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Anticancer activity of small amphipathic $\beta^{2,2}$-amino acid derivatives

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ABSTRACT

We report the anticancer activity from screening of a series of synthetic $\beta^{2,2}$-amino acid derivatives that were prepared to confirm the pharmacophore model of short cationic antimicrobial peptides with high anti-*Staphylococcal* activity. The most potent derivatives against human Burkitt's lymphoma (Ramos) cells displayed IC_{50} values below 8 μM, and low toxicity against human red blood cells ($EC_{50} > 200$ μM). A more than 5-fold preference for Ramos cancer cells compared to human lung fibroblasts (MRC-5 cells) was also obtained for the most promising $\beta^{2,2}$-amino acid derivative 3-amino-*N*-(2-aminoethyl)-2,2-bis(naphthalen-2-ylmethyl)propanamide (**5c**). Screening of **5c** at the National Cancer Institute (NCI, USA) confirmed its anticancer potency and revealed a very broad range of anticancer activity with IC_{50} values of 0.32–3.89 μM against 59 different cancer cell lines. Highest potency was obtained against the colon cancer cell lines, a non-small cell lung cancer, a melanoma, and three leukemia cell lines included in the NCI screening panel. The reported $\beta^{2,2}$-amino acid derivatives constitute a promising new class of anticancer agents based on their high anticancer potency, ease of synthesis, mode-of-action, and optimized pharmacokinetic properties compared to much larger antimicrobial peptides.

1. Introduction

Cationic antimicrobial peptides (AMPs) are a diverse group of peptides contributing to the innate immunity of mammals, amphibians and insects [1–3]. Excitingly, reports have also shown that the cationic and amphipathic nature of AMPs can be exploited to design AMP-based peptides and peptidomimetics that also kill cancer cells [4–7]. Anticancer AMPs exert a unique mode-of-action compared to traditional anticancer drugs by preferring binding to cancer cells with elevated levels of anionic surface molecules, such as phosphatidylserine, *O*-glycosylated mucins, and sialylated glycolipids and glycoproteins, followed by rapid lysis of the cancer cell membrane [4,8–14].

Intracellular targets have also been identified for anticancer AMPs *in vitro* [15]. Studies have shown that lactoferricin B (LfcinB) translocates across the cancer cell membrane and acts by disrupting the mitochondrial membrane, as observed by swelling of the mitochondria and induction of apoptosis [16]. Furthermore, *in vivo* studies have revealed that mice treated with a synthetic LfcinB derived peptide became resistant to reintroduction of the same cancer cell line, implying that the membranolytic mechanism of action of the anticancer AMPs may activate adaptive immunity against established tumors [17,18].

Although cancer cells are notorious for developing resistance against chemotherapeutic drugs, e.g. by increased expression of efflux pumps or mutations in key regulatory proteins, studies have shown that multi-drug resistant cancer cells are still susceptible to anticancer AMPs due to their membrane lytic mechanism of action [5,19,20]. Anticancer AMPs are therefore a promising class of molecules for future cancer therapy, either as therapeutic agents alone, or in combination therapy with traditional chemotherapeutic drugs against resistant tumors [11,20].

We have recently demonstrated that the minimal pharmacophore elements of small AMPs, i.e., amphipathic peptides enclosing two bulky lipophilic groups in combination with a net charge of +2, can be incorporated into a single $\beta^{2,2}$-amino acid derivative. The resulting β-peptidomimetics display high antimicrobial activity

Abbreviations: AMP, cationic antimicrobial peptide; BOC_2O, di-*tert*-butyl dicarbonate; DIPEA, di-isopropyl ethyl amine; 5-FU, 5-fluorouracil; LfcinB, bovine lactoferricin; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MD, molecular dynamics; MRC-5, human lung fibroblasts; MRSA, methicillin resistant *Staphylococcus aureus*; MRSE, methicillin resistant *Staphylococcus epidermidis*; NCI, National Cancer Institute; PBMCs, peripheral blood mononuclear cells; RBC, human red blood cells; TEA, triethyl amine; TFFH, fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate; TMRE, tetramethylrhodamine ethyl ester; SI, selectivity index.

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against methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant *Staphylococcus epidermidis* (MRSE), *Escherichia coli*, and also *Pseudomonas aeruginosa* for the overall most potent derivatives [21–23].

Compared with per orally administered commercial drugs the absorption properties of the $\beta^{2,2}$-amino acid derivatives were more than satisfactory both in theoretical calculations and *in vitro* studies [22]. The $\beta^{2,2}$-amino acid derivatives are also highly stable against proteolytic degradation, but are metabolized through CYP450 Phase I oxidations, and thereby follow common metabolic routes of many commercial drugs [24].

