Peptides 31 (2010) 2017–2026

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Antimicrobial cyclic decapeptides with anticancer activity

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ARTICLE INFO

Article history:
Received 23 June 2010
Received in revised form 30 July 2010
Accepted 30 July 2010
Available online 11 August 2010

Keywords:
Apoptosis
Cervical carcinoma
Cyclopeptides
Pharmacological synergism
Design of experiments

ABSTRACT

Antimicrobial peptides have been considered as potential candidates for cancer therapy. We report here the cytotoxicity of a library of 66 antibacterial cyclodecapeptides on human carcinoma cell lines, and their effects on apoptosis [as assessed by cleavage of poly(ADP-ribose) polymerase (PARP)] and cell signaling proteins (p53 and ERK1/2) in cultured human cervical carcinoma cells. A design of experiments approach permitted to analyze the results of a subset of 16 peptides and define rules for high anticancer activity against MDA-MB-231 breast carcinoma cells. Eight peptides were identified with IC_{50} values ranging from 18.5 to 57.5 μM against the five cell lines tested, being HeLa cells the most sensitive. Among these sequences, BPC88, BPC96, BPC98, and BPC194 displayed specificity and high cytotoxicity against HeLa cells (IC_{50} of 22.5–38.5 μM), showed low hemolytic activity and low cytotoxicity to non-malignant fibroblasts, and were stable to proteases in human serum. Induction of apoptosis by these peptides was observed and the apoptotic effect of BPC88 and BPC96 caused a marked decrease on the activated form of ERK1/2 kinase and an induction of p53. We further showed that BPC96 at low doses synergized the cytotoxic effect of cisplatin. These findings suggest that cyclic decapeptides may represent novel anticancer agents providing a new strategy in cancer therapy.

1. Introduction

Antimicrobial peptides have emerged as potential candidates for cancer therapy. They are cytotoxic not only against a wide range of bacteria, fungi, enveloped viruses and protozoa, but also against different types of human cancer cells, such as those from breast, bladder, ovarian and lung [15,17,23,25,31,46]. Moreover, antimicrobial peptides have a mode of action and cellular targets that are different from those of currently available anticancer drugs, and some of them show low eukaryotic cytotoxicity [15,17,23,25,31,46].

Several modes of action have been suggested for the anticancer activity of antimicrobial peptides. They affect cell membranes through a mechanism in which the presence of a net positive charge and the ability to assume an amphipatic structure are crucial peptide structural requirements [3,5,14,15,23,25,31,37]. The positively charged amino acids interact electrostatically with the negatively charged molecules present on the cell surface while the amphipaticity favors their insertion into the membrane. Antimicrobial peptides may kill cancer cells via membrane permeation, either by a detergent-like disruption of the cell membrane into peptide coated vesicles or by formation of transient transmembrane pores. Based on this mode of action, these peptides are unlikely to cause rapid emergence of resistance because it would require significant alteration of membrane composition, which is difficult to occur [33,45]. In addition, there is increasing evidence that apart from membrane damage, other mechanisms may be involved including intracellular targets, such as mitochondria and nucleic acids which may induce apoptosis in cancer cells [15,25,31].

Some antimicrobial peptides with anticancer activity do not show significant cytotoxicity against untransformed proliferating cells at peptide concentrations that are able to kill cancer cells [15,23,25,31]. Parameters that could account for this selectivity involve the higher net negative charge and membrane fluidity of cancer cells as compared to normal cells. The higher negative charge of cancer cells is due to a higher expression of anionic molecules

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0196-9781/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2010.07.027

such as phosphatidylserine and *O*-glycosylated mucins on the outer membrane leaflet, favoring the electrostatic interactions with cationic antimicrobial peptides. The increased membrane fluidity facilitates cancer cell membrane destabilization by membrane-bound antimicrobial peptides.

Although peptides with significant biological activities have been described, their therapeutic use is mainly limited by their low stability towards protease degradation [14,40]. Peptide cyclization constitutes a chemical strategy to overcome this limitation. In particular, cyclic peptides have reduced conformational freedom which, apart from increasing their stability to proteolysis, makes them potent and specific binding ligands to macromolecular receptors [7,9,21]. In recent years, there has been much interest in the development of cyclic peptides through modification of natural products or by *de novo* design, leading to the identification of compounds with improved or novel pharmacological activities [8,12,21]. For instance, potent cyclic antitumor peptides have been studied, such as Kahalalide F which is currently under clinical trials for several types of cancer [11,29], and novel anticancer peptide-based immunotherapies, such as peptide-based vaccines used in cancer clinical trials [27].

The process involved in the development of lead candidates is time-consuming and limited by the number of individual compounds that can be prepared. Synthesis of libraries of small cyclic peptides by combinatorial chemistry constitutes a suitable strategy for the rapid identification of effective compounds with pharmacological activity [10,28,34,38,39]. In fact, such an approach has led to the discovery of cyclic peptides as ligands targeted to different types of cancer cells [13,18,24,43,47]. However, a limitation of combinatorial chemistry to optimize molecular properties is the difficulty in determining cooperative effects among the molecular substitutions. Design of experiments (DOE) [4] constitutes a general statistical methodology able to grasp simultaneous, synergic and non-linear effects among experimental factors and to elucidate inner rules governing the system's behavior in order to assist an investigation course. This methodology has been successfully applied in the peptide design and activity prediction [1,28].

We have recently designed and synthesized a library of cyclic decapeptides with general structure c(X_5-Phe-X_3-Gln) where X is Lys or Leu that display high antibacterial activity and low hemolytic activity [28]. Based on these properties, we decided to evaluate the anticancer activity of these peptides. We first explored the cytotoxicity of the cyclic decapeptide library on human breast cancer cells. A DOE approach was applied to determine the cooperative effects among the molecular substitutions and get the associated statistical significance. The most active peptides were then tested for cytotoxicity on five different human carcinoma cell lines. Peptides markedly selective towards HeLa cells by inducing apoptosis, as evidenced by caspase activation, were identified. For the peptide with the optimal biological profile, we also evaluated its ability to synergize with cisplatin in HeLa cells.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

The 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid derivatives, coupling reagents, and 4-methylbenzhydrylamine (MBHA) resin hydrochloride (0.4 mmol/g) were obtained from Iris Biotech (Marktredwitz, Germany). Trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidinone (NMP), $Pd(PPh_3)_4$, sodium *N*,*N*-diethyldithiocarbamate, triisopropylsilane (TIS), and $CHCl_3$ were from Sigma–Aldrich Corporation (Madrid, Spain). Piperidine, *N*-methylmorpholine (NMM), and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Fluka (Buchs, Switzerland). Acetic acid was from Panreac (Castellar del Vallès, Spain). Solvents for reverse-phase high-performance liquid chromatography (RP-HPLC) were obtained from Scharlau (Sentmenat, Spain). Cisplatin (Pharmacia Spain S.A., Barcelona, Spain) was provided by the Girona Division of Catalan Institute of Oncology Hospital Pharmacy (ICO, Hospital Josep Trueta, Girona, Spain) and was stored at 4 °C. Human serum was supplied by *Banc de Sang i Teixits* of Hospital Josep Trueta and was stored at 20 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and monoclonal anti-β-actin mouse antibody (clone AC-15) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antibodies against PARP and mouse monoclonal antibodies against phospho-ERK1/2 were from Cell Signaling Technology (Beverly, MD, USA). Mouse monoclonal antibody against p53 (clone PAb 1801) was from Neomarkers (Fremont, CA, USA). Peroxidase conjugated secondary antibody was from Calbiochem (San Diego, CA, USA).

2.2. Synthesis of the cyclic decapeptide library

The MBHA resin (3.5 g, 0.4 mmol/g) was swollen with CH_2Cl_2 (1× 20 min) and DMF (1× 20 min), and washed with piperidine–DMF (3:7, 1× 5 min), DMF (6× 1 min) and CH_2Cl_2 (3× 1 min). Then, the resin was treated with Fmoc-Rink linker (5 equiv.), *N*-[1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) (5 equiv.) and DIEA (10 equiv.) in DMF overnight. After this time, the resin was washed with DMF (6× 1 min), CH_2Cl_2 (3× 1 min) and diethyl ether (3× 1 min), and air-dried.

