博士論文

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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Preface

This dissertation presents results of the studies carried out in Professor Kazunori Kataoka's laboratory and Associate Professor Horacio Cabral's laboratory at the University of Tokyo. In this thesis, I developed a two-drug co-delivery platform for Epirubicin, an anticancer drug, and staurosporine, a potent cancer stem cell inhibitor, and determined the anticancer efficacy of this multidrug delivery system against primary tumor model and drug resistant tumor model.

First and foremost, I would like to express my deep gratitude to Professor Kazunori Kataoka and Associate Professor Horacio Cabral for giving the best opportunity to pursue the present study, and their instructive guidance and mentorship, help advice and great encourage from beginning to the completion of this thesis.

I want to express my heartfelt gratitude to the colleagues of Kataoka Laboratory and current members of Cabral laboratory for giving kind help, assistance and constructive advice to this research and also for their invaluable love and friendship in this study life.

Finally, I want to express my hearty gratitude to my family for their support and encourage.

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Chapter 1 General Introduction

Chapter 2 Preparation and Characterization of dual drug Co-loaded Micelles

Chapter 3 Optimization of STS/EPI Micelles

Chapter 4 Antitumor Activity of STS/EPI/m against Breast Cancer

Chapter 5 Antitumor Activity of STS/EPI/m against recalcitrant cancers

Chapter 6 General conclusion and future perspective

Chapter 1.

General Introduction

Chapter 1. General Introduction

1. Challenge for cancer treatment

Cancer is one of the leading causes of death. With the countless efforts from doctors and scientists working on studying the cancer, there has been an increasing understanding about the development of cancer. From a very early age, where people confused the causes of untreatable diseases and cancer (1-9), to the development of modern knowledge on cancer, doctors and researchers have gained more correct and exact information on causes of cancer and properties of tumors. The development of medicines and biomaterials also provided great chance for improving diagnosis, treatment efficacy and therapeutic effects against cancer. Nowadays, cancer can be treated by many ways, such as surgery, chemotherapy, radiation therapy, therapy with hormones, and targeting treatment. The choice of therapy depends upon the location where tumor developed and phase of the tumor and the period of the illness, and the general health status of the patient. A number of clinical cancer treatment trials are also undergoing with various new strategies based on development of therapeutic agents and biomaterials.

Eradication of the tumor tissue with no damage or effect to the rest of the body is the aspiration of cancer treatments, though it is a tough goal in practice. While sometimes surgery can solve this barrier, the features of cancers to insult nearby organs and tissue or to move, as well as spread to further locations by metastasis usually decrease and limit the application of localized treatments. Therefore, chemotherapy and radiotherapy are major approaches for dealing with systemically

distributed disease, because they have the ability to remove cancer cells that otherwise would be undetectable. Unfortunately, there are side effects of these therapies.

First chemotherapy agents were considered at the beginning of the XX century. During World War II, mustard gas was found to cause toxic alterations in blood cells. At the meantime, a number of chemicals connected to mustard gas was being surveyed by the US Army to explore more effective drugs and exploit protective measures in war (10-11). From that study, a mixture named nitrogen mustard was discovered and detected to fight against lymphoma (12-14). Soon, followed the finding of nitrogen mustard, aminopterin, an agent connected with the vitamin folic acid, was illustrated by Sidney Farber (Boston), who pointed out that aminopterin protected children with acute leukemia, which inhibited a pivotal chemical reaction with requirement of DNA replication. Aminopterin was the precursor of methotrexate,which was usually employed nowadays (15-18). From then on, more and more drugs were detected by scientific researchers that inhibit various actions in cell growth and replication, and the chemotherapy entered in an era of unprecedented development.

After all these years, chemotherapy agents have resoundingly cured many cancer patients. With the development of medical research on tumor treatment, the drug activity of chemotherapeutic agents on cancer are better understanding and more drugs are available in treatment, at the meantime, the adverse effects of chemotherapy also required to decrease. They are shown as the followings:

- Newly-developed chemotherapy and delivery methods for combination.
- Specifically targeting and new approaches for eradication of tumor tissues with limited side effects.
- Drugs and drug delivery systems reducing adverse effects.

- Agents that overcome multi-drug resistance and repress tumor relapse (the usual cancer therapeutics have slight efficacy).

Complete removal of tumor tissue with near zero adverse effectiveness is an ideal and complicated goal. The developed treatment strategies for many cancers have more or less toxicities on normal tissue, and affect the recovery of patients from cancer treatment. To get an ideal treatment effect, doctors and researchers are working hard on finding new and efficient therapeutic methods for enormously improving therapeutic effects and decreasing the adverse effects in the meanwhile. The chemotherapy drugs commonly are exploited in cancer treatments by leading death to cells during replication to form two new cells. These chemotherapy drugs not only killed cancer cells in tumor tissue, but also killed part of normal tissue cells, even though these drugs lead greater toxic to tumor cells. Targeting treatments take function by affecting the proliferation procedures that control cell growth, cell dividing, and invading of tumor cells, and also all kinds of signals that cause tumor cells to natural death.

Cancer cells can grow fast, unstopping and pass to other organs in the body. Researchers and scientists found that growth factors, hormone-like substances affecting the growth and dividing of cells, have close relationship with the growth and spread of cancer cells in tumor tissue, especially the overwhelmingly high amount of growth factors would potentially give all kinds of affection to the tumor growth speed and spread sites. With increasing number of patients are escaping successfully away from various of cancers, life quality and long-term effects of these cancer survivors are the novel focuses. Behavioral researchers are paying hard effort and try their best to

make it clear of the problems survivors face. There are some medical problems, for example, long lasting adverse affection on therapies and other complications after various treatments, and the requirement for continuous treatment and a series of followed-up medical care. Emotional or social challenges are also huge problems, *e.g.* obtaining health insurance, distinction by employers, alterations in relationships, or deducing possibility of cancer recurred and relapsed.

1.1 Cancer stem cells (CSCs) in tumor malignancy

Despite all the major efforts in developing effective therapies, the failures in the therapies are frequent. One of the most common considerations associated with the treatment failure in cancer is linked with the inherent or developed resistance of the tumors to chemotherapy and radiotherapy, and the eventual relapse of aggressive tumor phenotypes after a first successful treatment. These events have been associated with a subpopulation in tumors, which present stem-like properties, thus, they have received the name of cancer stem-like cells (CSCs). The CSCs are a small fraction to the cancer cells found within tumor tissues that hold the ability of developing into special types of cancer sample. The influence of CSCs in biological features and treatments during the development of diseases, providing the possibility that focusing on these cells may be appropriate for providing long-term disease-free survival. However, such therapies represent a major challenge, as CSCs have shown to present lacking of reactive oxygen species (ROS), and to be able to overcome the radio- and chemotherapy by up-regulation of efflux pumps, DNA-repair proteins and thiol-based scavengers, and failure to eradicate CSCs can lead to recurrence of aggressive tumors and development of metastasis. Besides intensified defense mechanisms, CSCs have

shown deregulation in several pathways involved in self-renewal control and differentiation, such as PI3K/Akt, Wnt/β-catenin, PTEN, NF-κB, JAK/STAT, hedgehog, Notch, Bcl-2, and overexpression of transcription factors for stem cell pluripotency, including OCT4, NANOG and SOX2, which represent promising targets for developing clinically useful inhibitors. Nevertheless, complete eradication of CSCs with such inhibitors compels a challenging task due to the underlying risk of damage to healthy tissues, as many of these pathways are found in normal stem cells, while the toxicity of inhibitors of CSCs combined with other anticancer agents could be rendered intolerable.

CSCs are also phenotypically heterogeneous both inter- and intratumorally, which poses a significant challenge for developing targeted therapies. The heterogeneity of CSCs could be given by genetic mutations and epigenetic changes, or by microenvironmental differences, such as cell-cell interaction, cytokines and hypoxia. Increasing evidence is also indicating that CSCs exist in anatomically and physiologically specialized environments within tumors, constituting niches that favor their survival. The reliance of the CSCs on niche signals is a general phenomenon and has been demonstrated in several tumors. CSCs can in turn modulate their niche, and utilize cell-signaling pathways for maintaining internal equilibrium processes, including inflammation, epithelial-mesenchymal transition (EMT), lack of oxygen and newly-developed vessels. Consequently, the architecture and position of this niche are dynamic, and change with tumor development and progression, as well as with the applied treatments. Additionally, the microenvironment of the niche can revert non-tumorigenic cancer cells into CSCs by EMT-associated processes, increasing tumor invasion and metastasis. Such dynamic interchange between cancer cells and CSCs

populations suggests that therapies that are only active against CSCs may eventually fail, if the residual differentiated cancer cells can repopulate the niche of CSCs. Therefore, targeted therapeutics aimed for tumor suppression should be able to reach the entire tumor, including CSC niches, at any step of tumor development and eliminate both cancer cells and CSCs with sufficient selectivity, for achieving safe and robust long-term responses (19-23).

1.2 Therapies targeting CSCs

Nowadays, couples of new treatments strategies have been designed aim to eliminate CSCs and vary the living environment of CSCs. These alterations of markers located in surface of cells and changes happened during signaling transduction and on signaling pathways are promising targets for treatments. Scientists have made clear of a series of potential targets for CSC therapies, for example, the ATP Binding Cassette (ABC) huge family, factors of anti-apoptosis, enzymes of getting rid of toxicity and enzymes for DNA repair, also outstanding cancer caused cascades (including the Wnt/β-catenin, Notch signals, hedgehog, and EGFR) (24). Treatment strategies that can effectively kill CSCs in tumor tissues may be too toxic to healthy stem cells.

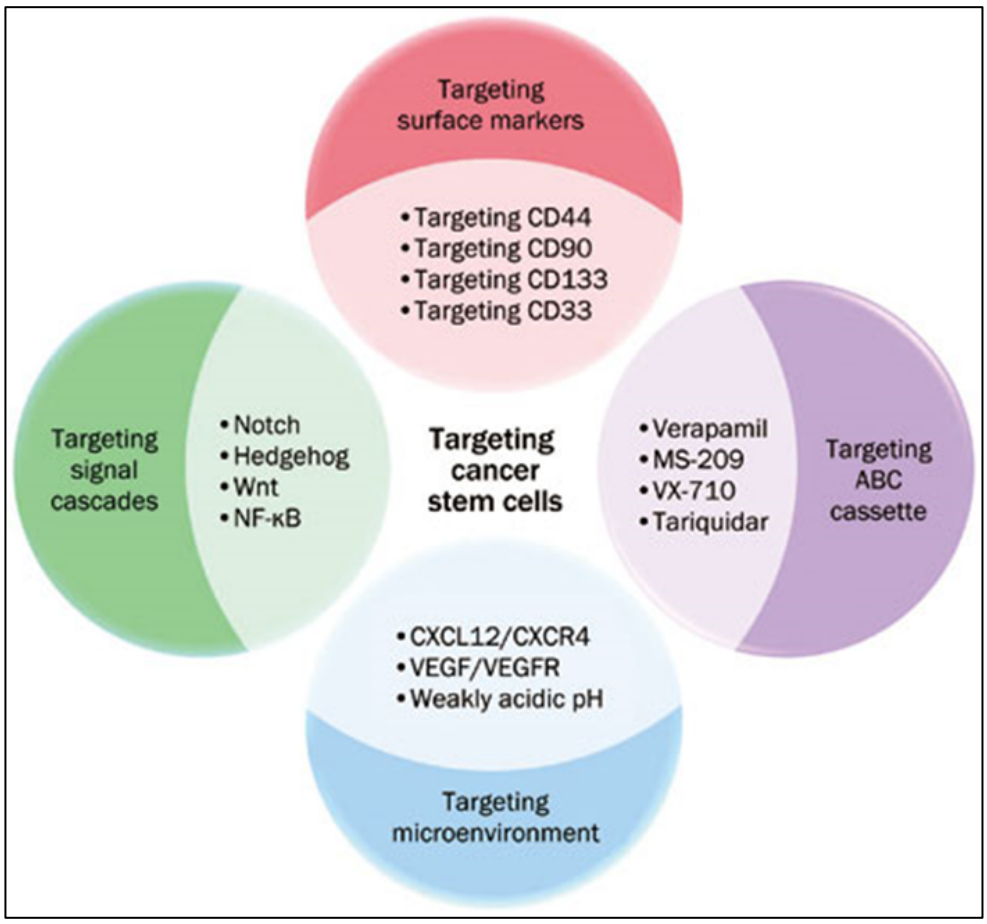

Figure 1. Cancer stem cells targeting therapies (23).

Figure 1 briefly summarizes the new CSC-targeted therapeutic strategies. An array of therapies directing at clearing CSCs have been designed and developed. There are few different fields which can sum up recent trends. By selectively and specifically targeting markers on cell surfaces (red part), targeted scenarios could be realized. With the development and aid of other disciplines skills and techniques especially the molecular biology, a branch of important transduction signaling pathways have been opened (green part). By disrupting specific and abnormal signaling pathways, particular features of CSCs are inhibited and potential outcome has been illustrated.

Anticancer agents suppressing ABC transporters (purple part) have developed into mature state (tariquidar) and these drugs are beening undergoing trial usage on patients.

1.3 Staurosporine (STS)

STS is a natural drug of microbial alkaloid having anti-fungal activity able to extract from the natural Streptomyces staurosporesa. STS is known as a broad-spectrum inhibitor, which easily interacts with many kinases with high activity, though with none specificity. STS is especially potent as inhibitor of phospholipid/calcium-dependent protein kinase with strong inhibition of the upregulation of VEGF expression in tumor cells (25).

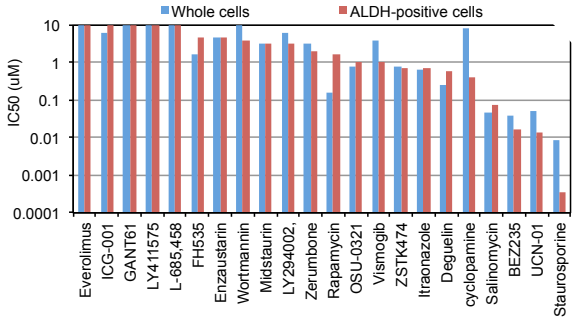

Figure 2.Cytotoicity against mesothelioma cells (MSTO) (27).

STS also inhibits phospholipid Ca^{2+} dependent protein kinase (protein kinase C) and acts as inducer of apoptosis (0.7 nM). STS also inhibits other kinases (1-20 nM), for instance, PKA, CAMKII, PKG, and Myosin light chain kinase (MLCK) (26). These kinases have been associated to support the survival of CSCs in several tumors. Moreover, STS induces cell apoptosis (0.2-1 μM) (27-30). STS takes its biological function mainly according to its affinity to inhibiting the ATP interaction with kinase make kinase lose its function. As STS is unspecific inhibitor of a wide range of kinase and signal pathways, it can induce apoptosis through different signaling pathways and

inhibit kinase activity. STS can induce cell apoptosis both through caspase-dependent way or independent way (29,30). With such strong cytotoxicity and broad kinase inhibitory ability, STS is important for depletion of CSCs. In fact, STS was shown to effectively eradicate CSC in mesothelioma cells with nM potency (Figure 2) (27). However, it is difficult for clinical usage of STS due to its potent cytotoxicity. On the other hand, the high affinity of STS to bind with proteins, namely, human asialoglycoprotein, also inactivates it in blood. Taken together, STS is a promising candidate for anti-CSC therapies, though its high toxicity and *in vivo* inactivation indicate a better strategy for delivering STS into tumor tissues.

