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Synthesis of anticancer heptapeptides containing a unique lipophilic $\beta^{2,2}$-amino acid building block

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We report a series of synthetic anticancer heptapeptides (H-KKW$\beta^{2,2}$WKK-NH_2) containing eight different central lipophilic $\beta^{2,2}$-amino acid building blocks, which have demonstrated high efficiency when used as scaffolds in small cationic antimicrobial peptides and peptidomimetics. The most potent peptides in the present study had IC_{50} values of 9–23 μM against human Burkitt's lymphoma and murine B-cell lymphoma and were all nonhaemolytic ($EC_{50} > 200$ μM). The most promising peptide 10e also demonstrated low toxicity against human embryonic lung fibroblast cells and peripheral blood mononuclear cells and exceptional proteolytic stability. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: peptide synthesis; anticancer peptides; $\beta^{2,2}$-amino acids; heptapeptides

Introduction

Alkylating agents, antimetabolites, and plant alkaloids are traditionally used as anticancer chemotherapeutics but are often associated with numerous side effects and low patient compliance [1]. Several targeted therapies such as monoclonal antibodies and small molecule inhibitors have also been introduced, which are less toxic and better tolerated than traditional anticancer chemotherapy [2]. A key concern in anticancer chemotherapy is, however, the often rapid development of resistance, involving expression of efflux pumps or mutations in intracellular or extracellular target proteins [3,4]. Clearly, there is a strong need for continued development of anticancer drugs with new modes of action, low toxicity, and low probability of inducing resistance.

Cationic antimicrobial peptides (CAPs) is a promising class of anticancer agents having a unique mode of action by targeting and disrupting the integrity of the cancer cell membrane or the mitochondrial membrane, the latter demonstrated by swelling of the mitochondria and induction of apoptosis [5–7]. Anticancer CAPs have also been shown to trigger innate immunity, kill resistant cancer cells, and enhance the cytotoxicity of traditional chemotherapeutics in multidrug-resistant tumours [8,9].

The preference of CAPs for cancer cells compared with normal cells has been attributed to an electrostatic attraction between the cationic residues of the peptides and the negatively charged outer membrane constituents of cancer cells [6]. The difference in susceptibility between normal and cancerous cells is explained by a 3–9% excess of the acidic phospholipid phosphatidylserine, elevated levels of O-glycosylated mucins, and increased sialylation of glycoproteins and glycolipids on cancer cells [10,11]. Additionally, increased fluidity of the cell membrane and increased surface area owing to more microvill on cancer cells is believed to affect the action of CAPs [12]. Thus, by evading the use of specific extracellular and intracellular target proteins, CAPs may have a lower probability of inducing resistance compared with traditional chemotherapeutics.

Although the literature on anticancer CAPs of less than nine residues is somewhat limited, an inspiring aspect when designing small anticancer CAPs is that the antimicrobial core sequence of bovine lactoferricin (LfcinB) (H-RRWQWR-NH_2) displays anticancer activity when delivered intracellularly to T-leukaemia cells [13]. Furthermore, the peptide contains a lipophilic core sequence with cationic ends, and despite its small size, it adopts a highly defined amphipathic structure upon interaction with negatively charged micelles [14].

We have recently investigated the antimicrobial activity of small CAP-based β-peptidomimetics consisting of a newly developed series of achiral, lipophilic $\beta^{2,2}$-amino acids, which were potent against both Gram-positive and Gram-negative bacteria and exceptionally potent against MRSA [15,16]. Noteworthy, $\beta^{2,2}$-amino acids have been shown to induce turns when incorporated into longer

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***Abbreviations used**: Ramos, human Burkitt's lymphoma; A20, murine B-cell lymphoma; MRC-5, human embryonic lung fibroblasts; CAPs, cationic antimicrobial peptides; RBC, red blood cells; PBMCs, human peripheral blood mononuclear cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRSA, methicillin-resistant Staphylococcus aureus; ATCC, American Type Culture Collection; HBTU, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate; HOBt, N-hydroxybenzotriazole; TFFH, fluoro-N,N,N′,N′-tetramethylformamidinium hexafluorophosphate; ITC, isothermal titration calorimetry; PI propidium iodide.*

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peptides, and by including bulky lipophilic substituents, the turn inducing effect may be enhanced [17,18]. To design an even more distinctive amphipathic motif around the $\beta^{2,2}$-amino acids, they were flanked by tryptophans to increase the bulkiness and then by lysines as cationic residues, resulting in a palindromic model heptapeptide H-KKW$\beta^{2,2}$WKK-NH_2. Lysine was preferred as cationic residue because Arg in CAPs has been reported to interact more strongly with zwitterionic phospholipids in healthy cells and thereby promote cell toxicity [19].

