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Enhanced Antitumour Activity of 15-residue Bovine Lactoferricin Derivatives Containing Bulky Aromatic Amino Acids and Lipophilic *N*-terminal Modifications

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Abstract: In a structure–antibacterial activity relationship study of a peptide fragment of bovine lactoferricin consisting of FKCRRWQWRMKKLGA (LFB 17–31), it was revealed that the two Trp residues were important for antibacterial activity. It has further been demonstrated that the size, shape and the aromatic character of the side chains were even more important than the Trp itself. In this study the antitumour effect of a series of LFB 17–31 derivatives are reported, in which the two Trp residues in position 6 and 8 were replaced with the larger non-coded aromatic amino acids Tbt, Tpc, Bip and Dip. The counterproductive Cys in position 3 was also substituted with these larger aromatic residues. In addition, the effect of introducing lipophilic groups of different size and shape in the *N*-terminal of the LFB 17–31 sequence was addressed. The resulting peptide derivatives were tested for activity against three human tumour cell lines and against normal human umbilical vein endothelial cells and fibroblasts. High antitumour activity by several of the peptides demonstrated that Trp successfully could be substituted by the bulky aromatic residues, and peptides containing the large and rigid Tbt residue in position 6 and/or 8 in LFB 17–31 were the most active candidates. The antitumour effect was even more increased by the Tbt-modified peptides when the three counterproductive amino acids Cys3, Gln7 and Gly14 were replaced by Ala. Enhanced antitumour activity was also obtained by modifying the *N*-terminal of LFB 17–31 with either long-chained fatty acids or bulky moieties. Thus, our results revealed that the size and shape of the lipophilic groups and their position in the peptide sequence were important for antitumour activity.

Keywords: antitumoural peptides; bovine lactoferricin; bulky aromatic amino acids; tryptophan

INTRODUCTION

Bovine lactoferricin (LfcinB) is a pepsin-derived fragment of the iron-binding protein lactoferrin possessing antitumour activity against certain solid tumours and experimental metastases [1–5]. Recently it was found that the antitumour effect of LfcinB was not species-specific and that its mechanism of action *in vivo* seemed to include a direct lytic effect against certain tumour cell lines [5]. LfcinB encompasses residues 17–41 of the solvent exposed *N*-terminal α-helical segment of the

Abbreviations: ATCC, American Type Culture Collection; Bip, β-(4,4′-biphenyl)alanine; Dip; β-diphenylalanine; LFB, bovine lactoferrin; ECGF, endothelial cell growth factor; FBS, fetal bovine serum; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IC_{50}, 50% inhibitory concentration; MEM, modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; Tbt, β-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine; Tpc, β-[2-(Pmc)-indol-3-yl]alanine, i.e. Trp(2-Pmc).

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lactoferrin protein [6]. The disulfide bond between the Cys19 and Cys36 gives the peptide a cyclic structure [7,8]. The 25-residue peptide fragment originally obtained attention as an antibacterial peptide and has a well-documented broad-spectrum antibacterial activity [8]. In contrast to what has been observed with respect to the antibacterial effect of LfcinB, in which both the linear and the cyclic peptide display almost equal antibiotic potency [8,9], only the cyclic LfcinB was found to have any effect on tumour cells [5]. These findings indicate that a stabilized secondary structure is more important for the antitumour activity of LfcinB than for its antibacterial activity. A recent study further demonstrated that the size of the cationic sector and the number and position of aromatic amino acids in a helical LFB 14–31 model peptide were crucial for antitumour activity and tumour cell specificity relative to normal cells [10,11].