2. Drug delivery systems for tumor targeted therapy

2.1 Concept of drug delivery

Drug delivery systems (DDS) have demonstrated potential for constructing safe and targeted strategies against cancer (31-34). DDS are utilized for improving the levels of therapeutic agents at the diseased site, thereby, increasing efficiency, and sometimes efficacy of treatments, as well as reducing side-effects of toxic agents and compounds. The potential of DDS to control drug release of therapeutic agents at the targeted sites, while keeping concentrations in the effective therapeutic range, have been attracted intensive attention (34). In the following sections, I described the basic concepts for drug delivery to solid tumors and the potential for developing therapies capable of eradicating both cancer cells and CSCs

2.2 Nanocarriers of drug delivery

With the adding number of cancer patients every year, cancer has threatened human being's health and wealth seriously, under this situation, a rapid soaring requirement for effective cancer therapies become urgent. There is great expectation on nanoparticles for providing enormous improvements in aspects of diagnosis, therapy and control of intractable diseases. With benefit of offering increased safety, reduced adverse effects, specific and selective targeting and decreased treatment cost. From this point of view, nano-scaled drug carrier systems, which can selectively deliver cytotoxic compounds and chemical agents, anticancer drugs and complex or DNA/RNA to targeted diseases, show promising for cancer detection and therapy, also enhance therapeutic efficacy (35-37). Macromolecules of nanoparticles can selectively reach targeting site in solid tumors by going through leaky blood vessels in cancer tissues. Also, these macromolecules get kept in the diseases parts owing to the damaged lymphatic tubes. These phenomena are described as the enhanced permeability and retention (EPR) effect (38). Hence, the success of such nanoparticles in the body circulation depends on their ability to keep stable while they running through the body without being recognized and caught by the reticuloendothelial system, also their ability for extravasation and go through in tumor tissues for controlled and targeted release of their cargos.

There are a series of enormous advantages of the approaches of nanotechnology in aspects of diagnosis, therapy, control and supervise of malignancies. Nano-sized drug delivery systems could function for monitoring pathophysiological alterations *in situ*, and altering its features according to the specific condition induced by the malignancy (37). These features are of great potential for evaluation of diseased tissues and taking charge of abnormal processes in biology (Table 1) (37). Besides, the

features of diseases on pathophysiological aspects, such as physiological obstacles, cell populations or marker expressions of special marker, can also take effect on the function of nanoparticles. The interaction between nano-pathophysiological aspects and nanocarriers can detected by installing indicators directly linked on nanoparticles.

Table 1. Nanodevices for studying nano-pathophysiology.

Nanodevice	Type	Size (nm)	Unique features
Top-down	Replication in template (Lithography)	80–	Precise replication
			Designable shape
Lipid	Liposomes	80–1000	Versatile loading
			Controlled release
			Membrane fusion
	Solid lipid nanoparticles	10–100	Versatile loading
Polymeric self-assemblies	Micelles	10–100	Versatile loading
			Controlled release
	Polymersomes	100–1000	Versatile loading
			Controlled release
Inorganic nanoparticles	Gold	2–200	Optical
	Iron oxide	5–	Magnetic
	Mesoporous silica	10–	Pores for incorporation
	Quantum dots	1–20	Bright luminescence

The capacity of nanocarriers to particularly and selectively target pathological conditions can improve treatment efficacies. On one hand, nanocarriers are gathering in the tumor tissue by a passive mechanism owing to the augmented vascular permeability in the tumor area which is known as EPR effect. On another hand, nanocarriers also can be activated by signals in tumor cells through active mechanisms depending on interactions of molecules installed on the surface of nanoparticles that

can be recognized specifically by receptors on the surface of cell membrane, or by chemical stimuli, such as low pH or enzymes, intracellularly (Figure 2-B).

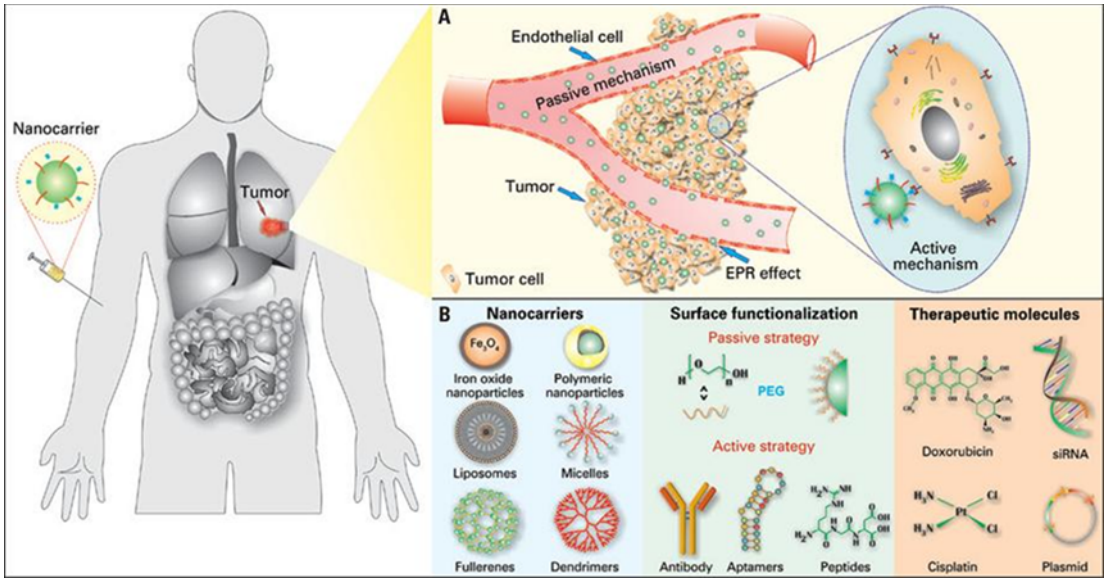

Figure 2. Nanocarriers for cancer treatment (41).

Nanocarriers are exploited for diagnosis and therapy of solid tumors (39-41), owing to their function of selectively and specifically gathering up in abnormal tumor tissues through the EPR effect. However, the leaky blood vessels and impaired lymphatic drainage of EPR effect could be heterogeneous and vary from tumor to tumor, and even within the tumors. The tumor stroma, i.e. the surrounding tissue tht host the cancer cells, is critical for the EPR effect, and the degree of collagen and hyaluronic acid, as well as the presence of particular cell populations, such as tumor associated macrophages and activated fibroblasts, could irecety affect the accumulation of nanocarriers in tumors. It has been recognized that the control of the size of the nanocarriers in the sub-100 nm range is essential for improving the tumor

targeting ability through the EPR effect (42). In addition, recent reports indicate that of the design features of the nanocarriers affect their capacity for targeting with effect on the physicochemical settings and treatment efficacy. The properties of nanocarriers for effectively targeting tumors greatly count on their particle size, surface charge and surface chemistry, as well as their stability and drug release rates.

2.3 Multidrug delivery

Single drug therapies are usually not sufficient for effectively treating tumors, thereby, in the clinic two or more drugs are combined for enhancing efficacy. However, drug combinations may not always result in long-term responses, and treatment failure may occur when there is at least one mutation that could confer resistance to one of the drugs. This is particularly important for tumors harboring significant fractions of CSCs, with the efficiency of therapies dropping in cancers with increased point mutation rates. Thus, combination of chemotherapy with CSC-inhibitors has gained much attraction for improving therapeutic outcomes (43). However, even though various selective inhibitors of survival pathways in CSCs have accomplished therapeutic improvements in the clinic, tumors may eventually develop resistance to such inhibitors, and tumor repopulation and recurrence would majorly occur from resistant-cancer cells and resistant-CSCs that survive repeated treatments.

Recent drug combinations have shown cooperative mechanisms of action, affecting multiple biological pathways in cooperative manners, such as prohibiting interaction between different membrane receptors, overcoming multi-drug resistance (MDR), and minimizing overlapping toxicity (39). Based on the safety and targeting ability of nanocarriers to tumor tissues, there is increasing interest in developing and

designing new approaches and platforms for multidrug delivery. Thus, enormous efforts have recently been devoted on to establish combination therapies using various drugs within single nanocarriers. Such co-loading of drugs into nanocarriers can facilitate the achievement of synergistic activities by delivering the appropriate ratio of drugs to tumors, as well as controlling the pharmacokinetics of the drugs contained inside the nanocarrier. Moreover, while combination therapies showed heterogeneous drug distribution in tumor tissues and differential uptake by cancer cells, which results in the synergistic activities happening in a limited fraction of cells, by using multidrug-loaded nanocarriers, it is possible to deliver the effective combination of drugs to each cell, increasing the therapeutic response.

Delivery of multidrug is defiant and challenging. There are six major desires and requirements to consider when measuring the successful vehicle development for multi-drug delivery of anticancer drugs. These necessities are as follows: (1) Carriers delivering multiple drugs should maintain long-term physical and chemical stability of drugs throughout manufacturing, storage, and administration; (2) The carriers must be biocompatible, biodegradable, and non-toxic. (3) The solubility of highly hydrophobic drugs should be enhanced by incorporation into vehicles in injectable aqueous solution. (4) The incorporated multiple drugs should be released from the carriers in a controlled pattern for maximizing efficacy, while avoiding toxicity. (5) The carriers should selectively accumulate in solid tumors with minimal off-targeting effects. (6) The carriers should be simple in design and have a facile manufacturing process.

2.4 Polymeric micelles for drug delivery

Polymeric micelles are established based on self-assembled core-shell nanostructures in aqueous condition, which usually consist of amphiphilic block copolymers including two parts of hydrophobic and hydrophilic blocks (40-42,44,45). These block copolymers which are hydrophilic spontaneously form core-shell structures over the critical micelle concentration (CMC) driven greatly by entropy gain from the released water molecules associated to the hydrophobic backbone. The hydrophobic core provides a basement for storage of water-insoluble agents, whereas the water affinity outside part interfaces with the aqueous medium, avoiding aggregation and precipitation. The most largely used hydrophilic block is poly(ethylene glycol) (PEG). Shield of PEG with high density assures micelle dissolve in an aqueous condition. Regarding the hydrophobic blocks, various polymers have been considered, such as poly(ester)s (46) and poly(l-amino acid)s (47), which provides versatility for designing drug loading and release strategies. The size of polymeric micelles is confined and controlled between 10 to 100 nm (48), which reduces distribution to parts of the reticuloendothelial system and allows fighting against physiological barriers for achieving escaping and thoroughly going inside the tissues and high gathering amount in tumors by the EPR effect. This broad and enhanced distribution of polymeric micelles in diseased tissues enables the transportation of drugs for effective treatment amount of agents into most cancer cells, which is crucial for avoiding sub-toxic concentrations within tumors that could lead to the development of resistant populations. Moreover, after reaching the tumors, polymeric micelles can be designed for selectively delivering their cargo to their subcellular targets after cellular internalization, thereby, overcoming drug resistance mechanisms and enhancing drug efficiency (49-52). Additionally, the cellular

recognition and intracellular delivery of nanoparticles can be further decorated through adding ligand molecules of their surface directed to specific biomarkers on cancer cells. Finally, after completing their operation, drugs inside of micelles get loaded off the polymer chains and out of the protected core to take function as killer of cells (53). Several polymeric micelle formulations incorporating anticancer drugs have proceeded to clinical trials, which demonstrated lessening of side effects and high efficacy even on treatment of recalcitrant diseases (54).

Among promising polymeric micelle formulations, epirubicin-loaded micelles (NC-6300), *i.e.* 50-nm core-shell nanostructures having a PEG shell and a core of poly (aspartate hydrazide) (PEG-b-p(Asp-Hyd)) with pendant epirubicin, are now being studied in Phase I clinical trials (55). These micelles are designed to release the drug selectively at endosomal pH, thereby, avoiding drug leakage during circulation and minimizing side effects. Moreover, due to their effective shielding and stable drug encapsulation, the micelles can deliver high quantities of drugs. NC-6300 is unique and outstanding in that epirubicin is covalently bound onto PEG-b-p(Asp-Hyd) by a pH-sensitive hydrazone linker, allowing stability in blood, but drug release in the interior of acidic endosomes and lysosomes of cancer cells (Figure 4) (55).

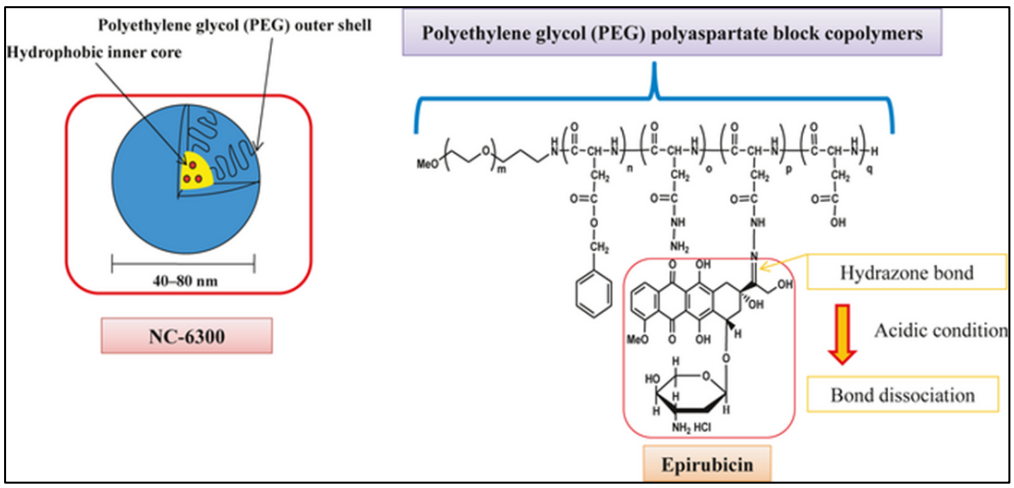

Figure 4. NC6300 micellar formulation (55).

2.5 Polymeric micelles for multidrug delivery

Anticancer agents can be encapsulated chemically or physically into polymeric micelles for concurrent multi-drug delivery (Figure 5). Clinically, many poorly water-soluble anticancer compounds are administrated sequentially. Concurrent carrying by polymeric micelles increases safety, and allow the anticancer therapies to take function against diseases simultaneously and more effectively with the aid of synergistic drug interaction (56-58). Corresponding multi-drug delivery may also be established by the conjugation of the drug in a polymeric micelle in the first step and the physical loading of an anticancer agent at a secondary step. This strategy has the advantage of being a one-step drug-loading process for two or three drugs (56). In both situations, chemical modification of drugs is not a necessity for drug loading.

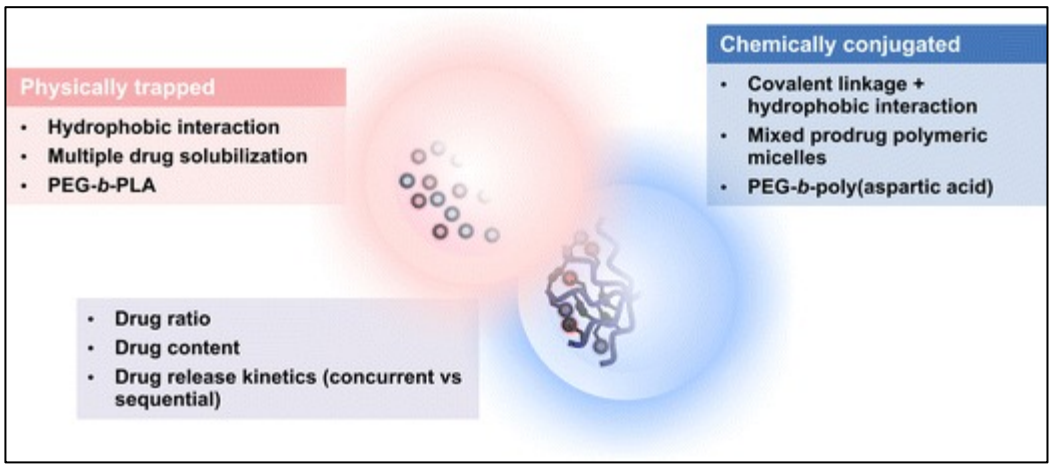

Figure 5. Multi-drug polymeric micelles for drug delivery (56).

