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Tumor targeted delivery of octreotide-periplogenin conjugate: Synthesis, *in vitro* and *in vivo* evaluation

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ABSTRACT

Periplogenin (PPG), a cardiac glycoside prepared from *Cortex periplocae*, with similar structure to bufalin, has been found to induce apoptosis in many tumor cells. However, lots of cardiac glycosides possessing strong antitumor activity *in vitro* have still not passed phase I clinical trials, mostly due to poor tumor selectivity and systemic toxicity. To overcome this drawback, we designed octreotide-periplogenin (OCT-PPG) conjugate by coupling PPG–succinate to the amino-terminal end of octreotide. In comparison with free PPG, the conjugate exhibited significantly stronger cytotoxicity on HepG2 cells (SSTRs over-expression) but much less toxicity in L-02 cells. After intravenous injection of OCT-PPG conjugate into H_{22} tumor-bearing mice, its total accumulation in tumor was 2.3 fold higher than that of free PPG, but was 0.71- and 0.84-fold lower in heart and liver, respectively, suggesting somatostatin-mediated target delivery of PPG into the tumor tissue and reduced distribution in heart and liver. *In vivo* studies using H_{22} tumor model in mice confirmed the remarkable therapeutic effect of this conjugate. These results suggested that OCT-PPG conjugate could provide a new approach for clinical application of cardiac glycosides and as a targeting agent for cancer therapy.

1. Introduction

The use of cardiac glycoside containing plants for medicinal purposes was first reported in ancient literature more than 1500 years ago. Cardiac glycosides are a class of natural products that are used clinically to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias (Newman et al., 2008; Spera et al., 2007). Cardiac glycosides, however, have a narrow therapeutic index, limiting their wider applications to the treatment of other diseases, such as cancer. Recently, Scandinavian oncologists suggested that the apoptosis produced by digitalis in human tumor cells could be achieved at concentrations with no toxicity in humans, and therefore, this agent might be useful for treatment of cancer (Haux, 1999). However, lots of cardiac glycosides with strong antitumor activity *in vitro* have still not passed phase I clinical trials, mostly due to lack of tumor selectivity and severe adverse effects (Tian et al., 2014).

Cortex periplocae (CP) is the dry root of the traditional Chinese herb *Periploca sepium bunge*, referred to as Xiangjiapi in Chinese. It is a traditional type of medicine commonly used for a variety of clinical effects including anti-inflammation, bones and muscles enhancement and nervous system stimulation (Zhao et al., 2010). Itokawa et al. (1987) first isolated periplogenin (PPG) from chloroform extract of CP which markedly inhibited the growth of ascite cancer S_{180} cells. It was reported that periplogenin strongly inhibited proliferation of PC3, U937, HCT-8, Bel-7402, BGC823, A549, and A2780 cell lines *in vitro* with IC_{50} values of 0.66–3.16 μM (Li et al., 2012). However, periplogenin, which belongs to cardiac glycosides, also has serious side effects such as cardiotoxicity, neurotoxicity and hepatotoxicity (Mijatovic et al., 2007). The future goal to consider cardiac glycosides as "lead compounds" and to obtain chemically modified non-cardioactive derivatives with enhanced anticancer potency has been proposed (Langenhan et al., 2005). Recently, targeted chemotherapy has become a novel approach to the treatment of cancers due to improved efficacy and reduced toxicity.

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Somatostatin is a neuropeptide that exerts a powerful inhibitory effect against various cellular functions including secretions, motility, and proliferation (Huo et al., 2012). Its actions are mediated by five specific somatostatin receptors subtypes (SSTR-1-5) which belong to the super-family of G-protein coupled receptors. Most neuroendocrine tumors and their metastases express SSTRs to a much greater extent than normal tissues (Huang et al., 2000). The majority of tumors express SSTR-2, followed by SSTR-1, SSTR-5, SSTR-3 and SSTR-4 (Klagge, 2010). However, the clinical usefulness of naturally occurring somatostatins is limited by its very short half-life in circulation (1–3 min). Therefore, synthetic derivatives including octreotide and lanreotide, have been created with improved metabolic stability and increased affinity to SSTRs (Sclafani et al., 2011).

Octreotide (OCT), one of the most extensively studied somatostatin analogues, is selectively binding to SSTR-2, SSTR-3 and SSTR-5, but has no binding affinity for receptors SSTR-1 and SSTR-4. Since octreotide is highly resistant to enzymatic degradation and has a prolonged plasma half-life of about 100 min in humans, it has been applied clinically to prevent carcinoid crisis (Marsh et al., 1987) and for scintigraphic visualization of tumors containing a high density of SSTRs (Breeman et al., 2009). Following its successful application on radio-oncology, OCT has been found to enhance the delivery of drug to tumor cells with reduced toxicity by chemically conjugating with the anti-tumor drugs (Niu et al., 2012; Park and Na, 2008; Shen et al., 2008).