The importance of Trp residues in antibacterial peptides has been reported [12,13] and by performing an Ala-scan experiment, Strøm *et al.* [14] recently revealed the necessity of the two tryptophans in LFB 17–31 (FKCRRWQWRMKKLGA) for the antibacterial activity of the peptide. Additional studies demonstrated that the shape and size of the aromatic side chains were the important factors rather than the presence of tryptophan itself [15]. It was also demonstrated that the antibacterial activity of LFB 17–31 could be substantially increased by replacing Trp with large unnatural aromatic amino acids [16]. Since it was recently found that a higher number of aromatic amino acids were mandatory for the antitumour activity of LFB 14–31 derivatives than was needed for its antibacterial activity [10], the antitumour effect of replacing Trp by large unnatural aromatic residues in LFB 17–31 peptides was investigated. A number of derivatives of the LFB 17–31 peptide were prepared in which the three residues, Cys3, Trp6 and 8 were successively replaced by larger non-coded aromatic amino acids. Additional Tbt-modified LFB 17–31 derivatives were designed in which the three counterproductive amino acids, Cys3, Glu7 and Gly14, [14] were replaced with the hydrophobic and helix-promoting amino acid alanine [17]. The effect of introducing lipophilic moieties, different from those incorporated within the peptide sequence, in the *N*-terminus of LFB 17–31 was also investigated. As test systems we used a human mammary carcinoma cell line (MT-1), a melanoma cell line (RMS), and a colorectal adenocarcinoma cell line (HT-29), and as controls, normal human umbilical vein endothelial cells (HUV-EC-C) and fibroblasts (MRC-5).

MATERIALS AND METHODS

Materials

All natural Fmoc-amino acids, Fmoc-resins and chemicals used during peptide synthesis, cleavage and precipitation were purchased from PerSeptive (Hertford, UK), Fluka (Buchs, Switzerland) and Sigma (St Louis, MO, USA). Fmoc-diphenylalanine and Fmoc-biphenylalanine were purchased from Synthetech (Albany, USA). β-(2,5,7-Tri-*tert*-butylindol-3-yl)alanine (Tbt) was prepared as described by Lôw *et al.* [18] and Fmoc-protected as previously described [16]. β-[2-(2,2,5,7,8-pentamethylchroman-6-sulfonyl)-indol-3-yl]alanine (Tpc) was prepared as described by Haug *et al.* [19]. Fetal bovine serum (FBS) was obtained from Biochrom KG (Berlin, Germany). The culture media prepared at our University and the endothelial cell growth factor (ECGF) were delivered from Sigma-Aldrich AS (Oslo, Norway). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St Louis, MO, USA). The human melanoma cell line RMS and mammary carcinoma MT-1 were kindly provided by Dr Ø. Fodstad, Department of Tumour Biology, the Norwegian Radium Hospital, Oslo, Norway. The human colorectal adenocarcinoma cell line HT-29 (HTB-38), the human umbilical vein endothelial cell line HUV-EC-C (CRL-1730) and the embryonic fibroblast cell line, MRC-5 (CCL-171) were all obtained from the American Type Culture Collection (ATCC).

Peptide Synthesis, Purification and Analysis

All peptides were synthesized on a Milligen 9050 Plus Pepsynthesizer (Milford, MA, USA) using standard Fmoc chemistry, as earlier described [14]. Crude peptides were purified by preparative RP-HPLC (Waters, Milford, MA, USA) using a C_{18} column (Delta-PAK™ C18, 100 Å, 15 μm, 25–100 mm) and analysed on an analytical C_{18} HPLC column (Delta-Pak™ C18, 100 Å, 5 μm, 3.9 × 150 mm, Waters Corp., Milford, MA, USA) and the purity of all peptides were found to be >95%. Peptide characterization was done by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK).