Multi-drug delivery micelles may release physically loaded drug(s) by disassembly of polymeric micelles in blood and/or by diffusion. Drug release tends to be rapid *in vivo*, and *in vitro* results must be translated with caution due to poor in vitro/in vivo correlation. Thus, while EPR targeting of solid tumors is feasible with physically loaded drug, e.g., PTX in NK-105, it is considered as difficult and challenging, and research on multi-drug polymeric micelles for tumor targeting deserves more attractions. There has been synthesized PEG-b-p(Asp-Hyd)s with pendant doxorubicin (anthracycline antibiotic) and wortmannin (WOR, PI3K inhibitor) and constructed multi-drug polymeric micelles (53-58) by adjusting the original feed ratio, drug ratios, *i.e.,* % WOR on PEG-b-p(Asp-Hyd), were 0, 20, 51, 79, and 100%. Drug ratios of PEG-b-p(Asp-Hyd) micelles were also adjusted by separate attachment of each drug on PEG-b-p(Asp-Hyd), and establish into micelles with mixed prodrugs. At 50% WOR, PEG-b-p(Asp-Hyd) with pendant DOX and WOR constructed micelles with the average size of ca. 90 nm, whereas mixed polymeric micelles with PEG-b-p(Asp-Hyd) with pendent DOX and PEG-b-p(Asp-Hyd) with pendent WR were larger,

ca. 220 nm. While a size difference was observed, equivalent toxicity against MCF-7, was conformed, suggesting a similar mechanism of action.

Table 2. Multi-Drug Polymeric Micelles for Concurrent Drug Delivery (56).

Polymer	Drugs	Indication	Status
PEG-*b*-p(Asp-Hyd)[a]	Doxorubicin/wortmannin	MCF-7 breast cancer	*In vitro*
PEG-*b*-p(Asp-Hyd)[a]	Doxorubicin/17-hydroxethylamino-17-demethoxygeldanamycin	MCF-7 breast cancer	*In vitro*
PEG-*b*-PLA	Paclitaxel/17-AAG/rapamycin	A549 NSCLC and MDA-MB-231 breast cancers	*In vivo*
PEG-*b*-p(γ-benzyl l-glutamate) + PEG-*b*-p(l-lactide)	Doxorubicin/etoposide, Doxorubicin/paclitaxel	CT-26 murine colorectal cancer	*In vivo*
PEG-*b*-PLGA	Doxorubicin/paclitaxel	A549 NSCLC, B16 mouse melanoma, and HepG2 liver cancers	*In vitro*
Poly(2-methyl-2-oxazoline)-*b*-poly(2-butyl-2-oxazoline)-*b*-poly(2-methyl-2-oxazoline)	Paclitaxel/17-AAG/etoposide, Paclitaxel/17-AAG/bortezomib	MCF-7 and MDA-MB-231 breast, PC3 prostate, and HepG2 liver cancers	*In vitro*
PEG-*b*-poly(carbonate-*co*-lactic acid)	Cyclopamine/gefitinib	L3.6pl and MIA PaCa-2 pancreatic cancers	*In vivo*
PEG-*b*-PCL	Paclitaxel/cyclopamine/gossypol	ES-2-*luc*and SKOV-3-*luc*ovarian cancers	*In vivo*
PEG-DSPE/TPGS	Paclitaxel/17-AAG	SKOV-3 ovarian cancer	*In vivo*
PEG-*b*-poly-(glutamic acid)-*b*-poly(phenylalanine)	Paclitaxel/cisplatin	A2780 ovarian cancer	*In vivo*
PLGA-*b*-PEG-*b*-PLGA	Paclitaxel/17-AAG/rapamycin	ES-2-*luc*ovarian cancer	*In vivo*

[a]Chemical loading (conjugation) of drugs

Multidrug-loaded nanocarriers have demonstrated effective regulation of pharmacokinetics, tumor accumulation of co-incorporated drugs, and in some cases, synergistic activity. However, for achieving cooperative therapies toward overcoming

intratumoral heterogeneity and CSCs, multidrug-loaded nanomedicines should surpass the mere co-delivery of therapeutic agents, and provide a platform capable of actively directing the intracellular interplay of drugs to achieve synergistic efficacies in different cell populations. Thus, polymeric micelles could be engineered for such purpose by precisely tailoring the interaction of cytotoxic agents and CSC-inhibitors within their compartmentalized structure to potentiate cooperative actions at the molecular level within cells. By considering the plasticity of cancer cells and CSCs, and the articulation between drug arrangements, such polymeric micelles would have the potential for improving treatment responses, eventually against refractory cancers, and avoiding recurrence.

3. Overview of this study

3.1 Significance of this study

In this thesis, I aim to develop clinically translationable nanocarrier approaches cooperatively delivering chemotherapeutic agents and anti-CSC drugs for robust and enduring therapeutic responses by treating both cancer cells and CSCs in tumors. Such nanocarrier approach will involve targeting of both CSCs inhibitors and cytotoxic drugs in a synergistic fashion by using polymeric micelles. Thus, these micelles will serve as an effective way for controlling side effects and improving treatment outcomes. While current drug combination approaches are aimed for maximal efficacy, such therapies will fail if the cancer cells develop resistance to at least one of the drugs. On the other hand, the proposed nanocarrier strategy will be capable of tolerating or rapidly adapting their function to therapeutic demands in cancer cells and CSCs,

thereby, articulating the functions of drugs to circumvent resistance mechanisms/development.

As the combination of EPI and STS shows synergistic effects against mesothelioma cells by treating both differentiated cancer cells and CSCs (27), we exploited the NC-6300 micelles, which are under phase I clinical evaluation, to concurrently load STS and EPI in a single nanocarrier platform. These micelles are expected to concurrently load STS by enhanced interaction with EPI molecules (27), and promote the concomitant release of STS and EPI after intracellular activation at endosomal pH. These micelles would be able to kill proliferating cells by action of EPI, kill CSC by STS, abrogate mechanisms of EPI resistance and eliminate the cells that escape the action of STS by differentiation. Thus, the main goals of this project can be described as follows:

1. To deliver cooperative drugs combination for eliminating both cancer cells and CSCs. The final goal of this study is to establish a dual drug co-loaded nanocarrier platform to treat cancer cells and cancer stem cells in the solid tumor tissues to achieve robust cancer therapeutic efficacy, extending the overall survival.

2. To develop a drug delivery system for STS. To achieve this goal, the cytotoxic anthracyclines anticancer drug of EPI and STS, broad spectrum inhibitor of multiple cell signaling and potent cancer stem cell inhibitor, have been co-loaded inside the hydrophobic core of PEG-b-poly (aspartate-hydrazide-EPI) copolymers in aqueous condition to develop a two-drug loaded micelles. The properties of STS allow both EPI and STS to encapsulate into the core structure of micelles. This will be the first polymeric micelle delivering STS.

3. To develop a safe and effective therapy against CSC-rich relapsed tumors. Several reports showed that the multidrug resistance, tumor metastasis and tumor relapse are highly related with CSC. Thus, CSC elimination in relapsed tumors could provide an effective strategy to prolong survival of patients. This approach was assessed by challenging the STS/EPI/m against EPI pre-treated breast tumors, which mimic the usual clinical situation, *i.e.* the possible recurrence of aggressive tumors having expanded CSC-populations after treatment with EPI.

4. Study the potential of STS/EPI/m against recalcitrant tumors, such as kidney cancer and pancreatic cancers. Recalcitrant tumors do not have any effective therapy at this moment and the overall survival is lower than 5-years. The possibility that STS/EPI/m is effective against these intractable tumors could bring hope to a large number of patients.

The nanocarrier design, together with the pharmacological effects and the antitumor activity results, may be applicable for future cancer therapies and may give a new understanding for developing nanocarriers for multiple drug delivery for synergistic cancer treatment (Figure 6).

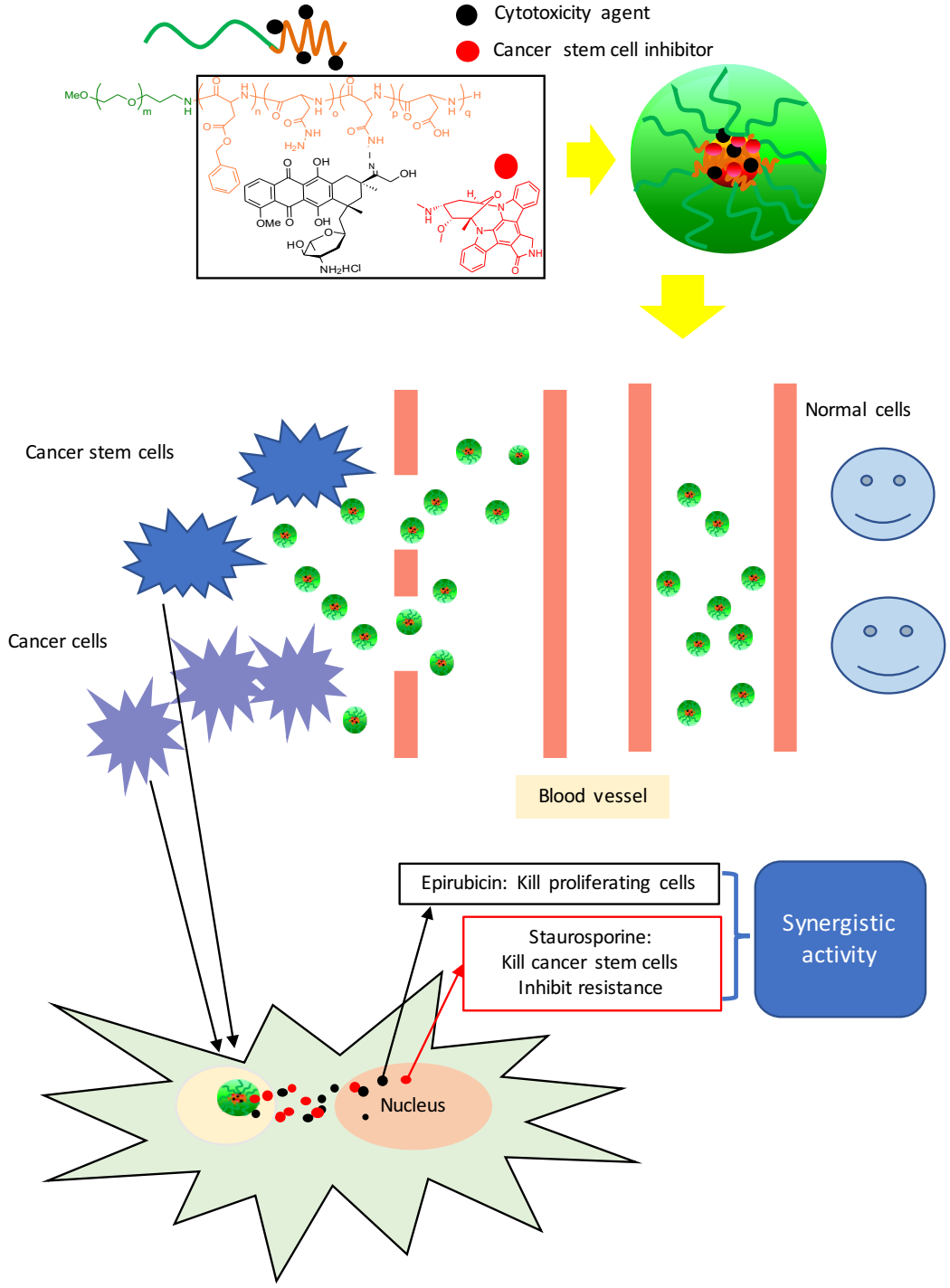

Figure 6. Concept of dual drug loaded micelles for eradicating both cancer cells and CSCs.

3.2 Outline of this dissertation

This dissertation consists of six chapters. **Chapter 1** and **Chapter 6** are the general introduction and the conclusion, respectively.

In **Chapter 2**, the preparation method in aqueous condition of a series of candidate drugs, as well as STS and EPI co-loaded micelles formed from PEG-b-poly (aspartate-hydrazide-EPI) copolymers, and the size and size distribution, as well as drug loading concentrations are reported. The results in this chapter indicate the uniqueness of combining STS and EPI through their enhanced molecular affinity (27).

Subsequently, in **Chapter 3**, I describe the optimization of STS/EPI/m, their drug loading efficiency and their size distribution after preparation in methanol or DMF. The developed STS/EPI/m presented 50-nm after optimization, which is comparable to the original EPI/m. Moreover, the release rate of the micelles was also evaluated

In **Chapter 4**, the antitumor effects of the STS/EPI/m were analyzed *in vitro* and *in vivo* against naïve breast tumor model, as well as in an EPI-pretreated tumor model, which simulates the relapse of aggressive tumors having expanded CSC-populations after treatment with EPI occurring in the clinic. The effects of the STS/EPI/m on the CSC fraction in tumors are evaluated in detail. Moreover, the ability of the micelles to overcome drug resistance is also determined.

In **Chapter 5**, the antitumor activity against recalcitrant cancers was studied. Thus, the antitumor activity experiments against tumor models of intractable cancers, i.e. pancreatic cancer and kidney cancer, are described.

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Chapter 2.

Preparation of dual drug co-loaded micelles

Chapter 2

Preparation and optimization of co-drugs loaded micelles

Abstract

STS, cyclopamine, 7-Hydroxystaurosporine (UCN-01), reserpine, lestaurtinib (CEP-701), and midosutaurin (PKC412) were loaded into the core structure of the poly (ethylene glycol)-b-poly(aspartate-hydrazide-epirubicin) copolymer. The various multi-drug loaded micelles were synthesized in the same condition and characterized. The micelle size ranged from 40 to 80 nm with narrow distribution determined by dynamic light scattering measurement. The drug loading amounts were determined by high performance of liquid chromatography. The results showed the enhanced drug loading capability of STS/EPI/m compared to the other drugs, probably due to the superior affinity of STS to EPI.

1. Introduction

Combination therapy is more fashionable for the treatment of cancer owing to synergistic anticancer effects produced by this kind of treatments. Synergistic effects have the advantage of reducing toxicity of individual drug and fighting against multi-drug resistance by cooperative action and different mechanisms.

Anthracyclines, red aromatic polyketides and present various forms according to the different aglycone and attached sugar residues, are anticancer drugs derived from Streptomyces and established in the 1960s (1, 2). Epirubicin, one of the most popular anthracycline antitumor drugs (Figure 1), has been extensively used for the therapy of many human cancers (3, 4). Epirubicin presents ~33% lower cardiotoxicity

than doxorubicin but the anthracyclines have severe dose-limiting cardiotoxicity, it remains a serious issue for clinical usage (5-7). Nowadays, it still remains unsolvable to inhibit or reduces this cardiotoxicity caused problem by epirubicin. Based on this situation, epirubicin loaded micelle of NC-6300 was established. In micelles, epirubicin is covalently conjugated to polyethyleneglycol–poly (aspartiate) block copolymer according to hydrazone bond (Figure 2A) which is super active at low pH value condition. The conjugate of poly (ethylene glycol)-b- poly (aspartate-hydrazide-epirubicin) copolymer in water is spontaneously gathering into a polymeric micellar formation. NC-6300, average diameter of 40–70 nano-meters demonstrated better ability of inhibition against Hep3B s.c. tumors than free drug of epirubicin. Moreover, NC-6300 also showed an obviously longer lasting survival against the liver cancers which was confirmed in vivo by establishing an orthotopic tumor mouse model of Hep3B than mice with the treatment of free epirubicin (6).

