論 文 の 内 容 の 要 旨

論文題目　A Study on Polymeric Micelles Directed to Achieve Synergistic Therapies against Solid Tumors by Cooperative Co-delivery of drugs
（固形がんに対する共働的な治療の実現のためのドラッグ内包した高分子ミセルの研究）

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Introduction

The development of drug resistance in tumors has been associated with recalcitrant cancer stem-like cell (CSC) sub-populations, which promote the relapse of tumors and treatment failure. With traditional chemotherapeutics being futile against the CSC population [1], the effective combination of CSC inhibitors with cytotoxic therapies emerges as crucial for achieving robust responses capable of long-term disease-free survival. Nevertheless, complete eradication of CSCs with such inhibitors compels a challenging task due to the underlying risk of damage to healthy tissues by CSCs inhibitors and anticancer agents. Thus, effective strategies should be designed for sufficient selectivity toward tumors and CSCs. Nanomedicine has demonstrated great potential for developing safe and targeted strategies against solid tumors [2]. Thus, nanomedicine approaches involving the targeting of both CSC inhibitors and cytotoxic drugs to tumor tissues could serve as an effective way for controlling side effects while improving treatment outcomes, eventually against recalcitrant cancers. However, beyond the mere co-incorporation of drugs, such nanomedicines should precisely tailor the intracellular interplay of CSC inhibitors and cytotoxic agents to achieve synchronized activities and potentiate cooperative synergistic efficacies.

Polymeric micelles, *i.e.* self-assembled core-shell nanomedicines, can be engineered for efficiently incorporating various payloads, as well as selectively releasing them under precise stimuli [3]. Thus, in this study, we have developed polymeric micelles designed for cooperative delivery of a cytotoxic agent directed to remove cancer cells and a CSCs inhibitor capable of eliminating CSCs to achieve synergistic efficacy against intractable tumors. For this purpose, staurosporine (STS), a multikinase inhibitor, especially of protein kinase C (PKC), Akt-1, Cdk-1–4, and Chk-1 [4], which suggests high potential for CSC inhibition, was selected as the CSC inhibitor in this study. Moreover, STS was found to have high affinity, as well as the synergistic efficacy, with the anthracycline epirubicin (Epi), a potent cytotoxic agent. Therefore, we exploited these features to encompass STS into the core of Epi-loaded polymeric micelles (Epi/m), which are being studied in phase I clinical studies, for intracellular synchronization of therapeutic effect. This polymeric micelle system was optimized to maximize loading ratio aimed for synergistic activity, release rate and safety. Moreover,

the efficacy of these micelles against highly relevant tumor models was evaluated. Thus, as Epi is used in the clinical treatment of breast tumors, which usually relapse showing increased fraction of CSCs, we used the STS/Epi/m to treat both naïve and Epi-pretreated orthotopic breast tumors. Particularly, we focused on the ability of STS/Epi/m to eradicate the CSC fraction, *i.e.* the $CD44^{+}/CD24^{-}$ and aldefluor dehydrogenase (ALDH)-positive sub-populations. In addition, we evaluated the potential of STS/Epi/m against recalcitrant pancreatic and kidney tumors, which imperatively require effective therapies.

Results and Discussion

STS/EPI/m were prepared by mixing STS with PEG-*b*-poly(aspartate-hydrazide-epirubicin) copolymer in methanol, followed by evaporation and re-suspension in HEPES buffer. STS was carried into the core of the micelles by the engineered interaction with EPI molecules conjugated on the polymer. To explore the suitable drug ratio in EPI and STS co-encapsulating micelles, various initial drug ratios of EPI to STS were used for micelles preparation and the maximum drug ratio of EPI to STS inside the micelles is around 1:1 despite the increase feeding of STS, suggesting that the interaction between Epi and STS successfully controls the loading of drugs in the micelles. The diameter of the micelles increased with the addition of STS from 50 nm without STS to 80 nm for micelles having a 1:1 STS/Epi ratio. For studying the activity of STS/EPI/m in biological studies, micelles with 5 Epi per 1 STS were selected due to their size in the 50-nm range and synergistic potency at this STS/EPI ratio. These micelles showed to release the drugs concomitantly at endosomal pH (pH 5), while at extracellular pH (pH 7.4) no drug release was observed, suggesting the selectivity of the system for intracellular release.

