European Journal of Medicinal Chemistry 89 (2015) 540–548

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis and biological evaluation of novel peptides with anti-cancer and drug resistance-reversing activities

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ARTICLE INFO

Article history:
Received 7 September 2014
Received in revised form
24 October 2014
Accepted 25 October 2014
Available online 28 October 2014

Keywords:
Anti-cancer
Drug resistance-reversing effects
Membrane-disruption
Peptides

ABSTRACT

Chemotherapy is an important approach used to treat cancer, but severe side effects and emerging drug resistance restrict its clinical application. In this present study, we found that peptide B1 showed specific cytotoxicity to tumor cells. Moreover, a helix-wheel plot predicted that the Ser14 in this peptide is located at the interface of the hydrophobic and hydrophilic faces of B1. Subsequently, we wondered whether replacing Ser14 would alter the activity of B1, and so a series of B1 analogs were synthesized where the Ser14 was replaced by amino acids with distinct physicochemical properties. Amongst them, peptides where Ser14 was substituted by a nonpolar and basic amino acid had improved anti-cancer activity. Further investigations revealed that B1 and its analogs were capable of penetrating into cytoplasm and triggering cytochrome C release from mitochondria, which ultimately resulted in apoptosis. Meanwhile, B1 and its analogs inhibited the migration of cancer cells. The peptides also acted against drug-resistant cells and had drug resistance-reversing effects. In conclusion, these peptides might be promising candidates for oncotherapy.

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1. Introduction

Cancer comprises a group of complicated diseases characterized by the unregulated growth of abnormal cells that have the capability of invading normal tissues, metastasizing to other organs and spreading to other body parts [1–3]. As a leading cause of death and disability, cancer is responsible for nearly 7.6 million deaths per year [4]. Chemotherapy is one of the important approaches used in cancer therapy, but severe side effects resulting from toxicity of chemotherapeutics on normal cells remains an important obstacle in clinical application [5–7]. Meanwhile, another problem encountered during cancer therapy is drug resistance resulting from changes in drug transporters or detoxifying enzymes of the cancer cells [2].

Antimicrobial peptides (AMPs) are ancient host defense compounds that have activity against a broad spectrum of microorganisms [8,9]. In general, AMPs possess 12–50 amino acids and, of these, 2–9 are typically positively charged while about 50% are hydrophobic amino acids. These residues are spatially organized to make AMPs amphipathic [10,11]. Recent studies have shown that several AMPs can exert anti-cancer effects [12].

As a novel type of promising chemotherapeutic, AMPs present significant advantages over traditional antitumor agents, such as higher specificity and circumvention of drug resistance [13,14]. There are essential differences between the cell membranes of neoplastic and normal cells and these differences might account for the selective cytotoxicity of AMPs to cancer cells while sparing healthy cells. Most cancer cells carry a net negative charge on the membrane due to the overexpression of various anionic molecules, such as phosphatidylserine and *O*-glycosylated mucins [15–18]. On the contrary, healthy eukaryotic cells possess high contents of zwitterionic membrane components, including phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, which confer an overall neutral charge on the cell surface [19]. As a consequence, an electrostatic interaction would occur between the AMPs and cancer cells rather than with untransformed counterparts.

Several presumptions have been proposed to explain the interactions between AMPs and cell membranes. In brief, the positively-charged peptides initially bind to and cover the cell

Abbreviations: 5-Fu, 5-fluorouracil; ADM, adriamycin; AMPs, antimicrobial peptides; AO, acridine orange; DMSO, dimethyl sulfoxide; EB, ethidium bromide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; JC-1, 5,5′,6,6′-tetrachloro-l,l′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide; MTT, thiazolyl blue; RBCs, red blood cells; RP-HPLC, reversed phase high-performance liquid chromatography; SEM, scanning electron microscope; UPLC, ultra-high performance liquid chromatography; Ver, Verapamil.

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http://dx.doi.org/10.1016/j.ejmech.2014.10.072
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membrane via electrostatic attraction. Then AMPs insert into membrane when a critical concentration is reached. At this point, two mechanisms have been proposed: (i) a membrane disruptive mechanism where the AMPs reorientate and disrupt membrane integrity resulting in leakage of metabolites and ions as well as depolarization; (ii) a non-membrane mechanism where the AMPs translocate into the cytoplasm by virtue of cell membrane perturbation or pore formation so they are free to act on intracellular targets and cause apoptosis [12,20].

Cathelicidin-BF15 (BF-15) is a 15-mer AMP with broad antimicrobial activity, but its C-terminal amidated derivative B1 (primary sequence: VKRFKKFFRKLKKSV-NH_2) has even greater antimicrobial potency [21]. The anti-cancer properties of B1 have not been reported previously. In this present study, we demonstrate the anti-cancer effects of B1 on the MCF-7 breast cancer and K562 leukemia cell lines. Additionally, we show that B1 has relatively low cytotoxicity against the normal cell line GES-1.

