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Enhancement of the cancer targeting specificity of buforin IIb by fusion with an anionic peptide via a matrix metalloproteinases-cleavable linker

Ju Hye Jang[a], Min Young Kim[a], Jin-Won Lee[b], Sun Chang Kim[c], Ju Hyun Cho[a,*]

[a] Department of Biology, Research Institute of Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660-701, Republic of Korea
[b] Department of Life Science and Research Center for Natural Sciences, Hanyang University, Seoul 133-791, Republic of Korea
[c] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea

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ABSTRACT

Buforin IIb is a novel cell-penetrating anticancer peptide derived from histone H2A. In this study, we enhanced the cancer targeting specificity of buforin IIb using a tumor-associated enzyme-controlled activation strategy. Buforin IIb was fused with an anionic peptide (modified magainin intervening sequence, MMIS), which neutralizes the positive charge of buforin IIb and thus renders it inactive, via a matrix metalloproteinases (MMPs)-cleavable linker. The resulting MMIS:buforin IIb fusion peptide was completely inactive against MMPs-nonproducing cells. However, when the fusion peptide was administrated to MMPs-producing cancer cells, it regained the killing activity by releasing free buforin IIb through MMPs-mediated cleavage. Moreover, the activity of the fusion peptide toward MMPs-producing cancer cells was significantly decreased when the cells were pretreated with a MMP inhibitor. Taken together, these data indicate that the cancer targeting specificity of MMIS:buforin IIb is enhanced compared to the parent peptide by reactivation at the specialized areas where MMPs are pathologically produced.

1. Introduction

Although numerous chemotherapeutic drugs, including alkylating agents, antimetabolites and hormone agonists/antagonists, have been developed and successfully used for the treatment of metastatic cancers, the efficacy of cancer chemotherapy is limited by severe side-effects and dose limitations [30]. Current chemotherapeutic drugs cannot distinguish between cancer cells and proliferating normal cells, and kill both. Moreover, cancer cells develop resistance to these drugs that is mediated by the overexpression of multidrug-resistance proteins that pump the drugs out of cells and thus render the drugs ineffective [28]. To overcome the limits of current chemotherapeutic drugs, many researchers have labored to identify new anticancer molecules. Recently, anticancer peptides, cationic antimicrobial peptides (AMPs) with cancer-selective toxicity, have received attention as alternative chemotherapeutic agents that overcome the limits of current drugs. These peptides have several advantages over currently used anticancer therapeutics, such as low intrinsic cytotoxicity, decreased likelihood of resistance development and additive effects in combination therapy [15,25].

Most anticancer peptides selectively kill cancer cells by disruption of the cancer cell membrane or permeation and swelling of mitochondria, where the electrostatic attraction between the negatively charged membrane components of cancer cells and the cationic anticancer peptides is believed to play a crucial role [29]. Among the anticancer peptides, buforin IIb-a synthetic analog of buforin II that contains a proline hinge between the two α-helices and a model α-helical sequence at the C-terminus (3×RLLR) [26]-selectively targets cancer cells through interaction with the cell-surface gangliosides. Buforin IIb then traverses cancer cell membranes without damaging them and induces mitochondria-dependent apoptosis [17]. Buforin IIb also displays powerful cytotoxic activity when injected into solid tumors in p53-deficient mice [6]. These results suggest that buforin IIb may be developed into a novel therapeutic agent for the treatment of cancers. However, preliminary study showed that buforin IIb also killed normal cells at higher concentrations, though not efficient compared to cancer cells. Moreover, neurons, of which gangliosides are abundant in the plasma membranes [31], may be vulnerable to buforin IIb. Therefore, the cancer-targeting specificity of buforin IIb should be enhanced to fully exploit its therapeutic potential.

In this study, we used a tumor-associated enzyme-controlled activation strategy to enhance the cancer targeting specificity of buforin IIb. Matrix metalloproteinases (MMPs) are a family of over

Abbreviations: AMP, antimicrobial peptide; MMIS, modified magainin intervening sequence; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

* Corresponding author. Tel.: +82 55 751 5950; fax: +82 55 754 0086.
E-mail address: juhyun.cho@gnu.kr (J.H. Cho).

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Table 1
Peptide sequences.

Peptide name	Peptide sequence
Buforin IIb	RAGLQFPVG[RLLR]$_3$
MMIS:buforin IIb	DAEAVGPEAADEEKDEDGPLG/IAGQRAGLQFPVG[RLLR]$_3$

Note: the MMP-2 cleavage site is underlined. The cleavage position is indicated by "/".

25 secreted and membrane-bound zinc endopeptidases that participate in numerous normal and pathologic extracellular matrix (ECM) remodeling events, including tumor progression, metastasis and angiogenesis [5,10,22,32]. They have also been implicated as key mediators of the cellular responses to inflammation and degeneration [16,27,34]. Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) especially play a critical role in ECM breakdown, and many type of cancer have shown increased levels of MMP-2 and MMP-9 [7–9,12,20,23]. MMP-2 and MMP-9 substrate sequences have successfully been used in prodrug strategies attempting to release doxorubicin, melittin and methotrexate as the cytotoxic agents to tumor tissues [3,14,21]. Therefore, we fused buforin IIb with an anionic peptide which can neutralize the positive charge of buforin IIb and thus render it inactive via a MMP-2/MMP-9-cleavable linker, and analyzed the anticancer activity of the fusion peptide using cell lines of different MMPs expression levels. Here we have shown that the cancer targeting specificity of the fusion peptide was enhanced compared to the parent peptide by reactivation through MMPs-mediated cleavage.