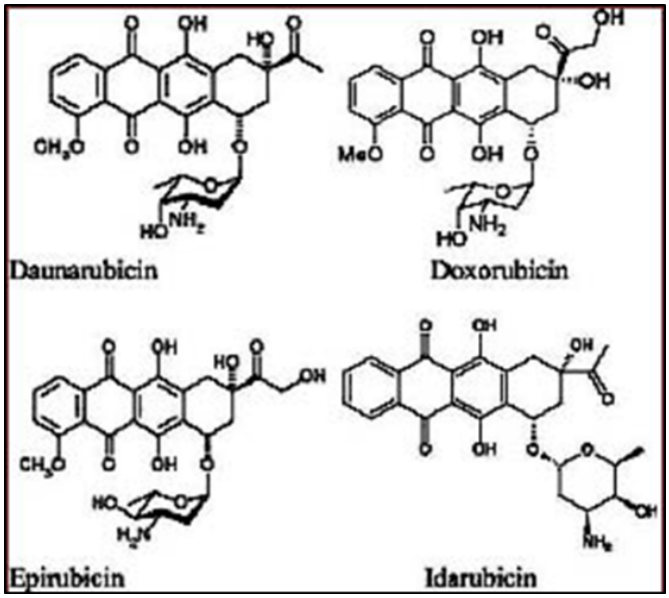


Figure 1. Chemical structure of Anthracyclines.

The hydrazine bond, a linker between copolymers and epirubicin, holds the characteristics of completely degradation within 200 min in pH 5.5, 10 times faster than in pH 7.5. In the NC6300 micelles, PEG is 12 kDa (weight-average molecular weight), consistent to the value of m = 272. There are 40 Asp residues, consistent to the sum of n, o, p and q = 40. In each NC-6300 micelles, there is about 8 epirubicin (EPI) molecules and 20 Bn groups, corresponding to the value of P = 8 and n = 20, respectively.

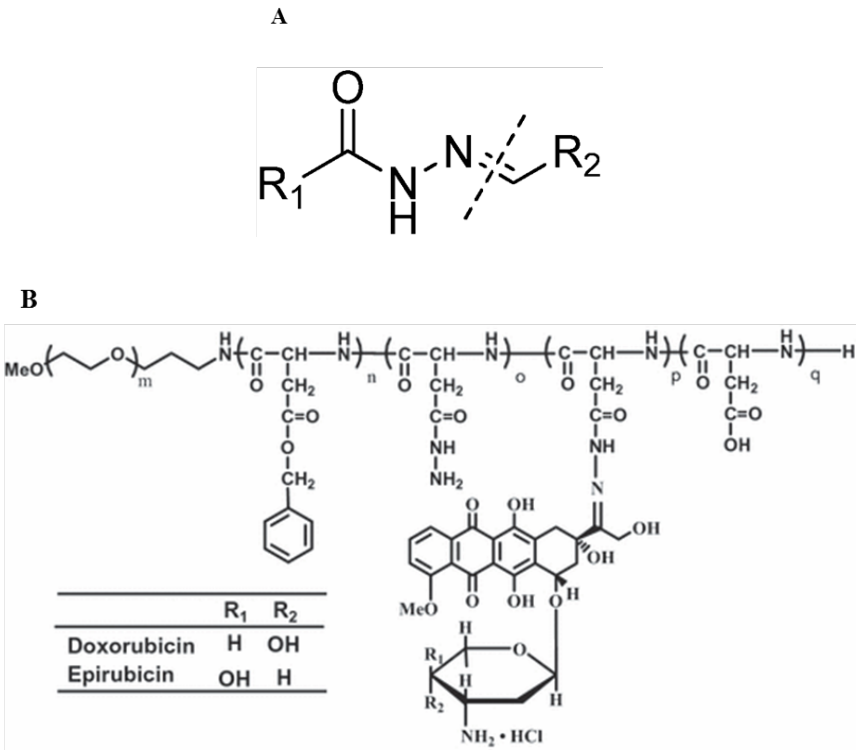


Figure 2. Chemical structure of hydrazine linker (A). Chemical structure of NC-6300 (B) (6).

Epirubicin conjugated polymeric micelles show improved antitumor activity and decreased adverse effects (8). Although chemotherapy eradicates most cells in the tumor tissue, tumor stem cells are still existing, which may be one of the main causes

of drug resistance. For instance, the ATP-binding cassette (ABC) drug transporters have been working on protection of cancer stem cells from chemotherapeutic treatment.

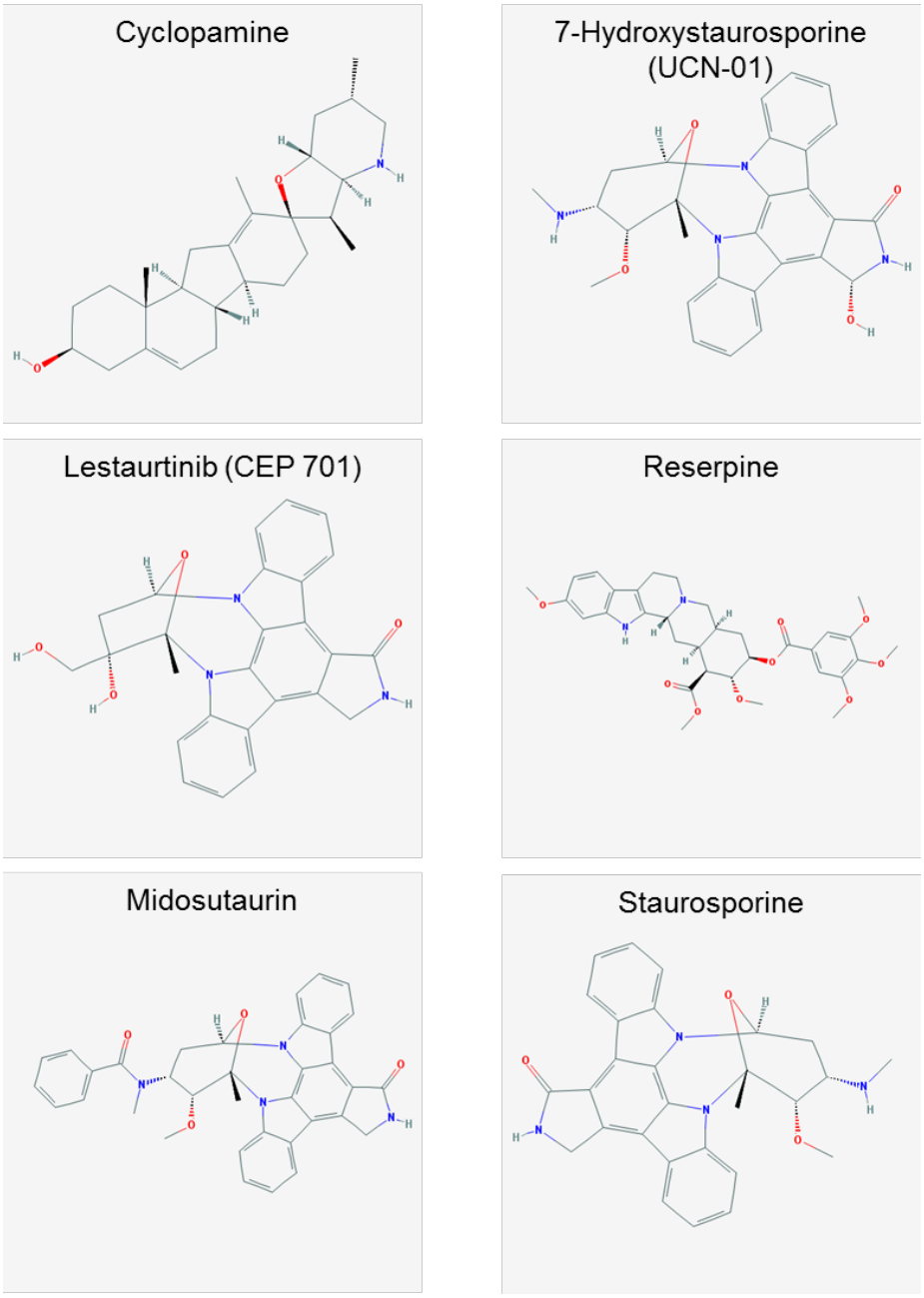


Figure 3. The chemical structures of candidate drugs for combination drug delivery in one delivery platform. The oxygen bonds and nitrogen bonds are highlighted in red and blue, respectively.

To further improve the antitumor effect of Epirubicin conjugated polymeric micelles, a series of toxic compounds (figure 3) including 7-Hydroxystaurosporine (UCN-01), Reserpine, Lestaurtinib (CEP-701), and Midosutaurin (PKC412), they share the similarity of structure and functions of STS, were added into the core structure of polymeric micelles in aqueous milieu as candidates from dual drug co-loaded micelles.

2. Experiment

2.1 Materials

Staurosporine and Cyclopamine were purchased from Funakoshi Co. (Tokyo, Japan). 7-Hydroxystaurosporine (UCN-01), Reserpine, Lestaurtinib (CEP-701), and Midosutaurin (PKC412) were bought from Sigma-Aldrich (St. Louis, MO, USA). 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, 0.22μm) were purchased from Millipore Co. (Massachusetts, USA). Blocking One Buffer (Tokyo, Japan).

2.2 Preparation of STS/EPI/micelles in methanol

The pH-sensitive Epi/m were prepared from PEG-b-poly (aspartate-hydrazide-epirubicin) (10 mg) (Mw of PEG = 12 000 Da; poly (aspartate) units = 40; Epi units = 8). The block copolymer, or the block copolymer plus STS, UCN-01, Reserpine, Lestaurtinib, and Midosutaurin (PKC412) (Table 1), was dissolved in MeOH (20 mL), respectively, shed away from light, and stirred for half of an hour around 30 Celsius degree. Then, the solvent was evaporated by using a rotatory evaporator, followed by addition of HEPES buffer with neutral pH value into the flask containing the thin film-formed dried sample, and then mixing and stirring for a whole night. The PEG-P (Asp-Epi)/STS micelles were prepared in a similar manner from PEG-b-poly (aspartate-amide-epirubicin) copolymer (Mw of PEG = 12 000 Da; poly (aspartate) units = 40; Epi units = 8) plus STS (Table 1).

2.3 Purification and size determination of STS/EPI/micelles

Micelles were then cleaned by filtration membrane (molecular weight cutoff (MWCO) = 30,000 Da) and concentrated to around 10 mL. Finally, the micelles were filtered by using a PES filter (0.22 μm). Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used for size distribution determination of the STS/EPI/micelles by dynamic light scattering (DLS) at 25 Celsius degree at the wavelength of 532 or 633 nano-meters as incident beam.

2.4 Drug concentration analysis of STS and EPI in STS/EPI/m

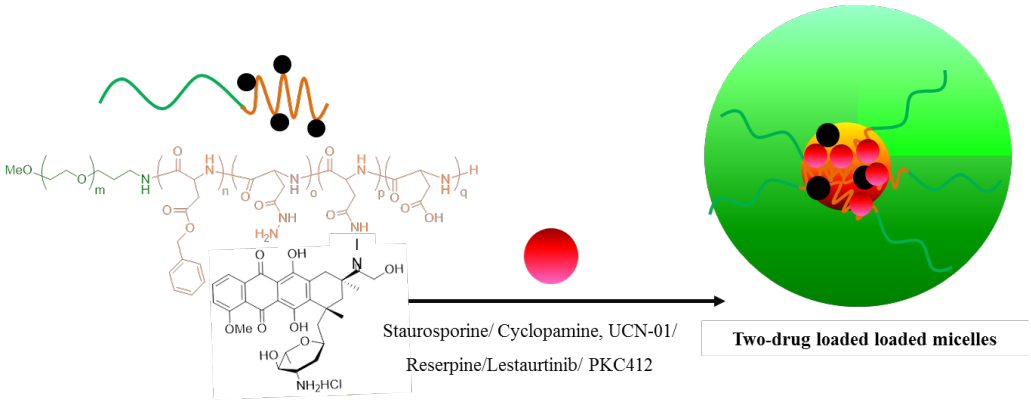
The concentration of the drugs inside STS/Epi co-loading micelles was determined by Liquid Chromatography (column: Tosoh with TSK gel 80-TM; flow

rate = 0.8 mL/min; temperature = 40 °C; mobile phase was 2:3 vol/vol mixture of 1 mM formic acid and methanol) by using a UV detector at 254 nm for Epi and 290 nm for STS.

3. Results

3.1 Synthesis of co-delivery of two drugs micelles

Two-drug encapsulated micelles synthesized with PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer and a series of drugs including Staurosporine, Cyclopamine, UCN-01, Reserpine, Lestaurtinib, and Midosutaurin (PKC412) (Scheme 1). The PEG-P(Asp-Epi)/STS micelles were prepared in a similar manner from PEG-b-poly(aspartate-amide-epirubicin) copolymer (Mw of PEG = 12 000 Da; poly(aspartate) units = 40; Epi units = 8) plus STS. The PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer and candidate drugs were dissolved in methanol (table 1) and the size of micelles was measured by dynamic light scattering (table 2). The loaded drug concentrations of each two-drug loaded micelles were determined (table 3) by using HPLC.



PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer + Candidate loading drug → Two drugs loaded micelles

Scheme 1. Preparation scheme of two-drug loaded micelles including EPI/STS loaded micelles, EPI/CEP-701(Lestaurtinib) loaded micelles, EPI/Cyclopamine loaded micelles, EPI/Reserpine loaded micelles, EPI/PKC-412 loaded micelles.

Table 1. Concentrations of PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer and drugs for preparation of co-delivery of two drugs micelles.

Micelles	Concentrations of drugs dissovled in methanol (mg)		Volume (ml)
EPI/Stau 1	EPI	0.5	20
	STS	1	2
PEG-P(Asp-Epi)/Stau	EPI	0.5	20
	STS	1	2
EPI/CEP-701(Lestaurtinib)	EPI	1	10
	CEP-701	1	2
EPI/Cyclopamine	EPI	1	10
	Cyclopamine	1	2
EPI/Reserpine	EPI	1	10
	Reserpine	0.5	10
EPI/PKC-412	EPI	1	10
	PKC-412	1	2

The pH-sensitive Epi/m were prepared. The block copolymer, or the block copolymer plus STS, UCN-01, Reserpine, Lestaurtinib, and Midosutaurin (PKC412), was dissolved in MeOH (20 mL), respectively.

3.2 Characterization of co-delivery of two drugs micelles

Average size and PDI of prepared micelles was measured by dynamic light scattering (DLS) (Figure 1).

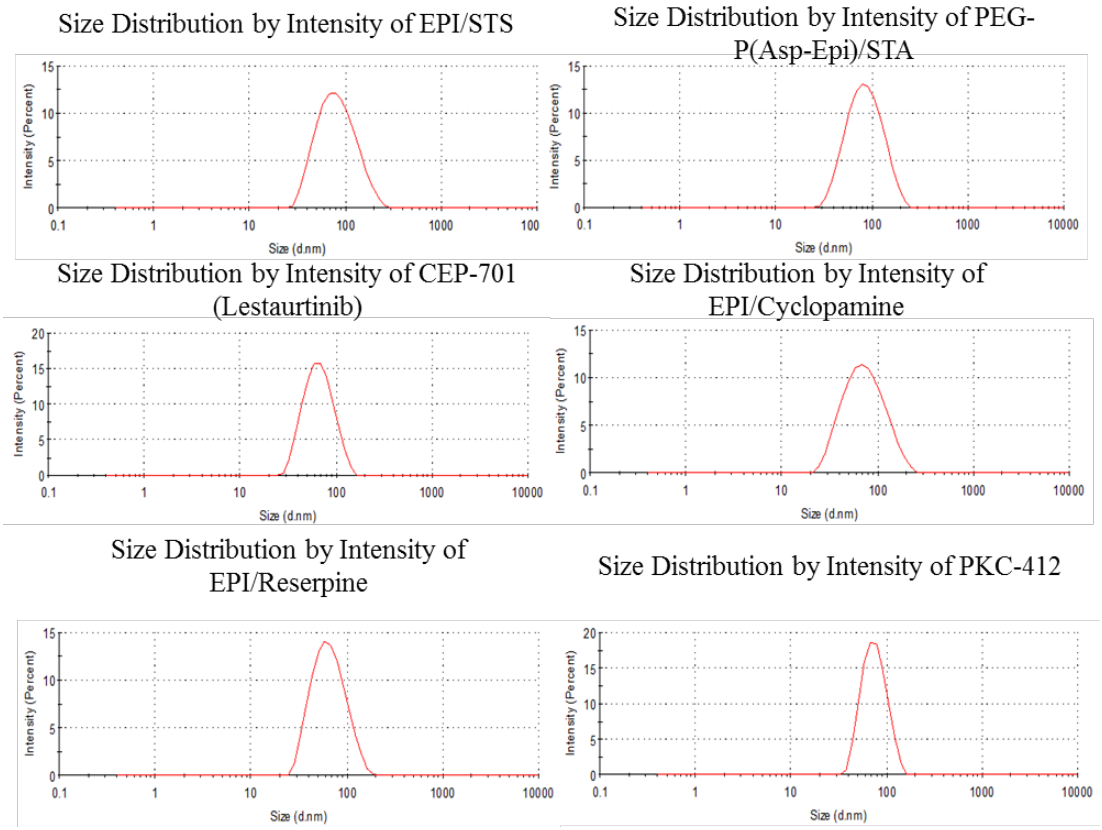


Figure 1. The size distribution of the STS/EPI/micelles. Data represent the means ± SE.

