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Effective treatment of drug resistant recurrent breast tumors harboring cancer stem-like cells by staurosporine/epirubicin co-loaded polymeric micelles

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ABSTRACT

Breast cancer recurrence and resistance are associated with cancer stem-like cell (CSC) sub-populations. As conventional therapies fail to treat CSCs, institution of novel therapeutic strategies capable of eradicating both cancer cells and CSCs is central for achieving effective treatments with long-term survival. Here, we studied the ability of polymeric micelles cooperatively loading the cytotoxic drug epirubicin (Epi) and the CSC inhibitor staurosporine (STS) to treat breast tumors, particularly when tumors relapsed after chemotherapy. The STS/Epi-loaded micelles (STS/Epi/m) demonstrated potent therapeutic efficacy against both naïve orthotopic 4T1-luc breast tumors and their recurrent Epi-resistant counterparts, significantly prolonging survival. This efficacy enhancement of STS/Epi/m was correlated with the ability of the micelles to suppress the CSC-associated sub-populations of breast cancer, *i.e.* the aldehyde dehydrogenase-positive ($ALDH^+$) population and the $CD44^+/CD24^-$ fraction, in Epi-resistant cells and tumors. These results demonstrated STS/Epi/m as a promising strategy for effective management of breast cancer.

1. Introduction

Breast cancer is the predominant cancer in women with 25% of new cases and 15% of deaths from all cancers [1]. Breast tumors are treated with various therapeutic strategies, including surgery, radiotherapy, immunotherapy and chemotherapy [2]. Particularly, treatment with epirubicin (Epi) offers considerable advantages for managing breast cancer, including its spectrum of activity, dosage, toxicity profile and the possibility to be combined with taxanes and/or trastuzumab for increasing efficacy [3,4]. However, despite showing good therapeutic responses, breast cancer patients present high relapse rate [5–7]. Such breast cancer recurrence has been associated with the presence of a subpopulation of cancer cells, so called cancer stem-like cells (CSCs), showing self-renewal ability, high proliferation rate and the capacity to produce heterogeneous cancer cells [8,9]. CSCs are also resistant to conventional therapies due to their enhanced detoxification mechanisms, including increased membrane transport, DNA repair and ROS scavenging systems [10,11]. Therefore, novel therapeutic approaches capable of eradicating both breast cancer cells and CSCs are needed to avoid recurrence and increase patient survival.

Nanomedicines have potential for enhancing therapeutic outcomes by concurrently treating both cancer cells and CSCs [12-15]. Among clinically translationable nanomedicines, polymeric micelles have shown unique properties for delivering drugs to solid tumors, including their stable and high drug loading, controlled drug release, relative small size, and high tumor accumulation [16,17]. Moreover, due to the core-shell compartmentalized structure of polymeric micelles, it is possible to cooperatively load various therapeutic agents aimed to exert synergistic efficacies. Thus, we have developed micellar nanomedicines capable of attaining coordinated effects of CSCs inhibitors and cytotoxic drugs within tumor cells to achieve synergistic activity against cancer cells and CSCs [18]. These nanomedicines profit from the synergistic anticancer effects of Epi and staurosporine (STS), a broadly multi-kinase inhibitor with high potency against CSCs [5], by co-incorporating STS in the core of our pH-sensitive Epi-loaded polymeric micelles (Epi/m) through manipulation of the interaction between STS and the polymer-conjugated Epi molecules [18]. The Epi/m used as platform have demonstrated high accumulation in tumor tissues, selective

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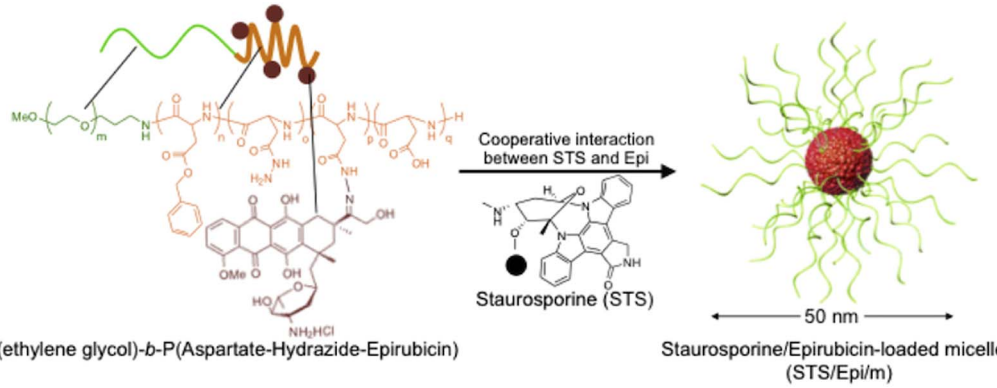

Fig. 1. Scheme of staurosporine/epirubicin-loaded micelles (STS/Epi/m). The micelles are assembled after mixing STS and Epi-conjugated block copolymers in DMF, which is then evaporated and replaced with HEPES buffer. The resulting self-assembled polymeric micelles are 50 nm in diameter.

intratumoral drug activation and potent antitumor effects [19,20], leading to their progress into Phase I/II clinical trials [17,21]. By co-operatively delivering both Epi and STS inside cancer cells, the STS/Epi-loaded micelles (STS/Epi/m; Fig. 1) facilitate the elimination of cancer cells and CSCs through endosomal pH-triggered drug release and reversal of drug resistance mechanisms [18]. These STS/Epi/m demonstrated enhanced antitumor activity in a model of recalcitrant mesothelioma, treating both cancer cells and CSCs, which resulted in tumor eradication [18]. In this way, STS/Epi/m appear as an attractive strategy for dealing with drug resistant tumors after relapse.