The linear peptide precursors were prepared on an ACT Multiple Peptide Synthesizer Apex 396 S (Aaptec). The above resin was split into 66 wells (50 mg). Couplings were conducted using the corresponding Fmoc-protected amino acid (0.6 M), HBTU (0.6 M), 1-hydroxybenzotriazole (HOBt) (0.6 M) and DIEA (1.2 M) in DMF for 1 h. Fmoc removal was performed with piperidine–DMF (3:7, 5 + 10 min). After each coupling and deprotection step the resin was washed with DMF (5× 1 min).

Once the chain assembly was completed, next steps were performed manually in 66 polypropylene syringes fitted with a polyethylene porous disk. The C-terminal allyl ester was cleaved by treatment with $Pd(PPh_3)_4$ (3 equiv.) in $CHCl_3$–acetic acid–NMM (37:2:1) under nitrogen for 3 h, and the resin was washed with tetrahydrofuran (3× 2 min), DMF (3× 2 min), DIEA–CH_2Cl_2 (1:19, 3× 2 min), sodium *N*,*N*-diethyldithiocarbamate (0.03 M in DMF, 3× 15 min), DMF (10× 1 min) and CH_2Cl_2 (3× 2 min). Following Fmoc removal with piperidine–DMF (3:7, 2 + 10 min), cyclization was carried out by treating the resin with benzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) (5 equiv.), HOBt (5 equiv.), and DIEA (10 equiv.) in NMP at 25 °C for 24 h. Following washes with NMP (6× 1 min) and CH_2Cl_2 (3× 1 min), cyclodecapeptides were cleaved from the resin by treatment with TFA–H_2O–TIS (95:2.5:2.5) for 1 h. The cleavage cocktail was then removed using a Thermo Savant SPD121P SpeedVac concentrator. After diethyl ether extraction, cyclic peptides were dissolved in H_2O, lyophilized, and analyzed by analytical RP-HPLC performed at 1.0 ml/min using a Kromasil (4.6× 40 mm; 3.5 μm particle size) C_{18} reverse-phase column. Linear gradients of 0.1% aqueous TFA and 0.1% TFA in CH_3CN were run from 0.98:0.02 to 0:1 over 7 min with UV detection at 220 nm. Final products were obtained in ~90% HPLC purity and were confirmed by electrospray ionization mass spectrometry.

2.3. Cell lines and cell culture

MDA-MB-231 human breast Caucasian adenocarcinoma and Panc-1 human pancreatic carcinoma cells were obtained from the ATCC (American Type Culture Collection Rockville, MD, USA). A431

human epidermoid carcinoma, HeLa human cervical carcinoma and HepG2 human hepatocellular carcinoma cells were obtained from Eucellbank (Universitat de Barcelona, Spain). Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Berlin, Germany) containing 10% fetal bovine serum (FBS, Bio-Whittaker, Walkersville, MD, USA), 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). The cells remained free of *Mycoplasma* and were propagated in adherent culture according to established protocols. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.4. Cell growth inhibition assay

Peptides were dissolved in sterile Phosphate Saline Buffer (PBS) to get a final stock solution of 9600 μM. Sensitivity was determined using a standard colorimetric MTT assay. Briefly, human cancer cells were plated out at a density of 7×10^3 cells/100 μl/well in 96-well microtiter plates and allowed an overnight period for attachment. Then, the medium was removed and fresh medium along with the corresponding peptide concentration (1–60 μM) were added to the cultures. For the drug-combination assay, a given concentration of peptide BPC96 (5–30 μM), of cisplatin (1.5, 2.5, 4.5 and 6.5 μM) or of BPC96 (5–30 μM) plus a fixed cisplatin dose (1.5, 2.5, 4.5 and 6.5 μM) were added to the cultures. Agents were not renewed during the 48 h of cell exposure, and control cells without agents were cultured under the same conditions. Following treatment, cells were fed with drug-free medium (100 μl/well) and MTT solution (10 μl, 5 mg/ml in PBS), and incubation was prolonged for 3 h at 37 °C. After carefully removing the supernatants, the MTT-formazan crystals formed by metabolically viable cells were dissolved in dimethyl sulphoxide (DMSO, 100 μl/well) and the absorbance was measured at 570 nm in a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). Using control optical density (OD) values (C), test OD values (T), and time zero OD values (T_0), the peptide concentration that caused 50% growth inhibition (IC_{50} value) was calculated from the equation, $100 \times [(T - T_0)/(C - T_0)] = 50$.

2.5. Isobologram analysis of drug interactions

The interaction between BPC96 and cisplatin was evaluated by the isobologram method as we have previously published [26,35]. Briefly, the concentration of one agent producing a 30% inhibitory effect (IC_{30}) is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a straight line joining these 2 points represents zero interaction (addition) between 2 agents. The experimental isoeffect points were the concentrations (expressed relative to the IC_{30} concentrations) of the 2 agents that when combined kill 30% of the cells. When the experimental isoeffect points felt below that line, combination effect of the 2 drugs was considered to be supra-additive or synergistic, whereas antagonism occurred if the experimental isoeffect points lay above it. A quantitative index of these interactions was provided by the equation $I_x = (A/a) + (B/b)$, being a and b the respective concentrations of BPC96 and cisplatin required to produce a fixed level of inhibition (IC_{30}) when administered alone; A and B, the concentrations required for the same effect when the drugs were administered in combination; and I_x, a drug interaction index. Values of $I_x < 1$ indicate synergy, a value of 1 represents addition, and values >1 indicate antagonism. To estimate I_x, isoboles were used only when intercept data for both axes were available.

2.6. Hemolytic activity

The data corresponding to the hemolytic activity of peptides was previously reported by Monroc et al. [28]. It was evaluated at 375 μM by determining the hemoglobin release from erythrocyte suspensions of fresh human blood (5%, v/v) using absorbance at 540 nm.

2.7. Design of experiments (DOE)

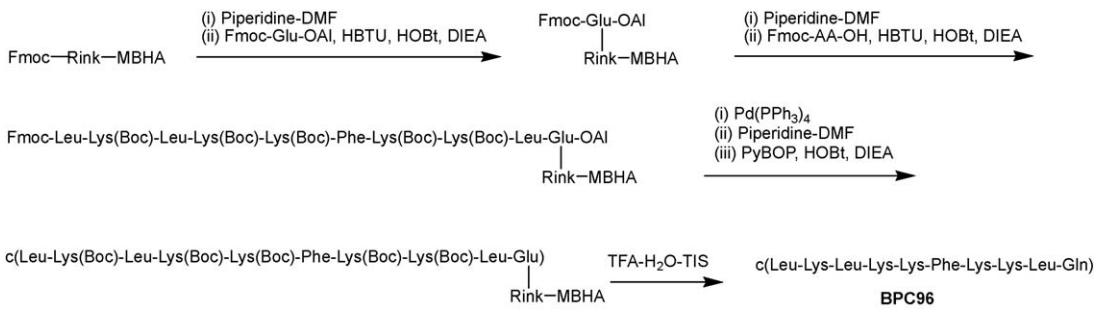
DOE was applied over a set of 16 cyclodecapeptides of general structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln) for their growth inhibition percentage of MDA-MB-231 breast carcinoma cells at 40 μM peptide concentration. Two residues (Leu or Lys) could be present at every molecular position coded with the letter X. Hence, every substitution point constitutes a two-level factor and a total of 2^4 structures were studied, being the calculation a full factorial design. All the molecular activity determinations were carried out in triplicate and in a random order. DOE calculations allowed not only to detect structural rules for high activity but also it helped to determine that these rules were statistically significant. This information was graphically represented in an effect plot. Data manipulations were carried out with the MINITAB program (MINITAB version 14 for Windows. Minitab Inc., State College, PA, 2004).

