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Enhanced antitumor efficacy of cisplatin by tirapazamine–transferrin conjugate

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ABSTRACT

Combination of tirapazamine (TPZ) with cisplatin has been studied extensively in clinical trial for tumor therapy. However, in phase III clinical trial, the combination therapy did not show overall survival improvement in patients. To decrease the side effects and increase the efficacy of the combination therapy, TPZ was conjugated with transferrin (Tf-G-TPZ) for targeted delivery and co-administered with cisplatin. In vitro toxicity study showed that the combination of Tf-G-TPZ with cisplatin induced substantially higher cytotoxicity of tumor cells than the combination of TPZ and cisplatin. After Tf-G-TPZ was intravenously injected into tumor-bearing mice, its total accumulation in tumor was 2.3 fold higher than that of the unmodified TPZ, suggesting transferrin-mediated target delivery of TPZ into the tumor tissue. With the increased accumulation of Tf-G-TPZ in tumor, the synergistic anti-tumor effects of Tf-G-TPZ and cisplatin were also enhanced as showed by the 53% tumor inhibition rate. Meanwhile, the side effects such as body weight lost were not significantly increased. Therefore, Tf-G-TPZ holds great promise to a better substitute for TPZ in the combination therapy with cisplatin.

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1. Introduction

Cisplatin is one of the most widely used chemical compounds in antitumor chemotherapy (Fruscio et al., 2011; Yamasaki et al., 2011; Ying et al., 2011). It was first synthesized by Mickäel Peyrone in 1844, however its antitumor activity was not discovered until 1960s (Rosenberg et al., 1965). In 1971, cisplatin was introduced into clinical trials and approved by FDA in 1979 for the anti-cancer therapies of ovarian, testis and head and neck cancers (Todd and Lippard, 2009). When exposed to cancer cells, the chloride ligands within cisplatin are slowly displaced by water, allowing the platinum atom to bind to the intracellular DNA bases resulting in the formation of several types of chemical bonds. The most abundant bonds are the intrastrand crosslinks between two adjacent DNA bases, which induce cell apoptosis and death (Crul et al., 2002). In clinic, cisplatin has been intravenously administered for treatment of solid malignancies (Dickson et al., 2011; Moriyama-Gonda et al., 2008). However, cisplatin-resistant disease always occurs during the treatment. The resistance of tumors to cisplatin might be attributed to the reduction in cellular uptake, the increased detoxification and the increased DNA repair of the cancer cells (Koberle et al., 2010; Stordal and Davey, 2007).

Tirapazamine (TPZ), a lead bioreductive agent, has been shown to potentiate the antitumor efficacy of cisplatin (Marcu and Olver, 2006). Under hypoxic conditions, TPZ is bioreduced to a nitroxide-base free radical that abstracts hydrogen from DNA strands, thereby causing breaks in DNA (Reddy and Williamson, 2009). Incubation of cancer cells with TPZ under hypoxia conditions prior to cisplatin treatment inhibited the self-repair of the DNA damage induced by cisplatin, thus enhancing its antitumor efficacy (Kovacs et al., 1999). The combination therapy of TPZ with cisplatin has also been extensively evaluated in clinical trial for cancer treatment. However, it did not improve overall survival in patients with advanced head and neck cancer in phase III clinical trial (Rischin et al., 2010). Furthermore, incorporation of TPZ to cisplatin treatment leads to a significant increase in unwanted toxicities which causes severe adverse effects including nausea, vomiting, myalgia, diarrhea and muscle spasms (Adam et al., 2006; Covens et al., 2006). The unwanted toxicity might be produced by the enhanced non-specific DNA crosslink to both the normal and cancer cells when TPZ is co-administered with cisplatin. Therefore, targeted delivery and treatment can improve the therapeutic efficacy of the combination treatment by reducing the adverse effect and increasing the overall survival in patients.

Transferrin, an 80 kDa glycoprotein, has been widely applied as a targeted drug delivery carrier (Amet et al., 2010; Hong et al., 2010; Park, 2010). It naturally functions as iron supply to cells in the process of DNA synthesis (Jensen et al., 2011; Wang et al., 2011). Transferrin has high affinity for transferrin receptor which

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mediates its internalization into endosomal acidic compartments via endocytosis. The expression of transferrin receptors is particularly prevalent on rapidly dividing cells such as tumor cells, which makes transferrin an ideal drug carrier for targeted anti-tumor therapy. Transferrin as drug carrier has been studied extensively and transferrin conjugated with a modified diphtheria toxin, named TransMID, has been shown to be effective in treating patients suffering from inoperable, recurrent high grade gliomas (Rainov and Soling, 2006).

The main aim of this study is to evaluate whether human serum transferrin can mediate the targeted delivery of TPZ to tumor and achieve a higher anti-tumor efficacy when co-administered with cisplatin. Transferrin–tirapazamine (Tf-G-TPZ) conjugate was synthesized by traditional bifunctional crosslinking method. A full characterization demonstrated TPZ was successfully conjugated with transferrin as the ratio of 6:1. Via transferrin mediated delivery, the concentration of Tf-G-TPZ in tumor tissue could be significantly increased and hence the antitumor effect of Tf-G-TPZ was enhanced when co-administrated with cisplatin.