2. Materials and methods

2.1. Peptides

Buforin IIb and MMIS:buforin IIb (Table 1) were synthesized on a MilliGen 9050 peptide synthesizer (Peptron, Korea). Synthetic peptides were purified by reversed-phase high performance liquid chromatography and characterized by mass spectroscopy and amino acid analysis. To test whether MMIS:buforin IIb is digested by MMPs, 10 μM of MMIS:buforin IIb was incubated with 10 nM of recombinant human active MMP-2 or MMP-9 (Calbiochem, San Diego, CA, USA) in the digestion buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$) at 37 °C. At the designated time points, the digestion mixture was sampled and analyzed by 15% SDS–PAGE.

2.2. Cells

FSF (normal human foreskin fibroblast) was obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (NIGMS). HeLa (human cervix adenocarcinoma), B16-F0 (mouse melanoma), HT1080 (human fibrosarcoma) and U87MG (human glioblastoma) were purchased from the American Tissue Cell Culture (ATCC). Cells were cultured in a complete medium [DMEM (FSF, HeLa, B16-F0 and U87MG) or RPMI-1640 (HT1080) supplemented with 10% FBS and 0.1% penicillin–streptomycin] in a humidified atmosphere of 5% CO_2 at 37 °C. Trypsin–EDTA (0.05%) was used to detach cells in subculturing. All the cell culture media and reagents were purchased from Lonza (Basel, Switzerland).

2.3. In vitro cytotoxicity assay

Cells were seeded onto 96-well plates at a density of 5000 cells/well (HeLa, B16-F0 and U87MG) or 10,000 cells/well (FSF and HT1080) in 0.1 ml of serum-free medium. In some experiments, MMP-2/MMP-9 inhibitor III (0–80 μM, Calbiochem) was included in the serum-free medium to inhibit endogenous MMPs expressed by the cells. After 48 h of incubation, cells were treated with peptides (buforin IIb or MMIS:buforin IIb, 0–8 μM) and incubated for another 24 h. Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using the CellTiter 96-cell proliferation assay kit (Promega, Madison, WI, USA). The percentage of cell viability was determined as follows:

$$\text{Viability}\ \ (\%) = \frac{A_s - A_0}{A_c - A_0} \times 100\%$$

where A_s is the absorbance value of the sample, A_c is the absorbance of control and A_0 is the background absorbance. Each experiment was repeated at least three times independently.

2.4. Hemolysis assay

Hemolytic activity was assayed as described by Aboudy et al. [1] with a slight modification. Three milliliters of freshly prepared human red blood cells (RBCs) were washed with isotonic phosphate-buffered saline (PBS), pH 7.4, until the color of the supernatant turned clear. The washed RBCs were then diluted to a final volume of 20 ml with the same buffer. Peptide samples (10 μl), serially diluted in PBS, were added to 190 μl of the cell suspension in microfuge tubes. Following gentle mixing, the tubes were incubated at 37 °C for 30 min and then centrifuged at 4000 × *g* for 5 min. One hundred microliters of supernatant were taken, diluted to 1 ml with PBS, and absorbance at 567 nm was measured to monitor the release of hemoglobin that indicated RBC membrane damage. Zero hemolysis and 100% hemolysis consisted of RBC suspended in PBS and 0.2% Triton X-100, respectively. The percentage of hemolysis was determined as follows:

$$\text{Hemolysis}\ \ (\%) = \frac{A_s - A_0}{A_{100} - A_0} \times 100\%$$

where A_s is the absorbance of the sample, A_{100} is the absorbance of completely lysed RBC in 0.2% Triton X-100, and A_0 is the absorbance of zero hemolysis.

2.5. Reverse transcription (RT)-PCR analysis

Total RNAs were extracted from cultured cells using a Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of each RNA was used for semi-quantitative RT-PCR. Three sets of gene-specific primers were used to specifically amplify MMP-2 and MMP-9 fragments [MMP-2F (5′-CAATACCTGAACACTTTCTATGG-3′)/MMP-2R (5′-CTGTATGTGATCTGGTTCTTG-3′) for human and mouse MMP-2 genes; hMMP-9F (5′-CCTGGAGACCTGAGAACCAA-3′)/hMMP-9R (5′-GGACCACAACTCGTCATCG-3′) for human MMP-9 gene; mMMP-9F (5′-CCCAAAGACCTGAAAACCTCCAA-3′)/mMMP-9R (5′-CGACCACAACTCGTCGTCG-3′) for mouse MMP-9 gene]. β-Actin gene expression, which was amplified by β-actin-F (5′-GCATCACACCTTCTACAATGAGC-3′)/β-actin-R (5′-GCTCATAGCTCTTCTCCAGGG-3′), was used as an internal control. The RT-PCR was performed using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) with a temperature profile of 50 °C for 30 min, 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, *T* °C (depending on the target gene) for 30 s, 72 °C for 60 s, and final extension at 72 °C for 10 min. The annealing temperature (*T*) was optimized for each pair of primers: MMP-2F/MMP-2R, 55 °C; hMMP-9F/hMMP-9R and mMMP-9F/mMMP-9R, 66 °C; β-actin-F/β-actin-R, 60 °C. After amplification, the PCR products were analyzed by 1% agarose gel electrophoresis, and bands were visualized by ethidium bromide staining. The predicted PCR product sizes for MMP-2, MMP-9 and β-actin are 227 bp, 539 bp and 467 bp, respectively.