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Enhancing the circulating half-life and the antitumor effects of a tumor-selective cytotoxic peptide by exploiting endogenous serum albumin as a drug carrier

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ABSTRACT

The elevated expression of bombesin receptors in many of the deadliest cancers has attracted special interest in developing bombesin-directed agents for tumor imaging and therapy. Previously, we constructed the chimeric peptide BB28 by fusing bombesin to a mitochondria-disrupting peptide. BB28 selectively induced the apoptosis of various tumor cells *in vitro* and showed promising *in vivo* antitumor effects. In general, a short circulating half-life limits the *in vivo* effect of peptides. To prolong the half-life of BB28, here, we generated the novel peptide ABB28 by fusing an albumin-binding domain (ABD) to the N-terminus of BB28. ABB28 exhibited much higher binding affinity for albumin than BB28, and this modification extended the peptide half-life from several minutes to 2 h. Optical imaging revealed that ABB28 accumulated in xenografted tumors within 1 h post-injection and persisted at an evident level for up to 24 h. ABB28 exerted stronger tumor-suppressive effects than BB28. Significant differences in the tumor volumes ($P < 0.001$) and the tumor weights ($P = 0.002$) were observed between ABB28- and BB28-treated mice. Moreover, ABB28 exhibited tumor suppression comparable to that of PEGylated 5K-BB28 *in vivo*. These results suggest that half-life extension *via* ABD fusion represents a useful strategy for optimizing bombesin-directed pharmaceuticals for cancer-targeted therapy.

1. Introduction

The recent cancer statistics in the United States have revealed that the cancer mortality rate has shown a declining trend during the past two decades (Siegel et al., 2015). However, cancer remains a major life-threatening disease worldwide, accounting for 8.2 million deaths in 2012 (de Martel et al., 2012). Based on a better understanding of the molecular mechanism underlying tumor progression, tumor-specific ligands such as antibodies, tumor-homing peptides and aptamers have been developed, thereby improving drug efficacy while reducing adverse off-target effects (Khawar et al., 2015; Svensen et al., 2012). Similar to well-known antibodies, tumor-homing peptides can specifically recognize and bind to their receptors, which are often overexpressed on tumor cell and tumor vasculature. As a drug delivery vehicle, tumor-homing peptides can be covalently conjugated to drugs or coadministered with drugs as separate entities without conjugation (Alberici et al., 2013; Roth et al., 2012). As a result, both methods selectively promote drug accumulation in tumors, even increasing their penetration deep into extravascular tumor tissues. Therefore, tumor-homing peptides are extensively used to deliver various cargoes such as radiolabeled probes, chemotherapeutic agents, proteins and nanoparticles for tumor imaging and treatment (Morgat et al., 2014; Shin et al., 2014; Zhong et al., 2014).

Bombesin is a tumor-homing peptide isolated from the skin of the frog *Bombina*. The C-terminal sequence of bombesin, which shares seven conserved amino acids with human gastrin-releasing peptide and neuromedin B, is principally responsible for its binding to bombesin receptors. Thus, bombesin can bind to three human receptors: gastrin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR) and bombesin receptor subtype 3 (BRS-3) (Jensen et al., 2008). Among them, GRPR is most frequently overexpressed in various cancer cell types, including some of the deadliest cancer types, such as lung cancer, breast cancer, colon cancer and prostate cancer (Mansi et al., 2013). To

Abbreviations: ABD, albumin-binding domain; B28, BMAP28 (amino acids 1–18); BB28, the conjugate of bombesin and B28; ABB28, the conjugate of ABD and BB28; HSA, human serum albumin; FcRn, the neonatal Fc receptor; GRPR, gastrin-releasing peptide receptor.

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exploit bombesin as a peptide drug carrier, in a previous study we constructed the anticancer peptide BB28 by fusing the mitochondria-disrupting peptide BMAP28 (amino acids 1–18, *i.e.*, B28) (Risso et al., 2002) to bombesin. The fusion peptide BB28 selectively induced cancer cell apoptosis *in vitro* and exhibited promising antitumor effects in mice bearing xenografted tumors (Cai et al., 2010).

It is well known that peptides often exhibit a short circulating half-life *in vivo* due to endogenous clearance mechanisms such as proteolytic degradation and renal clearance. This property typically results in the low therapeutic efficacy of such peptides in addition to potential side effects due to excessive and frequent administration. To improve the pharmacokinetics of these compounds, several approaches have been applied for retaining an effective blood concentration of the peptide. These methods include PEGylation, hyperglycosylation, PASylation, incorporation into various delivery systems, and fusion to IgG Fc, albumin and other serum proteins possessing a long half-life (Ibraheem et al., 2014; Kontermann, 2011; Morath et al., 2015). For instance, the PEGylation of peptide or protein can increase its hydrodynamic volume and thus reduce its renal clearance (Pasut and Veronese, 2012). In addition to PEGylation, Fc and albumin are broadly used for half-life extension *via* neonatal Fc receptor (FcRn)-mediated recycling. When Fc and albumin are internalized *via* endothelial cell pinocytosis, they can interact with FcRn in a pH-dependent manner. Following internalization, Fc or albumin binds to FcRn in acidified endosomes, subsequently returns to the cell surface *via* exocytosis, and finally dissociates from FcRn into the blood due to its physiological pH (Andersen et al., 2011).