Cell Cultures

The RMS, MT-1 and HT-29 cell lines were maintained as monolayer cultures in RPMI-1640 supplemented with 10% heat-inactivated FBS and 1% L-glutamine. The MRC-5 cells were cultured in MEM medium supplemented with 10% heat-inactivated FBS and 1% L-glutamine and the HUV-EC-C cells were cultured in MCDB105 without antibiotics containing 0.04 mg/ml ECGF and 10% heat-inactivated FBS. All cells were grown in tissue culture flasks in humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Before use, all cells were seeded at similar levels giving subconfluence into 96-well plates. MT-1, RMS and HUV-EC-C cells were seeded in a concentration of 2×10^4 cells/well, whereas HT-29 cells were seeded in a concentration of 4×10^4 cells/well and MRC-5 cells were seeded at a level of 1×10^4 cells/well, and allowed to adhere for about 16 h before assayed.

In vitro Cytotoxicity

The colorimetric MTT viability assay [20] was used to investigate the cytotoxic effect of the peptides. Different peptide concentrations (1–500 μg/ml) diluted in serum-free culture medium (100 μl) were added to the cells and incubated for 4 h at 37 °C. MTT solution (10 μl of 5 mg/ml stock in phosphate-buffered saline) was added to each well, and the incubation was continued for another 2 h at 37 °C. An aliquot of 70 μl was aspirated from each well and a solubilization agent, consisting of 100 μl 0.04 N HCl in isopropanol, was added and the plates were shaken for 1 h on a Thermolyne Roto Mix (Dubuque, Iowa, USA) at room temperature. The optical density was measured spectrophotometrically at 590 nm on a microtitre plate reader (Thermomax Molecular Devises, New Jersey, USA). As positive controls, cells treated with 1% solution of Triton X-100 were used, whereas cells and serum-free medium only were used as a negative control. Cell survival was determined from the ΔA_{590} nm relative to the negative control (100% living cells) and expressed as 50% inhibitory concentration (IC_{50}).

RESULTS

The LFB 17–31 derivatives containing the large non-coded aromatic amino acids β-(4,4′-biphenyl)alanine (Bip), β-diphenylalanine (Dip), β-(2,5,7-tri-*tert*-butyl-indol-3-yl)alanine (Tbt) and β-[2-(2,2,5,7,8-pentamethyl-chroman-6-sulfonyl)-indol-3-yl]alanine (Tpc) (Figure 1) were tested against mammary carcinoma (MT-1), melanoma (RMS) and colorectal adenocarcinoma (HT-29) cell lines and against normal umbilical vein endothelial (HUV-EC-C) and fibroblast (MRC-5) cells. The antitumour activities of all peptides are compiled in Table 1. The results revealed that by replacing Trp in position 6 and/or 8, or Cys in position 3 with larger aromatic amino acids, the antitumour activity of the peptides was considerably increased compared with the native LFB 17–31 peptide. The Tpc and Tbt modified LFB 17–31 derivatives appeared much more potent than the Bip and Dip modified peptides. The molecular volumes of the aromatic side chains of the four unnatural residues have been calculated [16,19] and are summarized in Table 2. The side chains of Tbt and Tpc are 2.5 and 2.9 fold larger than the molecular volume of the indole side chain of Trp, respectively, whereas the molecular volume of the side chains of both Bip and Dip is 1.3 fold larger than that of Trp. Hence, the antitumour activity of the peptides correlated with the molecular volumes of the side chains of the aromatic residues employed. Although the Bip and Dip-containing LFB 17–31 derivatives displayed low antitumour activity, peptides with the elongated and slim Bip residue appeared more active than peptides containing the isomeric Dip residue, which is shorter and broader,

