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Photosensitizer-conjugated tryptophan-containing peptide ligands as new dual-targeted theranostics for cancers

Jisu Kim[a], Jihyun Chae[b], Jun Soo Kim[b], Sung-Ho Goh[c], Yongdoo Choi, Ph.D.[a,c,*]

[a] Molecular Imaging & Therapy Branch, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10408, Republic of Korea
[b] Department of Chemistry & Nanoscience, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 03760, Republic of Korea
[c] Precision Medicine Branch, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10408, Republic of Korea

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ABSTRACT

Here we report that new dual-targeted theranostic anti-cancer agents can be produced by simple conjugation of photosensitizers with tryptophan-containing peptide ligands *via* cyclic disulfide linkages. In the proof-of-concept study, photosensitizers conjugated with EGFR-targeting peptide GE11 (C-EGFR) were in close proximity with tryptophan residues in the conjugate, resulting in quenching of its fluorescence and singlet oxygen generation. C-EGFR specifically binds to target receptors on the cancer cell surface, after which it is internalized *via* receptor-mediated endocytosis. Intracellular cleavage of cyclic disulfide bonds allows separation of the photosensitizers from the tryptophan residue, after which they emit near-infrared (NIR) fluorescence and produce a phototoxic effect in the target cells. This strategy enabled us to accomplish simultaneous real-time NIR fluorescence imaging of EGFR-overexpressing cancer cells with high contrast and selective photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) has been used successfully to treat various cancers and non-cancerous indications (Dolmans et al., 2003; Reinhard et al., 2015). However, the limited tumor selectivity and poor pharmacokinetics of PDT agents have been major hurdles in their clinical application as theranostic agents. In particular, prolonged skin photo-sensitivity is caused by accumulation of PDT agents in normal tissues such as the eye and skin; therefore, patients are recommended to avoid bright indoor light and sunlight for several weeks after PDT. Although targeted PDT agents have been developed as a strategy to solve these problems (Bugaj, 2011; Ongarora et al., 2012; Sharman et al., 2004; Solban et al., 2006), such "always on" targeted PDT agents have the fundamental disadvantage of emitting fluorescence regardless of their proximity to or binding status with target cells and thus produce unwanted phototoxicity in normal tissues. Therefore, development of PDT agents with improved target specificity to avoid off-target sensitization has proven challenging.

There has been significant effort to use peptides as a cancer-targeting ligands because, compared with antibodies and aptamers, they have several advantages, including low immunogenicity, low cost, good diffusibility, and simple methods of synthesis and conjugation with drugs, fluorophores, and photosensitizers (PS) (Cheetham et al., 2016; Lovell et al., 2010; Shadidi and Sioud, 2003; Sharman et al., 2004; Zhang et al., 2012). Currently, the only method of preparing peptide conjugates with controllable optical properties is the introduction of quenchers (*e.g.*, black hole quenchers) to photosensitizer-peptide conjugates (Lovell et al., 2010), although this method can reduce the binding specificity and water solubility of the conjugates, in addition to complicating the synthesis process. Therefore, an ideal method of preparing peptide-based activatable theranostic agents would not require the introduction of additional quenchers. Recently, we found that photosensitizers conjugated with tryptophan *via* short linkers are quenched, rendering them non-fluorescent and non-phototoxic (data now shown here). In quenching experiments performed with second-generation photosensitizers and tryptophan (Trp), we observed that the fluorescence of the photosensitizers was significantly decreased as the concentration of Trp was increased, indicating efficient quenching of the photosensitizers by photo-induced electron transfer (PET) (*e.g.*, Fig. S1). Inspired by these observations, we developed dual-targeted and activatable theranostic agents by conjugating photosensitizers

* Corresponding author at: Molecular Imaging & Therapy Branch, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, 10408, Republic of Korea.
E-mail addresses: ydchoi@ncc.re.kr, labjjang@daum.net (Y. Choi).