Recently, we have reported that anticancer activity can be achieved by using these $\beta^{2,2}$-amino acids as lipophilic building blocks in small synthetic peptides and shown how structural differences of the $\beta^{2,2}$-amino acids affect anticancer potency and toxicity against non-malignant cells [6,7]. The results show that these small peptides containing a $\beta^{2,2}$-amino acid building block with two (**a**) *tert*-butyl-benzyl, (**b**) *para*-trifluoromethyl-benzyl, or (**c**) 2-napthyl-methylene aromatic side-chains are highly potent. Importantly, one of the most promising peptides is a heptapeptide which is non-toxic against human red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs), and also shows low toxicity against human lung fibroblasts (MRC-5 cells) [6].

Through the discovery of anticancer activity of the small synthetic peptides, we have in the present study investigated if single $\beta^{2,2}$-amino acid derivatives enclosing the three most potent aromatic side-chains (**a**)–(**c**) from the larger synthetic peptides would display anticancer activity. We have previously shown how the *C*-terminal cationic group of such $\beta^{2,2}$-amino acid derivatives can tune both antimicrobial activity and cell toxicity [22]. In order to investigate the structural importance of the cationic groups, eight different *N*- and/or *C*-terminal amino and guanidinium groups were therefore selected in the present study that in total formed a test series of 24 different $\beta^{2,2}$-amino acid derivatives (Fig. 1).

The $\beta^{2,2}$-amino acid derivatives were screened for anticancer activity against human Burkitt's lymphoma (Ramos cells), and toxicity against human RBCs and MRC-5 cells. The current $\beta^{2,2}$-amino acid derivatives are to our knowledge among the smallest anticancer AMP-based peptidomimetics reported, and the anticancer activity of the most promising derivative **5c** was also confirmed by screening at the National Cancer Institute (NCI, USA) revealing high potency against 59 different cancer cell lines.

2. Results and discussion

2.1. Synthesis

Synthesis of the dicationic $\beta^{2,2}$-amino acid derivatives **5**–**8** and **10**–**12** was carried out as previously reported in a four step synthesis by first preparing the three Boc-protected $\beta^{2,2}$-amino acids **3a**–**c** (Scheme 1) [21,22]. Due to high steric hindrance of the $\beta^{2,2}$-amino acids, the following amide couplings were performed using TFFH as coupling reagent to give the final test series **5**–**8** and **10**–**12**.

The dicationic ester derivatives **9a**–**c** were on the other hand prepared by transesterification of the methyl ester of the precursors **1a**–**c** with 2-(dimethylamino)ethanol, followed by reduction of the nitrile group in **4a**–**c** (Supporting information). Synthesis of **9a**–**c** gave overall higher purity and yields of the crude products compared to the dicationic amide derivatives **5**–**8** and **10**–**12**. The major contaminant in the crude products of **9a**–**c** was approximately 10% of the non-hydrogenated nitriles **4a**–**c** (based on HPLC-UV chromatograms), which probably can be reduced by more careful monitoring of the reduction of the nitrile group. We also made an important alteration of the reported synthesis of the precursors **1a**–**c** by replacing sodium methoxide with potassium carbonate as base and stirring 2 eq. of the desired aryl bromide for 18 h (method a_2 in Scheme 1), in comparison to two sequential reactions with sodium methoxide and 1 eq. aryl bromide (method a_1 in Scheme 1). These adjustments were the key to both the improved crude purity and overall yields of **9a**–**c** (see the Supporting information for details).

2.2. Anticancer activity

The results revealed that all the prepared $\beta^{2,2}$-amino acid derivatives displayed anticancer activity against the Ramos cells, except for one, and that nine different derivatives displayed IC_{50} values below 8 μM (Table 1). The present $\beta^{2,2}$-amino acid derivatives are thereby amongst the smallest anticancer AMP-based peptidomimetics reported together with the lipopeptides patented by the research group of Shai and our own reported small synthetic peptides [5–7,25–28].

Among the most potent compounds, five were $\beta^{2,2}$-amino acid derivatives containing two lipophilic 2-napthyl-methylene side-chains (**5c**, **6c**, **9c**, **10c**, **11c**), which was clearly an efficient motif for anticancer activity. Also **11a** and **12a** having two *tert*-butyl-benzyl side-chains, and **5b** and **9b** comprising two *para*-trifluoromethyl-benzyl side-chains were highly potent and displayed IC_{50} values below 8 μM. It can be noted that all derivatives except for **7a**–**c** contained an *N*-terminal primary amino group, and all derivatives were expected to have a net charge of +2 at the test conditions used. However, by observing the variation in anticancer potency and side-chain structure, it was obvious that the potency also was influenced by the physicochemical nature of the cationic groups.

The overall most potent $\beta^{2,2}$-amino acid derivatives were **9b** and **9c**, which had a *C*-terminal tertiary amino group. However, **9b** and **9c** were also unique by being ester derivatives. Although it is difficult to determine the specific contribution of the ester group due to small differences in potency, the somewhat higher rotational freedom and flexibility of the ester derivatives **9b** and **9c** could be favorable with respect to cancer cell interactions, and compared to the double bond character of the bioisosteric amide derivatives **10b** and **10c**. The results nevertheless suggest the possibility of replacing both the ester and amide functionalities with other functional groups in order to further optimize the derivatives. It should however be noted that the ester derivate **9a** encompassing two *tert*-butyl-benzyl side-chains displayed similar potency against the Ramos cell line as the amide derivative **10a**. A *C*-terminal primary amino group (**5a**–**c** and **6a**–**c**) seemed furthermore to be more efficient than a *C*-terminal tertiary amino group (**10a**–**c**). Of note, the only difference between the two series **5a**–**c** and **6a**–**c** was a single methylene group, and a small effect favoring the smaller derivatives **5a**–**c** was also observed.