In the present study, we have synthesized and studied the effects of incorporating various lipophilic $\beta^{2,2}$-amino acid residues into the palindromic model heptapeptide to develop small anticancer CAPs (Figure 1). The anticancer potency of the peptides was investigated against human Burkitt's lymphoma (Ramos) and murine B-cell lymphoma (A20), and toxicity was examined against human red blood cells (RBC), healthy human embryonic lung fibroblasts (MRC-5), and healthy human peripheral blood mononuclear cells (PBMCs). High proteolytic stability was furthermore demonstrated by degradation studies using trypsin and α-chymotrypsin.

Materials and Methods

General

All chemicals were purchased from Sigma-Aldrich and used without further purification. HRMS spectra were recorded on a Waters Micromass LCT Premier time-of-flight mass spectrometer equipped with an electrospray ion source and analyzed using MassLynx v4.1 software (Waters Corporation, Milford, MA, USA). The samples were introduced to the mass spectrometer using a Waters 2795 analytical HPLC with an XTerra MS 3.5-μm C_{18} RP column (2.1 × 50 mm) (Waters Corporation). The mobile phase consisted of different combinations of Milli-Q water (Millipore Corporation, Billerica, MA, USA) and acetonitrile, both containing 0.1% formic acid, and a gradient running for 5 min was used. Leu-enkephalin was infused through the reference probe and used as lock mass for internal calibrations throughout the data acquisitions. All data were acquired in the positive ion mode. MS-spectra were recorded on a Waters Quattro LC quadropole MS equipped with an electrospray ion source (Micromass, Manchester, UK) and analyzed using the MassLynx v3.4 software. All samples were analyzed in the positive ion mode. ^{1}H-NMR and ^{13}C-NMR experiments were recorded on a Varian 400 NMR spectrometer or a Varian 600 NMR spectrometer with $CDCl_3$ or CD_3OD as solvents and analyzed using vnmr software (Varian Inc., Palo Alto, CA, USA). Chemical shifts are expressed in parts per million (ppm, δ) and referred to the solvent signal [20]. The preparative HPLC system consisted of a Waters 600 E System Controller, a Waters In-Line Degasser, a Waters 717 auto sampler, and a Waters 2487 Dual λ Absorbance Detector controlled by Empower Pro software (Waters Corporation). A SunFire Prep OBD 5-μm C_{18} RP column (19 × 250 mm) (Waters Corporation) was used. The absorbance detector was operated at 214 nm. The mobile phase was different combinations of 5% acetonitrile in Milli-Q water (A) and 95% acetonitrile in Milli-Q water (B), both containing 0.1% TFA, and the flow rate was 15 ml/min. The analytical HPLC system consisted of a Waters 2695 Separations Module and a Waters 996 Photodiode Array (PDA) Detector controlled by Empower Pro software. A SunFire 5 μm C_{18} RP column (4.6 × 250 mm) was used for purity analysis. A YMC-Pack Pro 5 μm C_{18} RP column (4.6 × 250 mm) was used for retention time analysis. The compounds were analyzed at wavelengths 214 and 254 nm with the PDA detector spanning from wavelengths 210 to 310 nm The mobile phase was different combinations of 5% acetonitrile in Milli-Q water (A) and 95% acetonitrile in Milli-Q water (B), both containing 0.1% TFA, and the flow rate was 1 ml/min.

Synthesis

For synthesis, yields, and characterization data of **1a**, **1c-1h**, **3a**, **3c**, **3d**, **3g**, **4a**, **4c**, **4d**, and **4g**, please see Hansen *et al.* [15]. Synthesis, yields, and characterization data of all other compounds are given in the Supporting Information. All peptides tested had a purity of >95% as determined by analytical RP-HPLC.

Maintenance of cell lines

Murine B-cell lymphoma, MRC-5, and Ramos cell lines were kindly provided by Prof. Øystein Rekdal (Department of Medical Biology,