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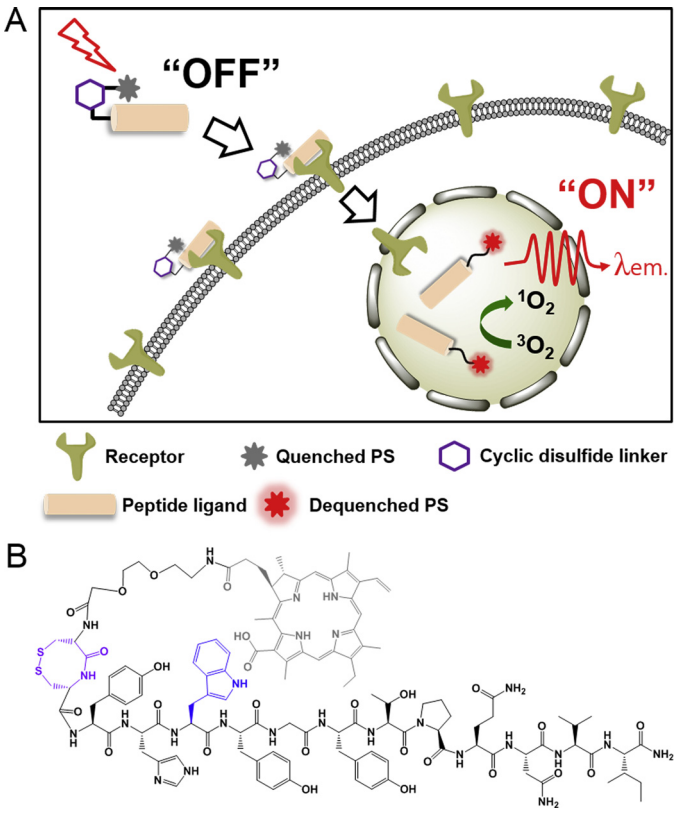

Fig. 1. (a) Schematic diagram of a photosensitizer-peptide conjugate with a cyclic disulfide linker for dual-targeted NIR fluorescence imaging and photodynamic therapy. (b) Chemical structure of C-EGFR.

with peptide ligands *via* cyclic disulfide linkers, which become cleavable inside cancer cells (Fig. 1A).

We hypothesized that, in the native state of the photosensitizer-peptide conjugate, the photosensitizer is within close proximity to a Trp residue in the peptide ligand (Doose et al., 2009), quenching NIR fluorescence emission by the photosensitizer and singlet oxygen generation (SOG) as a result of PET interaction. When the conjugate specifically binds with target receptors (1st target) on the cancer cell surface and is internalized into the cells *via* receptor-mediated endocytosis, cyclic disulfide bonds are cleaved by intracellular reductive agents at high concentrations (2nd target). As a result of this cleavage, the photosensitizer becomes more distant from the Trp residue, resulting in dequenching of NIR fluorescence and SOG production inside target cancer cells. Photosensitizer-peptide conjugates should enable simultaneous selective PDT and NIR fluorescence imaging of cancer cells with high specificity and contrast, with minor phototoxicity to normal cells.

As a proof-of-concept of this approach, a second generation photosensitizer, chlorin e4 (Ce4), was conjugated with epidermal growth factor receptor (EGFR)-targeting peptide GE11 *via* a cyclic disulfide linker to produce a Ce4-GE11 conjugate (C-EGFR) (Fig. 1B). EGFR activates downstream signaling pathways that are essential for cell proliferation, survival, adhesion, migration, and differentiation (Yewale et al., 2013). EGFR overexpression is frequently found in a variety of human cancers of epithelial origin, including breast, colorectal, gastric, non-small cell lung, and ovarian cancers, which are associated with poor clinical outcomes (Yewale et al., 2013). Therefore, EGFR has been considered as an important tumor-specific target with the potential to be utilized for imaging and therapy. A 12-residue linear peptide, GE11 (YHWYGYTPQNVI), has high binding specificity to EGFR and can be used for target-specific delivery of drugs and imaging agents *in vitro* and *in vivo* (Agnes et al., 2012; Li et al., 2005; Tang et al., 2014). Glutathione (GSH) is a protein disulfide bond reductase that is involved in many cellular processes (Traverso et al., 2013). Notably, cancer cells often have GSH concentrations much higher than those of normal cells, rendering neoplasms resistant to chemotherapy (Estrela et al., 2006). In addition, the intracellular concentration of GSH (approximately 2–10 mM) is much greater than its extracellular concentration (approximately 2 μM) (Estrela et al., 2006; Kim et al., 2010).

