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Improvement of the *in vitro* safety profile and cytoprotective efficacy of amifostine against chemotherapy by PEGylation strategy

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ABSTRACT

Amifostine, an organic thiophosphate prodrug, has been clinically utilized for selective protection of normal tissues with high expression of alkaline phosphatase from oxidative damage elicited by chemotherapy or radiotherapy. However, the patients receiving amifostine suffer from severe dose-dependent adverse effects. Strategies for improvement of the protective efficacy and toxicity profile of amifostine are urgently required. Here we constructed a PEGylated amifostine (PEG-amifostine) through conjugation of amifostine to the 4-arm PEG (5000 Da) by a mild one-step reaction. The relatively large PEG-amifostine molecules clustered into spherical nanoparticles, resulting in distinct hydrolysis properties, cell uptake profile and antioxidative activity compared with the free small molecules. PEGylation prolonged the hydrolysis time of amifostine, providing sustained transformation to its functional metabolites. PEG-amifostine could be internalized into cells and translocated to acidic organelles in a time-dependent manner. The intrinsic cytotoxicity of amifostine, which is related to the reductive reactivity of its metabolites and their ability to diffuse readily, was attenuated after PEGylation. This modification impeded the interaction between free sulfhydryls and functional biomolecules, providing PEG-amifostine with an improved safety profile *in vitro*. Moreover, PEG-amifostine showed higher efficiency in the elimination of reactive oxygen species and prevention of cisplatin-induced cytotoxicity compared with free amifostine. Overall, our study for the first time developed a PEGylated form of amifostine which significantly improved the efficacy and decreased the adverse effects of this antioxidant *in vitro* with great promise for clinical translation. *In vivo* study is urgently needed to confirm and redeem the cytoprotective effects of the PEG-amifostine in chemotherapy.

1. Introduction

Approved by the U.S. Food and Drug Administration (FDA), amifostine (trade name: Ethyol) has been clinically utilized for specific protection of normal tissues with high expression of alkaline phosphatase from oxidative damages induced by radiotherapy or chemotherapeutic drugs including alkylating agents and platinum-containing agents [1–3]. Amifostine, an organic thiophosphate prodrug, can be dephosphorylated quickly by alkaline phosphatase for conversion into its active metabolite with sulfhydryls which can scavenge oxygen-derived free radicals [4–7] and prevent DNA damage [8,9]. Preclinical and clinical investigations have suggested that the cytoprotective selectivity of amifostine is mostly caused by the variations in alkaline phosphatase expression and extracellular acidity between normal tissues and tumors. For a majority of neoplastic tissues, hypoxia, weakly-acidic interstitial pH, and reduced expression of alkaline phosphatase in the plasma membrane of tumor cells all limit generation of the active metabolite, and therefore, amifostine is typically incompetent to protect these tumors from oxidative damages. In contrast, amifostine can be efficiently hydrolyzed into its active metabolite in most normal tissues with high expression of alkaline phosphatase and physiological environment, leading to selective protection of these normal tissues from the cytotoxicity of radiotherapy and chemotherapy [4,10,11].

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In clinical practice, amifostine is intravenously administered at the dosage of 740 mg/m^2 which usually reaches the saturate drug concentration in blood [3,12]. Since the active metabolite of amifostine shows very limited stability *in vivo* due to the rapid formation of disulfide from free sulfhydryls, such a high dosage is required to achieve desirable protective effects. However, the extremely high drug concentration in blood results in inevitable adverse effects such as hypotension, nausea, and vomiting, which has significantly restricted the extensive application of amifostine [12]. Strategies that can ameliorate the efficacy and safety of amifostine are required for improvement of the therapeutic outcome.

PEGylation, covalently conjugating drug molecules with polyethylene glycol (PEG) chains, has shown great potential in improving the pharmacokinetics, pharmacodynamics and safety profiles of parent drugs [13]. Since PEG is commercially available and has been approved by FDA for clinical use [14], PEGylation has been widely applied for the development of therapeutic agents including protein and peptide drugs [15], small molecule drugs [16], and drug delivery systems [17]. PEGylation has the capability to change the unfavorable properties of drugs in solubility, biodistribution, immunogenicity, enzymatic degradation, renal filtration and reticuloendothelial system phagocytosis [13]. Therefore, the circulatory half-life and bioavailability of drugs can be elevated, while drug toxicity can be reduced. This prompted us to consider whether PEGylation of amifostine can improve drug stability and efficacy, thereby allowing low injection dosage for a favorable safety profile. So far, modification of amifostine by PEG has never been reported in the literature.

Considering the loading efficiency of small molecules to PEG polymers [18,19], we chose the 4-arm PEG with functional terminal for conjugation to amifostine. The physiochemical properties, capacity of hydrolysis by alkaline phosphatase, stability, *in vitro* toxicity and biological activities of PEGylated amifostine (PEG-amifostine) were evaluated. This study for the first time developed a PEGylated form of amifostine and demonstrated that PEG-amifostine had greater potency in antioxidant activity and safety profile compared with free amifostine *in vitro*.