We have recently reported a detailed mode-of-action study on **5c** that suggests that $\beta^{2,2}$-amino acid derivatives might not kill cancer cells exclusively by a membrane lytic mechanism [29]. In fact, incubation of Ramos cells with **5c** initiates controlled cell death involving the mitochondrial apoptotic pathway, and flow cytometry studies with tetramethylrhodamine ethyl ester (TMRE) show that **5c** is able to disrupt the mitochondrial potential of Ramos cancer cells within 1 h. Further investigations for hallmarks of apoptosis and electron microscopy studies were consistent with these findings. Previous results have also shown that **5c** is able to cross mimics of biological membranes by passive diffusion [22]. A different $\beta^{2,2}$-amino acid derivative with two phenyl-propyl side-chains did, however, not act by a similar mechanism as demonstrated for **5c**, but led to necrotic cell death by membrane destabilization as observed by increased propidium iodide uptake [29].

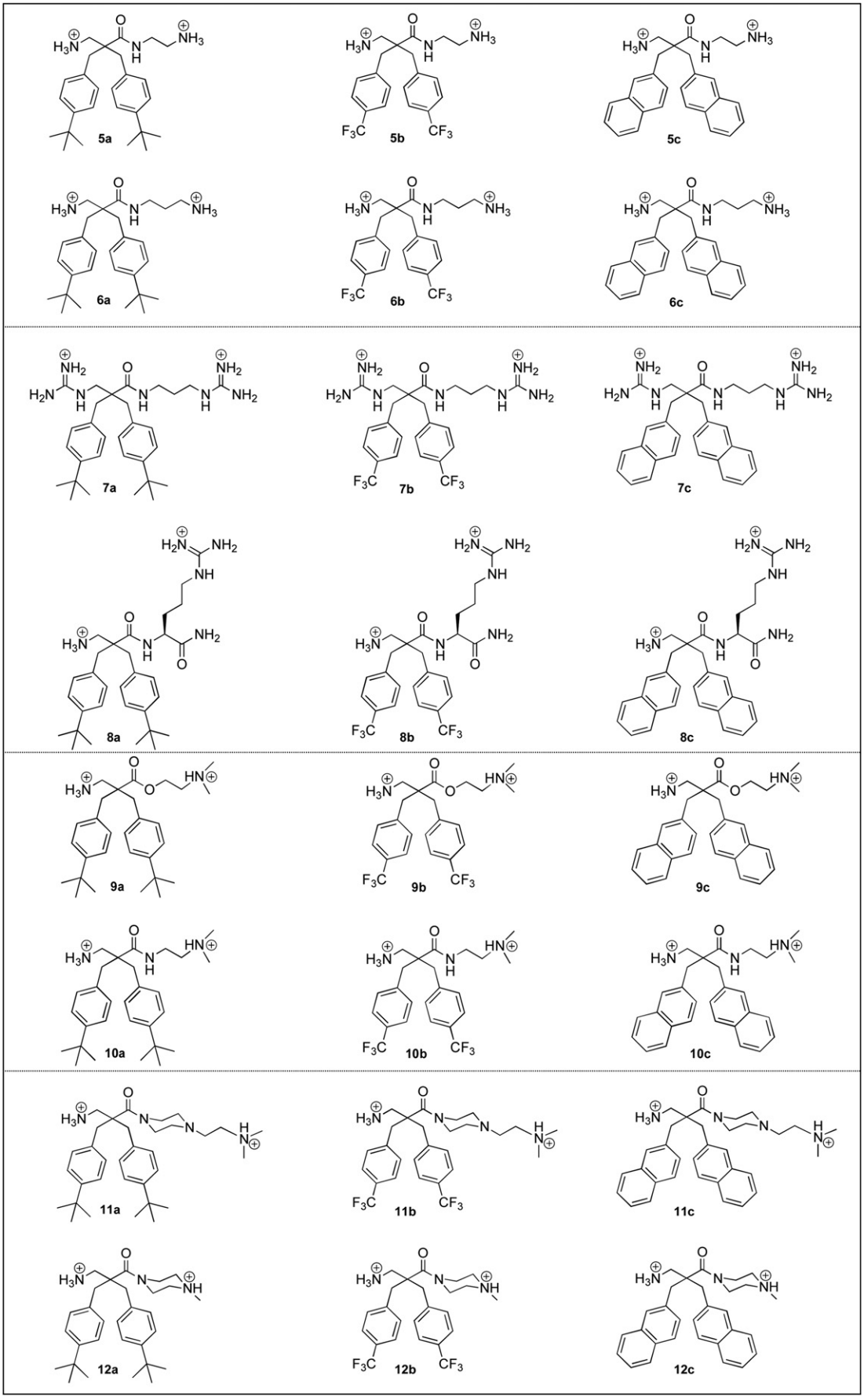
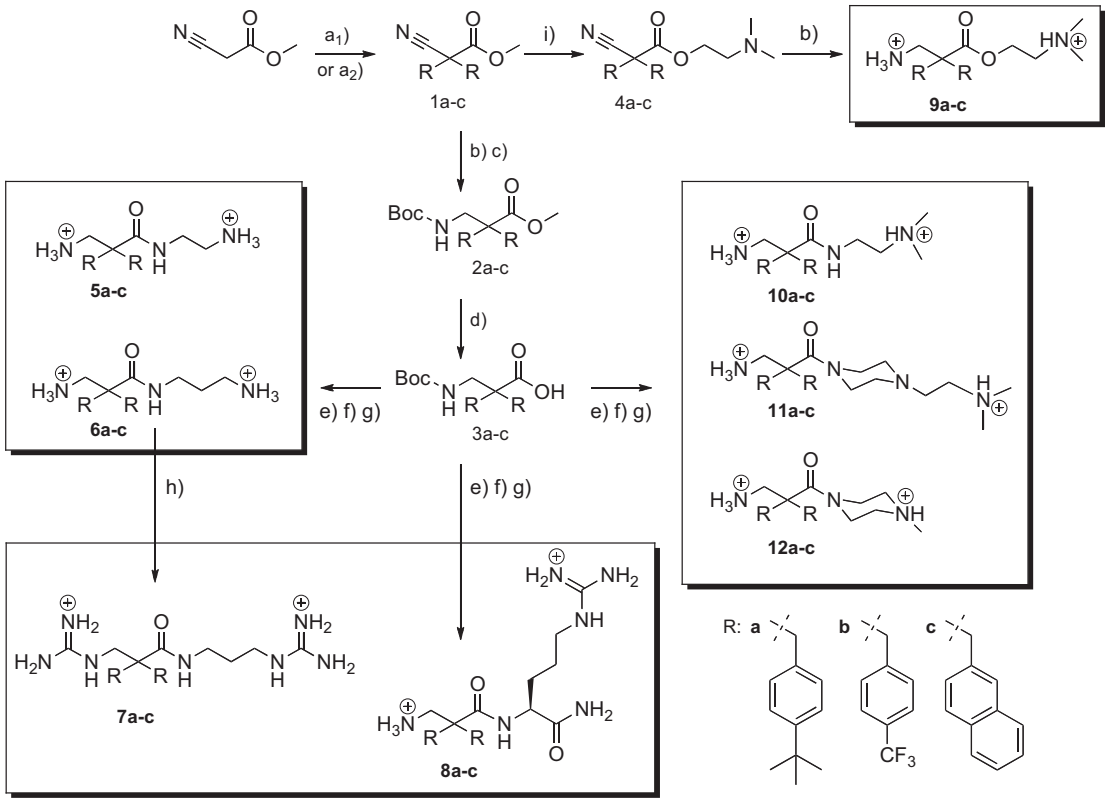