Table 2. Size of various two-drug loaded micelles

Micelles	Z-Average (d.nm)	PDI
EPI/STS	63	0.13
PEG-P(Asp-Epi)/STS	76	0.13
EPI/CEP-701(Lestaurtinib)	61	0.11
EPI/Cyclopamine	65	0.17
EPI/Reserpine	60	0.13
EPI/PKC-412	71	0.14

The average size of a series of two-drug loaded micelles was range from 60-80 nm. These micelles have narrow size distribution. The small size less than 100 nm could facilitate the cell uptake. Data represent the means ± SE.

3.3 Quantification of STS and EPI in STS/EPI/micelle

The concentration of the drugs inside STS/Epi co-loading micelles was determined by High Performance Liquid Chromatography (HPLC). The anount of various drugs loaded inside of core structure of EPI/m were determined (table 3).

Micelles	Concentrations of drugs in micelles	
EPI/STS	EPI	1.38mg/ml
	STS	0.56mg/ml
PEG-P(Asp-Epi)/STS	EPI	-
	STS	0.11mg/ml
EPI/CEP-701(Lestaurtinib)	EPI	0.15mg/ml
	CEP-701	0.006mg/ml
EPI/Cyclopamine	EPI	-
	Cyclopamine	-
EPI/Reserpine	EPI	0.003mg/ml
	Reserpine	0.12mg/ml
EPI/PKC-412	EPI	0.008mg/ml
	PKC-412	0.1mg/ml

Table 3. Concentrations of various drugs loaded inside of the core of co-delivery of two drugs micelles. Data represent the means ± SE.

4. Discussion

Drug combinations have been very successful in the treatment of cancer, and they continue to be widely researched in preclinical and clinical studies with expanding focus on targeting aberrant signaling pathways in solid tumors. The NC6300 micelles have been on the clinical phase one trial. This micelle successfully reduced the cardiotoxic and improved the treatment efficacy. Moreover, with EPI loaded in the core structure of the micelles, they would have lower cardiotoxicity and neurotoxicity than the corresponding free drugs. The combination of micelles was closely connected with high efficacy and favorable toxicities in the tumor models of human gastric, and

undertakes the conduct of clinical trials. Taken together, it is quite promising for developing multidrug delivery platforms to overcome a series of unsolved issues of free anticancer drug and agent on the therapies of malignancies.

5. Conclusion

A series of dual drug carried micelles established with PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer presented a size range around 40-80 nm, which is considered to be tiny enough to achieve elongated blood circulation avoiding caught by the RES and to overcome the permeation barrier of poorly permeable tumors. The combination of two free drugs encapsulated in a single core structure of micelles allow the two drugs get delivered and released synchronized in cells to take synergistic effect against both cancer cells and CSCs. These dual drug loaded micelles therapy after further optimization could provide a promising strategy for the construction of multidrug delivery system for enhancing cancer.

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Chapter 3.

Optimization of STS/EPI/m

Chapter 3. Optimization of STS/EPI/m

Abstract

We conceived a nanomedicine strategy capable of coordinating the interaction of cytotoxic drugs and CSC inhibitors within confined inner compartments to take function as an effective method for improving antitumor effects, inhibiting drug resistance and killing CSCs. Thus, STS got loaded into the core of previously developed EPI/m. The STS/EPI/m showed triggered release of both drugs at endosomal pH, which allowed intracellular coordinated and cooperative drug interactions. In this chapter, the STS/EPI co-loaded micelles were prepared in two different solvent, methanol and DMF, to further study the loading profile of this dual drug loaded micelles.

1 Introduction

Nanomedicines capable of manage over drug properties and controlling the intracellular interact effect of multi-targeted drug treatments could attain synergistic efficacies capable of eradicating both cancer cells and CSCs. Herein, we established a nanomedicine strategy for achieving coordinated effects of drugs within tumor cells by engineering the molecular interaction between therapeutics within inner compartments of nanomedicines.

With CSC sub-populations in tumors take main responsibility for resistance to therapies and tumor recurrence, institution of novel therapeutic strategies is vital for achieving significant responses capable of long-term patient survival (1-2).

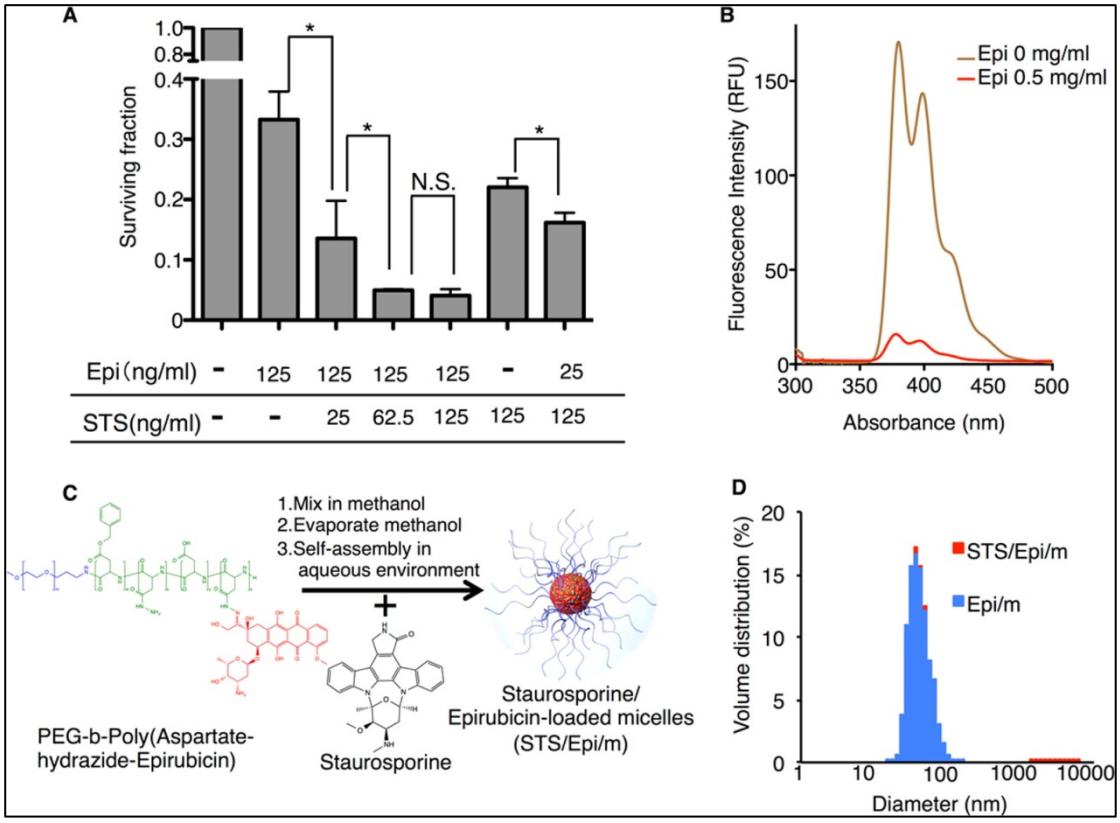

Figure 1. Staurosporine interaction with epirubicin facilitates its loading in Epi-loaded micelles (Epi/m) for synergistic efficacy. (A) Effect of the addition of STS on the cytotoxicity of Epi against MSTO-211H cells. Data are expressed as the mean ± SD (n = 3); *p < 0.01. (B) Quenching of the fluorescence of STS in methanol by addition of Epi (brown line, without Epi; red line, with 0.5 mg/mL Epi). (C) Scheme of the preparation of STS/Epi-loaded micelles. (D) Size distribution of Epi/m and STS/Epi/m by volume determined by dynamic light scattering. Blue histogram, Epi/m; red histogram, STS/Epi/m (3).

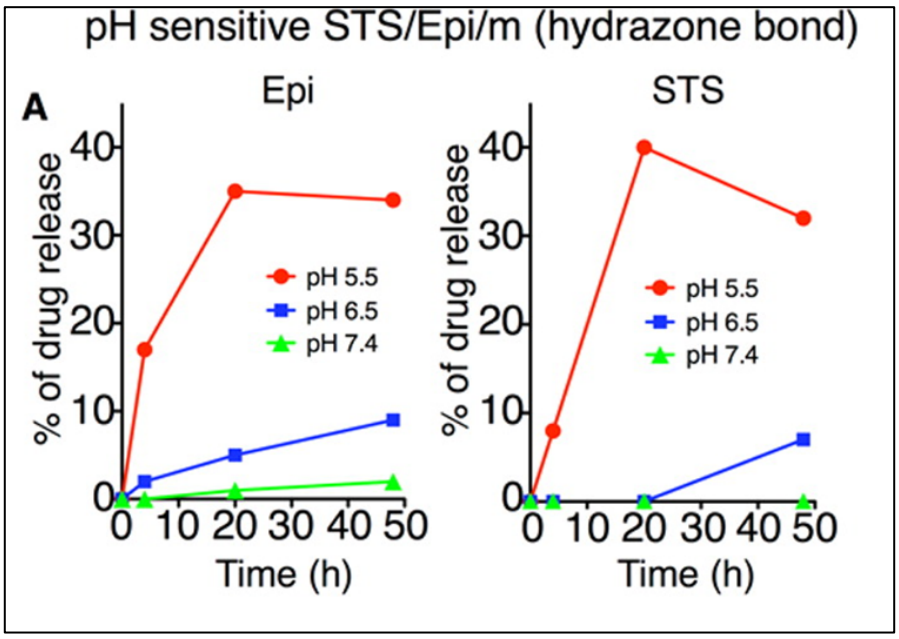

Figure 2. Effect of the pH-sensitive drug release of STS/Epi/m on their activity. (A) Drug release rate from the micelles under pH conditions of the bloodstream (pH 7.4), intratumoral space (pH 6.5), and endosomes (pH 5.5) (3).

Nanomedicine is promising for developing unprecedented therapies by controlling drug activities in a spatiotemporal manner. Herein, we conceived a nanomedicine strategy capable of coordinating the interaction of cytotoxic drugs and CSC inhibitors within confined inner compartments of nanomedicines designed to gain synchronized and synergistic drug co-operations within tumor cells, in ways of enhancing antitumor effects, overcoming drug resistance and eradicating CSCs. Thus, STS, which has been identified as a potent inhibitor of CSC with synergistic efficacy in combination with cytotoxic EPI, was loaded into the core of previously developed EPI/m. The STS/EPI/m showed triggered release of both drugs at endosomal pH,

which allowed intracellular coordinated and cooperative drug interactions (Figure1, 2) (3).

2. Experiment

2.1 Materials

Staurosporine 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, 0.22μm) were purchased from Millipore Co. (Massachusetts, USA). Blocking One Buffer (Tokyo, Japan).

2.2 Preparation of STS/EPI/micelles in methanol

The pH-sensitive Epi/m were established by using PEG-b-poly (aspartate-hydrazide-epirubicin) copolymer (10 mg) (Mw of PEG = 12 000 Da; poly (aspartate) units = 40; Epi units = 8). The the block copolymer plus STS was dissolved in methanol at different drug weight ratios of EPI: STS, shed away from light, and stirred for 30 min at room temperature. Methanol was evaporated from mixture to form a thin film at the surface of flask. Then, 10 ml of HEPES buffer was added in the flask and sonicated for 15 min. Micelles encapsulated with both EPI and STS were prepared

with different drug ratios. Micelle solution was collected and purified by ultrafiltration (molecular weight cut-off (MWCO: 100,000)) at 2,000 rpm for 5 times. The purified solution was filtered and the size (diameter, nm) and PDI of micelles were determined using Zetasizer.

2.3 Preparation of STS/EPI/micelles in DMF

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) and STS in DMF at different drug weight ratios of EPI: STS (16:1, 5:1, 4:1, 2.5:1, 0.8:1) 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 flask bottom, followed by addition of 10 mL buffer into the flask containing the dried sample. The mixture was sonicated 30 min. The resulting micelles were then purified by ultrafiltration (MWCO = 30,000 Da). Finally, the micelles were filtered by using a PES filter (0.22 μm). The size of the micelles was determined after purification.

2.4 Purification and size determination of STS/EPI co-loaded micelles

Micelles were then purified by ultrafiltration (molecular weight cutoff (MWCO) = 30,000 Da) and concentrated to a total volume of 10 mL. Finally, the micelles were filtered by using a PES filter (0.22 μm). Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used for size distribution determination of the STS/EPI/micelles at 25 °C at the wavelength of 532 or 633 nm as incident beam.

2.5 Drug concentration analysis of STS and EPI in STS/EPI/m

The concentration of the drugs inside STS/Epi co-loading micelles was determined by High Performance Liquid Chromatography (HPLC) (column: Tosoh with TSK gel 80-TM; temperature = 40 °C; flow rate = 0.8 mL/min; Elution was performed with a mobile phase composed of mobile phase = 2:3 vol/vol mixture of 1 mM formic acid and methanol) by using a UV detector at 254 nm for Epi and 290 nm for STS.

3. Results

3.1 Characterization of STS/EPI co-loaded micelles prepared in methanol

The STS/EPI co-loaded micelles were prepared in methanol at different drug weight ratios of EPI: STS. The various feeding amounts of STS were added to EPI-conjugated polymers dissolving in methanol (table 1).

Table 1. Initial drug solution and drug ratios of EPI/STS, EPI was dissolved in methanol, and then STS methanol solution (1mg/ml) was added following different drug ratios of EPI/STS, and the initial total volume was 5ml. Data represent the means ± SE.

Sample No.	Epirubicin (umol)	Staurosporine (umol)	Total Volume (ml)	Drug ratio of EPI/STS
A	3.4 (2mg)	0.11 (0.05mg)	5	31
B	3.4 (2mg)	0.21 (0.1mg)	5	16
C	3.4 (2mg)	0.42 (0.2mg)	5	8
D	3.4 (2mg)	0.85 (0.4mg)	5	4
E	2 (1.2mg)	0.8(0.4mg)	5	2.5
F	2 (1.2mg)	2 (0.93mg)	5	1
G	3.4 (2mg)	4.29 (2.0mg)	5	0.8
H	3.4 (2mg)	4.72 (2.2mg)	5	0.7
I	3.4 (2mg)	5.36 (2.5mg)	5	0.6

After evaporation of methanol, the mixture was dissolved in HEPES buffer and sonicated for 30 minutes, then the micelles solution was collected and purified. The various loading drug amount was determined by using HPLC (table 2).

Table 2. Concentrations of various drugs loaded inside of the core of STS/EPI loaded micelles. Data represent the means ± SE.