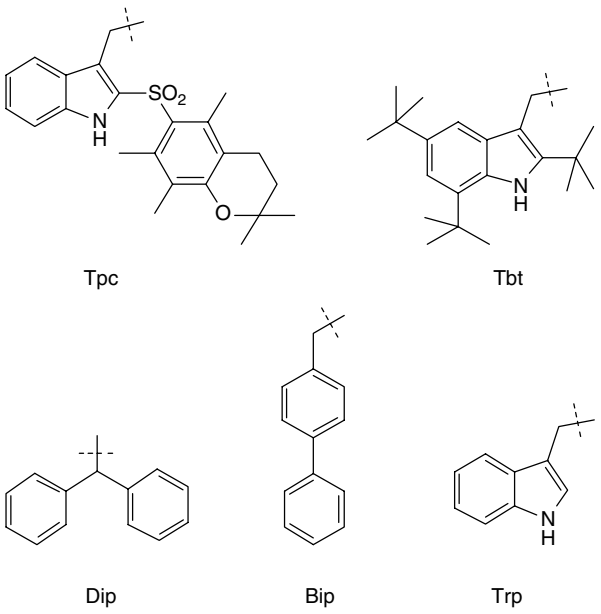

Figure 1 Structure of side chains of the aromatic amino acid residues employed.

Table 1 Antitumoural Effects of LFB 17–31 Peptide Derivatives Containing Bulky Non-coded Aromatic Amino Acids in Positions 3, 6 and 8 and Lipophilic Moieties in the *N*-terminal

Peptide name	IC_{50}MT-1 (μM)	IC_{50}RMS (μM)	IC_{50}HT-29 (μM)	IC_{50}HUV-EC-C (μM)	IC_{50}MRC-5 (μM)
LFB 17–31	>500	>500	>500	>500	>500
[Tpc^{3}]-LFB	21.8	23.5	24.5	33.3	32
[Tpc^{6}]-LFB	57.2	67.2	95.8	176.9	135.1
[Tpc^{8}]-LFB	50.1	52.9	53.2	120.2	90.1
[Tpc^{6,8}]-LFB	29.9	20.9	40.4	48.1	18.7
[Tbt^{3}]-LFB	21.1	35.5	25.7	53.4	33.3
[Tbt^{6}]-LFB	49.2	46.2	67	151.1	96.2
[Tbt^{8}]-LFB	53.5	41.3	63	127.6	63.4
[Tbt^{6,8}]-LFB	19.4	16.1	44	39.5	14.7
[Dip^{3}]-LFB	179.7	185	>500	235.3	>500
[Dip^{6}]-LFB	>500	>500	>500	224.9	>500
[Dip^{8}]-LFB	204.5	>500	>500	128.4	>500
[Dip^{6,8}]-LFB	>500	193.9	>500	>500	>500
[Bip^{3}]-LFB	177.3	115.9	147.8	194.6	179.7
[Bip^{6}]-LFB	>500	>500	>500	>500	>500
[Bip^{8}]-LFB	>500	>500	>500	>500	>500
[Bip^{6,8}]-LFB	200.9	190.5	156.6	>500	183.4
[A^{3,7,14}]-LFB	>500	>500	>500	>500	>500
[A^{3,7,14}Tbt^{6}]-LFB	10.3	11	22.3	23.5	21.6
[A^{3,7,14}Tbt^{8}]-LFB	37.4	26	43.1	27.8	21.6
[A^{3,7,14}Tbt^{6,8}]-LFB	30.8	19.9	24.1	31	35.5
Fmoc-LFB	72.6	50.3	45.9	70.8	63.7
Dodecyl-LFB	36.7	42.2	64.5	54.5	66.1
Adamantanoyl-LFB	72.3	86.8	90.5	165	107.8
Acetyl-LFB	>500	>500	>500	>500	>500

Table 2 Structural Properties of the Aromatic Amino Acids Used in the Present Study

Amino acid	Volume (Å^3)	Length[a] (Å)	Width[b] (Å)
Trp	129	4.3	2.4
Tpc	365	7.7	12.4
Tbt	325	7.6	8.9
Dip	172	4.3	7.1
Bip	172	8.7	2.4

[a] Measured as the maximum distance between the C-β and a carbon atom in the side chain.
[b] Measured as the maximum distance between two carbon atoms in the side chain.
Adapted from Haug *et al.* 2001. Bulky aromatic amino acids increase the antibacterial activity of 15-residue bovine lactoferricin derivatives. *J. Peptide Sci.* **7**: 425–432.

indicating that shape, and not merely the volume, influenced on the antitumour activity.