Fig. 1. Structure of the antimicrobial $\beta^{2,2}$-amino acid derivatives that were prepared for investigation of anticancer activity against human Burkitt's lymphoma (Ramos). Derivative **5c** was also subjected to an extensive screening against 59 cancer cell lines at the National Cancer Institute (NCI). All the $\beta^{2,2}$-amino acid derivatives were isolated as di-trifluoroacetate salts.



Scheme 1. Overview of the $\beta^{2,2}$-amino acid derivatives that were synthesized for investigation of anticancer activity. (a_1) NaOMe (1 eq), R–Br (1 eq), performed twice, 78 °C s: MeOH (a_2) K_2CO_3 (3 eq), R–Br (2 eq), 50 °C, 18 h, s: DMF (b) Ra/Ni, H_2 (g), 45 °C, 5 days, s: MeOH. (c) TEA, pH 8, Boc_2O (1.2 eq), rt, 18 h, s: H_2O:dioxane (1:5). (d) LiOH (6 eq), 18 h, 100 °C, s: H_2O:dioxane (1:3). (e) DIPEA (3 eq), TFFH (1.2 eq), rt, s: DMF. (f) H–Arg–NH_2 × 2 HCl or desired amine (2 eq), DIPEA, rt, 7 days, s: DMF. (g) TFA:TIS:H_2O (95:2.5:2.5), rt 2 h, s: DCM. (h) DIPEA (8 eq), 1*H*-1,2,4-triazole-1-carboxamidine × HCl (5 eq), r.t. 2 h, s: DMF, (i) 0.1 eq NaOMe, s: *N*,*N*-dimethyl 2-aminoethanol 50 °C, 18 h.