2. Materials and methods

2.1. Materials

Ce4 was obtained from Frontier Scientific, Inc. (Logan, UT, USA). Dithiothreitol (DTT) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). SOSG reagent was obtained from Molecular Probes® (Eugene, OR, USA). Anti-EGFR antibodies were obtained from Santa Cruz Biotechnology, Inc. (sc-120, Santa Cruz, CA, USA). Bovine serum albumin (BSA) was obtained from Merck/Millipore (Darmstadt, Germany). Tween 20 was obtained from Biosesang (Seongnam, Korea). CCK-8 cell viability assay kits were obtained from Dojindo (Kumamoto, Japan).

2.2. Methods

2.2.1. C-EGFR synthesis

C-EGFR was synthesized by 9-fluorenylmethoxycarbonyl solid phase peptide synthesis (Fmoc-SPPS) using ASP48S (Peptron Inc. Daejeon, Korea). 8 eq. Fmoc- amino acid and 8 eq. 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/8 eq. N-Hydroxybenzotriazole (HOBt)/16 eq. 4-Methylmorpholine (NMM) in dimethylformamide (DMF) were added to H-Ile-2-chloro-Trityl Resin (Anaspec, CA, USA). The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20% piperidine in DMF was added to the reaction mixture and reacted for 5 min. The previous step was repeated once more before the product was washed with DMF, MeOH, and DMF. Peptide backbone attached resin (H-miniPEG2-Cys(Trt)-Cys(Trt)-Tyr(t-Bu)-His(Trt)-Trp(Boc)-Tyr(t-Bu)-Gly-Tyr(t-Bu)-Thr(t-Bu)-Pro-Gln(Trt)-Asn(Trt)-Val-Ile-2-chloro-Trityl Resin) was prepared by repeating the above procedure. 4 eq. Ce4 and 4 eq. HBTU/4 eq. HOBt/8 eq. NMM in dimethylsulfoxide (DMSO) was added to the peptide backbone attached resin. The mixture was reacted for 12 h, suctioned, and washed with DMF, MeOH, and DMF. Prepared Ce4-peptide conjugate was cleaved from the resin by treatment with trifluoroacetic acid (TFA)/EDT/thioanisole/triisopropylsilane (TIS)/H_2O (90/2.5/2.5/2.5/2.5). The reaction mixture was added to 10-fold cold diethyl ether for precipitation and centrifuged at 3000 rpm for 10 min. Supernatant was removed and the procedure was repeated 2 more times. Collected peptides were purified by prep-LC and lyophilized. For cyclic disulfide bond formation, peptide dissolved in water containing 0.1% ammonium acetate was stirred vigorously for 24 h. Synthesized C-EGFRs were purified by reversed-phase high performance liquid chromatography (HPLC) using a Vydac C4, 5u, 300 A column (4.6 × 50 mm). Elution was carried out with a water-acetonitrile linear gradient (30–60% (v/v) of acetonitrile) containing 0.1% (v/v) TFA.

2.2.2. Characterization of C-EGFR

The molecular weight of the synthesized C-EGFR was measured by LC/MSD (Agilent Hewlett Packard 1100 series, California, USA). The UV–vis spectra of free Ce4 and C-EGFR were measured with a