The [Tbt^{6,8}]-LFB and [Tpc^{6,8}]-LFB, in which both Trp were replaced, were 2–3 fold more active against the tumour cell lines than the peptide derivatives with only one of the two Trp residues replaced with Tbt or Tpc, and displayed a more than 25 and 17 fold higher antitumour activity against the MT-1 tumour cell line, respectively, than did LFB 17–31. [Tbt^{6,8}]-LFB was more active against the MT-1 and RMS tumour cell lines than was [Tpc^{6,8}]-LFB, whereas they were equally active against the HT-29 cell line, which generally was shown to be the least sensitive tumour cell line in the study.

The replacement of the counterproductive amino acid Cys3 with the Tbt, Tpc, Bip and Dip amino acids revealed that peptide derivatives containing Tpc and Tbt in position 3 possessed similar and in some cases better antitumour activity than did [Tbt$^{6.8}$]-LFB and [Tpc$^{6.8}$]-LFB. The introduction of an extra aromatic residue seemed to be favourable for the antitumour activity of the LFB 17–31 peptide derivatives. Additional Tbt-modified peptides were

prepared in which all three counterproductive amino acids, Cys3, Gln7 and Gly14 were replaced with Ala. The results showed that [$A^{3,7,14}$]-LFB did not display any antitumour activity, while the introduction of Tbt in the peptide resulted in a dramatic increase in antitumour activity. [$A^{3,7,14}Tbt^{6}$]-LFB turned out to be the most active peptide in this study with a 3 and 4 fold higher antitumour activity than the [$A^{3,7,14}Tbt^{8}$]-LFB and [$A^{3,7,14}Tbt^{6,8}$]-LFB derivatives, respectively, and with a 50 fold higher activity than [$A^{3,7,14}$]-LFB against the MT-1 tumour cells.

The effect of introducing lipophilic groups of different length and volume (Figure 2) at the *N*-terminal of the LFB 17–31 peptide was also investigated. The results showed that dodecyl-LFB displayed the highest activity against the MT-1 and RMS tumour cell lines, whereas Fmoc-LFB was the most active peptide against the HT-29 cell line, among the four *N*-terminal modified peptides tested (Table 1). Fmoc-LFB displayed an up to 2-fold higher antitumour activity than did adamantanoyl-LFB, while acetyl-LFB did not show any antitumour activity in the concentration range tested. Hence, the introduction of either long or bulky lipophilic *N*-terminal groups in the LFB 17–31 peptide led to a strong enhancement of antitumour activity.

The LFB 17–31 peptides containing either Tbt or Tpc were the most selective peptides displaying a 2- to 3-fold selectivity for tumour cells compared with the normal endothelial cells and fibroblasts. The most potent antitumoural peptide in the study, [$A^{3,7,14}Tbt^{6}$]-LFB displayed a 2-fold selectivity for the MT-1 tumour cells versus the endothelial cells, which in general was the least sensitive of the two normal cell types tested in this study.

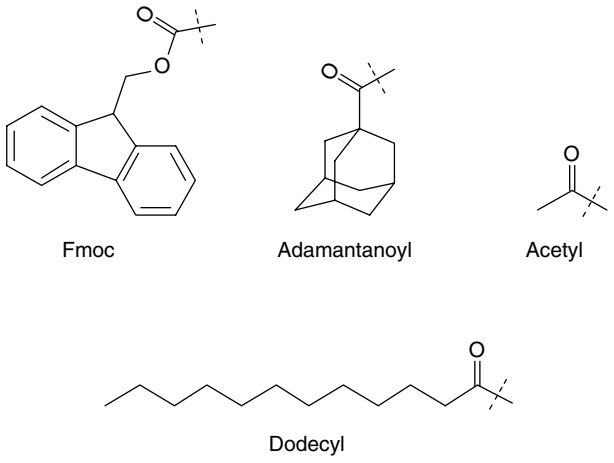

Figure 2 Structure of the *N*-terminal moieties employed.