In particular, albumin is a remarkably promising drug carrier because it is by far the most abundant protein in blood. Therapeutic molecules can be either covalently conjugated to exogenous albumin or non-covalently bound to endogenous albumin. In this fashion, albumin-based drugs have been successfully translated into commercial products, including antidiabetic drugs (Levemir®, Tresiba®, and Victoza®), an anticancer product (Abraxane®) and radiolabeled diagnostic molecules (Nanocoll® and Albures®) (Kratz, 2014; Sleep et al., 2013). Notably, an albumin-binding domain (ABD) is typically a small amino acid motif that can non-covalently associate with albumin. Therefore, the fusion of ABD to therapeutic proteins or peptides might prolong their circulating half-life by inducing their binding to endogenous albumin *in vivo* (Sleep et al., 2013).

The heptapeptide WQRPSSW is an ABD that was identified by phage display screening. This 7-mer peptide binds to human serum albumin (HSA), mouse serum albumin (MSA) and rabbit serum albumin (RSA) with comparable affinity. Upon the fusion of the ABD WQRPSSW to an insulinotropic peptide, the fusion peptide exhibits dramatically delayed clearance in animal models and sustained control of the blood glucose level (Ma et al., 2015). The fusion of this ABD to BB28 might enhance its *in vivo* antitumor effects by prolonging its serum half-life. Here, we designed a chimeric peptide, referred to as ABB28, by fusing the albumin-binding peptide WQRPSSW to the N-terminus of BB28. The serum half-lives and antitumor effects of ABB28 and BB28 were compared. The results demonstrated that fusion to this ABD significantly extended the half-life and thus the enhanced antitumor effects of BB28.

2. Materials and methods

2.1. Materials

Methoxy-polyethylene glycol-butyraldehyde (mPEG-butyr-ALD; >95% purity, molecular weight, 5 kDa, 10 kDa) was purchased from Kaizheng Biotech Development Co., Ltd. (Beijing, China). The LIVE/DEAD BacLight Bacterial Viability Kit containing SYTO 9 and propidium iodide (PI) was purchased from Life Technologies (Thermo Fisher Scientific, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan). Horseradish peroxidase (HRP)-labeled streptavidin was purchased from BioLegend (USA). Near-infrared (NIR) CF750 succinimidyl ester, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and other reagents used in this study were obtained from Sigma–Aldrich (USA).

2.2. Cell culture

Human colon adenocarcinoma cells (COLO 205) and human bone marrow chronic myelogenous leukemia cells (K562) were purchased from the American Type Culture Collection. Human hepatocellular carcinoma cells (SMMC-7721) were obtained from the Cell Bank of the Chinese Academy of Science. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Peptide design and synthesis

BB28 (GGLRSLGRKILRAWKKYGQRLGNQWAVGHLM) is a chimeric peptide designed by conjugating a truncated antimicrobial peptide, B28 (GGLRSLGRKILRAWKKYG), to the N-terminus of the tumor-homing peptide bombesin (QRLGNQWAVGHLM). Our previous study showed that BB28 selectively induced tumor cell apoptosis *in vitro* and *in vivo*. To improve the pharmacokinetics of BB28, a 7-mer peptide albumin-binding domain (ABD, WQRPSSW) displaying high affinity for albumin was coupled to the N-terminus of BB28 to generate the novel chimeric peptide ABB28 (WQRPSSWGGLRSLGRKILRAWKKYGQRLGNQWAVGHLM-amide). CABB28 (WQRPSSWGQRL GNQWAVGHLMGGLRSLGRKILRAWKKY-amide) was also generated by flanking bombesin with ABD and B28, which resulted in the blockade of the receptor-binding C-terminus of bombesin. The predicted secondary structure of BB28 and ABB28 was obtained from a series of online programs on the Pole Bio-Informatique Lyonnais-Network Protein Sequence Analysis Web Server (http://npsa-pbil.ibcp.fr) according to the online guide. The three-dimensional (3D) structure of these peptides was predicted and constructed using the online tool QUARK (http://zhanglab.ccmb.med.umich.edu/QUARK/) as described by Xu and Zhang (2013). All peptides were synthesized using standard solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry techniques and were custom-made by Genescript Inc. (Nanjing, China). Biotinylation was performed by conjugating biotin to the N-terminus of the peptide during peptide synthesis. The peptides were purified (>90% purity) *via* reverse-phase high performance liquid chromatography (RP-HPLC) and were identified *via* matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Peptides were aliquoted into 1 mg samples, subjected to amino acid analysis, lyophilized and stored at −20 °C for further use.

2.4. Circular dichroism analysis of peptides

The peptide was dissolved either in phosphate buffer (PBS, 137 mM NaCl, 2.68 mM KCl, 8.09 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4) or in egg-derived L-α-phosphatidylcholine (EPC) solution to a final concentration from 0.1–0.5 mg/mL. The EPC lipid solution was prepared in chloroform as described by Haney et al. (2012) and resuspended in PBS before use. The circular dichroism (CD) spectra of the peptides were recorded at 25 °C from 190 nm to 260 nm with a bandwidth of 1 nm. Each sample