Sample No.	Epirubicin (umol)	Yield of EPI (%)	Staurosporine (umol)	Yield of STS (%)	Drug ratio of EPI /STS	Total Volume (ml)	PDI	Size (nm)
A	1.5	44.1	0.021	19.1	69	0.88	0.19	76
B	1.4	41.2	0.055	26.2	26	0.23	0.16	47
C	1.2	35.3	0.055	13.1	22	0.75	0.16	68
D	1.3	38.2	0.14	16.5	9	0.62	0.19	73
E	0.7	35	0.1	12.5	7.2	0.5	0.19	80
F	1.1	55	0.52	26	2.6	0.5	0.17	66
G	1.4	41.2	1.13	26.3	1.3	0.6	0.19	84
H	1.5	44.1	1.19	25.2	1.2	0.8	0.19	84
I	1.9	55.9	2	37.3	0.96	0.8	0.28	112

Yield of EPI = Encapsulated EPI / Initial EPI*100%

Yield of STS = Encapsulated STS / Initial STS*100%

The initial amount of STS vs. the final drug ratio of EPI/STS, the results indicated that almost all the feeding amount of STS was loaded inside of the core structure of EPI-conjugated polymer in methanol if the original feeding drug ratio of EPI:STS was less than 1 (Figure 3).

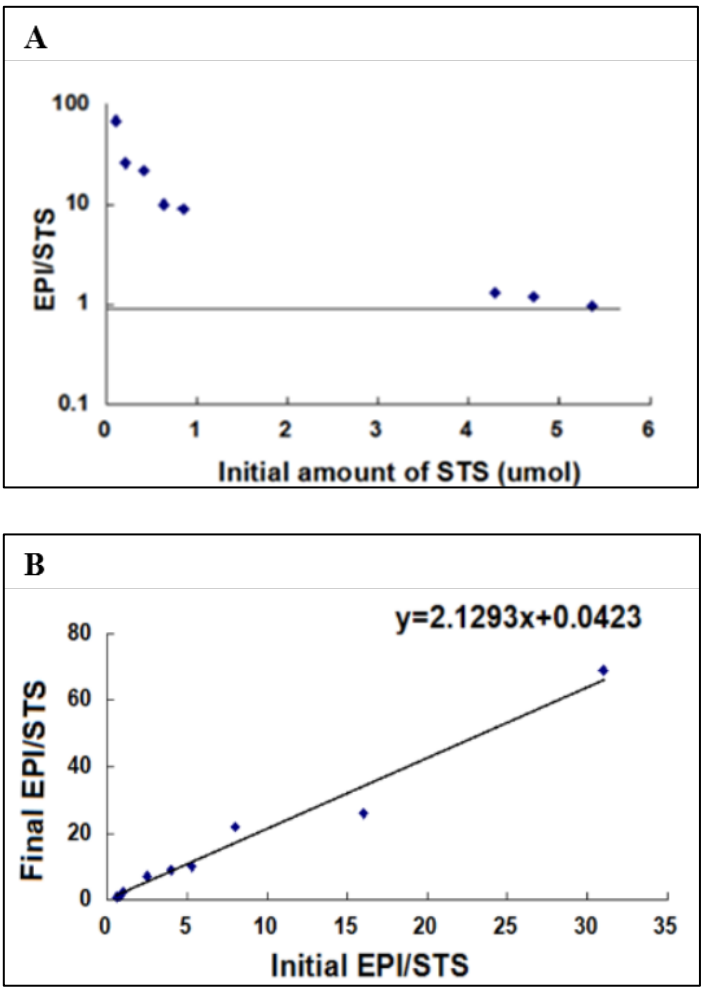

Figure 3. A. The initial amount of STS vs. the final drug ratio of EPI/STS. B. The initial drug ratio of EPI/STS vs. the final drug ratio of EPI/STS. Data represent the means ± SE.

The initial drug ratio of EPI/STS vs. the final drug ratio of EPI/STS. These results demonstrate that final drug ratio of EPI to STS around 1:1 encapsulated in the same drug carrier system can be successfully achieved (Figure 3-A).

3.2 Characterization of STS/EPI co-loaded micelles prepared in DMF

The STS/EPI co-loaded micelles prepared in DMF followed the same preparation pressure as preparation in methanol. The original drug ratio of EPI: STS was shown as Figure 4. The characterization of these micelles with different loading amount of STS was conducted. The size and PDI of STS/EPI/m were determined by DLS, the size was increasing with the adding amount of STS increased (Table 3).

Figure 4. 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) and STS in DMF at different drug weight ratios of EPI: STS (16:1, 5:1, 4:1, 2.5:1, 0.8:1)

Table 3. The original and final drug ratio of EPI: STS, the size and PDI of STS/EPI/m.

STS/Epi feeding ratio in weight	STS/Epi loading ratio in the micelles in weight[a]	Diameter (nm)[b]	PDI
0.06	0.06	48	0.1
0.2	0.2	53	0.1
0.3	0.3	77	0.2
0.5	0.4	78	0.2
1.25	1	119	0.2

[a] Z-averaged hydrodynamic diameter measured by dynamic light scattering (DLS) at 25ºC.

[b] Polydispersity index (PDI) measured by DLS.

The release Profile of STS/EPI/m made from drug ratio of EPI: STS. The STS/Epi/m were incubated under pH conditions of the bloodstream (pH 7.4), intratumoral space (pH 6.5), and endosomes (pH 5.5), and the concentration of released drugs was determined by method of liquid chromatography of high-performance in short as HPLC.

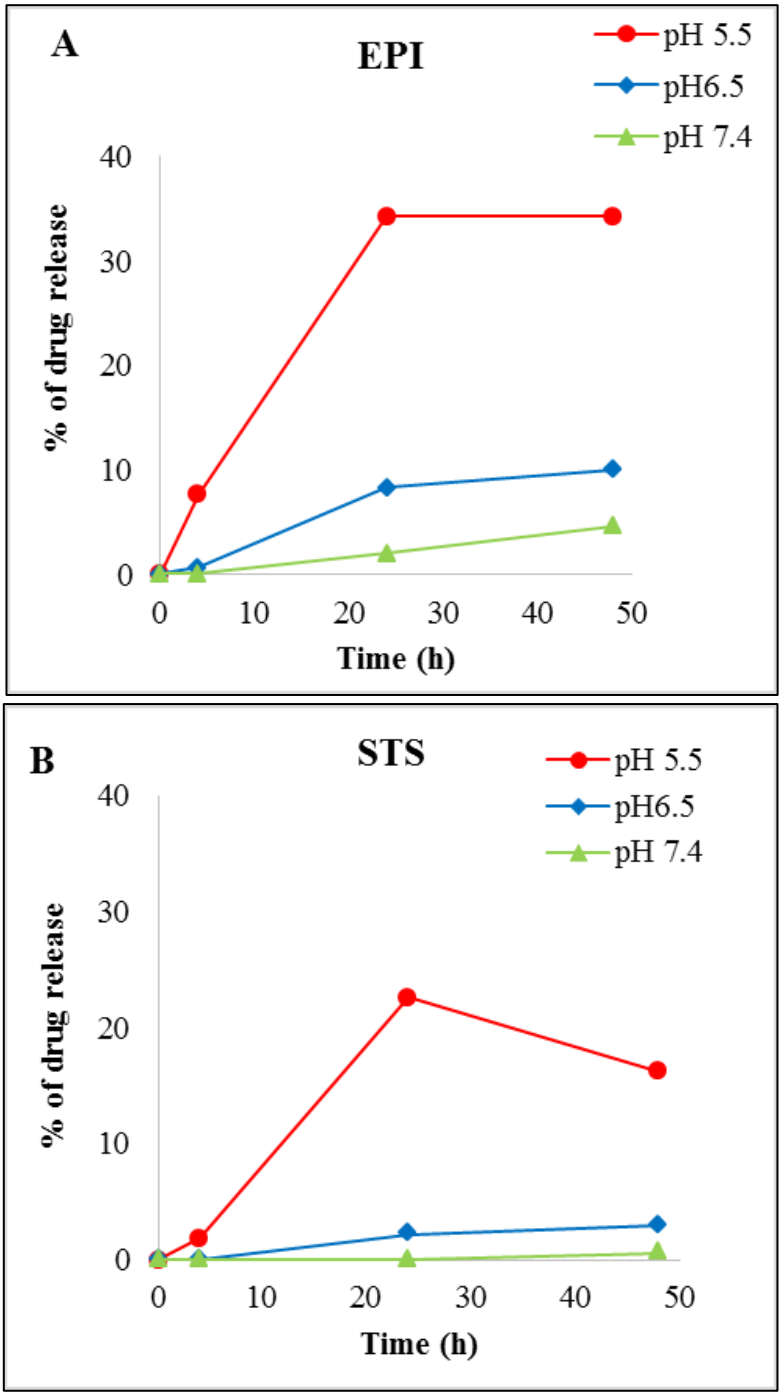

Figure 5. Release profiles of STS/EPI co-loaded micelles prepared in DMF at pH7.4 (green triangle), pH6.5 (blue diamond), and pH5.5 (red dot). Data are expressed as the mean ± SD.

This result was consistent with our group published study about this dual drug loaded micelles. Kinoh et al. showed the release profile of STS/EPI/m prepared in methanol, in agreement with the pH-sensitive characteristics. The STS/Epi/m did not release the drug at pH 7.4, which could avoid drug leakage from the micelles during circulation in the body, while the Epi release was increased as the pH decreased (Figure 5A). Interestingly, STS followed the pH-sensitive release profiles of Epi from STS/Epi/m (Figure 5B), which may be related with the drug interaction.

4. Discussion

Corresponding multi-drug delivery may be realized by the physical loading of an anticancer agent in a polymeric micelle in a first step and of a second anticancer agent in a second polymeric micelle in a second step, followed by mixing and concurrent drug delivery (4). Alternatively, multi-drug-loaded polymeric micelles are viable. In the preparation of STS/EPI co-loaded micelles preparation, the EPI conjugated to the polymeric micelles chain by hydrazine bond. The free drug of STS is not water-soluble. To get free drug of STS loaded inside of the EPI polymers, we chose methanol and DMF as solvent. Nanomedicines capable of broad distribution in tumor tissues could further ease reaching CSCs. In this way, the 50nm diameter of STS/EPi/m is a substantial advantage for achieving effective targeting, as our group has been working on the effect of the size of polymeric micelles which is essential for their extravasation and deep penetration within tumors (5). The micelles prepared in DMF have smaller average size compared with STS/EPI/m prepared in methanol. Poorly water-soluble anticancer agents can be loaded chemically or physically into

polymeric micelles for concurrent multi-drug delivery (6). Clinically, many poorly water-soluble anticancer agents are infused sequentially or are orally administered and separately infused. In The STS/EPI/m preparation, the EPI polymeric micelles and free STS were dissolved together. By adding a series of the initial feed ratio, we found that the final loading drug ratios of EPI: STS was around 1, it is may depend on drug interaction between STS and EPI. Concurrent delivery by polymeric micelles simplifies the task of multi-drug delivery, improves safety, and may give permission of anticancer agents to take function at solid tumors at the same time, aiming for synergistic drug interaction (7,8). The STS/EPI/m shared the same release profile and both STS/EPI/m were released in the stimuli- /environment-dependent release, no matter this dual drug loaded micelles prepared from methanol or DMF.

5. Conclusion

The STS/EPI/m was prepared successfully in methanol and DMF. The micelles achieved small size and narrow size distribution, which could facilitate the micelles uptake in cells and tumor tissues for further enhancement of the synergistic effects. The STS/EPI/m shared synchronized release profile of the drugs at endosomal pH for functioning as both toxic agent against cancer cells and CSCs inhibitor, which may further improve treatment effect on cancers. Because of micelles with drug ratio of 0.2 (STS/EPI) have the same size as EPI/m, they were chosen for further biological studies in the next chapters. The findings in this chapter indicate that the interaction of STS with EPI was key for achieving the incorporation of the drug in the core of the micelles.

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Chapter 4.

Antitumor Activity of STS/EPI/m against Breast Cancer

Chapter 4. Antitumor Activity of STS/EPI/m against Breast Cancer

Abstract

CSCs are closely correlated with drug resistance and relapse in breast cancer. Therefore, in order to properly treat such cancers, killing both CSCs and cancer cells are necessary to suppress cancer progression and to extend survival. In this section, we evaluated the efficacy of a micelle coloaded with staurosporine(STS) and epirubicin(Epi) in treating drug resistant tumors and CSC populations. Our STS/Epi micelles indicated significant treatment efficacy against naïve orthotopic 4T1-luc breast cancer tumors as well as their relapsed Epi-resistant tumors, extending overall survival significantly. We further demonstrated the treatment was successful in inhibiting the CSCs of breast cancer, specifically influencing the CD44+/CD24- fraction and aldehyde dehydrogenase positive (ALDH+) population in drug resistant tumors. From the results in this section, we demonstrate significant suppression of both naïve and drug resistant 4T1-luc tumors, with a significant extension of survival when compared to alternative methods of administering epirubicin and stuarosporine. This data demonstrates that combining staurosporine and epirubicin into a micelle can treat cancer tumors more effectively than administering these drugs separately. Furthermore, our results confirm our hypothesis of CSC suppression to enhance epirubicin efficacy and extend survival.

1. Introduction

Breast cancer is the leading cause of cancer death in women worldwide (1). After decades of research, various therapeutic strategies have been developed to treat breast cancer, including surgery, radiotherapy, immunotherapy, and chemotherapy (2). Epirubicin is currently one of the most commonly prescribed chemotherapeutic agents for treating breast cancer. Despite good therapeutic responses and rates of remission, there is a high risk of recurrence among patients who use epirubicin to treat their breast cancer (3-5). There is now mounting evidence that suggests that recurrence of breast cancer is associated with small subpopulations of cancerous stem cells that can successfully survive epirubicin treatment. These cells have the ability of self-renewal, controlled proliferation, and maintain the ability to differentiate into heterogenous cancer cells (6-7). CSCs are resistant to conventional therapies by a variety of mechanisms, which include efflux pumps to remove drugs from the cell, upregulation of DNA repair proteins, ROS scavenging, and suppressed proliferation (8-11). Together, these mechanisms prevent epirubicin from properly intercalating the cell's DNA, allowing CSCs to avoid apoptosis during treatment of epirubicin. Thus, new therapeutic approaches are needed to treat CSCs along with cancer cells to avoid recurrence, drug resistance, and to increase patient survival.

We have developed polymeric micelles with core-shell structures which are loaded with drugs to kill both cancer cells and CSCs. We loaded our micelles with staurosporine, a potent protein kinase inhibitor capable of killing CSCs, along with epirubicin, a DNA intercalator used to kill rapidly proliferating cancer cells, to create a micelle that can kill any type of malignant cell that may be present in breast cancer. In

this section, we demonstrate the antitumor activity of our coloaded micelles both in vitro and in vivo against 4T1-luc tumors and drug resistant 4T1-luc tumors.

2. Experiments

2.1 Materials and methods

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, 0.22μm) were purchased from Millipore Co. (Massachusetts, USA). RPMI 1640-cell culture medium for various cell lines culture were obtained from Sigma-Aldrich Co., Inc. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cell Proliferation Kit I, Sigma, USA) for cell viability assay were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO). Microplate Readers: Infinite M1000 PRO Microplate Readers, Tecan Group Ltd. (Männedorf, Switzerland). 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

(Tokyo, Japan). ALDEFLUOR Kit and ALDEFLUOR DEAB reagent were purchased from STEMCELL Technologies Inc. (Vancouver, Canada).

2.2 Cell line

Murine breast adenocarnicoma 4T1 cells expressing luciferase (4T1-luc), were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). For the cells culture, RPMI-1640 medium adding with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin (P/S) were used and cells during the experiment were keep at 37 °C in 5% CO2 humidified atmosphere.

2.3 Animals

Balb-c mice (female; 6week-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) and STS 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 flask bottom, followed by addition of 10 mL of buffer into the flask containing the dried sample. The mixture was sonicated 30 min. The resulting micelles were then purified by ultrafiltration (MWCO = 30,000 Da).

Finally, the micelles were filtered by using a PES filter (0.22 μm). Zetasizer Nano ZS (Malvern, UK.) was used for size determination.