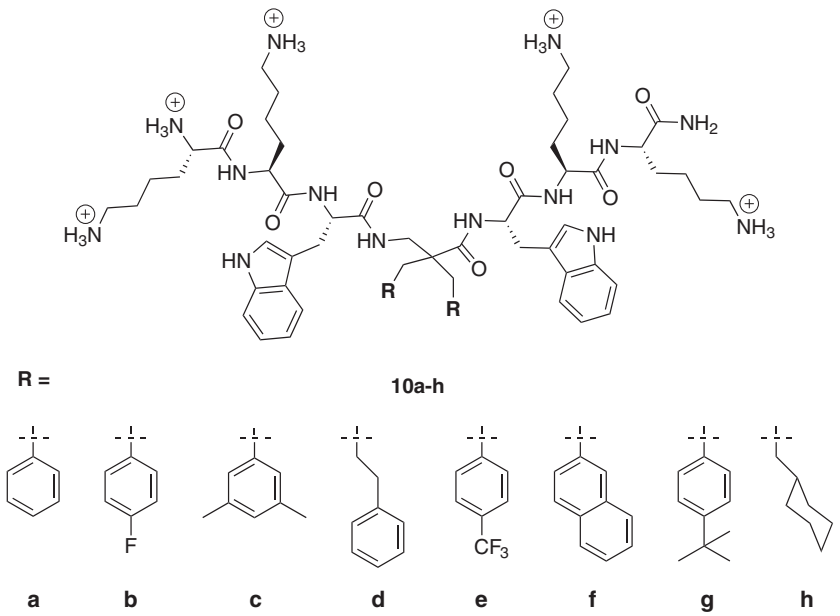

Figure 1. General structure of the synthesized heptapeptides **10a-10h** (H-KKW$\beta^{2,2}$WKK-NH_2).

University of Tromsø). A20, a B-cell lymphoma of BALB/c mice origin (TIB-208, ATCC, Manassas, VA, USA), and the Ramos cell line, a human Burkitt's lymphoma B lymphocyte, were maintained in RPMI-1640 medium containing 10% heat-inactivated foetal bovine serum (FBS). The human embryonic fibroblast cell line MRC-5 (CCL-171, ATCC) was maintained in Minimal Essential Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. Both culture media were without antibiotics. The cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and all cell lines were regularly tested for viruses (RapidMAP™-27, Taconic, Europe) and mycoplasma (MycoAlert Assay Control Set, Cat # NLT07-518, Lonza Rockland Inc., Rockland, ME, USA). The adherent cell line MRC-5 was detached from the solid support using trypsin and suspended in RPMI-1640 medium with 10% FBS and 1% L-glutamine prior to use.

PBMCs separation

Buffy coat samples were obtained from the University Hospital of North Norway Blood Bank. PBMCs were isolated by centrifugation in Lymphoprep (1.077 g/ml; Nycomed Pharma, Oslo, Norway). Briefly, buffy coat was diluted 1 : 4 in PBSA (0.2% bovine serum albumin in PBS) without calcium or magnesium. Afterwards, 35 ml of the diluted buffy coat was layered over 15 ml of Lymphoprep, followed by a 15-min centrifugation at 800 *g* at room temperature. The interface was collected and mixed gently with 40 ml of PBSA. The sample was centrifuged two times at 400 *g*, for 6 min, at room temperature. After each centrifugation, the supernatant was eliminated and another 40 ml of PBSA was added. The pellet, containing the PBMCs, was resuspended in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) and immediately used on MTT assay.

MTT Assay

All cells, including the suspension cells, were counted and centrifuged, and the resulting pellets were suspended in serum-free RPMI-1640 medium [21]. The cells were seeded in 96-well plates, 100 µl per well of cell suspension. A20 cancer cells were seeded at a concentration of 6×10^5 cells/ml, MRC-5 cells at 1×10^5 cells/ml, Ramos cancer cells at 4×10^5 cells/ml, and PBMCs at 2×10^6 cells/ml. Ramos, A20, and PBMCs were stimulated immediately, whereas the MRC-5 cells were incubated for 24 h and washed twice with serum-free RPMI-1640 medium before stimulation.

To determine the cytotoxic effect of the peptide, we used the colorimetric MTT viability assay, and 100 µl of different concentrations of peptides were added to all the cell lines. Cells added with 100 µl serum-free medium were used as negative controls, and cells added with 100 µl 1% Triton X-100 in serum-free medium were used as positive controls. After 4 h of incubation, 10 µl MTT solution (5 mg MTT per mL PBS) was added to the MRC-5 cells, whereas 20 µl was added to the Ramos, A20, and PBMCs, followed by another 2 h of incubation. A volume of 70 µl was removed from the MRC-5 cells, and 130 µl was removed from Ramos, A20, and PBMCs, before 100 µl of isopropanol with 0.04 N HCl was added, and the plates were shaken for 1 h at room temperature. The absorbance at 590 nm was measured on a microtitre plate reader. Cell survival was determined from the $\Delta A_{590\,nm}$ relative to the negative control, and the 50% inhibitory concentrations (IC_{50}) were determined from the dose–response curves. The procedures per sample were carried out in triplicates.