The recent studies on **5c** may also explain the surprisingly low potency of the guanidinium derivatives **7a**–**c** and **8a**–**c**. Our previous investigations of antimicrobial activity have revealed similar potencies for amino and arginine $\beta^{2,2}$-amino acid derivatives, but the present results showed that anticancer activity was much more sensitive to such structural alterations [21,22]. The low potency of the guanidinium derivatives was suspected to be the result of a more dispersed cationic charge and thereby reduced electrostatic interaction with the cancer cell membrane of the guanidinium groups than for the primary and tertiary amino groups [30,31]. However, another explanation may be that the much higher pK_a of the guanidinium groups may prevent **7a**–**c** and **8a**–**c** from interacting efficiently with the membrane and entering the cancer cells through passive diffusion due to permanent ionization, and thereby preclude interaction with important intracellular targets such as the mitochondria.

While rotational freedom was increased by the highly potent ester derivatives **9b**–**c**, introduction of a piperazine ring in the cationic *C*-terminal end restricted the flexibility of **11a**–**c** and **12a**–**c**. Of note, **11a**, **11c**, and **12a** were still among the nine highly potent $\beta^{2,2}$-amino acid derivatives displaying anticancer potencies below 8 μM against Ramos cancer cells. When similar derivatives with respect to side-chain structure were compared, the *C*-terminal modified piperazine derivatives revealed a fairly higher anticancer potency for the largest but more flexible derivatives **11a**–**c**, compared to the smaller and more restricted analogs **12a**–**c**. Furthermore, derivatives **11a**–**c** contained in theory three basic amino groups, but due to probable charge repulsion between the *C*-terminal basic amino groups, we expect these derivatives to be only di-cationic and have a net charge of +2.

Small differences in the structures of the $\beta^{2,2}$-amino acid derivatives therefore seemed to have a major impact on their interactions with cancer cell membranes and their detailed mode-of-action. With respect to the macroscopic physiochemical properties of the derivatives and their membrane interactions, we have recently concluded a study on small synthetic linear and cyclic peptides containing a $\beta^{2,2}$-amino acid residue where these interactions were studied through nuclear magnetic resonance (NMR) spectroscopy and molecular dynamics (MD) simulations [7]. The results show that adjacent α-amino acid residues and their overall structures affected both the orientation and depth of peptide insertion into the model membranes. Thus, in a linear synthetic hexapeptide only one of the lipophilic $\beta^{2,2}$-amino acid side-chains was in fact inserted into the bilayer together with an adjacent lysine residue, which probably explained its low anticancer potency. However, in a cyclic hexapeptide with the same amino acid sequence but with an overall more amphipathic structure, both the lipophilic $\beta^{2,2}$-amino acid side-chains together with a nearby tryptophan residue were inserted deep into the bilayer surface and resulted in a highly potent membranolytic peptide. (In this particular study peptides with a $\beta^{2,2}$-amino acid having two side-chains of structure **b**, *para*-trifluoromethyl-benzyl were used). The present $\beta^{2,2}$-amino acid derivatives are too small and flexible for similar studies. However, the results from comparison of the linear and cyclic hexapeptides imply that the overall macroscopic physiochemical properties of the $\beta^{2,2}$-amino acid residues, i.e. steric effects of the lipophilic side-chains, pK_a and structure of the cationic groups, amphipathicity and overall conformation have a substantial influence on membrane activity. This affects how the derivatives orient into a membrane, how deep they insert, and

Table 1
Anticancer potency of $\beta^{2,2}$-amino acid derivatives against human Burkitt's lymphoma (Ramos) cells, and toxicity against human RBC and MRC-5 cells. All potencies are in μM.

Entry	IC_{50}	EC_{50}		Selectivity index	
	Ramos[a]	RBC[b]	MRC-5[c]	RBC/Ramos[d]	MRC-5/Ramos[e]
5a	10.6	18	6.1	1.7	0.6
5b	7.1	425	17.8	60	2.5
5c	4.1	457	22	112	5.4
6a	14.4	254	35	17.6	2.4
6b	11.0	790	30	72	2.8
6c	4.3	447	6.6	104	1.5
7a	63	n.s.	64		1.0
7b	78	n.s.	171		2.2
7c	79	n.s.	760		9.6
8a	136	>654	158	>4.8	1.2
8b	>254	990	>634		
8c	107	425	552	4.0	5.2
9a	31	52	90	1.7	2.9
9b	2.3	437	8.2	190	3.6
9c	3.1	458	12.7	148	4.1
10a	28	274	49	9.8	1.8
10b	11.4	468	47	41	4.1
10c	6.6	217	4.9	33	0.7
11a	5.2	337	12.0	65	2.3
11b	19	1116	69	58	3.6
11c	7.2	409	18.5	57	2.6
12a	6.1	349	16.6	57	2.7
12b	27	526	182	19.6	6.7
12c	14.0	303	41	22	2.9

n.s.: not soluble to required concentration in test media.
[a] IC_{50} values against the Ramos cancer cell line.
[b] EC_{50} values against human RBC.
[c] EC_{50} values against the MRC-5 cell line.
[d] Selectivity index calculated as the EC_{50} value against human RBC divided by the IC_{50} value against the Ramos cell line.
[e] Selectivity index calculated as the EC_{50} value against normal MRC-5 cells divided by the IC_{50} value against the Ramos cancer cell line.

whether they penetrate the membrane. In total, these properties determine whether the derivatives kill cancer cells through a membranolytic mode-of-action or interaction with intracellular targets, as demonstrated for the antimicrobial and anticancer peptide LfcinB [16]. However, extensive future experiments such as calcein release studies from model liposomes are required to establish the details of membrane interactions for individual $\beta^{2,2}$-amino acid derivatives.