2.8. Analysis of apoptosis

Apoptosis and induction of caspase activity were evaluated by Western blotting analysis of PARP cleavage. Following treatment of HeLa cells with the corresponding selected peptide (20 μM) during 12 h, cells were harvested with trypsin–EDTA, washed twice with PBS, lysed in a lysis buffer (1 mM EDTA, 150 mM NaCl, 100 μg/ml PMSF, 50 mM Tris–HCl (pH 7.5)) and kept at 4 °C while they were routinely mixed every 5 min on a vortex during 30 min. A sample was taken for measurement of protein content by the Lowry-based BioRad assay. Total protein extracts were immunoblotted using 4–12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE, Invitrogen, Van Alley Way, CA, EUA), transferred to nitrocellulose membranes and blocked for 1 h at room temperature in blocking buffer (2.5% powdered-skim milk in PBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.05% Tween-20]) to prevent non-specific antibody binding. Blots were incubated overnight at 4 °C with the primary antibody (PARP) diluted in blocking buffer. After washes in PBS-T (3× 5 min), blots were incubated for 1 h with the corresponding peroxidase conjugated secondary antibody and developed employing a commercial kit (West Pico chemiluminescent substrate). Blots were reproved with an antibody for β-actin as control for protein loading and transfer.

2.9. Immunoblot analysis of p53 and phospho-ERK1/2

The effect of selected peptides on the tumor suppressor factor p53 and on phospho-ERK1/2 was investigated. Following treatment of HeLa cells with the corresponding selected peptide (20 μM) during 12 or 24 h, cells were lysed in a buffer containing 1 mM EDTA, 150 mM NaCl, 100 μg/ml PMSF, 50 mM Tris–HCl (pH 7.5), protease and phosphatase inhibitor cocktails. A sample was taken for measurement of protein content by the Lowry-based BioRad assay. Total protein extracts were immunoblotted using 4–12% SDS-PAGE, transferred to nitrocellulose membranes and blocked for 1 h at room temperature in blocking buffer (2.5% powdered-skim milk in PBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.05% Tween-20]) to prevent non-specific antibody binding. Blots were incubated overnight at 4 °C with the corresponding primary antibody diluted in blocking buffer. After washes in PBS-T (3× 5 min), blots were incubated for 1 h with the corresponding secondary antibody cho-



Scheme 1. General synthetic route for the synthesis of the cyclic decapeptides depicted for BPC96.

sen according to the species of origin of the primary antibody (p53 and phospo-ERK1/2), and developed employing a commercial kit (West Pico chemiluminescent substrate). Blots were reproved with an antibody for β-actin as control for protein loading and transfer.

2.10. Stability of peptides in serum

The stability of selected peptides was evaluated in human serum. Each peptide (2.5 mg) was exposed to 25% aqueous filtered human serum (1 ml) at 37 °C. After 30 min, 1, 1.5 and 2 h exposure, aliquots (200 μl) were removed and proteins were precipitated with acetonitrile (200 μl). Samples were cooled to 0 °C for 15 min and centrifuged (11,000 rpm, 5 min). The supernatant was frozen, freeze-dried and analyzed by matrix-assisted laser desorption ionization with time-of-flight analysis (MALDI-TOF, ULTRAflex TOF, Bruker) using an α-cyano-4-hydroxycinnamic acid matrix.

3. Results

3.1. Library design and synthesis

The library comprised 66 cyclic decapeptides which incorporated a Phe and a Gln residue at positions 6 and 10, respectively, and alternating Lys and Leu at the other positions (Table 1). Among them, 50 peptides incorporated all combinations of three Leu and five Lys, and 16 cyclopeptides consisted of the substructure $Lys^5PheLysLysLeuGln^{10}$ and all possible combinations of Leu and Lys at positions 1–4. This cyclic decapeptide library was synthesized by carrying out solid-phase synthesis of linear sequences on an ACT Multiple Peptide Synthesizer Apex 396 S, followed by manually on-resin cyclization. A three-dimensional orthogonal Fmoc/^{t}Butyl/Allyl strategy was used [20]. Side-chain protection for Lys was as *tert*-butyl carbamate (Boc). A Fmoc-Glu-OAl residue was introduced as trifunctional amino acid to allow peptide anchoring onto the resin, which resulted in a Gln after peptide cleavage from the solid support. Peptides were obtained with ~90% purity as determined with high-performance liquid chromatography (HPLC). Electrospray ionization mass spectrometry was used to confirm peptide identity. The general strategy for the synthesis of the peptide library is depicted in Scheme 1 for BPC96.

3.2. Cytotoxic effect of cyclic peptides against a breast carcinoma cancer cell line and design of experiments (DOE)

The anticancer activity of the full cyclic decapeptide library was first screened in MDA-MB-231 human breast adenocarcinoma cells by determining the growth inhibition percentage at a peptide concentration of 40 μM by an MTT assay. Growth inhibition values are summarized in Table 1. Eleven peptides produced an inhibition percentage of breast carcinoma cell growth between 20 and 50%, and eight sequences caused ≥50% inhibition, indicating that these peptides exhibit a potential anticancer effect. All the latter

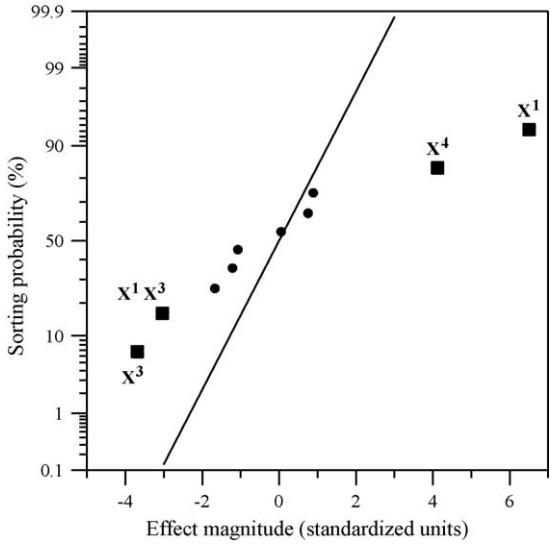


Fig. 1. Effects plot obtained when single and two-residues interactions are being considered. The statistically significant and dominant factors combinations are represented by squares, labeled as single (X^1, X^4, X^3) and double interactions (X^1X^3).

peptides (BPC88, BPC94, BPC96, BPC98, BPC184, BPC194, BPC198, and BPC202) have the substructure $Lys^5PheLysLysLeuGln^{10}$ as common structural feature. The most active peptides were BPC94 and BPC184, with a growth inhibition percentage of 95 and 88%, respectively.

The above eight peptides belong to a set of sixteen sequences that were previously designed to be analyzed by design of experiments (DOE) [28]. We decided to apply this methodology to study the influence of the residues X^1 to X^4 in the general structure $c(X^1X^2X^3X^4LysPheLysLysLeuGln)$ on the growth inhibition percentage of the MDA-MB-231 cells. Representative data series are shown in Table 2.

A full two-level factorial design was performed. In a first calculation, only first order (single residues) and two-order interactions (pairs of residues) were analyzed. Results are represented by means of the effects plot (Fig. 1), a probabilistic scale graph commonly used to represent the influential factors obtained from DOE calculations. The points depicted in plots represent calculated effects (increase or decrease of the measured activity) associated to different factor combinations (single, double or higher order interactions). This magnitude, once expressed in standardized deviation units, constitutes the abscissas axis variable. The vertical axis variable is the left queue cumulated probability to find a data point in the sequence of points once sorted by its effect magnitude. The straight line in Fig. 1 shows the ideal fitting line to which the points should approach if a random Gaussian distribution is followed. The black points around this line represent effects that are not significant. The points that

Table 1
Antitumor activity against MDA-MB-231 cells and hemolysis of the cyclic decapeptide library.

