EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Proapoptotic Cyclic Peptide BC71 Targets Cell-Surface GRP78 and Functions as an Anticancer Therapeutic in Mice

Chieh Kao [a], Ritu Chandna [a], Abhijeet Ghode [a], Charlotte Dsouza [a], Mo Chen [a], Andreas Larsson [b], Siau Hoi Lim [b], Minjun Wang [c], Zhonglian Cao [c], Yizhun Zhu [c], Ganesh S. Anand [a], Ruowen Ge [a,*]

[a] Department of Biological Sciences, National University of Singapore, 16 Science Drive 4, 117558, Singapore
[b] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 639798, Singapore
[c] School of Pharmacy, Fudan University, 826 Zhangheng Rd, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 2 March 2018
Received in revised form 25 May 2018
Accepted 6 June 2018
Available online xxxx

ABSTRACT

Glucose regulated protein 78 kDa (GRP78) is a recently emerged target for cancer therapy and a biomarker for cancer prognosis. Overexpression of GRP78 is observed in many types of cancers, with the cell-surface GRP78 being preferentially present in cancer cells and cancer blood vessel endothelial cells. Isthmin (ISM) is a secreted high-affinity proapoptotic protein ligand of cell-surface GRP78 that suppresses angiogenesis and tumor growth in mice. The C-terminal AMOP (adhesion-associated domain in MUC4 and other proteins) domain of ISM is critical in mediating its interaction with human umbilical vein endothelial cells (HUVECs). In this work, we report novel cyclic peptides harboring the RKD motif in the ISM AMOP domain that function as proapoptotic ligands of cell-surface GRP78. The most potent peptide, BC71, binds to GRP78 and converge to tumor in mice. Intravenous administration of BC71 suppressed xenograft tumor growth in mice as a single agent, with significant reduction in tumor angiogenesis and upsurge in apoptosis. Fluorescent-labeled BC71 accumulates in tumor in mice by targeting cell-surface GRP78. We show that BC71 triggers apoptosis via cell-surface GRP78 and activates caspase-8 and p53 signaling pathways in HUVECs. Using amide hydrogen-deuterium exchange mass spectrometry (HDXMS), we identified that BC71 preferentially binds to ATP-bound GRP78 via amino acid residues 244–257 of GRP78. Hence, BC71 serves as a valuable prototype for further development of peptidomimetic anticancer drugs targeting cell-surface GRP78 as well as PET imaging agents for cancer prognosis.

1. Introduction

GRP78 is a member of the heat-shock protein family and a major endoplasmic reticulum (ER) chaperone protein which modulate protein folding. It is a stress-response protein whose expression is up-regulated in cells under stress. Cancer cells are under constant stress caused by their microenvironment of hypoxia, acidity and metabolic toxicity. It has been reported that GRP78 is overexpressed in various human cancers including breast cancer, prostate cancer, lung cancer, ovarian cancer, and melanoma. Overexpression of GRP78 in cancer is linked to chemoresistance, increased malignancy, and poor patient outcomes [16, 17, 25].

Overexpression of GRP78 leads to its translocation to the cell-surface in cancer cells and cancer blood vessel ECs. Cell-surface GRP78 has been shown to function as a receptor for proapoptotic ligands such as Kringle-5 and Par-4, mediating apoptosis [21, 28]. Phage-display library screening has identified several synthetic peptides that bind to cell-surface GRP78 [1, 12]. Conjugation of a proapoptotic peptide to a GRP78 binding peptide resulted in a GRP78-targeting proapoptotic peptide that potently suppressed tumor growth in mice [22]. Meanwhile, anti-GRP78 monoclonal antibodies also function as potent anticancer therapeutics in mice or human clinical trials [18, 23, 24]. Nevertheless, no anticancer therapeutics targeting GRP78 have reached the clinics as of now.

GRP78 is overexpressed in both cancer cells and cancer blood vessel endothelial cells (ECs) [4, 26, 31]. Excessive angiogenesis and resistance to apoptosis are two hallmarks of cancer [9]. Inducing EC apoptosis can suppress angiogenesis and induce vessel regression, thus is a useful strategy for antiangiogenic cancer therapy [34]. Meanwhile, inducing cancer cell apoptosis has also been actively pursued in anticancer therapeutic development [5, 7, 11].