2.3. Hemolytic activity and toxicity against MRC-5 cell

The $\beta^{2,2}$-amino acid derivatives were in general non-toxic to human RBC and displayed EC_{50} values above 200 μM, which was well above their anticancer potencies. The only exception was **5a**, which previously has been shown to be surprisingly hemolytic with an EC_{50} value of 18 μM [22].

The MRC-5 cells were in general less susceptible to the $\beta^{2,2}$-amino acid derivatives compared to the Ramos cells. In general, the most potent derivatives against the Ramos cancer cells were also the most potent derivatives against the MRC-5 cells, but **5c**, **9b**, and **9c** were anyway 3.6–5.4 fold more potent against the Ramos cancer cell line than the MRC-5 cells. Of note, **10c** showed the highest toxicity of all derivatives against the non-malignant MRC-5 cell line, and was in fact more toxic against MRC-5 cells than against Ramos cells.

In order to compare the toxicity and the potency of the $\beta^{2,2}$-amino acid derivatives, we calculated a selectivity index (SI) by dividing the IC_{50} value against the Ramos cancer cell line with the EC_{50} values against RBCs and MRC-5 cells (Table 1). Among derivatives with IC_{50} values below 8 μM, highest selectivity for the Ramos cancer cell line compared to RBCs was **9b**, whereas highest selectivity compared to MRC-5 cells was **5c**.

2.4. NCI screening of *5c*

The specificity of AMP derived anticancer peptides for cancer cell lines over non-transformed cell lines, or for specific cancer cell lines, is based on different charge of the outside of the membrane and the fluidity of the membrane as well as increased surface of the membrane due to increased number of microvilli on cancer cells [4]. Cancer cells are reported to have increased negatively charged membranes due to a 3–9% excess of phosphatidylserine, increased sialylation of membrane bound glycoproteins and glycolipids as well as increased level of *O*-glycosylated mucins [32,33].

The $\beta^{2,2}$-amino acid derivative **5c** was selected based on its structural simplicity, high potency and selectivity to obtain information concerning its activity against different cancer cell lines, and was screened against 59 cancer cell lines at the National Cancer Institute (NCI), USA. The results revealed that **5c** was active against all 59 cancer cell lines tested, and displayed IC_{50} values between 0.32 μM and 3.89 μM (Fig. 2). Compound **5c** was exceptionally potent against all the colon cancer cell lines COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, and SW-620, the non-small cell lung cancer cell line NCI-H460, the melanoma cell line LOX IMVI, and the three leukemia cell lines HL-60(TB), K-562, and SR. The IC_{50} values against all these cell lines were below 1 μM.

In addition to the IC_{50} results, the NCI also provided an LC_{50} value, corresponding to the concentration where 50% of the starting cells were killed (Figures for the LC_{50} results from NCI are shown in the Supporting information). The LC_{50} results were below 8 μM for all the cell lines, except for the leukemia cell lines, which displayed LC_{50} values of 9.1 μM for one cell line and above 10 μM for the remaining five cell lines. Despite the low IC_{50} values against the colon cancer cell lines, the LC_{50} values were in the same range as against the other cancer cell lines, resulting in a noticeable difference between the LC_{50} and the IC_{50} values for the colon cancers.

Furthermore, a TGI value (total growth inhibition) was provided by NCI and corresponded to the concentration where there was no detectable growth during the course of the screening (Figures of TGI are shown in the Supporting information). The TGI values were in average 1.3 times higher than the IC_{50} values, but still below 5 μM. Also for the TGI values, the leukemia cell lines and the colon cancer cell lines displayed the largest differences when compared to the IC_{50} values. The small differences between the IC_{50} and the TGI and LC_{50} (Figures enclosed in the Supporting information) were consistent with our previous data on bacteria, in which very small differences were seen between the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) [21]. Larger AMPs are known to exert their antibacterial effects through a killing mode of action, and the small differences observed in this study between the IC_{50}, the TGI, and the LC_{50} values supported a cytotoxic mode-of-action for the $\beta^{2,2}$-amino acid derivatives, and not a cytostatic mechanism [34].

The COMPARE database supplied by the NCI revealed furthermore that **5c** was on average almost 4 times more potent than tamoxifen against 58 of the 59 cell lines. In addition, **5c** was on average 50 times more potent than the widely used antimetabolite 5-fluorouracil (5-FU). It should, however, be noted that the potencies of 5-FU displayed more variation than for both **5c** and tamoxifen, probably due to a different mode of action for 5-FU.