Herein, we studied the capability of STS/Epi/m for treating breast tumors, particularly after developing resistance to free Epi *in vivo*. The loading of STS in the micelles was tuned to maximize the STS/Epi incorporation ratio, while preserving the original diameter of Epi/m. By evaluating the ability of STS/Epi/m to treat both naïve and Epi-pretreated orthotopic breast tumors, we demonstrated the effective suppression of tumor growth of STS/Epi/m through enhanced efficacy against the CSC fraction.

2. Materials and methods

2.1. Materials

STS was purchased from Funakoshi Co. (Tokyo, Japan). Epi and poly(ethylene glycol)-*b*-poly(aspartate-hydrazide-epirubicin) copolymer were obtained from Nanocarrier Co. (Tsukuba, Japan). *N*, *N*-Dimethylformamide (DMF), methanol (MeOH), dimethyl sulfoxide (DMSO), penicillin-streptomycin, Dulbecco's phosphate buffered saline (D-PBS), hydrochloric acid (HCl) and other common use chemicals were purchased from Wako Pure Chemicals Industries, Ltd. (Tokyo, Japan). Amicon stirred cells, ultrafilter tubes and dialysis membrane (molecular weight cut-off size (MWCO): 30,000), Syringe Filters (Polyethersulfone (PES), Sterile, 0.22 μm) were purchased from Millipore Co. (Massachusetts, USA). Cell culture medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Sigma-St. Louis, USA). Fetal bovine serum (FBS) was bought from Invitrogen (Carlsbad, CA). Anti-CD44 antibody (ab112178), Anti-CD24 antibody (ab202963), Donkey Anti-Rabbit IgG H & L (Alexa Fluor 488) (ab150073) and Goat Anti-Rat IgG H & L (Alexa Fluor 594) (ab150160) were purchased from Abcam (Cambridge, UK). Blocking One Buffer was purchased from Nakalai Tesque Co., Ltd. (Tokyo, Japan). ALDEFLUOR Kit and ALDEFLUOR DEAB reagent were purchased from STEMCELL Technologies Inc. (Vancouver, Canada).

2.2. Cell lines

Murine breast adenocarnicoma 4T1 cells expressing luciferase (4T1-luc) were purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS and 1% streptomycin/penicillin, and maintained at 37 °C and 5% CO_2.

2.3. Animals

Balb-c mice (female; 6 week-old) were purchased from Charles River Co. (Tokyo, Japan). All the experiments were conducted under the ethical guidelines of The University of Tokyo.

2.4. Preparation of STS/Epi/m

pH-sensitive STS/Epi/m having different loading of STS were prepared by mixing PEG-*b*-poly(aspartate-hydrazide-epirubicin) copolymer (Mw of PEG = 12,000 Da; poly(aspartate) units = 40; Epi units = 8) at 1 mg/ml on an Epi-basis with STS at different drug weight ratios of STS/Epi (0.0625:1, 0.2:1, 0.3:1, 0.5:1 and 1.25:1) in DMF, and stirred for 30 min at room temperature in dark. Then, DMF was evaporated using a rotatory evaporator to form a thin film on the surface of the flask, followed by addition of 10 mL of HEPES buffer (10 mM, pH 7.4) into the flask containing the dried sample. The mixture was sonicated for 30 min. The resulting micelles were then purified by ultrafiltration (MWCO = 30,000). Finally, the micelles were filtered by using a PES filter (0.22 μm). The size of the micelles was measured by dynamic light scattering (DLS) by using a Zetasizer Nano ZS (Malvern, UK).

2.5. Drug loading of STS/Epi/m

pH-sensitive STS/Epi/m were disrupted by incubation in 1 mol/L HCl for 1 h at 37 °C to release both STS and Epi. The concentration of the drugs inside micelles was determined by HPLC (Column: Tosoh with TSK gel 80-TM; injection volume: 10 μL; mobile phase; 40% 1 mM formic acid (pH = 3) and 60% HPLC grade methanol; flow rate: 0.8 mL/min; temperature: 40 °C). STS and Epi were detected by UV absorption at 290 nm and 254 nm, respectively. The concentration of STS was interpolated from a standard curve, and the concentration of Epi was calculated according to area of Epi standard solution determined each time.

2.6. In vitro cytotoxicity study against 4T1-luc cells

The cytotoxic effects of the drugs, including free Epi, Epi/m, free STS plus free Epi, free STS plus Epi/m and STS/Epi/m, on 4T1-luc cells was evaluated, as follows: 4T1-luc cells were seeded in 96-well plates in DMEM medium with 10% fetal bovine serum at 37 °C. Twenty-four hours later, the drugs were added and the cells were incubated for