2.5 Purification and size determination of STS/EPI/m

Micelles were then purified by ultrafiltration (molecular weight cutoff (MWCO) = 30,000 Da) and concentrated to a total volume of 10 mL. Finally, the micelles were filtered by using a PES filter (0.22 μm). By using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) with a laser, the size distribution of the STS/EPI/micelles was measured at 25 °C at the wavelength of 532 or 633 nm as incident beam.

2.6 Drug concentration analysis of STS and EPI in STS/EPI/m

pH-Sensitive STS/Epi/m incubating were disrupted by incubation in 1 N HCl of for 1 h at 37 °C to release both STS and Epi. The concentration of the drugs inside STS/Epi co-loading micelles was determined by High Performance Liquid Chromatography (HPLC) (column: Tosoh with TSK gel 80-TM; flow rate = 0.8 mL/min; temperature = 40 °C; mobile phase = 2:3 vol/vol mixture of 1 mM formic acid and methanol) by using a UV detector at 254 nm for Epi and 290 nm for STS. 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.

2.7 *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 cells were evaluated. Thus, 4T1 cells were grown in the RPMI-1640 medium plus 10 percent of fetal bovine serum at 37 Celsius degree in five percent of CO2 humidified atmosphere. The confluent monolayer cells were collected and evenly spread into 96 wells microplates with 5000 cells per well. The cells were cultured overnight in the incubator for cell attachment and cell recovery after trypsinization before subjected to various treatments in one hundred microliter culture medium and incubation for 48 h. Then, ten microliter Cell Proliferation Kit I solution was put into each well for 4h, 100ml of the solubilization solution was directly added to wells containing culture medium and MTT for solubilization of cells. Add 100ml of 20% SDS-Hcl was added. The intensity of final solvent was determined by using a microplate reader of Infinite M1000 PRO (Tecan, Männedorf, Switzerland) at 570 nano-meters. Wells with no cells which were regarded as the zero of the absorbance were used as a blank control, and taking cells without any treatment but with MTT and SDS-Hcl, as control. (9).

2.8 Antitumor activity evaluation against orthotopic 4T1 tumors

Syngeneic orthotopic breast tumors were prepared by inoculating 4T1-luc cells (ten to six times cells suspended in 100 μl PBS) into the mammary fat pad of Balb/c mice (Female, 18-20 g; 6week old). When tumors diameter reached 6 mm, it was ready for various treatments. Then, mice were divided into 5 groups (n = 8) randomly. Mice were injected intravenously 3 times with free Epi, Epi/m, free STS plus free Epi, free STS plus Epi/m and STS/Epi/m every 4 days, i.e. on days 0, 4 and 8. The dose of the drugs was fixed at 6 mg/kg based on Epi and 1.2 mg/kg based on STS. The tumor

volume was measured each other day and the size of tumor was calculated based on the following equation V = (Length × Width× Width)/2, where L presents the longest dimension and W is the shortest dimension. The body weight was measured to evaluate the systemic toxicities.

2.9 Immunostaining of tumor tissues

Tumor tissues were collected. The tissues were fixed in paraffin and transferred into 30% sucrose solution overnight at 4 Celsius degree. To make frozen block of tumor tissues, tumors were dried and put into block molds filled with Tissue Tek O.C.T. Then by immersing the OCT cylinder into liquid nitrogen for about 2-3 min until the liquid nitrogen not boiling, the tissues were becoming frozen blocks and stored at -80 Celsius degree for cryostat section. For immunofluorescence studies, tumor tissue slides were added with anti-44 antibody and anti-24 antibody after incubation with blocking one buffer, and then after washing, the tissue slides were added and incubated with secondary anti-rat/rabbit IgG antibodies conjugated with Alexa 647 or 488. For analysis of immunofluorescence, Images were collected by using a Zeiss LSM780 Meta confocal microscope.

2.10 *In vitro* cytotoxicity against cells from naïve tumors and Epi-treated tumors

Cells of naïve tumors or Epi-treated tumors were collected from the mice of above described models, i.e. with and without Epi pretreatment. The tumor tissues were dissociated by 0.05 percentage of collagenase at 37 Celsius degree and by pipetting the tumor debris several times. Thereafter, filtered cell suspensions were prepared by using cell strainers (40 μm) and washed three times by repeated

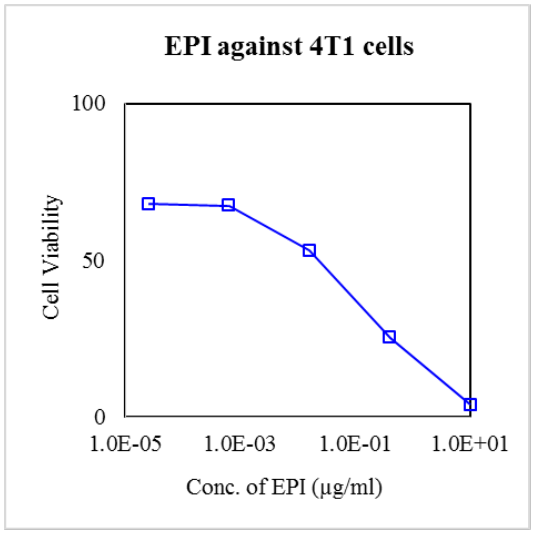
centrifugation in PBS. Cells were transferred into a flask with DMEM medium plus 10 percentage of FBS and 1 percentage of streptomycin/penicillin. The cells were cultured at 37 Celsius degree for 1 week, and their proliferation rate was confirmed to be comparable to 4T1-luc cells. Then, the cells from naïve tumors and Epi-treated tumors were transferred into 96-well plates (5000 cells/well). Twenty-four hours later, 100 μL medium containing free Epi, Epi/m, free STS plus free Epi, and STS/Epi/m were added. After 48 h incubation, the cytotoxicity was measured as described previously.

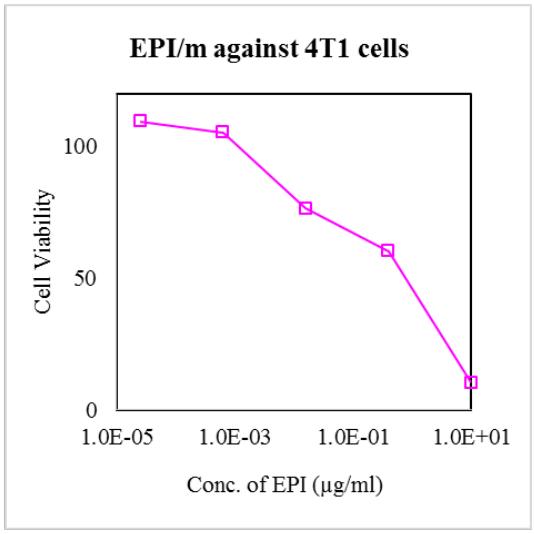
2.11 Aldehyde Dehydrogenase (ALDH) Activity Assay

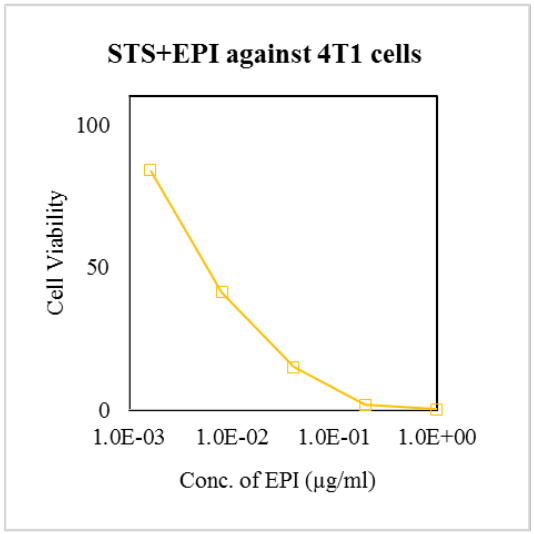
Cells of Epi-treated 4T1-luc tumor were collected as described above. The cells were seeded in six wells plates and incubated for 48h with free Epi, free Epi plus free STS, EPI/m, and STS/Epi/m at the 90% inhibitory concentration of each drug. The cells were then collected for the measurement of activity of the subpopulation with high aldehyde dehydrogenase (ALDH) by using the Aldefluor kit (StemCell Technologies, Durham, NC, USA). Accordingly, cells were dealt with the direction of the assay kit and as for negative control was prepared by adding diethylaminobenzaldehyde (DEAB) into each sample. The samples were then kept for 30 minutes at 37 Celsius degree in dark. The sorting of the ALDH+ cell subpopulations was done by using a MoFlo Astrios FACS instrument (Beckman Coulter, Brea, CA, USA).

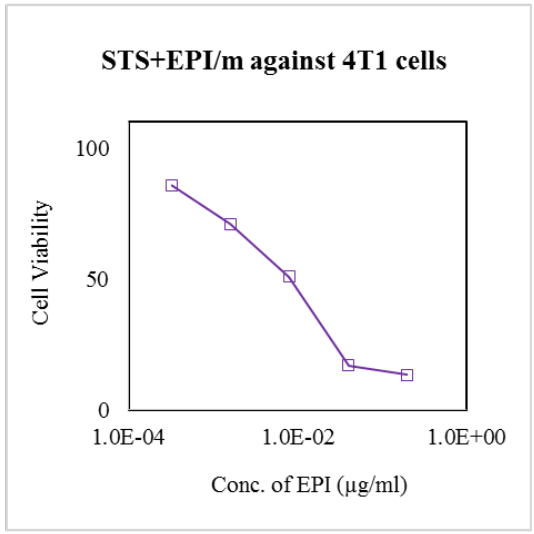
3. Results

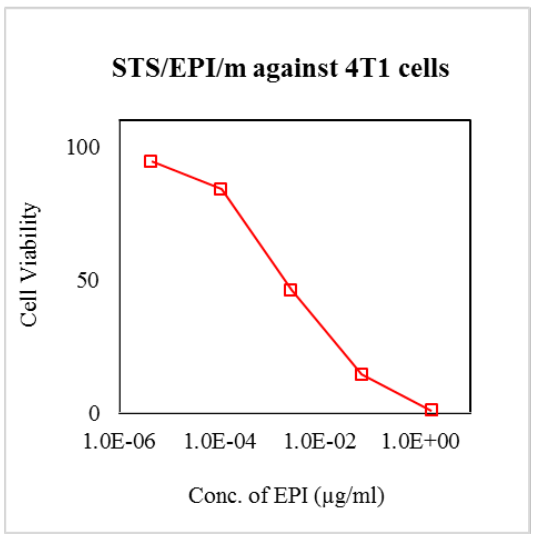
3.1 *In Vitro* Cytotoxicity of Various Treatments against 4T1-luc cells











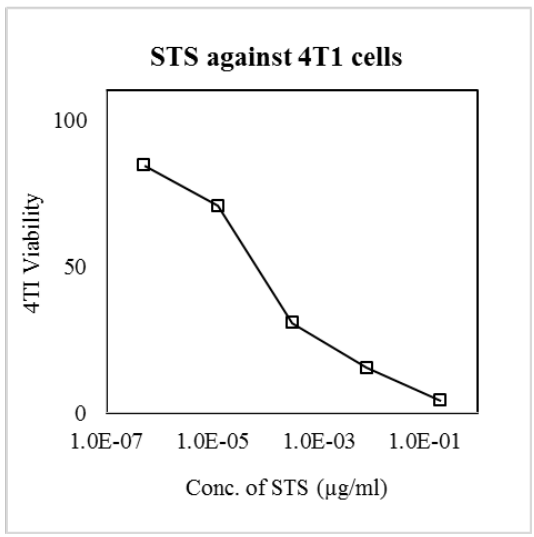


Figure 1. Cell viability for 4T1 cancer cells exposed to various treatments for 48 h, including free drug of EPI (blue), free drug of STS (black), combination of free drugs of STS plus EPI (orange), EPI/m (pink), EPI/m plus STS and STS/EPI/m (red).

In vitro cytotoxicity of STS/EPI/m against 4T1 murine breast cancer cells was studied after cells exposed to various treatments for 48 h, including free drug of EPI, free drug of STS, combination of free drugs of STS plus EPI, micelles of EPI/m, STS+EPI/m and STS/EPI/m (Figure 1).

Table 1. Fifty-percent inhibitory concentration of free Epi, free Epi plus free STS, Epi/m and STS/Epi/m against 4T1-luc cells based of Epi and STS concentrations. STS concentration was fixed at the ratio of 5:1 of Epi: STS. Data are expressed as the mean ± S.D. (n = 4).

		Epi	Epi/m	Epi+STS	Epi/m+STS	STS/Epi/m
IC_{50} (ng/ml)	Based on Epi	60 ± 7	2400 ± 300	10 ± 1	10 ± 1	2 ± 0.1
	Based on STS	-	-	2± 0.2	2± 0.2	0.4±0.02

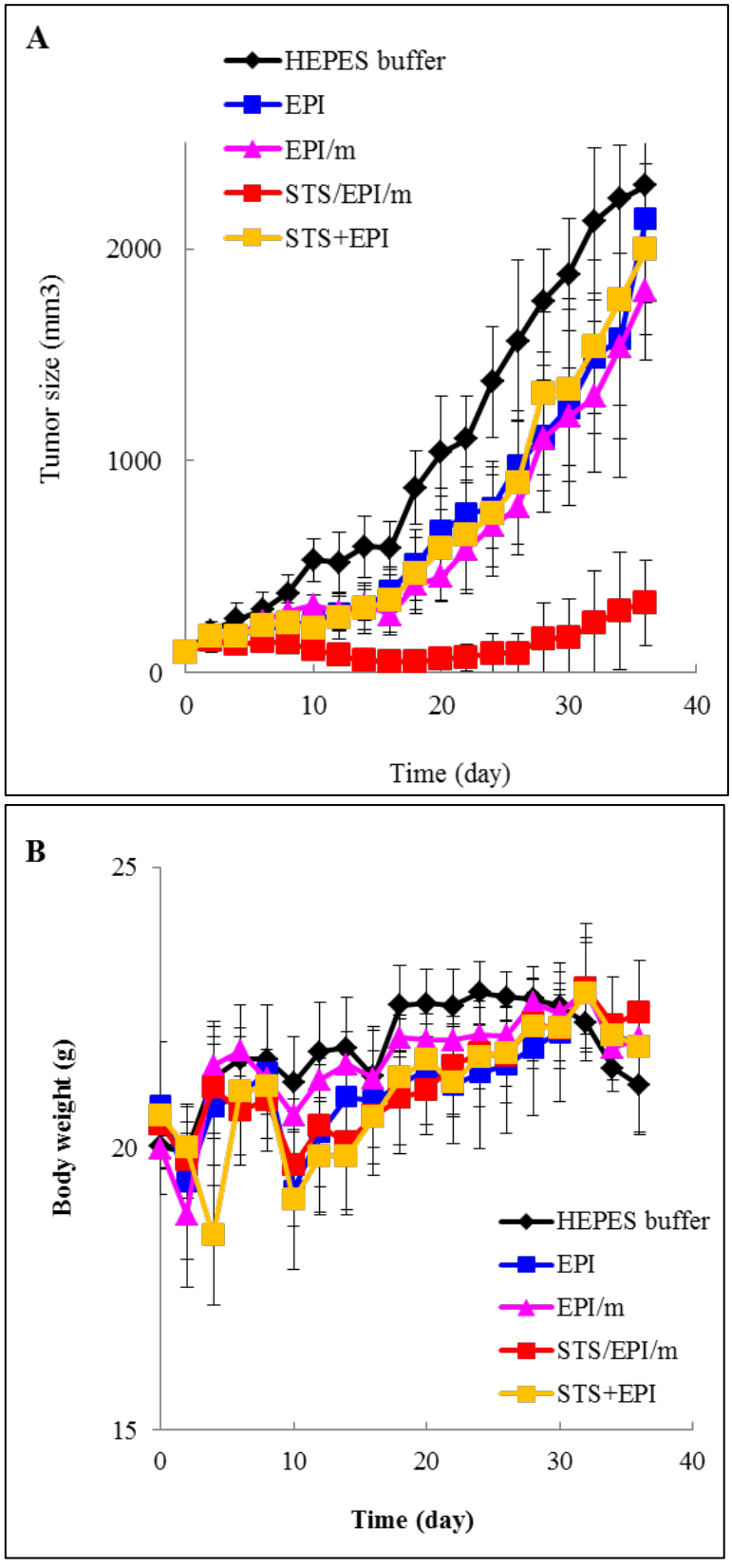
STS/Epi/m presented the great cytotoxicity against 4T1-luc cells, with an IC50 value 5-fold lower than free Epi plus STS or Epi/m plus free STS (Table 1). The enhancement of cytotoxicity could be associated with the overcoming drug resistance ability of STS to benefit EPI to take its function to eliminating cancer cells (12).

