C*-terminal end of S3 resulted in a slight reduction in antitumour activity, in which the most pronounced reduction was observed when the first Ala residue was deleted. The resulting peptides S6 and S7 were close to non-toxic against fibroblasts, and thereby gave the most selective peptides prepared with a 7-fold IC_{50} ratio between fibroblasts and A549 cells. The complete loss of antitumour activity observed when the *C*-terminal Lys residue of S7 was deleted giving S8, as well as the low antitumour activity of S5, implied that a net charge of +7 was required in order to exert a high antitumour activity and selectivity. A reduction in antimicrobial activity was also observed for S8, but it was much less affected than the antitumour activity and supported that the antitumour activity was dependent on a relatively higher net positive charge than the antimicrobial activity. None of the peptides S9–S11 showed antitumour activity or were toxic against fibroblasts within the concentration range tested. A gradual reduction in antimicrobial activity was also observed for these peptides. Hence, no further deletions in the *N*- or *C*-terminal ends could be performed without deleting residues critical for both the antitumour and antimicrobial activity.

Based on previous studies describing the design of short antimicrobial peptides [5,19,25] the effects of deleting residues that are indifferent or counter-productive was explored by designing the M-series of peptides. Thus, the M1 peptide was designed by deleting both of the internal Ala residues of S7. Ala is demonstrated to have a relative small effect on the antimicrobial activity of short peptides [27], but the deletions of the Ala residues resulted in a total loss of antitumour activity for M1 (a possible explanation to this is described below). The M2 peptide was designed by deleting the Lys residues in positions 3 and 12 of M1 and amidating the *C*-terminal carboxylate group in order to compensate for one of the two positively charged residues deleted. Amidation had a significant impact on antitumour activity, and besides S5 only the *C*-terminal amidated peptides M2, M3 and M6 displayed antitumour activity among the peptides of charge less than +7.

It was decided to delete Lys and not Arg in M1 since Arg is more important for the antimicrobial activity than Lys in short peptides [4,19,28]. This is due to the ability of Arg to participate in cation-π interactions with aromatic residues, which may result in a deeper insertion of the peptides into the cell membrane [29]. Arg is also positively charged at all physiological conditions and forms a more extensive hydrogen-bonding array with the negatively charged phospholipids of the cell

membrane. All the peptides in the M-series were highly active against bacteria. It was noteworthy that M6 was the most active antimicrobial peptide prepared and the only peptide that fulfilled the minimal structural requirement of short highly active antimicrobial peptides, which should include three Trp and three Arg residues [19]. The increased hydrophobicity by the replacement of the *C*-terminal Lys residue of M5 with a Trp residue also rendered M6 with slight antitumour activity and detectable toxicity against fibroblasts. This was consistent with our experience with longer lactoferrin derivatives, in which bulkiness of the amino acid residues is an important parameter in peptides with low hydrophobicity [18]. Thus, as observed for M6, by increasing the content of bulky residues in short peptides an increased antitumour activity with low toxicity against fibroblasts can be obtained.

Tumour cell membranes are often more negatively charged relative to normal cell membranes [12–14] and it has been shown that the L12 peptide possesses a considerably higher degree of α-helicity in negatively charged sodium dodecyl sulfate micelle (SDS) solutions than in buffer [16]. Since α-helical structures also have been identified in conjunction with binding of relatively short peptides to negatively charged membranes [1,30], it is also possible that short analogues of L12 may adopt an α-helical structure upon interaction with the negatively charged tumour cell membrane. Thus, the larger changes in antitumour activity than tumour cell specificity compared with normal cells could therefore be a result of reduced α-helical propensity of the M-derivatives due to the deletion of the internal Ala residues of S7, which have a high α-helical stabilizing propensity [31].