Peptide		Growth inhibition (%)[a]	Hemolysis (%)[b]
Code	Sequence		
BPC10L	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	0	84 ± 6.9
BPC58	c(Lys-Lys-Lys-Lys-Lys-Phe-Leu-Leu-Leu-Gln)	0	6 ± 0.5
BPC60	c(Lys-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Leu-Gln)	0	72 ± 5.5
BPC62	c(Lys-Lys-Lys-Leu-Lys-Phe-Lys-Leu-Leu-Gln)	0	6 ± 0.6
BPC64	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	0	10 ± 2.0
BPC66	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	4 ± 0.11	22 ± 4.0
BPC68	c(Leu-Lys-Lys-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	0	0 ± 0.5
BPC70	c(Lys-Lys-Lys-Lys-Leu-Phe-Leu-Lys-Leu-Gln)	4 ± 0.07	19 ± 0.5
BPC72	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Lys-Leu-Gln)	2 ± 0.08	7 ± 2.0
BPC74	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	22 ± 0.08	36 ± 1.7
BPC76	c(Lys-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	14 ± 0.05	13 ± 1.9
BPC78	c(Leu-Lys-Lys-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	13 ± 0.05	0 ± 0.4
BPC80	c(Lys-Lys-Lys-Leu-Leu-Phe-Lys-Lys-Leu-Gln)	9 ± 0.11	19 ± 0.7
BPC82	c(Lys-Lys-Leu-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	25 ± 0.03	36 ± 2.5
BPC84	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	27 ± 0.11	45 ± 3.5
BPC86	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	8 ± 0.21	8 ± 0.9
BPC100	c(Lys-Lys-Lys-Lys-Leu-Phe-Leu-Leu-Lys-Gln)	6 ± 0.10	1.8 ± 0.8
BPC102	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Leu-Lys-Gln)	23 ± 0.08	26 ± 3.0
BPC104	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	11 ± 0.08	15 ± 2.2
BPC106	c(Lys-Leu-Lys-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	17 ± 0.10	0 ± 0.1
BPC108	c(Leu-Lys-Lys-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	9 ± 0.09	2 ± 0.2
BPC110	c(Lys-Lys-Lys-Leu-Leu-Phe-Lys-Leu-Lys-Gln)	14 ± 0.15	9 ± 0.4
BPC112	c(Lys-Lys-Leu-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	9 ± 0.06	1 ± 0.1
BPC114	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	17 ± 0.05	24 ± 1.8
BPC116	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	5 ± 0.24	13 ± 0.9
BPC118	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	19 ± 0.01	6 ± 0.6
BPC120	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12 ± 0.02	8 ± 1.6
BPC122	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	7 ± 0.08	4 ± 1.3
BPC124	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	4 ± 0.07	3 ± 0.1
BPC126	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	8 ± 0.03	4 ± 0.6
BPC128	c(Lys-Lys-Lys-Leu-Leu-Phe-Leu-Lys-Lys-Gln)	22 ± 0.06	1 ± 0.3
BPC130	c(Lys-Lys-Leu-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	15 ± 0.20	6 ± 3.0
BPC132	c(Lys-Leu-Lys-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	18 ± 0.18	4 ± 0.9
BPC134	c(Leu-Lys-Lys-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	3 ± 0.15	2 ± 0.3
BPC136	c(Lys-Lys-Leu-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	29 ± 0.05	41 ± 1.8
BPC138	c(Lys-Leu-Lys-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	0	5 ± 0.9
BPC140	c(Leu-Lys-Lys-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	0	23 ± 1.1
BPC142	c(Lys-Leu-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	0	14 ± 1.6
BPC144	c(Leu-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	7 ± 0.17	9 ± 1.9
BPC146	c(Leu-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	12 ± 0.19	3 ± 0.5
BPC148	c(Lys-Lys-Leu-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	19 ± 0.09	1 ± 0.2
BPC150	c(Lys-Leu-Lys-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	11 ± 0.22	4 ± 0.2
BPC152	c(Leu-Lys-Lys-Leu-Leu-Phe-Lys-Lys-Lys-Gln)	0	2 ± 0.1
BPC154	c(Lys-Leu-Leu-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	28 ± 0.05	11 ± 1.7
BPC156	c(Leu-Lys-Leu-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	1 ± 0.26	3 ± 0.4
BPC158	c(Leu-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	2 ± 0.13	9 ± 0.7
BPC160	c(Lys-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	20 ± 0.26	3 ± 0.4
BPC162	c(Leu-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	16 ± 0.31	2 ± 0.8
BPC164	c(Leu-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Lys-Gln)	16 ± 0.15	4 ± 0.5
BPC166	c(Leu-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Lys-Gln)	5 ± 0.24	0 ± 0.1
BPC88	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	50 ± 0.10	33 ± 3.3
BPC90	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	23 ± 0.17	30 ± 4.1
BPC92	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	13 ± 0.08	7 ± 0.7
BPC94	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	95 ± 0.01	73 ± 1.6
BPC96	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	59 ± 0.08	32 ± 7.2
BPC98	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	60 ± 0.07	36 ± 3.7
BPC184	c(Lys-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	88 ± 0.01	89 ± 5.3
BPC186	c(Lys-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	22 ± 0.09	0 ± 0.4
BPC188	c(Leu-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	14 ± 0.01	87 ± 6.5
BPC190	c(Leu-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	0	0
BPC192	c(Leu-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	20 ± 0.05	49 ± 7.7
BPC194	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	70 ± 0.01	17 ± 1.7
BPC196	c(Leu-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	18 ± 0.09	47 ± 7.2
BPC198	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	56 ± 0.06	14 ± 1.4
BPC200	c(Leu-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	11 ± 0.27	71 ± 11.7
BPC202	c(Lys-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	63 ± 0.02	2 ± 0.2

[a] Percentage of MDA-MB-231 human breast cancer cell growth inhibition at 40 μM dose plus standard deviation.
[b] Percentage of erythrocytes hemolysis at 375 μM plus confidence interval.

move far away from the ideal line (squares) reveal which kind of interactions are statistically significant.

DOE calculations indicated that X^1 exerted the greatest influence on the activity (Fig. 1), being a Lys the optimal residue. This first rule accounted for eight molecules, six of which produced a growth inhibition percentage ≥50% (BPC88, BPC94, BPC184, BPC194, BPC198, and BPC202). Next residue crucial on the activity was X^4, and a Lys was also preferred. This second rule led to select

Table 2
Data used for DOE analysis (changes of residues X^1 to X^4 in the cyclic decapeptides c($X^1X^2X^3X^4$LysPheLysLysLeuGln) and growth inhibition of MDA-MB-231 cells).

Residues				Peptide	Growth inhibition (%)[a]		
X^1	X^2	X^3	X^4				
Leu	Leu	Leu	Leu	BPC200	7.2	0.0	29.5
Lys	Leu	Leu	Leu	BPC184	88.0	88.6	87.4
Leu	Lys	Leu	Leu	BPC192	24.2	18.2	17.4
Lys	Lys	Leu	Leu	BPC88	42.4	51.2	57.5
Leu	Leu	Lys	Leu	BPC196	15.3	12.7	26.2
Lys	Leu	Lys	Leu	BPC90	35.0	16.5	16.4
Leu	Lys	Lys	Leu	BPC92	19.8	11.2	8.9
Lys	Lys	Lys	Leu	BPC186	16.4	21.6	27.1
Leu	Leu	Leu	Lys	BPC188	13.6	14.6	13.8
Lys	Leu	Leu	Lys	BPC94	95.1	93.6	94.9
Leu	Lys	Leu	Lys	BPC96	54.3	56.9	65.9
Lys	Lys	Leu	Lys	BPC194	70.5	68.9	70.6
Leu	Leu	Lys	Lys	BPC98	55.9	58.6	66.5
Lys	Leu	Lys	Lys	BPC198	52.0	57.5	59.8
Leu	Lys	Lys	Lys	BPC190	0.0	0.0	0.0
Lys	Lys	Lys	Lys	BPC202	63.8	62.8	61.7

[a] Percentage of MDA-MB-231 human breast cancer cell growth inhibition at 40 μM dose. Data are obtained from three independent experiments as required by DOE.

six active compounds (BPC94, BPC96, BPC98, BPC194, BPC198, and BPC202) out of eight. A third rule referred to X^3, being Leu the preferred residue and selecting five active peptides (BPC88, BPC94, BPC96, BPC184, and BPC194). Finally, a double interaction X^1X^3 was worth of mention, being the optimal combination the Lys-Leu pair. This significant effect pointed to consider four active compounds, BPC88, BPC94, BPC184, and BPC194. The simultaneous application of these four rules accounted for two of the most active peptides: BPC94 and BPC194. These four effects were statistically significant with p values much lesser than 0.001. For all cases, the respective standardized effects, in terms of t-Student variable, were greater than 3 units with absolute values of 6.50, 4.12, 3.69, and 3.04 for the effects X^1, X^4, X^3, and X^1X^3, respectively (Fig. 1).