2. Materials and methods

2.1. Reagents and animals

Amifostine was a generous gift from Merro Pharmaceuticals (Dalian, China). Four-arm poly (ethylene glycol) succinimidyl carboxy methyl ester (4-arm-PEG-SCM; molecular weight, 5000 Da) was supplied by JenKem technology (Beijing, China). Alkaline phosphatase was obtained from Takara (Dalian, China). 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Solarbio (Beijing, China). Aminofluorescein was from Acros Organics (Morris Plains, NJ, USA). LysoTracker Red DND-99 was supplied by Invitrogen (Carlsbad, CA, USA). Collagenase IV, DNase and H_2-DCFDA probe was obtained from Sigma Aldrich (St. Louis, MO, USA). Cisplatin was purchased from Alfa Aesar (Ward Hill, MA, USA). Alkaline phosphatase activity assay kit was purchased from Beyotime (Shanghai, China). Non-essential amino acids (NEAA), basic fibroblast growth factor (bFGF), and kits for membrane protein extraction or BCA protein assay were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Annexin V-FITC apoptosis detection kit was supplied by BD Biosciences (San Jose, CA, USA). Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose), fetal bovine serum (FBS), trypsin/EDTA, penicillin, and streptomycin were provided by Wisent (St-Bruno, Canada). BALB/c mice (male, 4–6 weeks) were provided by Vital River Laboratory Animal Technology (Beijing, China). All animal studies were approved by the Institutional Animal Care and Use Committee of Peking University.

2.2. Cell culture

Human cervical carcinoma cell line (HeLa) and Human lung carcinoma cell line A549 were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells used were those frozen within 6 months of purchase from the cell bank (authenticated using short tandem repeat DNA profiling analysis). Primary mouse testicular stromal cells (MTS) were isolated and cultured as previously described [20]. In brief, testes were separated from BALB/c mice (4–6 week old). The seminiferous tubules were digested successively by collagenase/DNase solution for 20 min and trypsin/EDTA solution for 5 min at 37 °C. Single cell suspension was obtained by filtering through a nylon mesh with 100 μm pore size and the resulting cells were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, NEAA, and 10 ng/mL bFGF. After 24 h, MTS cells were further purified by removal of the suspending cells. Cells within 5 passages were utilized.

2.3. Preparation and characterization of PEG-amifostine and PEGylated fluorescein

Amifostine (100 μmol) or aminofluorescein (100 μmol) was dissolved in 1 mL of disodium hydrogen phosphate buffer (0.2 M). About 20 μmol of 4-arm-PEG-SCM was added to the solution for conjugation. After the pH was adjusted to 7.2–7.4, the reaction was continued by stirring at room temperature for 4 h. Then the solution was dialyzed (molecular weight cutoff, 1000 Da) against deionized water for 24 h and was subsequently lyophilized. The final product was characterized by Fourier-transform infrared (FTIR) spectrometry (Spectrum One, PerkinElmer, MA, USA) with 16 scans taken for each sample, ^{1}H nuclear magnetic resonance (NMR) spectrometry (Bruker AVANCE 400 NMR spectrometer, Billerica, MA, USA) at 400 MHz in D_2O, and MALDI-TOF mass spectrometry (Microflex LRF, Bruker Daltonics, USA) in reflection and positive ion mode with sinapic acid as the matrix.

2.4. Morphological characterization of PEG-amifostine

Lyophilized PEG-amifostine was dissolved in deionized water and the solution was dropped onto a carbon coated copper grid. After the residual liquid being evaporated, the absorbed sample was stained by 2% sodium phosphotungstate for 10 min. The morphology of PEG-amifostine was examined by Transmission Electron Microscopy (TEM, Tecnai G2 20 S-TWIN, FEI, USA).

2.5. Thermal analysis

The thermal properties of amifostine, PEG and PEG-amifostine were evaluated using differential scanning calorimetry (Diamond DSC, PerkinElmer, MA, USA). Approximately 2 mg of each sample was placed into an aluminum pan and crimp sealed. The samples were then heated at the rate of 10 °C/min from 0 to 200 °C.

2.6. Efficiency of PEG conjugation to amifostine

The conjugation efficiency was determined by evaluation of free sulfhydryl groups in amifostine and PEG-amifostine as described previously [21]. In brief, 1 μmol of amifostine or PEG-amifostine was hydrolyzed by 500 μL of hydrochloric acid solution (0.15 M) at 60 °C for 40 min, followed by neutralization using 500 μL of sodium hydroxide (0.15 M). Then the generated free sulfhydryl groups were measured by Ellman's reagent, DTNB. According to a previous study [22], DTNB was dissolved in the alkaline buffer containing 100 mM Na_3PO_4 and 1 mM EDTA (pH = 8.0). About 40 μL of