RBC Toxicity Assay

The RBC toxicity testing was conducted as previously described [15,22,23]. In brief, 8 ml of blood was collected from adult male donors, and the blood was divided equally into a commercially available test tube containing EDTA (BD vacutainer, 7.2 mg K2 EDTA, BD, Heidelberg, Germany) and into a 10 ml reaction vial containing 40 µl of a heparin solution (1000 U/ml). After 30 min, the haematocrit (Hct) of the EDTA-treated blood was determined (Sysmex K-1000, Sysmex Corporation, Kobe, Japan), and according to the measured value, the necessary amounts of blood and PBS for a suspension with 10% Hct were calculated. The heparinized blood sample was centrifuged for 10 min at 437 g (1500 r.p.m)., and the supernatant was carefully removed and replaced with prewarmed (37 °C) PBS. The procedure was repeated three times, and the sample was finally diluted with PBS to 10% Hct. The peptides were dissolved in PBS and were pipetted in reaction vials in end concentrations from 1 µg/ml up to 1000 µg/ml. A positive control with an end concentration of 0.1% Triton X-100 and a negative control containing pure PBS buffer were included. The erythrocytes (1% v/v) were added, and the vials were incubated under agitation at 37 °C for 1 h. The samples were centrifuged for 5 min at 3112 g (4000 r.p.m)., and 100 µl of each reaction vial was transferred to a 96-well plate, and intensely coloured samples were diluted with PBS. After measuring the absorbance at 405 nm with a microplate reader (VersaMax™, Molecular Devices, Sunnyvale, CA, USA), the percentage of haemolysis was calculated as the ratio of the absorbance in the peptide-treated and surfactant-treated samples. The absorbance of the negative control was used for baseline corrections.

Proteolytic Stability Assays

The proteolytic stability of **10e** against trypsin and α-chymotrypsin was determined in a solution of 100 µg/ml of **10e**, 2 µg/ml of trypsin or α-chymotrypsin, 100 mM tris-HCl and 10 mM $CaCl_2$. The enzyme digestions were incubated at 37 °C. Samples of 15 µl were collected at different time intervals, and 100 µl 10% (v/v) formic acid was added to stop the enzyme activity. A negative control without enzyme was incubated to determine whether the degradation was due to the enzyme or other factors. As positive control for α-chymotrypsin, a solution of succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline (0.62 mg/ml) was added to a final concentration of 0.48 mM instead of the peptide solution. Similarly, 0.5-mM Nα-Benzoyl-L-arginine ethyl ester hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 11.8 µg/ml was used as positive control for trypsin. Propranolol hydrochloride was used as internal standard and added to the 200 mM tris-HCl buffer to a final concentration of 57.8 µM. In the digestions containing succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline, the internal standard was 5-nitroimidazole at a final concentration of 40 µg/ml in the buffer solution. All samples were run on an analytical HPLC-PDA (Waters 2695 Separations Module and a Waters 996 PDA Detector controlled by Empower Pro software with an YMC-Pack Pro 5 µm C_{18} RP column (4.6 × 250 mm)) immediately after preparation for quantitative analyses of remaining **10e**. The gradient for separation started with an isocratic elution with 80% A and 20% B for 3 min, then a linear gradient to 40% A and 60% B over the next 17 min. Solvent A consisted of 95% water, 5% acetonitrile, and 0.1% TFA, whereas solvent B consisted of 5% water, 95% acetonitrile, and 0.1% TFA. Detection of **10e**, Nα-Benzoyl-L-arginine ethyl ester hydrochloride and propranolol was performed at 260 nm, and detection of succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline and 5-nitroimidazole was performed at 350 nm. The relative ratio of the area of **10e** over the area of propranolol was determined, and the degree of degradation of **10e** was calculated using the ratio at $t = 0$ as reference. To determine the degradation product of trypsin, we purified the enzyme digestion on a preparative HPLC-UV and analyzed the collected fractions using MS.