The numerical analysis revealed that peptides mainly showed individual effects on substitution. Cooperative effects were stated only for the X^1X^3 interaction, but it was the last rule in the hierarchic path. A second calculation including all the possible orders of interaction effects showed that the triple interaction $X^1X^2X^3$ was also important, being Lys-Leu-Leu the preferred residues. This interaction pointed to the two most active structures BPC94 and BPC184. This proposal was consistent with the above single-effect preferences and, together with the Lys substitution at position X^4, led to identify the most active peptide BPC94.

In view of the above results, we checked the degree of coherence among the data and the prediction capacity. A simpler fractional factorial design was performed considering only a subset of eight molecules (BPC184, BPC186, BPC188, BPC190, BPC192, BPC194, BPC196, and BPC198). Deliberately, the most active compound BPC94 was not included in the calculations. This partial data revealed that many single effects and some two-factor interactions were relevant. The most preferential rule (t value greater than 30.0) was to set X^1 = Lys, followed by the condition X^3 = Leu (t = 15.69). These two rules led to identify the active peptides BPC184 and BPC194 (both were included into the calculations). They also predicted the design of other compounds which were not present in the analyzed data set, i.e., the result gave a clue to consider peptides BPC88 and BPC94. Additionally, the next relevant rule suggested to set X^2 = Leu (t = 10.65), definitively pointing out to peptide BPC94. The remaining rules were also coherent with this choice. These results showed that DOE is a useful tool in molecular design.

3.3. Cytotoxic effects of cyclic peptides against a panel of human cancer cells

The eight best peptides in terms of cancer growth inhibition of MDA-MB-231 cells were selected to determine the IC_{50} against this cell line and also against HeLa cervical, HepG2 hepatocellular, A431 epidermoid, and Panc-1 pancreatic carcinoma cells (Table 3). Peptides were active at the concentrations tested against all cell lines with IC_{50} values ranging from 18.5 to 57.5 μM, except for BPC198 in HepG2. In these experiments, BPC94 was active against all cell lines, with an IC_{50} comprised between 18.5 and 23 μM. BPC88 was also effective although at higher concentrations (IC_{50} of 22.5–32.5 μM), as were peptides BPC96, BPC98, BPC184 and BPC194, with IC_{50} values from 24.5 to 51 μM. Interestingly, in HeLa cells except for BPC202 (IC_{50} of 54 μM) and BPC98 (IC_{50} of 38.5 μM), the other peptides exhibited an $IC_{50} \leq 35$ μM.

3.4. Hemolysis and growth inhibition assay of peptides in normal mammalian cells

The hemolytic activity of the cyclic peptides was evaluated at 375 μM (Tables 1 and 3). Results showed that two of the eight selected peptides, BPC94 and BPC184, showed significant hemolysis (73 and 89%, respectively). In contrast, the other peptides exhibited a low hemolytic activity, ranging from 2 to 36% (Table 3).

The activity on non-malignant human cells (fibroblasts N1) was assessed for peptides that displayed high cytotoxic activity against HeLa cells and also low hemolysis: BPC88, BPC96, BPC98, BPC194 and BPC198. Peptides BPC94 and BPC184 were not included due to their high hemolysis and BPC202 due to its low activity against HeLa cells. Results showed that BPC198 was highly cytotoxic in non-malignant cells (IC_{50} of 7.5 μM in N1 *vs.* IC_{50} of 35 μM in HeLa cells). By contrast, the IC_{50} values of BPC88, BPC96, BPC98 and BPC194 in non-malignant fibroblasts were up to two fold their IC_{50} values in malignant HeLa cells. This trend is particularly important for BPC96, which only caused a letality of 13% in non-malignant cells (data not shown) at its IC_{50} value in HeLa cells (24.5 μM).

3.5. Stability of peptides in human serum

The stability in human serum of the cyclic decapeptides BPC88, BPC96, BPC98, BPC194 and BPC198 was tested next (see Supporting Information). After exposure to 25% aqueous human serum at different time intervals, the presence of peptide was analyzed by MALDI-TOF. Results showed that all five cyclic peptides were stable in human serum. The least stable was BPC88 which after a 30 min exposure was almost completely degraded. In contrast, BPC96 and BPC98 were still detected up to 1.5 and 1 h, respectively. The most stable peptides were BPC194 and BPC198 which were observed after 2 h.

Table 3
Cytotoxicity (IC_{50})[a] of selected cyclic decapeptides against a panel of human carcinoma cells and non-malignant fibroblasts (N1), and hemolytic activity.

Code	MDA-MB-231	HeLa	HepG2	A431	Panc-1	Hemolysis[b]	N1
BPC88	31.2 ± 5	22.5 ± 0	32.5 ± 4	28.0 ± 3	32.5 ± 11	33 ± 3.3	42.0 ± 7.1
BPC94	22.0 ± 0	23.0 ± 3	20.5 ± 6	21.5 ± 8	18.5 ± 8	73 ± 1.6	nd[c]
BPC96	40.0 ± 7	24.5 ± 0.7	34.5 ± 2	35.0 ± 7	51.0 ± 6	32 ± 7.2	39.0 ± 9.9
BPC98	40.7 ± 3	38.5 ± 4	44.0 ± 3	47.5 ± 4	44.5 ± 0.7	36 ± 3.7	58.5 ± 2.1
BPC184	32.2 ± 7	30.5 ± 12	42.2 ± 2	24.5 ± 0.7	41.5 ± 11	89 ± 5.3	nd
BPC194	32.5 ± 0.5	29.5 ± 2	46.0 ± 3	50.0 ± 10	40.0 ± 3	17 ± 1.7	56.5 ± 0.7
BPC198	53.2 ± 13	35.0 ± 1	>60	49.5 ± 6	57.5 ± 6	14 ± 1.4	7.5 ± 0.7
BPC202	40.5 ± 9	54.0 ± 6	46.0 ± 8	35.5 ± 2	43.5 ± 0.7	2 ± 0.2	nd

[a] Cells were treated for 48 h. Cytotoxicity (μM) was determined using the MTT assay. Each value represents the mean ± SD from three independent experiments performed in triplicate.
[b] Percentage of erythrocytes hemolysis at 375 μM plus confidence interval.
[c] Not determined.

3.6. Analysis of apoptosis induced by peptides

The cell death mechanism displayed by peptides BPC88, BPC96, BPC98, BPC194 and BPC198 was examined using HeLa cells. First, apoptosis and induction of caspase activity were checked by Western blotting analysis showing cleavage of poly-ADP-ribose polymerase (PARP) (Fig. 2A). Treatment of HeLa cells with peptides at 20 μM during 12 h induced a marked increase on the levels of the PARP cleavage product (89 kDa band) that, compared to untreated cells, ranged between 32- (BPC88) and 684-fold (BPC194).

3.7. Effect of peptides on the expression levels of cellular proteins p53 and phospho-ERK1/2

We also examined the effect of peptides BPC88, BPC96, BPC98, BPC194 and BPC198 on the two related mediators involved on two different targets: the oncogen p53 and the ERK1/2, a Mitogen-Activated Protein Kinase pathway (Fig. 2B and C). Treatment of HeLa cells with BPC98, BPC194 and BPC198 at 20 μM for 12 h had little effect on the cellular protein levels of the tumor suppressor factor p53 (Fig. 2B). In contrast, incubation of cells with BPC88 and BPC96 at 20 μM for 12 h noticeably increased the protein levels of p53 compared to untreated cells (1.8- and 1.5-fold, respectively). Concerning ERK1/2-related pathway, incubation of cells with BPC88, BPC96, and BPC198 at 20 μM for 12 and 24 h substantially blocked this survival pathway based on the decrease in the activated form of ERK1/2 (p-ERK1/2) compared to untreated cells (Fig. 2C). During this experiment, there was no significant change in the total level of ERK1/2 protein as assessed by Western blotting analysis (data not shown).