Isthmin (ISM) is a secreted proapoptotic protein recently identified by us [36]. It contains two important domains, a central thrombospondin type I repeat (TSR) domain and a C-terminal AMOP (adhesion associated

Abbreviations: GRP78, Glucose regulated protein 78 kDa; ISM, Isthmin; AMOP, Adhesion-associated domain in MUC4 and other proteins; HUVECs, Human umbilical vein endothelial cells; TSR, Thrombospondin type I repeat; ER, Endoplasmic reticulum.

* Corresponding author.
E-mail address: dbsgerw@nus.edu.sg (R. Ge).

https://doi.org/10.1016/j.ebiom.2018.06.004

Please cite this article as: Kao C, et al, Proapoptotic Cyclic Peptide BC71 Targets Cell-Surface GRP78 and Functions as an Anticancer Therapeutic in Mice, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.06.004

domain in MUC4 and other proteins) domain. Bacterially expressed recombinant ISM (rISM) induced human umbilical vein endothelial cell (HUVEC) apoptosis and suppressed angiogenesis in vitro and in vivo [36]. Cell-surface GRP78 is a high-affinity receptor for ISM (K_d of 8.6 nM) [4]. ISM selectively induces apoptosis in cells harboring high level cell-surface GRP78 including activated endothelial cells and highly metastatic and aggressive cancer cells [4]. As a potent proapoptotic ligand of cell-surface GRP78, ISM has the potential to be a drug specifically targeting chemoresistant and aggressive cancers.

Peptide-based drugs possess several advantages over small molecule drugs including higher target specificity, no accumulation in tissues and organs, less side effects and less long-term toxicity. Compared to recombinant proteins and antibodies, peptides are less immunogenic and potentially cheaper to manufacture [32].

In this study, we report a GRP78-specific cyclic peptide BC71 designed based on the AMOP domain of ISM which induces apoptosis in HUVECs. Systemic delivery of BC71 effectively suppressed subcutaneous tumor growth in mice as a single agent. We show that BC71 accumulates in tumors likely by binding to cell-surface GRP78 overexpressed on the surfaces of tumor cells and tumor blood vessel endothelial cells. Hence, BC71 is a valuable prototype peptide which can be further developed into GRP78-targeted anticancer therapeutic as well as an imaging probe for GRP78 for cancer prognosis.

2. Materials and Methods

2.1. Cell Lines and Reagents

HUVECs were purchased from Lonza and cultured in EndoGro-LS (Millipore, Bradford, MA, USA) supplemented with FBS and gentamycin (Sigma-Aldrich, St. Louis, MO, USA) on pre-coated culture dishes/inserts/slides. All experiments were performed on cells from passages 5–10. 4 T1 cells were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). Antibodies against integrin αvβ5 (P1F76; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GRP78 (A-10, Santa Cruz Biotechnology) and GRP78 (C-20, Santa Cruz Biotechnology) were used for neutralization. P53 inhibitor (Pifithrin-α hydrobromide, PFT-α sc-45050A) and caspase-8 inhibitor (z-IETD-fmk, sc-3084) were purchased from Santa Cruz Biotechnology. Synthetic ISM-related peptides were synthesized by Ontores (Hangzhou, China), Mimotopes (Melbourne, Australia) and China Peptide (Shanghai, China). The Cy7 labeled peptides was synthesized by Cambridge Research Biochemicals Limited (Cleveland, UK).

2.2. Cell Viability Assay

HUVECs were plated at 5×10^3–1×10^4 cells per well of 96-well plates and were treated with 100 μM ISM peptide in 2% FBS EndoGro medium for 24 h or 48 h before being used in the assays. The inhibitory effects of ISM peptide on the growth of HUVECs were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (Sigma-Aldrich, St. Louis, MO, USA). The optical density was measured at 570 nm using an automated scanning multiwall spectrophotometer.

2.3. Lactate Dehydrogenase (LDH) Assays

The cytotoxicity of ISM-peptide on HUVECs was investigated. Cells were plated at 5×10^3–1×10^4 cells per well of 96-well plates and treated with 100 μM ISM peptide in 2% FBS EndoGro medium for 24 h or 48 h before being used in the assays. To calculate the activity of LDH, 100 μl of a reaction mixture (Cytotoxicity Detection Kit; Sigma-Aldrich, St. Louis, MO, USA) and the conditional medium were added to each well and incubated in the dark for 10–30 min. The water-soluble formazan dye exhibited the broad absorption maximum at approximately 490 nm in the spectrophotometer.