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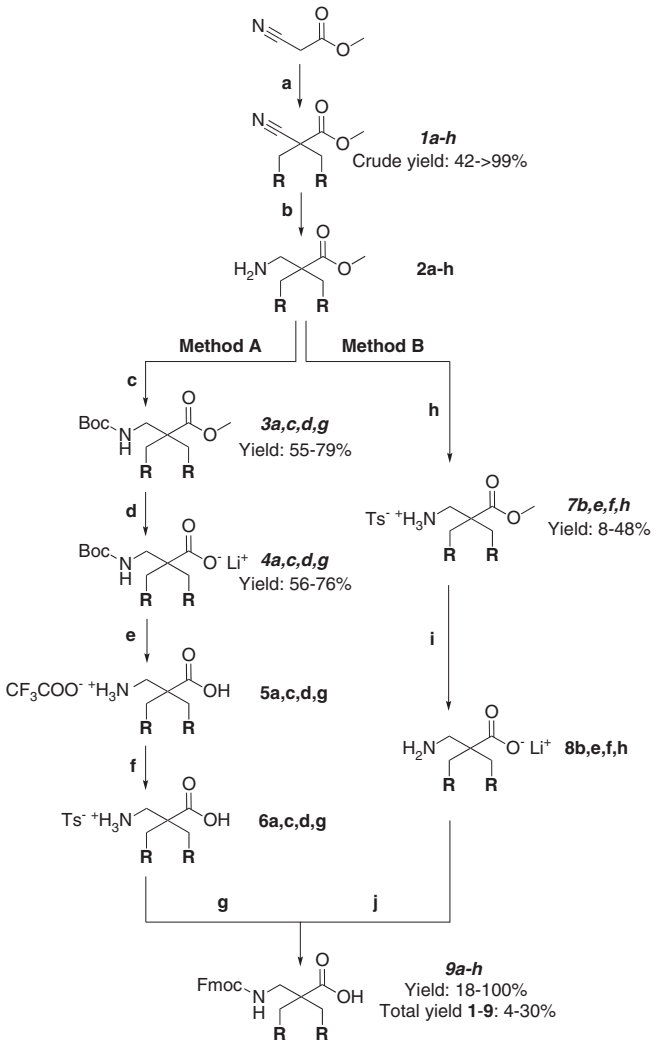
Results and Discussion

Synthesis

Synthesis of the Fmoc-$\beta^{2,2}$-amino acids **9a-9h** was performed by two different methods, A and B, as shown in Scheme 1. Both methods started with dialkylation of methyl cyanoacetate and reduction of the nitrile group by catalytic hydrogenation to achieve the unprotected $\beta^{2,2}$-amino acid methyl esters **2a-2h** [24]. Method A was based on our previous synthesis of antimicrobial β-peptidomimetics in which the $\beta^{2,2}$-amino acid methyl esters **2** were further Boc-protected before the methyl esters were hydrolyzed [15]. The Boc protections were removed, and the resulting $\beta^{2,2}$-amino acids (**5**) were precipitated as tosylate salts (**6**) before the amino groups were protected with Fmoc groups (**9**) [25].

A more efficient method was developed during the project, method B, which involved precipitation of the $\beta^{2,2}$-amino acid methyl esters **2** as tosylate salts (**7**) before hydrolysis of the methyl esters and subsequent Fmoc protection of the amino groups.



Scheme 1. Synthesis of the Fmoc-protected $\beta^{2,2}$-amino acids **9a-9h**. Method A gave total yields of 4–9%, and method B gave total yields of 10–30% for synthesis of **9a-9h**. R groups are shown in Figure 1 and in the Supporting Information. Isolated compounds are italicized. **a**: NaOMe, R-Br (performed twice). **b**: Ra/Ni, H_2(g), HOAc. **c**: TEA, Boc_2O. **d**: LiOH, H_2O/dioxane. **e**: TFA:TIS:H_2O (95:2.5:2.5). **f**: TsOH. **g**: TEA, Fmoc-OSu. **h**: TsOH. **i**: LiOH, H_2O/dioxane. **j**: Fmoc-OSu.

Although different $\beta^{2,2}$-amino acids were prepared, method B gave, in general, higher yields, involved fewer steps, and was quicker and easier to perform than method A. The final peptides **10a-10h** (Figure 1) were synthesized by Fmoc SPPS using HBTU/HOBt or TFFH as activating agents. (See Supporting Information for experimental details and comparison of yields.)

Anticancer Activity

The peptides were screened for anticancer activity against Ramos and A20 cells. As a measurement of toxicity, the peptides were tested for haemolytic activity against human RBC, and for cytotoxicity, against normal MRC-5 and human PBMCs.

Although only the side chains of the central $\beta^{2,2}$-amino acid residue were altered in the eight peptides prepared, the results nevertheless revealed a substantial variation in anticancer potency and toxicity. We also found that the anticancer potency of the peptides was comparable with potencies reported for longer anticancer CAPs, thereby supporting the hypothesis on the efficiency of including a central lipophilic core sequence with cationic N-terminal and C-terminal ends in our model heptapeptides [9,26].