3.8. Cytotoxicity of BPC96 and cisplatin co-treatment in HeLa cells

Peptide BPC96 was selected to study its ability to synergize with cisplatin in HeLa cells. Cells were co-treated for 48 h with different concentrations of BPC96 (5–30 μM) along with a fixed cisplatin dose (1.5, 2.5, 4.5, and 6.5 μM). We previously performed dose-response experiments (MTT assay) of cisplatin in HeLa cells to determine these fixed cisplatin concentrations required to perform the combination experiments (data not shown). The percentage of surviving cells after treatment with 1.5, 2.5, 4.5, and 6.5 μM cisplatin was of 94 ± 2, 72 ± 3, 50 ± 7, 45 ± 6 and 40 ± 4%, respectively (Fig. 3). The co-treatment of HeLa cells with BPC96 (5–30 μM) and a fixed dose of cisplatin showed a strong synergistic cytotoxicity in all cisplatin concentrations assayed (Fig. 3). Interestingly, this synergistic effect was even significant when the lowest BPC96 concentration tested (5 μM) was combined with the lowest cisplatin dose assayed (1.5 μM). In summary, the letality of HeLa cells treated with 5 μM BPC96 or with 1.5 μM cisplatin was 8 and 5%, respectively, while letality of HeLa cells co-treated with 1.5 μM cisplatin plus 5 μM BPC96 increased to 25% (Fig. 3).

We further examined the degree of synergistic interaction between BPC96 and cisplatin in HeLa cells. The combined effect was analyzed by the isobole method, using a series of isobologram transformations of multiple dose-response curves at an effect level of 30% (IC_{30}) [26,35]. Based on the median interaction index value (I_x), co-treatment of HeLa cells with BPC96 plus cisplatin resulted in a marked synergistic interaction ($I_x = 0.74 \pm 0.17$).

4. Discussion

Several natural antimicrobial peptides are known to exhibit anticancer activity. Particularly those with no or little hemolytic activity can be considered as lead molecules for the development of new anticancer agents [15,23,25]. We have recently documented the antibacterial activity of a library of 66 *de novo* designed cyclic decapeptides with general structure c(X_5-Phe-X_3-Gln) where X is Lys or Leu [28]. Best peptides were active against the phytopathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria* (3.1–25 μM) and displayed low hemolysis (~15% at 375 μM). In the present study, we examined the anticancer activity of this peptide library.

Antitumor activity of the cyclic decapeptide library against MDA-MB-231 human breast adenocarcinoma cells correlated with the previously observed antimicrobial activity against the above bacteria [28]. In both cases, the most active compounds were identified within a subset of 16 peptides that share the substructure $Lys^5PheLysLysLeuGln^{10}$. The most potent antitumor peptides caused a growth inhibition percentage of MDA-MB-231 cells between 70 and 95%.

In the present study, DOE was a useful tool to define general rules leading to the selection of optimal compounds against MDA-MB-231 cells. This approach has been previously used in peptide chemistry and allows to simultaneously study all the effects of each residue in the peptide sequence and to analyze and discriminate single or multiple interactions between them. Recently, the analysis using DOE of the peptide subset with the general structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln), with X being Lys or Leu, led to the identification of the optimal amino acids at positions 1–4 for antibacterial activity [28]. It was found that the general rule to follow was to set $X^2 \neq X^3$. In contrast, the numerical analysis of the antitumor activity of the same peptide subset showed that substitutions at X^1, X^3 and X^4 had the highest influence on activity. The optimal residues found were Lys at positions 1 and 4, and Leu at position 3. These rules led to the identification of the peptides with the best antibacterial (BPC194 and BPC198) and antitumor (BPC94 and BPC184) activities. Therefore, these results suggest that cyclic decapeptides with selective antibacterial or antitumor activity could be designed.

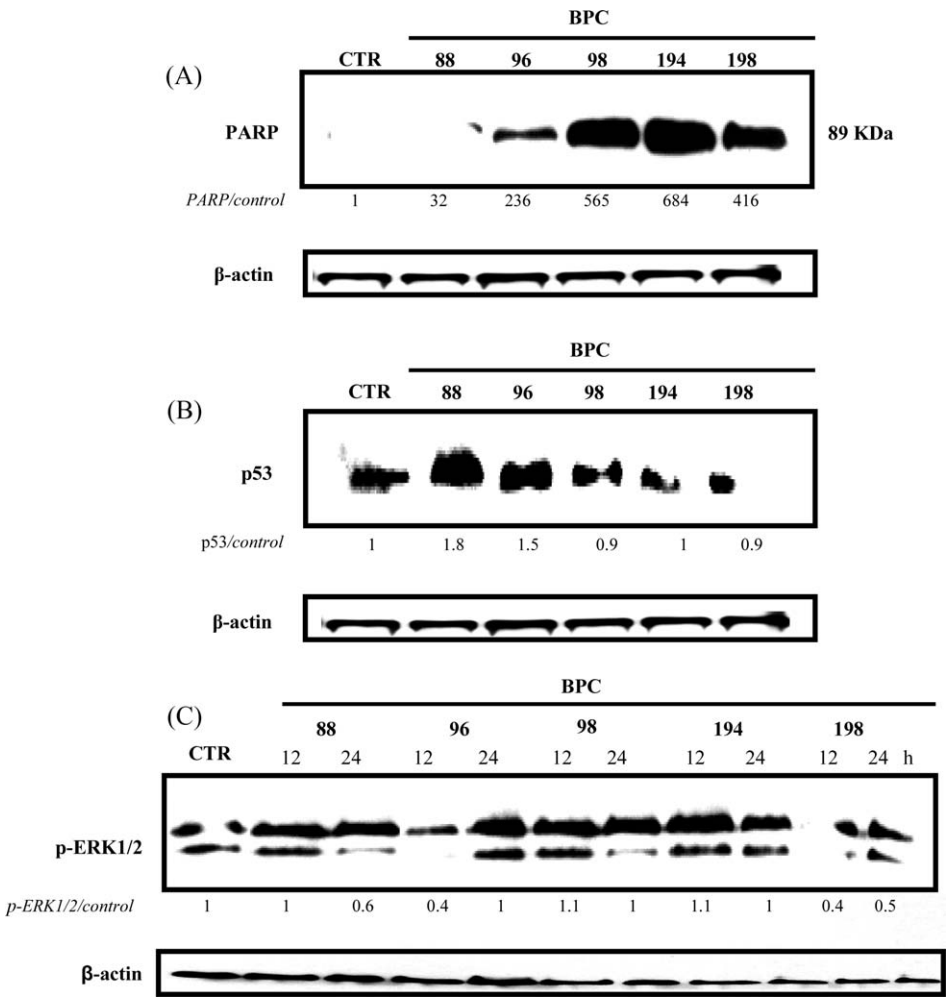


Fig. 2. Effect of cyclic peptides on the activity of caspase, p53 and ERK1/2 in HeLa cells (Western blotting and densitometry). (A) Effect of peptides BPC88, BPC96, BPC98, BPC194 and BPC198 on caspase activity by PARP cleavage. HeLa cells were treated with peptides (20 μM) for 12 h, and equal amounts of lysates were immunoblotted with anti-PARP antibody to identify the 89 kDa band (cleavage product). Blots were reproved for β-actin as loading control. Gels shown are representative of those obtained from two independent experiments. (B) Effect of BPC88, BPC96, BPC98, BPC194 and BPC198 on p53 levels. HeLa cells were treated with peptides (20 μM) for 12 h, and equal amounts of lysates were subjected to Western blot analyses with an anti-p53 antibody. Gels shown are representative of those obtained from two independent experiments. Blots were reproved for β-actin as loading control. (C) Effect of BPC88, BPC96, BPC98, BPC194 and BPC198 on p-ERK1/2. HeLa cells were treated with peptides (20 μM) for 12 and 24 h, and equal amounts of lysates were subjected to Western blot. Activation of ERK1/2 was analyzed by assessing the phosphorylation status of ERK1/2 using the corresponding phospho-specific antibody (phospo-ERK1/2). Blots were reproved for β-actin as loading control. Gels shown are representative of those obtained from two independent experiments.

The DOE approach was also considered for the above subset of peptides but for the hemolytic activity [28]. It was found that the simultaneous presence of a Lys at positions 2 and 3 accounted for many of the less hemolytic peptides (BPC92, BPC186, BPC190, and BPC202). This rule was not exactly coincident with the peptide sequence pattern associated to high antitumor activity. This was also observed when comparing the rules for antibacterial and hemolytic activities. However, except for peptides BPC94 and BPC184, many of the compounds with the highest antitumor activity (BPC88, BPC96, BPC98, BPC194, BPC198, and BPC202) displayed low levels of hemolysis.