2.4. Cell Death ELISA Assay

HUVECs were seeded at 1–1.5 × 10^4 cell per well of 96-well plates and were co-treated with 100 μM ISM peptide +15 ng/ml VEGF and 5, 10, 20 μM z-IETD-fmk or 7.5, 15, 30 μM PFT-α, or pretreated with anti-GRP78 antibody for 60 min after pre-starved in 2% FBS medium for 3 h. The Cell Death Detection ELISA photometric enzyme immunoassay (Sigma-Aldrich, St. Louis, MO, USA) was used for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) as an indicator of apoptosis. The absorbance was measured at 405 nm.

2.5. ATP Colorimetric Assay

The ATP concentrations in whole-cell lysate, mitochondrial and cytosolic (without mitochondria) fractions in HUVECs with indicated treatment of 100 μM BC71 for 24 h, were analyzed using the ATP colorimetric assay kit (BioVision, Milpitas, CA, USA).

2.6. Surface Plasmon Resonance (SPR) Binding Assays

The experiments were performed using a BIACORE T200 instrument (GE healthcare) equipped with research-grade NTA-S sensor chip. GRP78 protein was immobilized using the capture-coupling method performed by a slight modification of the method as described previously [33]. The surfaces of flow cells 1 and 2 were activated for 3 min with 0.5 mM $NiCl_2$, followed by 7 min with a 1:1 mixture of 0.1 M NHS and 0.1 M EDC and finally by 1 min 0.5 mM $NiCl_2$ at a flow rate of 5 μl/min. The ligand, GRP78 at a concentration of 60 μg/ml in 10 mM sodium acetate, pH 6.0, was immobilized at a density of 2500–3000 RU on flow cell 2; flow cell 1 was left blank to serve as a reference surface. All the surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.0 and washed for 2 min with 0.35 M EDTA solution.

Peptides of interest (the analytes) dissolved or exchanged into HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA 0.005% P20, pH 7.4), and injected over the two flow cells at a flow rate of 30 μl/min and at a temperature of 25 °C. Data were collected at a rate of 1 Hz. Each sample series consisted of single injections of the analyte at 1.25 μM and 200 μM followed by duplicate injections of buffer blank to allow double referencing. The complexes were allowed to associate for 30 s and dissociate for 120 s and the surfaces were regenerated with a 60 s injection of a 1:1 mixture of 20 mM sodium hydroxide and running buffer. The binding data were analyzed and fitted using the software Scrubber 2.0 (BioLogic software). At higher concentrations many of the peptides exhibited problematic injections likely due to low solubility or aggregation so a full, saturated isotherm couldn't be recorded in many cases. To ensure fair comparability of the different peptides and a good relative ranking we fitted RU_{max} globally which was then used in the subsequent steady-state fits of the peptides.

2.7. HDXMS

Recombinant GRP78 ATPase domain (26–410) was expressed and purified in *E. coli* as previously described [35] and reconstituted in buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 5% glycerol, 5 mM DTT). Stock ADP and AMPPNP (Sigma-Aldrich, St. Louis, MO, USA) solutions at 4.8 mM were prepared in buffer A with 2 mM Mg^{2+} added. Lyophilized BC71 peptide was dissolved in autoclaved deionized water, and its pH adjusted to ~7.5. For pepsin fragment peptides identification, aqueous samples (undeuterated reactions), were diluted in buffer A, and a quench solution prepared using trifluoroacetic acid (TFA) and 1 M GnHCl was added to bring final pH to 2.5. Deuterium exchange reactions were initiated with buffer A reconstituted in 99.9% D_2O to generate a final D_2O concentration of 90% followed by incubation at 25 °C for different time points (t = 0.5, 1, 5, 10 and 100 mins). Deuteration reactions were quenched with pre-chilled quench solution. Samples were

Please cite this article as: Kao C, et al, Proapoptotic Cyclic Peptide BC71 Targets Cell-Surface GRP78 and Functions as an Anticancer Therapeutic in Mice, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.06.004