DISCUSSION

In the present study the antitumour activity was investigated of a series of lactoferricin peptide derivatives in which Trp in position 6 and/or position 8 in the LFB 17–31 sequence was replaced by the larger aromatic amino acids Tbt, Tpc, Bip and Dip (Figure 1). To elucidate the importance of removing a counterproductive amino acid for the benefit of introducing a fourth aromatic residue in the peptide, Cys in position 3 was also replaced by these large aromatic residues. Additionally, to check whether the antitumour effect of the Tbt-modified peptides could be improved, the three counterproductive amino acids were replaced with Ala. The effect of increasing the hydrophobicity with respect to antitumour activity was further probed by introducing lipophilic moieties (Figure 2) *N*-terminally in the LFB 17–31 peptide. The modified LFB derivatives were tested against the three different human malignant cell lines; MT-1, RMS and HT-29 and against normal human endothelial cells (HUV-EC-C) and fibroblasts (MRC-5).

The current study revealed that the replacement of Trp by the Tbt, Tpc, Bip and Dip residues enhanced the antitumour activity of the LFB 17–31 peptide considerably, showing that Trp successfully could be substituted by large and bulky aromatic amino acids. This observation is consistent with former studies on bacteria performed by Haug *et al.* [16,19], showing that the antibacterial activity was strongly enhanced by introducing the aromatic amino acids in the LFB 17–31 peptides. Further, our study demonstrated that the peptide derivatives containing the Tbt and Tpc residues were invariably more potent against the tumour cell lines than were the Bip and Dip peptides. Hence, these results differ from the former study in which the effect of Bip and Dip substitutions in some cases resulted in peptides with higher activity against certain bacteria than the peptide derivatives containing Tbt [16]. By comparing the structures and the calculated molecular volumes of the side chains of the aromatic amino acids (Figure 1 and Table 2) with the antitumour potency of the different peptide derivatives tested (Table 1), it is evident that both volume and shape are important factors. The side chains of Tbt and Tpc residues are 2.5 and 2.9 times larger than the side chain volume of Trp, respectively, while Dip and Bip are 1.3 times larger. From Figure 1 it can be seen that Tbt and Tpc are both broad and long, Bip is elongated and slim while Dip is short and wedge shaped.

Even though low antitumour activity was obtained for both Bip and Dip containing peptides, the effect on the antitumour activity of introducing Bip in the LFB 17–31 sequence was stronger than that obtained by introducing Dip. The LFB 17–31 peptides containing Bip are previously found to be most potent against the Gram-positive bacteria *S. aureus*, whereas the Dip-containing LFB 17–31 peptides were more active against the Gram-negative *E. coli* [16]. These differences in antitumour and antibacterial activity between the Bip and Dip peptides are consistent with a recent study showing that the antitumoural activity of a series of 18-residue lactoferricin derivatives correlated better with their antibacterial activity against the Gram-positive *S. aureus* than against the Gram-negative *E. coli* [11]. This correlation was also confirmed by quantitative structure–activity relationship studies [11]. Hence, it seems that by introducing longer aromatic side chains in membrane active peptides, the cytoplasmic membrane of tumour cells and Gram-positive bacteria may be more efficiently disrupted, whereas broader and shorter aromatic side chains with a similar molecular volume may be more optimal for disrupting the more complex membrane of Gram-negative bacteria [16], which has an outer membrane exterior to the cytoplasmic membrane [21].