A variability in peptide cytotoxicity was observed when they were tested against different types of cancer cells. Among the human cancer cell lines tested, data pointed to HeLa cells as the most sensitive cell line to the selected peptides. This different sensitivity may be attributed to differences in cell membrane composition, fluidity, and surface area between these cell lines [15]. Notably, cyclic peptides selective against HeLa cells exhibited low toxicity against erythrocytes and normal mammalian cells. Peptides BPC88, BPC96, BPC194, and BPC198 did not show a significant hemolytic activity, even at a much higher concentration than the IC_{50} values against all tumor cell lines tested (14–33% hemolysis at 375 μM). Moreover, BPC88, BPC96, and BPC194 were less cytotoxic against non-malignant fibroblasts N1 than against tumor cells. In particular, a mortality of only 13% in N1 cells was observed for BPC96 at its IC_{50} value towards HeLa cells (24.5 μM, data not shown). The basis of the selective killing of tumor cells by antimicrobial peptides has been attributed to their mode of action [15,23,25,31,36,42,45]. While much is known about the general mechanism by which antimicrobial peptides are active against microorganisms, less is known concerning their anticancer mechanism of action. Generally, antimicrobial peptides function by disrupting cell membranes, being the positive charge of the peptides and the different composition of the cell membrane bilayers the most critical factors for the activity. Due to the fact that the amount of phosphatidylserine located in the outer leaflet of the membrane is higher in cancer cells than in normal cells, the former are more susceptible to the lytic action of antimicrobial peptides [36].

Susceptibility to enzymatic cleavage is one of the limitations of using peptides as therapeutic agents. Cyclization has been applied to improve peptide stability against metabolic degradation. In a previous report, we observed this tendency when analyzing the

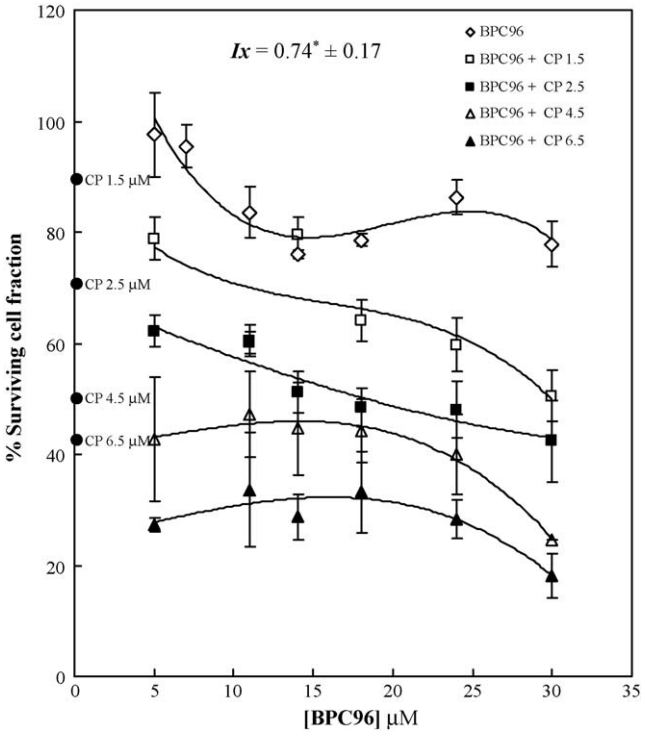


Fig. 3. Cytoxicity in HeLa cells following BPC96 and cisplatin (CP) combination treatment. HeLa cells were treated with different concentrations of BPC96 (5–30 μM) or with different combinations of BPC96 (5–30 μM) and a fixed cisplatin concentration (1.5, 2.5, 4.5 and 6.5 μM) for 48 h. Black circles represent the percentage of surviving cells after 48 h in cisplatin treatment, which was determined using the MTT assay. Results are expressed as percentage of surviving cells from three independent experiments performed in triplicate. The interaction index (I_x) for the two-drug effect in HeLa cells was calculated using isobologram analysis. The I_x parameter indicate whether the doses of the two drugs required to produce a given degree of cytotoxicity are greater than ($I_x > 1$ or antagonism) equal to ($I_x = 1$ or additivism) or less than ($I_x < 1$ or synergism) the doses that would be required if the effect of two agents were strictly additive. I_x values for the two-drug treatment were obtained from duplicate studies. * ($p < 0.05$) Indicate the level of statistical significance of the I_x compared with an I_x of 1.

stability of BPC10L to protease degradation. This cyclic peptide was degraded significantly slower than its linear counterpart [28]. In the present study, the stability of cyclic peptides in human serum was checked. Notably, the cyclic peptides BPC96 and BPC194 with an optimal biological profile in terms of anticancer activity, hemolysis and cytotoxicity against non-malignant fibroblasts also showed good stability in human serum.

Apart from disrupting cell membranes, it has been reported that antimicrobial peptides are endowed with cytotoxic mechanisms in cancer cells involving apoptosis via mitochondrial pathway [15,22,31]. This mode of action was observed for the above selected peptides, which induced PARP cleavage indicating their apoptotic activity in HeLa cells. The anticancer activity of these peptides was accompanied by changes in cell growth and proliferation signaling pathways. BPC96 exhibited an optimal balance between PARP cleavage, activation of p53 and blockage of ERK1/2. On the other hand, peptides had different effects on the proliferation signaling pathways. In particular, BPC88 and BPC96 induced apoptosis in HeLa cells through activation of p53 mediated by ERK1/2 kinases. These findings are consistent with previous reports showing that ERK1/2 kinases target the transcriptional regulatory domain of p53 responding to several anticancer drugs, such as cisplatin [19,32,41]. In contrast, the induction of apoptosis by BPC198 was dependent on ERK1/2 kinases but was not related with the p53 pathway. Further molecular studies with these peptides are needed with the objective of gaining additional insights into the molecular mechanisms underlying the observed cytotoxicity.

A synergistic effect between low doses of BPC96 and cisplatin was strongly evident. This effect was observed even at the lowest cisplatin concentration assayed. These synergistic results are promising, since there is a lack of specific and non-toxic therapies to treat human cervical carcinomas [2,6]. Current therapies use high doses of cisplatin causing important undesirable collateral effects such as nephrotoxicity [44]. The synergism between antimicrobial peptides and existing chemotherapeutic agents, which may decrease the required dosage of these drugs to achieve a positive response and thereby reduce drug-related side effects, has been previously documented [25]. In fact, it has been described that antimicrobial peptides with anticancer activity would supplement rather than replace the conventional chemotherapeutics. For example, cecropin A enhances the cytotoxic effect of 5-fluorouracil or cytarabine on lymphoblastic leukemia cells [16]. A similar additive inhibitory effect on the growth of small cell lung cancer cells has been demonstrated when synthetic magainin analogues are used in combination with cisplatin or etoposide [30].

5. Conclusions

Taken together, a number of our designed cyclic decapeptides displayed a favorable profile for future therapeutic development against cancer. In particular, BPC96 exhibited a marked anticancer activity, high stability to protease degradation, induced apoptosis in human cancer cells, and showed synergistic antitumor effects when combined with cisplatin. These findings suggest that BPC96 may represent a novel therapeutic strategy for the treatment of cervical carcinoma. Future experiments will investigate the efficacy of BPC96 in a pre-clinical model of HeLa cisplatin resistant cells and the anticancer efficacy and long-term toxicity of the combination treatments *in vivo* using a murine cervical tumor model.

Acknowledgments

Financial support was provided by grant from the University of Girona (Grant for R+D Projects on Health Sciences). We are also grateful to the *Serveis Tècnics de Recerca* of the University of Girona for their support with the mass spectrometry analysis. We acknowledge the *Banc de Sang i Teixits* of Josep Trueta Hospital of Girona for supplying us the human serum and to Girona Division of Catalan Institute of Oncology (ICO) Hospital Pharmacy for providing us the cisplatin. E. Besalú thanks the project CTQ2009-09370 from the Ministry of Science and Innovation of the Spanish Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2010.07.027.