2. Material and methods

2.1. Material

Transferrin used in the synthesis process was purified in our lab. More details about the purification and characterization of transferrin can be found in our previous publication (Wu et al., 2008). Porcine serum was obtained from HyClone Bio-Engineering Co., Ltd. (Lanzhou, China). TPZ was obtained from Shunlong Chemical Co., Ltd. (Shangyu, China). Glutaric anhydride, NHS (hydroxysuccinimide) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) were purchased from Medpep Co., Ltd. (Shanghai, China). The BCA (bicinchoninic acid) protein assay kit was purchased from Kengentec Co., Ltd. (Nanjing, China). Cell culture media (DMEM, Dulbecco's modified Eagle's medium) was obtained from Invitrogen (CA, USA) and fetal calf serum (FCS) was purchased from National HyClone Bio-Engineering Co., Ltd. (Lanzhou, China). All culture flasks were obtained from Corning (USA).

2.2. Synthesis of Tf-G-TPZ

Tf-G-TPZ was prepared according to the method of Kato et al. (1983). Briefly, TPZ was first reacted with glutaric anhydride to form N-glutaryl-TPZ. Then 14.6 mg N-glutaryl-TPZ, 10 mg EDC and 6 mg NHS were dissolved in 2 ml DMF (N,N-dimethylformamide) and mixed well by a vertical mixer for 4 h. After 12 h incubation at 4 °C in the dark, the suspension was centrifuged and the supernatant was collected. Then 133 mg Tf dissolved in 5 ml carbonate buffer (0.05 M, pH 9.6) was added into the supernatant. A little amount of insoluble material was formed and removed by centrifugation, and the supernatant was concentrated to approximately 2.0 ml by centrifugation at 4 °C and 4000 rpm with CENTRIPREP-10-concentrators (Millipore, MA, USA) (Beyer et al., 1998). The concentrated sample was subjected to gel filtration on a Sephadex G-25 column (2.0 cm × 20 cm) with 0.02 M phosphate buffer to get rid of the unreacted TPZ derivative. The macromolecular fractions containing Tf-G-TPZ were collected, dialyzed against distilled water and lyophilized.

2.3. Characterization of Tf-G-TPZ

Total protein concentration of the purified Tf-G-TPZ was determined using a bicinchoninic acid (BCA) protein assay kit. A stock solution (C_{TPZ} = 300 μM) of Tf-G-TPZ was subsequently employed in all of the characteristic experiments. The characteristic absorption peak of conjugate was determined using a UV-visible spectrophotometer (SHIMADZU, Japan).

The purity of Tf-G-TPZ was determined at 254 nm with an analytical HPLC-Gel-Filtration column (Bio-Gel® SEC 50-XL, 300 mm × 7.8 mm) from Bio-Rad (mobile phase: 0.15 M NaCl, 0.01 M NaH_2PO_4, 5% CH_3CN, pH 7.0) (Kratz et al., 1998). The integrity and aggregation of Tf-G-TPZ were evaluated by SDS-PAGE, using 12% polyacrylamide gel with 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie Blue R-250 and destained in 45:10:45 (methanol:glacial acetic acid:water).

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry with time-of-fight (TOF) was performed using an ultraflex II TOF/TOF instrument (Bruker-Daltonics, Germany) equipped with a smart beam laser operating at 200 Hz. A saturated solution of 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile and 0.1% trifluoroacetic acid was prepared as a matrix solution. An aliquot (1 μl) of the sample solution was mixed with an equal aliquot of the matrix solution, and 1 μl of the matrix solution was spotted onto the target plate and evaporated under a gentle stream of warm air. Mass spectra were acquired in positive ion reflector mode using a reflectron voltage of 25000 V, accelerating voltage at 92.8%, lens voltage at 26% and pulsed ion extraction of 150 ns. External calibration was performed for molecular assignments using a standard of bovine serum albumin with $[M+]^+$ at 66.5 kDa.

2.4. Release profile of Tf-G-TPZ

Tf-G-TPZ was dissolved in 0.02 M phosphate buffer (pH 7.4 and 6.5) or 0.02 M acetate buffer (pH 5.2 and 4.0) both containing 0.15 M NaCl and 0.02% sodium azide in a total volume of 2 ml to give a final concentration of 5 mg/ml. Then the sample solution was put into a dialysis bag. Each bag was placed into capped, wide-mouth jars containing 50 ml of the appropriate release medium incubated at 37 °C. For each trial with Tf-G-TPZ, a corresponding trial with un-conjugated TPZ and a physical mixture of transferrin/TPZ were conducted as controls. After varying periods of incubation (0, 1, 2, 3, 4, 5, 7, 9, 11, 13, 24, 28, 32, 36, 48 h), 1 ml of release medium was withdrawn and replaced with fresh medium to maintain a constant volume (Ofner et al., 2006). Samples removed at intervals were immediately frozen. All samples were collected and analyzed by HPLC. The HPLC analysis for TPZ was performed on a Symmetry® C18 column (5 μm, 250 mm × 4.6 mm) and recorded at 254 nm. The mobile phase was 14% (v/v) acetonitrile containing 0.1% acetic acid.

2.5. Cell culture

The SW1116 (Human Colorectal adenocarcinoma cells) were purchased from Kengentec Co., Ltd. (Nanjing, China). Cells were maintained as monolayer cultures in RPMI 1640 culture medium with 10% (v/v) heat-inactivated FCS (Fetal calf serum), 2 mM glutamine, and 0.1 mg/ml of penicillin/streptomycin. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Medium was routinely changed every 3 days.

2.6. In vitro cytotoxicity

100 μl cell suspensions were seeded in 96-well flat-bottomed microtiter plate at a density of 1×10^5 cells/ml. After pre-incubating for 12 h, medium was removed by aspiration and replaced by TPZ (50–400 μM), Tf-G-TPZ (12.5–100 μM) or Cisplatin (20 μM). After 48 h culture at 37 °C, cells were stained by 10 μl MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS) and re-incubated for an additional 3 h. Subsequently, the

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