Although Tpc has a larger volume than Tbt, the Tbt replacements resulted in general in more active peptide derivatives than did the Tpc replacements. The Tbt structure is, however, more rigid compared with the Tpc residue, and therefore the Tbt side chain might disturb the packing of the phospholipids in the cell membrane more readily than Tpc. Additionally, the polar sulfonyl moiety in Tpc could interfere with membrane interactions. When both Trp residues were replaced by Tpc or Tbt, the antitumour activity was enhanced relative to the peptides with only one Trp replacement. Interestingly, when the counterproductive Cys in position 3 was substituted by Tbt or Tpc the antitumour activity was even higher than when the Trp residues were substituted. This is probably due to the introduction of an additional aromatic amino acid making the peptides overall more hydrophobic. [Tbt^{3}]-LFB was the second most active peptide after [$Tbt^{6,8}$]-LFB of all the Tbt and Tpc-modified peptides tested so far. Even though [Bip^{3}]-LFB and [Dip^{3}]-LFB displayed much lower antitumour activity than [Tpc^{3}]-LFB and [Tbt^{3}]-LFB, these peptides were still the most active derivatives within the groups of Bip and Dip containing peptides, supporting earlier findings that the number of aromatic residues or the total molecular volume of the aromatic side chains in LFB derived peptides is important for antitumour activity [16].

Strøm *et al.* [14] has earlier reported three counterproductive residues in the LFB 17–31 sequence that could be replaced by Ala residues to obtain higher antibacterial activity. In order to check whether the antitumour activity of the most active LFB 17–31 peptides could be further improved, the effect of replacing the counterproductive amino acids, Cys3, Glu7 and Gly14 with Ala in the Tbt modified peptides was investigated. Our study found that the LFB 17–31 peptide containing the three alanines did not display any detectable antitumour activity. But, a major increase in activity was obtained when Tbt was introduced in position 6 and/or 8, and the increase in antitumour activity of [$A^{3,7,14}Tbt^{6}$]-LFB compared with [Tbt^{6}]-LFB does indicate that the three Ala residues are more favourable than the combination of the Cys, Gln and Gly residues. The peptides [$A^{3,7,14}Tbt^{6}$]-LFB and [$A^{3,7,14}Tbt^{8}$]-LFB were 5 and 1.5 fold more effective than [Tbt^{6}]-LFB and [Tbt^{8}]-LFB against the MT-1 cell line, respectively. When Tbt was introduced in both position 6 and 8 in the [$A^{3,7,14}$]-LFB peptide, the antitumour activity toward MT-1 cells was, however, decreased 1.5 fold compared with the [$Tbt^{6,8}$]-LFB and was even 3 fold lower than that of [$A^{3,7,14}Tbt^{6}$]-LFB. These results also indicate that for the Ala LFB 17–31 derivatives, the incorporation of Tbt in position 6 was more favourable than in position 8, which was opposite of what was observed for the native LFB 17–31 peptide with respect to antibacterial activity [19]. Noteworthy, the tumour cell specificity versus normal cells was also markedly higher for [$A^{3,7,14}Tbt^{6}$]-LFB than for [$A^{3,7,14}Tbt^{8}$]-LFB. A possible explanation of the difference in activity of the LFB 17–31 peptide derivatives observed when Tbt was introduced may be that the 6 and 8 residues have different orientations or are positioned in different environments in the active conformation of the peptides. Thus, different secondary structures of [$A^{3,7,14}$]-LFB and LFB 17–31 may well be an explanation as to why it was more favourable to replace Trp 6 by Tbt in the Ala-LFB 17–31 derivative, than in LFB 17–31 itself. Also the [Tbt^{6}]-LFB and [$A^{3,7,14}Tbt^{6}$]-LFB may have different secondary structures. Since Ala has a relative high helical propensity [17], the incorporation of multiple Ala residues may give the peptide a more α-helical structure. In our group, a circular dichroism (CD) study of LFB 17–31 showed that the peptide displayed low α-helical propensity [14]. However, another CD-study demonstrated by