References

[1] Barroso JM, Besalú E. Design of experiments applied to QSAR: ranking a set of compounds and establishing a statistical significance test. J Mol Struct (Theochem) 2005;727:89–96.
[2] Bar-Sela G, Epelbaum R, Schaffer M. Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. Curr Med Chem 2009 [Epub ahead of print].
[3] Bechinger B. Structure and function of membrane-lytic peptides. Crit Rev Plant Sci 2004;23:271–92.
[4] Box GEP, Hunter WG, Hunter JS. Statistics for experimenters. An introduction to design, data analysis, and model building. New York: John Wiley & Sons, Inc.; 1978.
[5] Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 2005;3:238–50.
[6] Chung SH, Lambert PF. Prevention and treatment of cervical cancer in mice using estrogen receptor antagonists. Proc Natl Acad Sci USA 2009;106:19467–72.

[7] Cowell SM, Lee YS, Cain JP, Hruby VJ. Exploring Ramachandran and Chi space: conformationally constrained amino acids and peptides in the design of bioactive polypeptide ligands. Curr Med Chem 2004;11:2785–98.
[8] Cruz LJ, Luque-Ortega JR, Ribas L, Albericio F, Kahalalide F. An antitumor depsipeptide in clinical trials, and its analogues as effective antileishmanial agents. Mol Pharmaceut 2009;6:813–24.
[9] Dathe M, Nikolenko H, Klose G, Bienert M. Cyclization increases the antimicrobial activity and selectivity of arginine- and tryptophan-containing hexapeptides. Biochemistry 2004;43:9140–50.
[10] Duléry V, Uhlich NA, Maillard N, Fluxá VS, Garcia J, Dumy P, et al. A cyclodecapeptide ligand to vitamin B_{12}. Org Biomol Chem 2008;6:4134–41.
[11] Faircloth G, Cuevas C, Kahalalide F. ES285: potent anticancer agents from marine moluscs. Prog Mol Subcell Biol 2006;43:363–79.
[12] Frecer V, Ho B, Ding JL. De novo design of potent antimicrobial peptides. Antimicrob Agents Chemother 2004;48:3349–57.
[13] Garcia-Martin F, Cruz LJ, Rodriguez-Mias RA, Giralt E, Albericio F. Design and synthesis of FAJANU: a de novo C_2 symmetric cyclopeptide family. J Med Chem 2008;51:3194–202.
[14] Hancock REW, Sahl HG. Antimicrobial and host defense peptides as new anti-infective therapeutic strategies. Nature Biotechnol 2006;24:1551–7.
[15] Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta 2008;1778:357–75.
[16] Hui L, Leung K, Chen HM. The combined effects of antibacterial peptide cecropin A and anticancer agents on leukemia cells. Anticancer Res 2002;22:2811–6.
[17] Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19:491–511.
[18] Jiang S, Liao C, Bindu L, Yin B, Worthy KW, Fisher RJ, et al. Discovery of thioether-bridged cyclic pentapeptides binding to Grb2-SH2 domain with high affinity. Bioorg Med Chem Lett 2009;19:2693–8.
[19] Johnson CL, Lu D, Huang J, Basu A. Regulation of p53 stabilization by DNA damage and protein kinase. Mol Cancer Ther 2002;1:861–7.
[20] Kates SA, Solé NA, Johnson CR, Hudson D, Barany G, Albericio F. A novel, convenient, three-dimensional orthogonal strategy for solid-phase synthesis of cyclic peptides. Tetrahedron Lett 1993;34:1549–52.
[21] Katsara M, Tselios T, Deraos S, Deraos G, Minos-Timotheos M, Lazoura E, et al. Round and round we go: cyclic peptides in disease. Curr Med Chem 2006;13:2221–32.
[22] Kim S, Kim SS, Bang Y-J, Kim S-J, Lee BJ. In vitro activities of native and design peptide antibiotics against drug sensitive and resistant tumor cell lines. Peptides 2003;24:945–53.
[23] Leuschner C, Hansel W. Membrane disrupting lytic peptides for cancer treatments. Curr Pham Design 2004;10:2299–310.
[24] Liu T, Joo SH, Voorhees JL, Brooks CL, Pei D. Synthesis and screening of a cyclic peptide library: discovery of small-molecule ligands against human prolactin receptor. Bioorg Med Chem 2009;17:1026–33.
[25] Mader JS, Hoskin DW. Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment. Expert Opin Investig Drugs 2006;15:933–46.
[26] Menendez JA, Lupu R, Colomer R. Inhibition of tumor-associated fatty acid synthase hyperactivity induces synergistic chemosensitization of HER-2/neu-overexpressing human breast cancer cells to docetaxel (taxotere). Breast Cancer Res Treat 2004;84:183–95.
[27] Mocellin S, Pilati P, Nitti D. Peptide-based anticancer vaccines: recent advances and future perspectives. Curr Med Chem 2009;16:4779–96.
[28] Monroc S, Badosa E, Besalú E, Planas M, Bardají E, Montesinos E, et al. Improvement of cyclic decapeptides against plant pathogenic bacteria using a combinatorial chemistry approach. Peptides 2006;27:2575–84.
[29] Morita H, Takeya K. Bioactive cyclic peptides from higher plants. Heterocycles 2010;80:739–64.
[30] Ohsaki Y, Gazdar AF, Chen H-C, Johnson BE. Antitumor activity of magainin analogues against human lung cancer cell lines. Cancer Res 1992;52:3534–8.
[31] Papo N, Shai Y. Host defense peptides as new weapons in cancer treatment. Cell Mol Life Sci 2005;62:784–90.
[32] Park SA, Park HJ, Lee BI, Ahn YH, Kim SU, Choi KS. Bcl-2 blocks cisplatin-induced apoptosis by suppression of ERK-mediated p53 accumulation in B104 cells. Mol Brain Res 2001;93:18–26.
[33] Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 2006;4:529–36.
[34] Qin CH, Bu X, Zhong X, Ng NLJ, Guo Z. Optimization of antibacterial cyclic decapeptides. J Comb Chem 2004;6:398–406.
[35] Ropero S, Menéndez JA, Vázquez-Martín A, Montero S, Cortés-Funes H, Colomer R. Trastuzumab plus tamoxifen: anti-proliferative and molecular interactions in breast carcinoma. Breast Cancer Res Treat 2004;86:125–37.
[36] Schröder-Borm H, Bakalova R, Andrä J. The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine. FEBS Lett 2005;579:6128–34.
[37] Shai Y. Mode of action of membrane active antimicrobial peptides. Biopolymers 2002;66:236–48.
[38] Takahashi T, Nagamiya H, Doi T, Griffiths PG, Bray AM. Solid phase library synthesis of cyclic depsipeptides: aurilide and aurilide analogues. J Comb Chem 2003;5:414–28.
[39] Ueda S, Oishi S, Wang Z, Araki T, Tamamura H, Cluzeau J, et al. Structure-activity relationships of cyclic peptide-based chemokine receptor CXCR4 antagonists: disclosing the importance of side-chain and backbone functionalties. J Med Chem 2007;50:192–8.
[40] Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug Discov Today 2010;15:40–56.
[41] Woessmann W, Chen X, Borkhardt A. Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines. Cancer Chemother Pharmacol 2002;50:397–404.
[42] Won H-S, Seo M-D, Jung S-J, Lee S-J, Kang S-J, Son W-S, et al. Structural determinants for the membrane interaction of novel bioactive undecapeptides derived from gaegurin 5. J Med Chem 2006;49:4886–95.
[43] Yao N, Xiao W, Wang X, Marik J, Park SH, Takada Y, et al. Discovery of targeting ligands for breast cancer cells using the one-bead one-compound combinatorial method. J Med Chem 2009;52:126–33.
[44] Yao X, Panichpisal K, Kurtzman N, Nugent K. Cisplatin nephrotoxicity: a review. Am J Med Sci 2007;334:115–24.
[45] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27–55.
[46] Zhang L, Falla TJ. Antimicrobial peptides: therapeutic potential. Expert Opin Pharmacother 2006;7:653–63.
[47] Zhang Y, Zhou S, Wavreille A-S, DeWille J, Pei D. Cyclic peptidyl inhibitors of Grb2 and tensin SH2 domains identified from combinatorial libraries. J Comb Chem 2008;10:247–55.