The amphipathic orientation of the residues in an α-helical structure is critical for the antitumour activity of 18-mer lactoferrin derivatives [16,32]. Based on an Edmundson helical-wheel projection of M1 (Figure 1), an amphipathic structure is lost by the deletion of the two Ala residues of S7. Thus, the loss of antitumour activity may have been caused by reduced ability to adopt an active amphipathic α-helical conformation of the M-series. It was noteworthy that the deletion of the two internal Ala residues did not result in a similar change in antimicrobial activity demonstrating that a higher number of active conformations or a higher flexibility was tolerated for antimicrobial activity. In order to test the hypothesis that an α-helical amphipathic structure was an important parameter for antitumour activity, the O1 and O2 peptides were designed by changing the order of the amino acids in the inactive peptide M1 (Figure 1 and Table 3). It was noteworthy that the O2 peptide displayed an augmented antitumour activity relative to the M1 peptide, whereas only a slight improvement in antimicrobial activity against *E. coli* was obtained (Table 4). These findings suggest that in addition to a certain net positive charge and peptide length, the ability to adopt an amphipathic α-helical conformation is a more important structural parameter for antitumour activity than for antimicrobial activity, implying a higher tolerated flexibility or number of active conformations for antimicrobial activity.

Several peptide parameters have been reported to influence the activity of peptides against mammalian cells [11,16,32,33]. These parameters include α-helicity, amphipathicity, overall hydrophobicity, hydrophobic moment, the nature of charged amino acid, and the angle subtended by the cationic residues. Therefore, further modifications of O2 by applying this information may form the basis for designing shorter highly active antitumour peptides. It is, however, clear that a number of structural parameters need to be included in a membrane active antitumour peptide. To the list of essential parameters deduced from our study of larger lactoferrin derivatives concerning bulk and lipophilicity [5,25], we can add for short peptides the importance of a net charge close to +7, an optimized α-helical amphipathic structure, and a thorough investigation of the effects of altering the order of the amino acid residues. Hence, it may not be possible to make as short membrane active antitumour peptides as highly active antimicrobial peptides in order to fulfil these structural requirements.

Shin *et al.* [11] reported the antitumour activity of P18, KWKLFKKIPKFLHLAKKF-NH_2, and derivatives ranging in size from 18 to 13 amino acid residues. Among these, the shortest peptide that displays high antitumour activity is N-5L, WKKIPKFLHLLKKF-NH_2, which consists of 14 amino acid residues and displays IC_{50} values in the range from 10 to 17 μM against three tumour cell lines, and 50 μM against the normal murine cell line NIH 3T3 [11]. Sequence comparisons between N-5L and S7 reveals that both peptides contain three aromatic amino acid residues, a net positive charge of +7, and are amphipathic when adopting an α-helical structure. Thus, these sequence features may constitute the requirements for designing highly active and selective antitumour 14-mer peptides.

The indolicidin-derived extended helical 13-mer peptide OLP-1, Ac-ILKKWPWWPWRRK, and its diastereomeric derivative OLP-4, Ac-iLKKWPWWPWRRk, are reported to have high antitumour activity, with a tumour to non-malignant cell ratio above 5-fold [15]. Thus, an active conformation of short antitumour peptides is not restricted to α-helical structures. The results from the present study show, however, that even shorter peptides than N-5L, OLP-1 and OLP-4 display antitumour activity and may constitute a novel class of antitumour agents with the ability to adopt an α-helical amphipathic conformation.

In conclusion, the results revealed that there was a profound difference between what are the structural requirements for short antimicrobial peptides and short peptides with activity against tumour cells. Small changes in the primary sequence had a much higher impact on the antitumour activity than on the antimicrobial activity of the peptides. Several of the hydrophobic residues could be deleted without losing antimicrobial activity, whereas antitumour activity was totally lost. Even though cationic residues are critical for both applications, a net charge of +7 was required for a high antitumour activity, whereas a net charge of only +4 is reported to be necessary for a high activity against prokaryotic cells [19]. Hence, the minimum peptide length of antitumour peptides must be longer than that of antimicrobial peptides. Moreover, the ability of a membrane active peptide to adopt an amphipathic structure was more critical for its antitumour activity relative to its antimicrobial activity. The fact that small changes in the primary sequence also had a significant influence on tumour cell specificity compared with normal cells, sheds light on the greater challenge of designing antitumour peptides than antimicrobial peptides due to the rather narrow borderline between peptides that are toxic against normal cells and peptides with an enhanced tumour cell specificity.

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