introduction of multiple Ala residues in LFB 14–31 peptide derivatives, the degree of α-helicity was increased compared with the native LFB 14–31 peptide, both in a sodium dodecyl sulfate (SDS) micellar solution and in a more hydrophobic hexafluoroisopropanol (HFIP) solution [10]. These α-helical LFB 14–31 derivatives, which also contained additional aromatic amino acids than those present in the native sequence, were highly active against certain tumour cell lines [10]. Thus, further studies need to be done in order to reveal whether the differences in activity between [$A^{3,7,14}Tbt^{6}$]-LFB and [$A^{3,7,14}Tbt^{8}$]-LFB not found between [Tbt^{6}]-LFB and [Tbt^{8}]-LFB is due to a relative higher degree of α-helicity by the two former peptides.

The effect of modifying the *N*-terminal of LFB 17–31 was also addressed in the present study. Lipophilic groups of different size and length were coupled to the *N*-terminal of the peptide (Figure 2). Evidently, the length of the *N*-terminal moiety seemed to be an important factor for obtaining enhanced antitumour activity, as dodecyl-LFB was the most active of the four peptide derivatives tested. The dodecyl fatty acid is elongated and slim and in combination with the LFB 17–31 peptide, this peptide derivative may exhibit binding probabilities similar to that of lipopeptaibols, a novel group of naturally occurring short antimicrobial peptides isolated from fungi [22,23]. These peptides are characterized by a lipophilic acyl chain at the *N*-terminal, a high content of the turn/helix forming α-aminoisobutyric acid and a *C*-terminal alcohol [24]. One of the most studied lipopeptaibol, trichogin GA IV, has a considerable membrane perturbing activity against bacteria [24], implying that the lipidation may be essential for the membrane lytic activity. A possible role for acylation could be that it facilitates the partitioning of the lipopeptide from water into the membrane. The length of the acyl chain is reported to be important for activity, as the acylation of trichogin GA IV with carboxylic acids that are shorter than four carbons gave inactive peptaibol derivatives [25]. This is consistent with the lack of antitumour activity for acetyl-LFB observed in our study. Furthermore, both adamantanoyl-LFB and Fmoc-LFB displayed 2-fold lower activity than did dodecyl-LFB against MT-1 and RMS, but against HT-29 Fmoc-LFB exhibited 1.4- and 2-fold higher effect than dodecyl-LFB and adamantanoyl-LFB, respectively, indicating that also *N*-terminal bulkiness displays some favourable properties for antitumoural activity. But, also here it seems that a limit regarding size is reached since adamantanoyl-LFB, the largest lipophilic group exhibited a lower effect than the smaller Fmoc structure.

It was recently reported that both antitumour activity and selectivity for tumour cells compared with normal fibroblasts could be enhanced by modulating the angle of the cationic sector of helical LFB derived peptides [10]. Johnstone *et al.* [26] have also reported high tumour cell specificity relative to normal cells by extended helical indolicidin derived peptides. Compared with the selectivity reported in these two studies, the selectivity for tumour cells relative to normal cells for the peptides in the present study was moderate. The highest selectivity was obtained by the Tbt and Tpc containing peptides with a 2–3 fold selectivity for the tumour cell lines compared with the normal cells. Hence, in order to enhance the selectivity of peptides containing non-coded aromatic amino acids, the interplay between cationic and hydrophobic residues must be optimized. Furthermore, since a strong enhancement in antitumour activity was obtained by introducing non-coded aromatic residues that are larger than Trp, it should be possible to construct shorter highly active and selective peptides by combining an appropriate cationic sector with non-coded aromatic residues.

In conclusion, the introduction of hydrophobic residues into the LFB 17–31 peptide dramatically increased the antitumour potency of the resulting peptides. By further replacing counterproductive residues with Ala, a more than 50-fold increase in antitumour activity compared with the starting peptide was obtained. The volume and shape of these large residues in the peptide sequence were also found to be important. Further manipulations of the net charge and distribution of charged residues combined with a proper number of large bulky amino acids might provide peptides with higher selectivity and are in progress in our laboratory.

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