When investigating the individual peptides, **10h** was the overall most cytotoxic peptide prepared, followed by **10g** and **10f** (Table 1). Peptide **10h** was the only peptide containing a $\beta^{2,2}$-amino acid residue with two purely aliphatic lipophilic side chains (Figure 1), and its high cytotoxicity against all cell types may be a result of a deeper penetration into the phospholipid acyl chain region of both normal and cancer cell membranes.

With respect to the remaining peptides containing $\beta^{2,2}$-amino acids with aromatic side chains, we observed that the trends in anticancer potency followed the same trends as observed for our small antimicrobial β-peptidomimetics with similar $\beta^{2,2}$-amino acid derivatives in the order from high to low potency: **10g**, **10f**, **10e**, **10d**, and **10c**, and with peptides **10b** and **10a** showing poor or no anticancer potency (Table 1) [15,16]. Importantly, peptides **10f** and **10g** contained the most bulky $\beta^{2,2}$-amino acid derivatives, which might explain their high anticancer potency when presuming a membrane-disrupting mode of action as reported for larger CAPs [6].

Although only being the fourth most potent peptide, **10e** was perhaps the most promising peptide prepared when considering overall anticancer potency and toxicity, that is, nontoxic against RBC and PBMCs and low toxicity against MRC-5 cells (Table 1). Peptide **10e** contained a $\beta^{2,2}$-amino acid derivative with two aromatic *p*-trifluoromethyl benzylic side chains, which also has proven highly efficient when incorporated into short antimicrobial peptidomimetics [15,16]. Noteworthy, incorporation of aromatic or benzylic fluorine substituents, as found in **10e**, is widely used during drug development to improve the pharmacokinetic properties of drug candidates [27]. The effects on anticancer potency and toxicity of the trifluoromethyl substituents of the $\beta^{2,2}$-amino acid side chains of **10e** are not fully understood. However, the trifluoromethyl groups may alter the electronic distribution of the aromatic side chains and contribute to increased lipophilicity, which together can affect the peptides' interaction with various cell membrane constituents, improve membrane permeability, and thereby permit interaction with intracellular targets as reported for LfcinB [7,28]. Importantly, we observed an increased influx of the noncell membrane permeable dye PI in Ramos cells treated for 4 h with **10e** at its IC_{50} concentration, indicating loss of membrane integrity (see Supporting Information for details). However, more extensive studies are needed to elucidate the detailed mechanism of action.

Table 1. Anticancer potency of peptides **10a-10h** against human Ramos and murine A20 cancer cells (IC_{50}) and toxicity against normal human RBC (EC_{50}), MRC-5 cells (IC_{50}), and PBMCs (EC_{50})

	Anticancer potency		Toxicity			Selectivity index[i]			Rt[j]
Entry	Ramos[a,d]	A20[a,e]	RBC[c,f]	MRC-5[b,g]	PBMCs[a,h]	RBC/Ramos	MRC-5/Ramos	PBMCs/Ramos	(min)
10a	—	—	—	—	n.d.	—	—	—	3.24
10b	—	113 ± 7	—	—	n.d.	—	—	—	3.13
10c	44 ± 3	24 ± 2	—	115 ± 9	—	>13	2.6	>2.6	4.69
10d	33 ± 1	24 ± 1	—	124 ±18	10 ± 3	>17	3.8	0.3	4.99
10e	23 ± 3	22 ± 2	—	140 ± 9	—	>23	6.1	>4.7	5.95
10f	22 ± 5	14 ± 2	425	58 ± 6	31 ±14	19	2.6	1.4	4.82
10g	16 ± 1	10 ± 1	232	29 ± 5	7 ± 2	15	1.8	0.4	9.44
10h	9 ± 2	9 ± 1	214	27 ± 6	12 ± 3	24	3.0	1.3	9.29

All values are in micromolar. Highest concentrations tested were the following: [a]200 μg/ml, [b]500 μg/ml, and [c]1000 μg/ml. All peptides were isolated as their pentatrifluoroacetate salts, and the molar concentrations were calculated as such. [d]Human Burkitt's lymphoma B-lymphocyte [e]B-cell lymphoma of BALB/c mice origin (ATCC TIB-208). [f]Human red blood cells. [g]Normal human embryonic lung fibroblast cells (ATCC CCL-171). [h]Healthy human peripheral blood mononuclear cells. [i]The selectivity index was calculated as the IC_{50} values against PBMCs, MRC-5 cells, or RBC divided by the IC_{50} values against Ramos cancer cells. [j]Retention time on an analytical RP-HPLC C_{18} column. The symbol '—'denotes no detectable activity (IC_{50} or EC_{50}) within the concentration range tested, and 'n.d.' denotes value not determined.