2.5. Conclusion

Design of small amphipathic scaffolds is an extensively used strategy for preparing small AMP-based antimicrobial peptidomimetics with improved pharmacokinetic properties, but the literature is limited with respect to applying the same strategy to design small anticancer peptidomimetics [2,21,22,27,35–39]. We have,

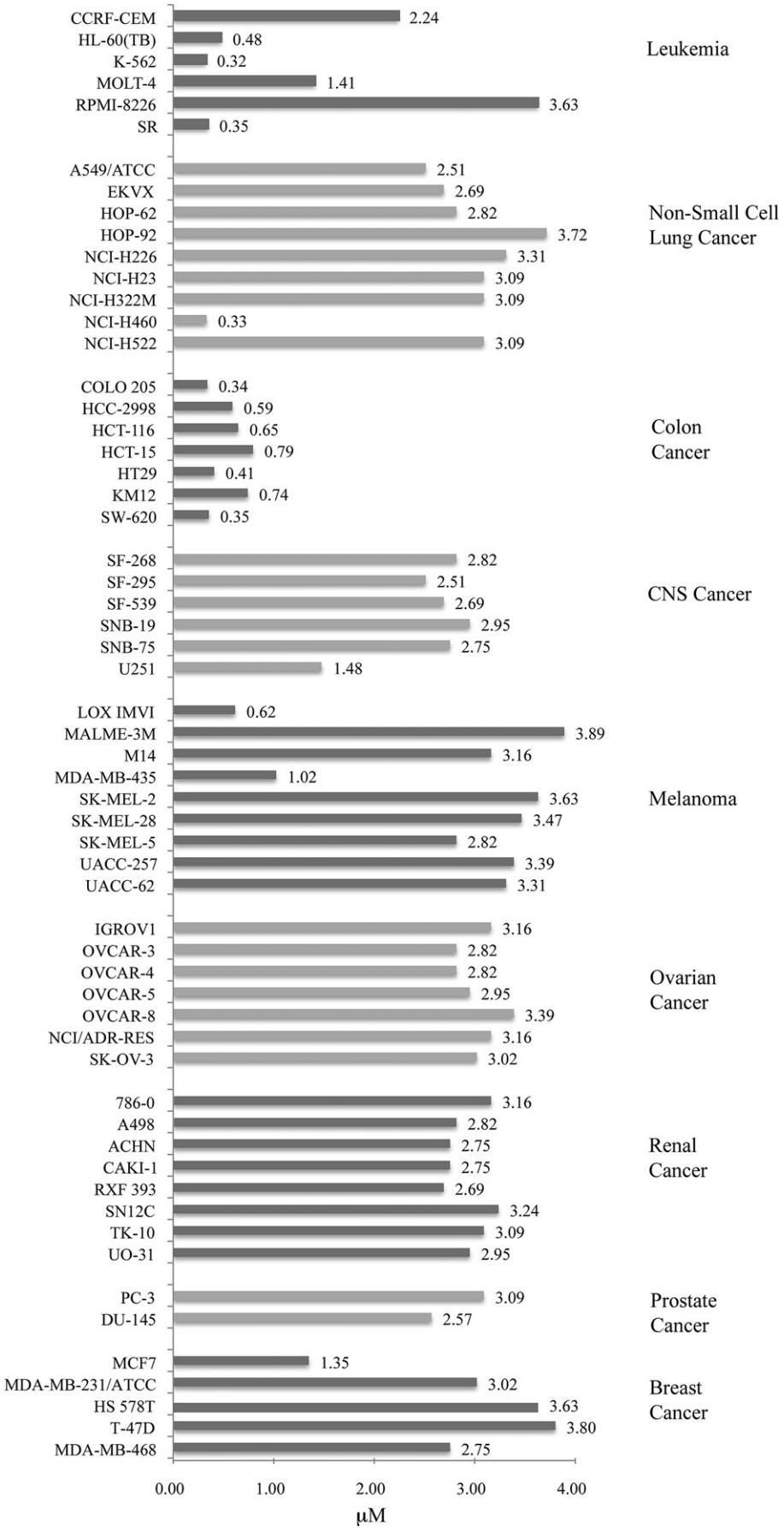


Fig. 2. Results from screening of **5c** against 59 cancer cell lines at the NCI. Derivative **5c** showed exceptional potency with IC_{50} values below 1 μM against the colon cancer cell lines, the non-small cell lung cancer NCI-H460, the melanoma LOX IMVI cell line, and the three leukemia cell lines HL-60(TB), K-562, and SR.

however, in the present study shown that small amphipathic $\beta^{2,2}$-amino acid derivatives fulfilling the pharmacophore model of small cationic AMPs are highly potent against cancer cells. A recent mode-of-action study has also shown that both a membranolytic mechanism and interaction with intracellular targets are possible based on the amphipathic nature of the $\beta^{2,2}$-amino acid derivatives [29]. The results of these studies show that **5c** is a promising anticancer peptidomimetic with high anticancer potency, and high selectivity for Ramos cancer cells compared to RBCs and MRC-5 cells. Screening of **5c** against 59 different cancer cell lines at the NCI confirmed the anticancer potency, and revealed that **5c** displays a broad range of activity against different cancer cell lines. Thus, design of small amphipathic $\beta^{2,2}$-amino acids and related scaffolds is a promising strategy also for developing new anticancer agents. An important future project in addition to further toxicity studies, is to investigate if such small anticancer $\beta^{2,2}$-amino acid derivatives are able to activate adaptive immunity *in vivo* against established tumors, as reported for a LfcinB derivative [17].