For the first time, this study was able to design cardiac glycosides conjugated with polypeptide. Octreotide conjugated with periplogenin was synthesized by coupling PPG–succinate to the amino-terminal end of octreotide. Using HepG2 and L-02 cells as models, cytotoxicity comparison between periplogenin and octreotide-periplogenin conjugate was made. Our aim was to investigate the efficiency of octreotide-periplogenin conjugate on improved anti-cancer efficacy and reduced toxicity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

OCT was purchased from HuaJin Pharmaceutical Co., Ltd. (HangZhou, China). Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Aladdin Industrial Corporation (ShangHai, China). Naringenin was obtained from J&K scientific Co., Ltd., (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) and trypsin were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Company (Grand Island, NY). Chromatographically pure methanol and acetonitrile were obtained from Hanbon Technology Co., Ltd. (Jiangsu, China).

2.2. Preparation of periplogenin from C. periplocae

The dry *C. Periplocae* (400 g) were powdered and extracted twice under reflux with water for 3 h. The combined solution was concentrated in *vacuo* to give a dark brown residue. Then 85% ethanol was added to dissolve residue and to remove the insoluble parts. The ethanol solution was further concentrated *in vacuo* to obtain a dark brown residue which was then suspended in water and extracted thrice with diethyl ether, ethyl acetate and n-butyl alcohol, successively. The n-butyl alcohol extraction (15.6 g) was hydrolyzed with 0.05 N H_2SO_4 in 50% MeOH(1000 mL) at 90 °C for 1 h. MeOH was evaporated off *in vacuo* at room temperature. The aqueous residue was extracted with CH_2Cl_2(×3) and CH_2Cl_2 layer was washed with water. After removal of the solvent, the residue was purified by means of C_8 column chromatography eluting with MeOH/H_2O (40:60–90:10 v/v gradient) to yield compound 1 (310 mg). Compound 1 was identified as periplogenin by spectroscopic analyses and compared with published data.

2.3. Synthesis and purification of suc-periplogenin (PPS)

Periplogenin (20 mg, 0.051 mmol) and succinic anhydride (30 mg, 0.3 mmol) were dissolved in 3 mL anhydrous methylene chloride (CH_2Cl_2), followed by the addition of appropriate amount of triethylamine and 4-dimethyl-aminopyridine (DMAP). The reaction mixture was stirred at room temperature for 24 h under a nitrogen purging. The reaction was monitored by TLC with the solvent system of dichloromethane-methanol (10:1, v/v), and the spots were detected by charring with 5% phosphomolybdic acid-ethanol solution followed by heating. After drying and concentrating under vacuum, the obtained crude PPS was further purified by C_8 column chromatography eluting with MeOH/0.05% Formic acid-H_2O (50:60–60:40 v/v gradient), to afford 12.1 mg (48.41% yield) PPS as a pale white solid.

2.4. Synthesis of NHS activated PPG (NHS-PPG)

PPS (10 mg, 0.0204 mmol), DCC (7.79 mg, 0.0408 mmol, 2 × excess), NHS (5.0 mg, 0.0408 mmol, 2 × excess), 2 mL of dichloromethane and 1 mL of Tetrahydrofuran was added to a round-bottom flask equipped a magnetic stirring bar, attached to a nitrogen line and a bubbler. The reaction was maintained for 24 h at room temperature. The reaction mixture was then filtered, concentrated under reduced pressure, and was subjected to C_8 column chromatography eluting with 0.05% TFA-acetonitrile-water (40:60 to 70:30 v/v gradient) to obtain NHS-PPG (9 mg).

2.5. Synthesis of OCT(Phe)-S-PPG, OCT(Lys)-S-PPG and OCT-2S-2PPG

OCT (12.1 mg, 0.0119 mmol, 2 × excess) was added to a solution of NHS-PPG (3.5 mg, 0.0059 mmol) in 50% acetonitrile-0.1 M HEPES, adjusting to pH 8.4 with N-methylmorpholine. The reaction was maintained for 24 h at room temperature under moderate stirring and monitored by LC-ESI-MS. The purification of the crude product was carried out on a semipreparative reversed-phase HPLC system using a Shimadzu instrument, equipped with Inertsil ODS-SP C_{18} column (5 μm, 10 × 250 mm), an LC-20 AT pump coupled to SPD-M10AVP diode array detector, column compartment and SPD-20A UV/Vis Detector (Shimadzu, Kyoto, Japan). The flow was 4 mL/min, with the mobile phase starting from 30% solvent A (0.05% TFA in water) and 70% solvent B (0.0.5% TFA in acetonitrile) at 0.01 min to 80% solvent A and 20% solvent B at 90 min. The fractions containing the OCT conjugate were collected and lyophilized.

2.6. Characterization of OCT(Phe)-S-PPG, OCT(Lys)-S-PPG and OCT-2S-2PPG

The characterization of the product was confirmed by LC-MS, MS and ^{1}H NMR. The ESI-MS data was acquired in a positive or negative ion mode using a Thermo Finnigan LXQ Dec XP and Ion Trap Mass Spectrometer instrument (Thermo Finningan; San Jose, CA). The instrument was equipped with an electrospray ion source (ESI) and an Xcalibur® system manager data acquisition software. Sample solutions (2 μL, 100 μg/mL) were injected into the ESI source using a syringe pump with a mass scan (100–2000 *m*/*z*). The conditions of the ion trap mass spectrometer were as follows: spray voltage, 4.5 kV; source current, 80 μA; capillary temperature, 325 °C; capillary voltage, 30 V; tube lens offset, 120 V; multi-pole