Peptides **10c** and **10d** displayed similar potencies against the A20 cancer cell line as **10e** but were a little less potent against Ramos cancer cells. However, the differences in structure of the $\beta^{2,2}$-amino acids resulted in a somewhat higher potency of **10d** against Ramos cancer cells compared with **10c**. As described previously, **10a** was inactive against all cell lines tested, whereas **10b** showed detectable activity against the A20 cancer cell line and thereby revealed a small effect of the fluorine substituents in **10b**.

Toxicity

Haemolytic activity is a widespread method for briefly assessing the toxicity of CAPs and is often used when designing peptide-based drugs. In general, a peptide is considered to be nonhaemolytic if it displays less than 10% lysis of RBC at therapeutic concentrations [29]. In the present study, only peptides **10f**, **10g**, and **10h** displayed measurable haemolytic activity but with EC_{50} values above 200 μM and well above their IC_{50} values against the Ramos and A20 cancer cell lines (Table 1). Noteworthy, even the most haemolytic peptide **10h** still displayed more than 27-fold preference for both cancer cell lines compared with human RBC.

Because only three of the peptides displayed measurable EC_{50} values against human RBC, the data set was fairly insufficient to rank all eight peptides with respect to a selectivity index (Table 1). Given that RBCs are fragile, nondividing cells, a measurement of toxicity with respect to metabolic active cells was therefore obtained by measuring the toxicity against normal human MRC-5 cells and PBMCs.

The results showed that the anticancer potency against the human Ramos cancer cell line was approximately twofold to more than sixfold higher than the toxicity against normal MRC-5 cells depending on the peptide in question and with **10c**, **10d**, and **10e** displaying the lowest measured toxicity against MRC-5 cells (Table 1). Similar results were also obtained with respect to the murine A20 cancer cell line (calculations not shown). Furthermore, **10e** was nontoxic against PBMCs resulting in the highest selectivity index of all peptides tested, whereas peptides **10d**, **10f**, **10g**, and **10h** with similar or higher anticancer potencies were rather cytotoxic against the PBMCs. Although **10c** also was nontoxic against PBMC, peptide **10e** was nevertheless considered the most promising peptide for further studies on the basis of its higher anticancer potency.

Proteolytic Stability

A major obstacle when designing peptide-based drugs is often their intrinsically low *in vivo* stability. Unmodified α-peptides are in general unable to survive systemic circulation for more than a few minutes because of extensive proteolytic degradation [30]. On the other hand, studies involving pure β-peptides show that these peptides are highly resistant to proteolytic degradation by a wide range of proteases [31].

By incorporating a disubstituted $\beta^{2,2}$-amino acid into an otherwise pure α-peptide, an extra methylene group was added to the current peptide backbone, which in combination with two bulky residues was suspected to improve proteolytic stability compared with pure α-peptides. To investigate how the introduction of the $\beta^{2,2}$-amino acids affected proteolytic stability, we examined our most promising peptide **10e** by ITC and degradation by trypsin and α-chymotrypsin. Trypsin cleaves C-terminal to cationic amino acids such as Lys and Arg, whereas α-chymotrypsin cleaves C-terminal to large lipophilic amino acids such as Trp, Phe, and Tyr. Thus, several of the amide bonds in the present peptides were theoretically susceptible to hydrolysis by these proteases.

The results from the stability experiments on **10e** were rather striking. No binding interactions at all were detected in the ITC studies (see Supporting Information for details). However, the proteolytic stability assay showed that **10e** was in fact slowly degraded by trypsin, with a half-life of about 22 h and with approximately 13% of the peptide remaining after 4 days (Figure 2). Studies by preparative RP-HPLC-UV and MS revealed that only the N-terminal Lys-Lys bond in H-KKW$\beta^{2,2}$WKK-NH_2 was susceptible to hydrolysis by trypsin, resulting in the two fragments H-K-OH and H-KW$\beta^{2,2}$WKK-NH_2. No other amide bonds C-terminal to the remaining Lys residues were hydrolyzed.

Peptide **10e** was seemingly completely stable against proteolytic degradation by α-chymotrypsin, and after 4 days, 92% of the peptide was still intact. The results thereby demonstrated how a single $\beta^{2,2}$-amino acid could protect at least the two adjacent residues on each side from being hydrolyzed. Thus, lipophilic $\beta^{2,2}$-amino acids may be valuable building blocks also in other classes of peptides by inducing unique conformations, altered lipophilicity, and biological profiles, as well as protecting scissile peptide bonds against proteolytic degradation.