The cytotoxicity of STS/EPI/m against 4T1-luc cells was determined. The 50% *in vitro* inhibitory concentration (IC_{50}) of STS/EPI/m was much lower that the IC_{50} of EPI/m, which demonstrates the synergistic effects of STS/EPI/m. In addition, the higher efficacy of STS/EPI/m over free Epi plus STS or EPI/m plus STS could be related to the protection of STS within the core of the micelles at the extracellular space, and the co-delivery of both STS and Epi for cooperative actions once inside the cancer cells [4].

The antitumor activity of STS/EPI/m against naïve orthotopic breast tumors prepared from murine mammary carcinoma 4T1-luc cells was conducted. STS/EPI/m effectively inhibited the growth of the tumors (Figure 1A), and mice experienced no body weight loss during the experiment, even after the repeated administration of STS/Epi/m (Figure 1B). These results demonstrate the high efficacy and safety of the micelles. Moreover, STS/Epi/m significantly improved the overall survival, with all mice living for more than 2 months (Figure 1C). On the other hand, all mice treated with the free Epi, STS plus free Epi and Epi/m died at approximately 40 days after starting the treatment. This outstanding antitumor activity of STS/Epi/m could be associated with the effective elimination of both cancer cells and CSCs through cooperative drug interactions [4].

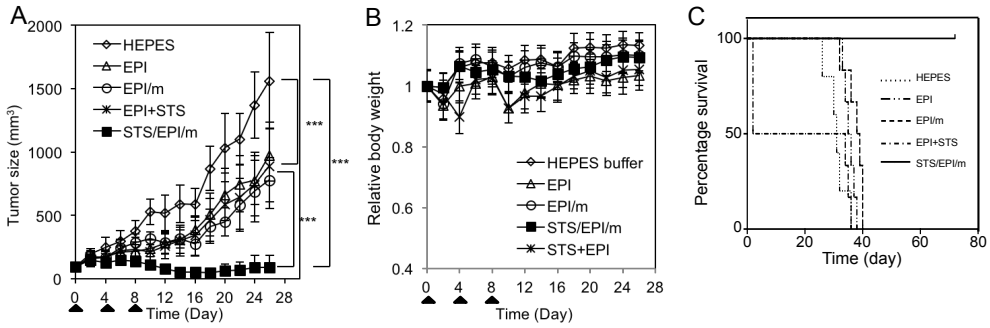

Figure 1. A. Tumor growth of tumors. The concentration of Epi was fixed at 6 mg/kg and the dose of STS was 1.2 mg/kg. Arrowheads: injection points. B. Relative Body weight changes during the antitumor experiment. C. Survival of mice during the experiment.

To confirm this efficacy of STS/Epi/m against the CSC fraction, the effect of the treatments on the CSC population within tumors was evaluated. Thus, the $CD44^{+}/CD24^{-}$ population, a reliable phenotype of CSCs from breast cancer, was determined by immunofluorescence microscopy in the cyro-sectioned tissues, which were collected from mice bearing 4T1-luc tumors at 48 h after intravenously injection with HEPES, Epi, Epi/m, STS plus Epi, and STS/Epi/m. STS/Epi/m significantly reduced the $CD44^{+}/CD24^{-}$ population in 4T1-luc tumors, verifying their enhanced inhibitory capacity against CSCs and their powerful antitumor effect against naïve tumors.

The ability of STS/Epi/m for suppressing relapsed tumors was evaluated by using tumors pretreated with Epi. The STS/Epi/m effectively reduced the growth rate of the relapsed tumors, significantly increasing the survival of mice compared to the other groups, with 50 % of mice alive for more than 2 months. Conversely, the other drug treatments (Epi, STS plus EPi and Epi/m) failed to show strong antitumor effects, and all mice died within 30 days. These results indicate the high potential of STS/Epi/m to inhibit reoccurrence and increase the overall survival, which have been considered main purposes for anti-CSCs therapies. These results confirm synergistic effects of STS and Epi within a single micelle platform, allowing effective inhibition of both cancer cells and the CSC fraction.