Two common traits, hydrophobicity and positive charge, play pivotal roles in the activity of AMPs [22,23]. According to a helix-wheel plot (http://heliquest.ipmc.cnrs.fr/), we found that the serine residue at position 14 (Ser14), an uncharged polar amino acid, is located between the hydrophobic and cationic surfaces of B1 (Fig. 1). Therefore, we wondered whether replacement of Ser14 by distinct amino acids would alter the anti-cancer activity of B1. Alanine substitution is a common approach when verifying the active site of peptides [24], and so we synthesized B1-Ala14 (VKRFKKFFRKLKKAV-NH_2) where Ser14 was replaced by alanine and assessed the antitumor activity. B1-Ala14 exhibited increased anti-cancer activity compared to B1. Subsequently, we synthesized 11 other peptides and assessed their antitumor activities using the MTT assay. Peptides where the Ser14 was replaced by a nonpolar or basic amino acid exhibited more favorable activity against some cancer cell lines, while all the peptides had lower toxicity against benign cells.

In order to clarify the anti-cancer mechanism, B1 and two of its analogs (B1-Leu and B1-Lys) were chosen for further investigation. Meanwhile, anti-cancer activity was also assessed against adriamycin (ADM)-resistant cell lines.

2. Materials and methods

2.1. Peptide synthesis, purification and analysis

The synthesis of B1 and its analogs was accomplished by solid-phase methods on a microwave synthesizer (CEM, NC, USA), and the general procedure has been reported previously [25]. Crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC; Shimadzu LC-10) using a C18 column (5 μm, 340 × 28 mm). Purity analysis and characterization was performed by ultra-high performance liquid chromatography/mass spectrometry (UPLC/MS; Waters UPLC with the ACQUITY TQD; Waters Corporation, Milford, MA, USA) on a Waters ACQUITY UPLC BEH C18 column (1.7 × 50 mm, Waters). The purity of the peptides was above 95%.

2.2. Helix-wheel plot

A helix-wheel plot was achieved using the software package provided by The Expert Protein Analysis System (ExPASy) proteomics server as described elsewhere (http://heliquest.ipmc.cnrs.fr/) [26].

2.3. Cell cultures

The MCF-7 breast cancer cell line, an ADM-resistant sub-line MCF-7/ADM, leukemia K562 cell line, an ADM-resistant sub-line K562/ADM, and the GES-1 gastric epithelial cell line were used in this study. All the cell lines were obtained from KeyGEN BioTECH (Nanjing, China). Cells were cultured in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories) and 1% penicillin–streptomycin antibiotic mixture (Gibco BRL). All the cell lines were cultured at 37 °C in a humidified atmosphere at 5% CO_2 and 95% air.

2.4. Cell proliferation and viability assay

Anti-cancer activity of B1 and its analogs was assessed by the MTT assay. Cells in the logarithmic phase of growth were collected and seeded at 5×10^3 cells/well in 96-well plates 24-h before peptide treatment. The cells were treated with various concentrations of peptides while controls were not exposed to peptide. After incubating for 48 h, 20 μL of 5 mg/mL MTT solution was added to each well and allowed to incubate for 4 h. Then, 150 μL DMSO was added to dissolve the MTT formazan precipitate. The absorbance at 490 nm of the mixture in each well was measured with a Bio-Rad microtiter plate reader. Average cell survival rate was calculated by the following formula: cell survival rate (%) $= (A'_{490nm} - A_{490nm})/A'_{490nm} \times 100\%$, where A'_{490nm} is absorbance at 490 nm of the sample without peptide and A_{490nm} is absorbance at 490 nm of the sample with peptide. Survival rates were plotted against peptide concentrations and the IC_{50} value for each cell line was calculated based on the survival curve.

2.5. Hemolysis assay

Rabbit red blood cells (RBCs) were used for hemolysis assay follow the process described by Chen et al. [21]. In brief, 100 μL of peptide solution was added to 100 μL of RBCs PBS suspension in 96-well plates, which were incubated for 1 h at 37 °C and centrifuged at 1000× *g* for 5 min. Supernatant were transferred to fresh 96-well plates and hemoglobin release was measured by microplate reader (Bio-Rad, iMark 680) at 540 nm. Zero and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

2.6. Acridine orange/ethidium bromide (AO/EB) double staining

The MCF-7 cell line was used for AO/EB double staining. Briefly, MCF-7 cells were grown in a 24-well microtiter plate at 1×10^5 cells

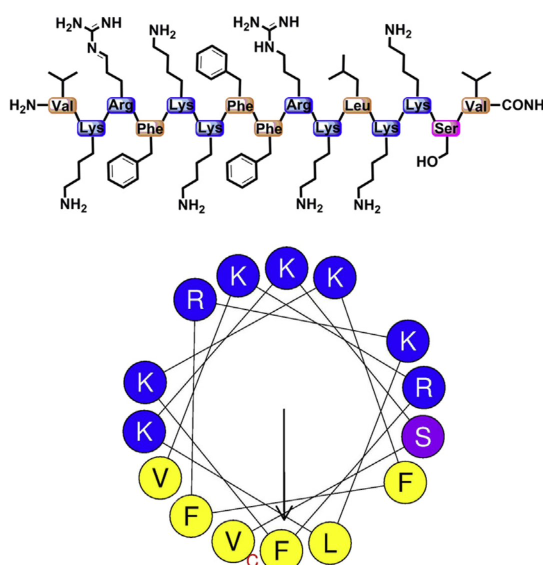

Fig. 1. The helix-wheel plot of B1.

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