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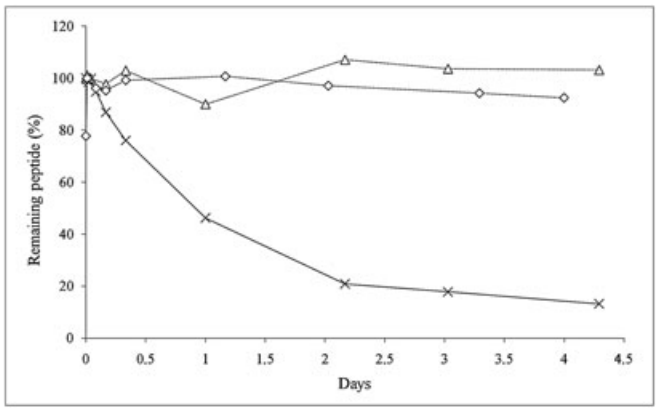

Figure 2. Proteolytic stability of peptide **10e**. The peptide was digested with trypsin (crosses) or α-chymotrypsin (diamonds). A peptide sample without enzyme was used as negative control (triangles). Percentage of the remaining peptide **10e** was measured using analytical RP-HPLC-UV.

Structural Considerations

When considering the primary structure of the peptides, the central $\beta^{2,2}$-amino acid was flanked by two tryptophan residues and thereby formed a lipophilic core sequence with cationic Lys residues at the N-terminal and C-terminal ends, similar to the core structure of LfcinB. Linear α-peptides are in general unstructured in solution, but β-peptides consisting of only 3–4 residues have been shown to form highly stable secondary conformations even in aqueous environment [17,32]. Because $\beta^{2,2}$-amino acids have been shown to be turn-inducing residues [17,18], the present peptides were designed to be able to adopt a wedge-shaped amphipathic secondary conformation upon interaction with cancer cell membranes.

However, the most obvious parameters that varied between the peptides were lipophilicity and the structures of the side chains within the central $\beta^{2,2}$-amino acid building blocks. As a measurement of lipophilicity of the peptides, we compared their retention time on an analytical C_{18}-RP-HPLC column eluted with a linear gradient of acetonitrile in water (Table 1) in which a correlation between overall lipophilicity and anticancer potency as well as toxicity was revealed (Supporting Information). However, the correlation was not completely linear because **10e**, being the third overall most lipophilic peptide, showed lower toxicity against MRC-5 cells and PBMCs than expected from its retention time. Also, **10f** was more potent than **10c** and **10d**, although they had similar retention times. Thus, these results emphasized how retention time alone was unable to explain all the observed effects of the peptides. Clearly, size and bulkiness had a vital effect on anticancer potency.

There was also another important aspect of the present peptides related to the close proximity of the aromatic side chains of the $\beta^{2,2}$-amino acids, especially in peptides **10a-10c** and **10e-10g**. The *ortho*-protons in these side chains are likely to cause steric repulsions, which may affect both the bulkiness of the $\beta^{2,2}$-amino acids and the overall peptide conformations. This may further reinforce the turn-inducing potential of the $\beta^{2,2}$-amino acids through a nearly perpendicular orientation of these side chains. In addition to peptide **10e**, an especially intriguing structure to solve is that of **10f**, which contained a $\beta^{2,2}$-amino acid with two 2-naphthyl methylene side chains and, as described previously, was more potent than presumed from its retention time. A quite complex π–π stacking pattern involving the tryptophan residues and the 2-naphthyl methylene side chains, in combination with side-chain repulsive forces within the $\beta^{2,2}$-amino acid building block could be imagined for this specific peptide. Elucidating the details of these interactions are challenging, but high-resolution NMR experiments are in progress to reveal possible active conformations of the peptides upon interaction with model micelles.

Conclusions

The study demonstrates how a recently developed series of lipophilic $\beta^{2,2}$-amino acids can be used to design small anticancer peptides. The most promising peptide was **10e**, which showed profound potency against Ramos and A20 cancer cell lines, low toxicity against human RBC, MRC-5, and PBMCs, and exceptional proteolytic stability against trypsin and α-chymotrypsin. Peptide **10e** may therefore form the basis for future anticancer drug candidates that can either be used individually or in combination with existing anticancer chemotherapeutics. Because of the relative ease of synthesis of the Fmoc-protected $\beta^{2,2}$-amino acids **9a-9h**, these lipophilic building blocks may also find applications in other biological active peptides involving optimization of pharmacokinetic properties and formation of extraordinary secondary conformations.

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