3. Experimental section

3.1. Chemistry in general

Chemicals and solvents were purchased from Sigma–Aldrich and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Varian spectrometer. Chemical shifts are expressed in ppm relative to $CHCl_3$ (^{1}H 7.26 ppm, ^{13}C 77.0 ppm) or methanol (^{1}H 3.31 ppm, ^{13}C 39.0 ppm). The values are given in δ scale. Mass spectra were obtained on a Micromass Quattro LC (Micromass, Manchester, UK). High-resolution mass spectra were obtained on a Waters Micromass LCT Premier mass spectrometer (Micromass, Manchester, UK). Preparative RP-HPLC was carried out on a Waters 616 system equipped with a XBridge, 5 μm, 19 × 250 mm C_{18}-column, and eluted with acetonitrile and water, both containing 0.1% TFA. Analytical HPLC was carried out on a Waters 2695 HPLC equipped with a Sunfire, 4.6 × 250 mm C_{18}-column and analyzed at wavelengths 214 and 254 nm with a PDA detector spanning from wavelengths 210 nm–310 nm. All compounds were prepared by using parallel reaction carousels from Radleys. All compounds undergoing biological evaluation possessed purity above 95% as determined by analytical RP-HPLC-PDA. For synthesis of compounds **1a**–**c**, **2a**–**c**, **3a**–**c**, **5a**–**c**, **8a**–**c**, **10a**–**c**, **11a**–**c** and **12a**–**c** please see our previous publications in Refs. [21,22]. Please see the Supporting information for details regarding synthesis and *in vitro* testing of the new compounds **6a**–**c**, **7a**–**c** and **9a**–**c** described in the paper.

3.2. In vitro testing

3.2.1. Hemolytic activity

Hemolytic activity was measured as previously described in Ref. [21]. Briefly, heparinized human blood was centrifuged and washed three times with 37 °C pre-warmed phosphate buffered saline (PBS) in order to remove the plasma fraction. The red blood cells (RBCs) were diluted to 10% the hematocrit value in PBS and the compounds under investigation were dissolved in the same buffer. Concentrations of the compounds ranged from 1 μg/ml to 1000 μg/ml. The diluted RBCs and the compound solutions were mixed to yield a final erythrocyte concentration of 1% v/v. PBS was used as negative control and a final concentration of 0.1% v/v Triton X-100 was included as positive control. The samples were centrifuged at 4000 rpm for 5 min after 1 h agitated incubation at 37 °C. Measuring the absorbance of the supernatant at 405 nm determined the release of hemoglobin and the hemolytic activity was calculated as the ratio of compound treated sample to the Triton X-100 treated sample.

3.2.2. Cell lines and cultures

Ramos human Burkitt's lymphoma cells were grown in suspension using RPMI 1640-medium supplemented with 10% fetal bovine serum (FBS). The MRC-5 human embryonic lung fibroblasts were cultured as monolayer with 10% FBS supplemented Minimal Essential Medium (MEM). Both cell lines were maintained in a humidified incubator at 37 °C and 5% CO_2. Both cell lines were kindly provided by Prof. Øystein Rekdal.

3.2.3. MTT assay

A colorimetric cell viability assay under usage of the tetrazole 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was used to evaluate the cytotoxic effects of the test compounds [40]. Ramos cells were counted and 0.1 ml of a 4×10^5 cells/ml cell suspension was seeded in 96-well plates using serum free RPMI-1640 medium. The cell concentration of MRC-5 cells was adjusted to 1×10^5 cells/ml and 0.1 ml seeded with MEM medium supplemented with 10% FBS. In RPMI-1640 dissolved compounds were added immediately to the Ramos cells in a volume of 0.1 ml, while the MRC-5 cells were first cultured overnight, the medium removed and 0.1 ml of compounds added. A 1% solution of Triton X-100 in RPMI-1640 medium and pure RPMI-1640 medium were used as positive and negative controls. The cells were incubated at 37 °C and 5% CO_2 in a humidified incubator for 4 h and subsequently the MTT work solution added. Incubation continued for additional 2 h. For Ramos cells 0.13 ml, for MRC-5 cells 0.7 ml incubation medium was removed and 0.1 ml acidic isopropanol (0.04 N HCl) added. After resuspending all wells, the plates were set on an orbital shaker rotating at 400 rpm and further incubated at room temperature until the formazan crystals were completely dissolved. Absorbance was measured via a multiwell spectrophotometer at 590 nm. The cell survival rate was calculated as ratio of the background corrected absorbance values of compound treated cells and the absorbance of the negative control (100% living cells). Following the half inhibitory concentration (IC_{50}) was determined.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.09.048.

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