To further study the efficacy enhancement of STS/Epi/m against the relapsed tumors, we evaluated the cytotoxicity of the drugs against the cells collected from naïve and Epi-pretreated 4T1-luc tumors, and determined the remaining CSC fraction, that is, the cells with high ALDH activity, upon drug exposure *in vitro*. STS/Epi/m showed the highest cytotoxicity and was the only treatment to effectively maintain the activity against the cells from relapsed tumors, with their IC_{50} values in cells from naïve and pretreated tumors being comparable. These results are coincident with our previous report showing that STS/Epi/m can effectively suppress drug-resistance mechanisms [4]. For analysis of ALDH-positive population, the cells from Epi-pretreated 4T1-luc tumors were incubated with free Epi, free Epi plus free STS, Epi/m, and STS/Epi/m at the 10% inhibitory concentration of each treatment for 48 h. Only the cells treated with STS/Epi/m showed decreased levels of ALDH-positive cells (Figure 2). This result further demonstrates that STS/Epi/m effectively decreased the CSC population in the Epi-pretreated cells, which could lead to improved

therapeutic outcomes even after tumor relapse following Epi treatment.

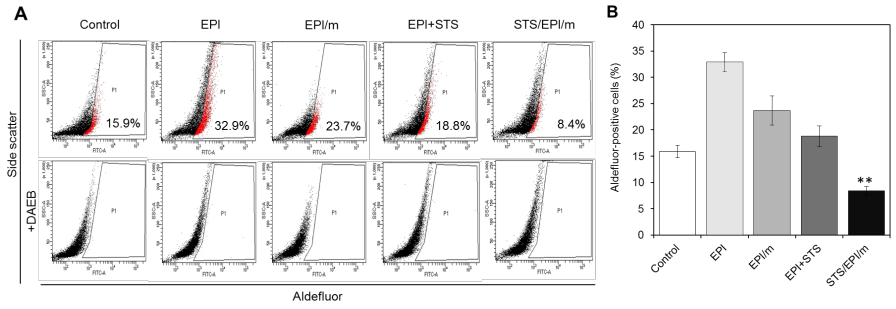

Figure 2. A. Flow cytometry study of Aldefluor stained cells. B. Quantification of the ALDH+ positive population.

The potential of STS/Epi/m against recalcitrant tumors (pancreatic and kidney tumors) was also investigated. In the case of kidney tumors, we prepared an orthotopic model of kidney cancer by implantation of RenCa cells in the kidney of Balb-c mice, and treated the mice with the series of drugs. Results showed that STS/Epi/m significantly prolonged mice survival compared to all other treatment groups. In addition, the efficacy of STS/Epi/m against pancreatic tumors prepared from BxPC3 cells, which are known to have low permeability to nanomedicines, was shown to be highly significant, suppressing the growth of the tumors for more than a month. These results indicate the promising therapeutic profile of STS/Epi/m against intractable tumors, and suggest further investigation in this direction.

Conclusion

We have developed a polymeric micelle system capable of cooperative delivery of a cytotoxic agent (Epi) and a CSCs inhibitor (STS), which synchronized the activity of the drugs inside the cells for eradicating both differentiated cancer cells and CSCs. The STS/Epi/m effectively treated breast tumors bearing recalcitrant CSC subpopulation, demonstrating potent therapeutic effects by eradicating primary orthotopic breast tumors, inhibiting the growth rate of Epi-resistant breast tumors and extending the survival. The results not only pose STS/Epi/m as a promising therapeutic strategy with potential for clinical translation (Nanocarrier, Co., recently announced the clinical development of these micelles), but also provide a new strategy for developing nanomedicines with synergistic activity through the manipulation of drug interactions within nanomedicines compartments, as an effective modality for designing therapies eliminating both cancer cells and CSC sub-populations in tumors.

References

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