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Antimicrobial cyclic decapeptides with anticancer activity

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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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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