The IC50 value was calculated according to the cell viability (table 1). The different cell uptake ways of EPI and EPI/m, including diffusion through the cellular membrane

for free Epi and endocytosis for the micelles, as well as with the gradual drug release from the micelles at endosomal conditions to attain active Epi molecules, are the possible reasons of difference in the cytotoxicity between free Epi and Epi/m. The combination of free Epi plus STS was more cytotoxic than the Epi formulations alone, showing IC50 values of around 0.01 μg/ml on an Epi-basis (Table 1). These results are part because of the high potency of STS against cancer cells, as well as the synergistic efficacy of Epi with STS. Remarkably,

3.2 *In vivo* antitumor activity against orthotopic breast tumors

To evaluate the antitumor activity of STS/EPI co-loaded micelles against breast tumor model, 4T1 tumor model was established and mice were separated into six groups in random (n = 8). Mice were treated with EPI, EPI + STS, EPI/m, STS/EPI/m and 10Mm HEPES buffer intravenously via tail vein every four days for twelve days (6 mg/kg EPI, 1.2 mg/kg STS, total: 3 injections,.). Thus, STS/Epi/m significantly inhibited the growth of the naïve 4T1-luc tumors, on the other hand, mice treated with free Epi, Epi/m, or the combination of free Epi and free STS failed to effectively suppress the tumor growth (Figure 2A). In addition, mice were repeatedly administrated of STS/Epi/m, but the body weight of mice did not lose during the experiment (Figure 2B), proving the safe profile of the micelles. According to survival curves, 4 mice treated with the combination of free Epi and free STS died soon after the first injection (Figure 2C), the strong toxicity of the combination of free STS and free EPI may be the main reason of death. Survival curves also indicated that STS/Epi/m significantly prolonged the overall survival, with all mice living for more than 2 months (Figure 2C).


A
HEPES buffer
EPI
EPI/m
STS/EPI/m
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Tumor size (mm3)
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Time (day)
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25
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Body weight (g)
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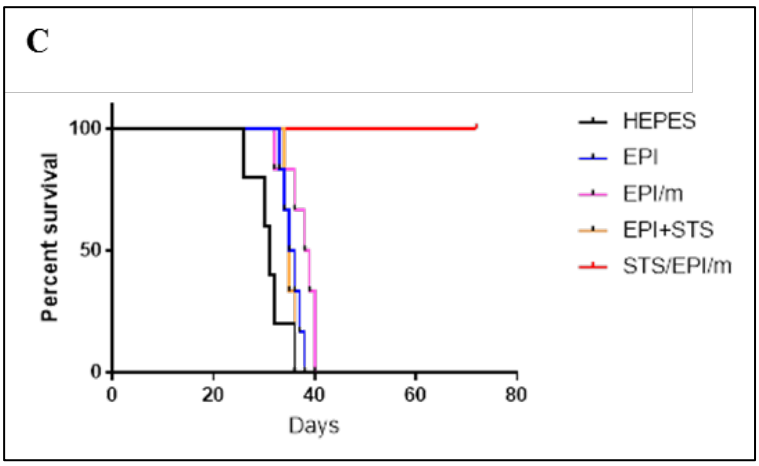
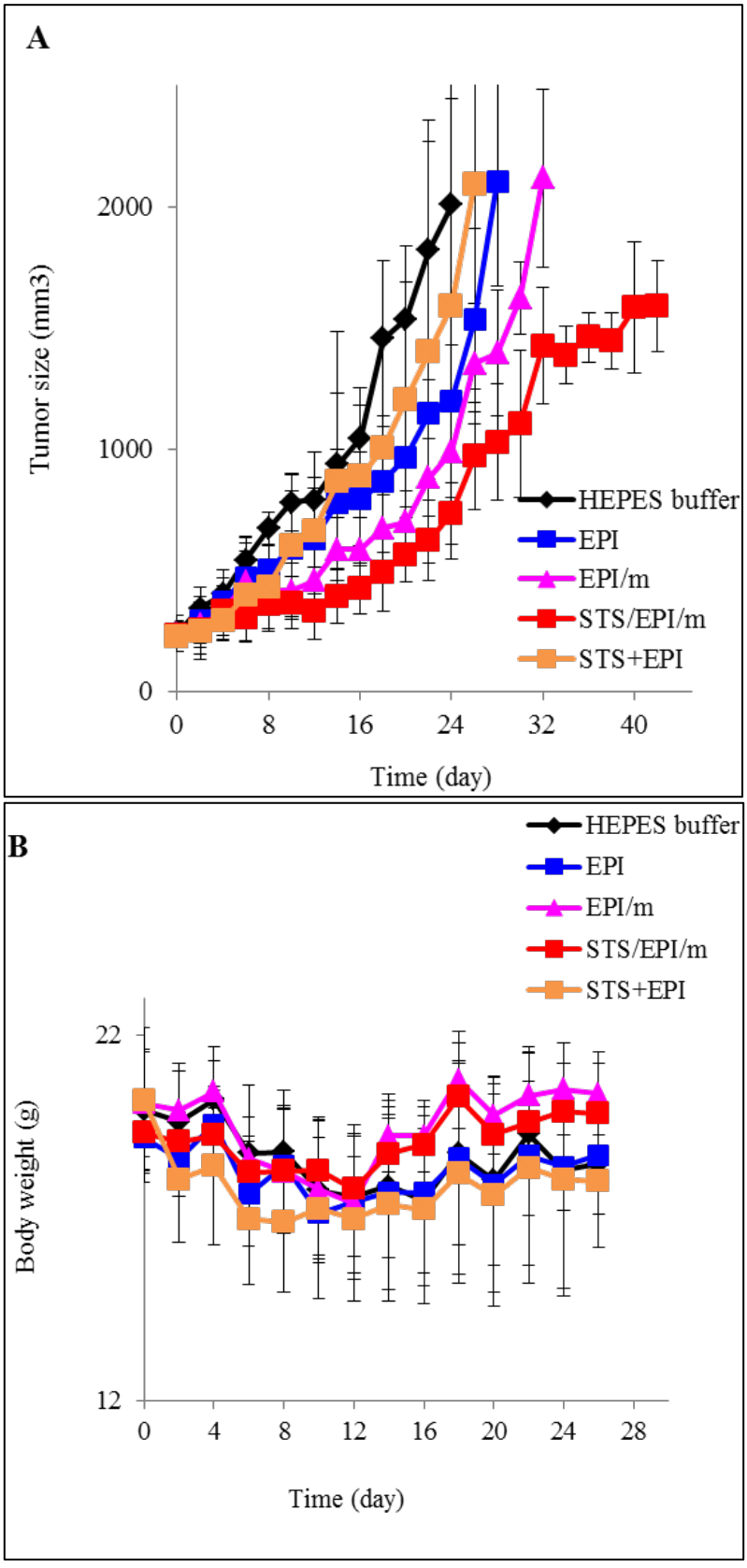


Figure 2. Antitumor activity of EPI/m and STS/EPI/m against 4T1 tumor model HEPES (black); EPI (blue), EPI/m (pink); STS+EPI (orange), STS/EPI/m (red) A. Tumor volume (mm^3); B. Body weight of mice for HEPES, EPI/m and STS/EPI/m treatment. C. Survival rate. Data are expressed as the mean ± SD.

These results supported that STS/Epi/m were enhanced the antitumor activity against the naïve 4T1-luc tumors. Therefore, 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 in EPI-pretreated 4T1-luc tumors. Tumor volume and body weight were measured every two days to evaluate the antitumor activities and systematic toxicities of various formulations. Data after treatments were collected and shown in Figure 2.

3.3 STS/Epi/m against epirubicin-resistant 4T1 tumor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Tumor size (mm3)
2000
1000
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8
16
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40
Time (day)
HEPES buffer
EPI
EPI/m
STS/EPI/m
STS+EPI
B
Body weight (g)
22
12
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4
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20
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28
Time (day)
HEPES buffer
EPI
EPI/m
STS/EPI/m
STS+EPI

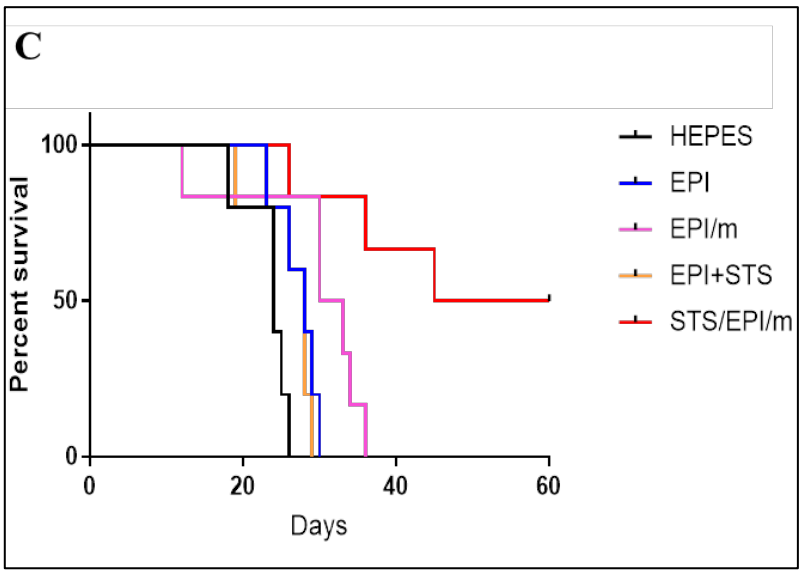


Figure 3. Antitumor activity of EPI/m and STS/EPI/m against epirubicin-resistant 4T1 tumor model HEPES (black); EPI (blue), EPI/m (pink); STS+EPI (orange), STS/EPI/m (red) A. Tumor volume (mm^3); B. Body weight of mice for HEPES, EPI, EPI/m, STS+EPI and STS/EPI/m treatment. C. Survival rate. Data are expressed as the mean ± SD.

Mice bearing orthotopic 4T1 tumors with 6 mm diameter were treated with free EPI (6 mg/kg) by intravenous injections 3 times on days 0, 4 and 8. Then, the mice were randomly divided into 5 groups (n = 7). Mice were treated intravenously 3 times with free Epi, Epi/m, free STS plus free Epi, free STS plus Epi/m and STS/Epi/m every 4 days, i.e. on days 12, 16 and 20. The drug dosage was fixed at 6 mg/kg based on Epi and 1.2 mg/kg based on STS. Tumor volume and body weight were measured every 2 days to evaluate the antitumor activities and systematic toxicities of various formulations. Data were collected and shown as Figure 3.

The STS/EPI/m effectively decreased the tumor growth rate of EPI-pretreated tumors compared with other treatments, and significantly extended the overall survival of mice bearing relapsed tumors after pretreatment with EPI. Moreover, despite repeated injection with the STS/EPI/m, the body weight of mice just slight decreased and kept the mice in better condition than free drugs treated groups. These data demonstrated that the cooperatively co-deliver STS and EPI in a single carrier to tumor tissues improved the therapeutic efficacy for relapsed tumors.

3.4 *In vitro* cytotoxicity against 4T1 cells from naïve tumors and Epi-treated tumors

For further study the antitumor activity of STS/EPI/m, the cytotoxicity of STS/EPI/m against 4T1 cells extracted from primary and epirubicin-resistant 4T1 tumors was conducted. Tumor cells of naïve 4T1 tumors or Epi-treated 4T1 tumor were collected from tumor tissues from the mice of above described models, i.e. with and without Epi pretreatment. The cells from naïve 4T1 tumors and Epi-treated tumors were seeded into 96 wells plates (5000 cells/well) Twenty-four hours later, 100 μL medium containing free Epi, Epi/m, free STS plus free Epi, free STS plus Epi/m and STS/Epi/m were added. After 48 h incubation, the cytotoxicity was measured using a MTT assay kit.

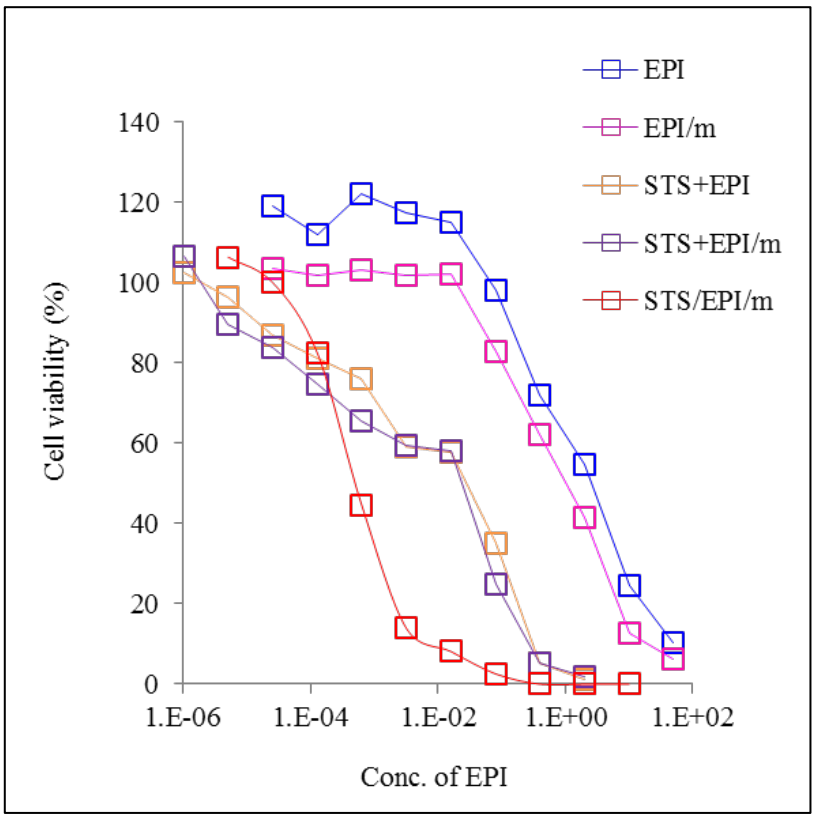


Figure 4. Cell viability for cells collected from naive 4T1 tumor cells exposed to various treatments for 48 h, including free drug of EPI (blue), micelles of EPI/m (pink), STS+EPI (orange), STS plus EPI/m (purple) and STS/EPI/m (red). Data are expressed as the mean ± SD.

Table 2. *In vitro* cytotoxicity of including EPI, STS+EPI, EPI/m, STS+EPI/m and STS/EPI/m against naive 4T1 tumor cells for 48 h.

IC_{50} of various treatments against Primary 4T1 tumor cells based on EPI					
Treatment	EPI	EPI/m	EPI+STS	EPI/m+STS	STS/EPI/m
μg/ml	1.3	3.2	0.04	0.03	0.006

The results showed that the STS/EPI/m significantly inhibited the cell growth and proliferation of cells collected from 4T1-luc naïve tumor cells (Figure 4), the IC50 value was calculated according to the cell viability (table 2). The STS/EPI/m also significantly inhibited the cell growth and proliferation of 4T1 EPI–resistant tumor cells (Figure 5), the IC50 value was calculated according to the cell viability shown as table 3.

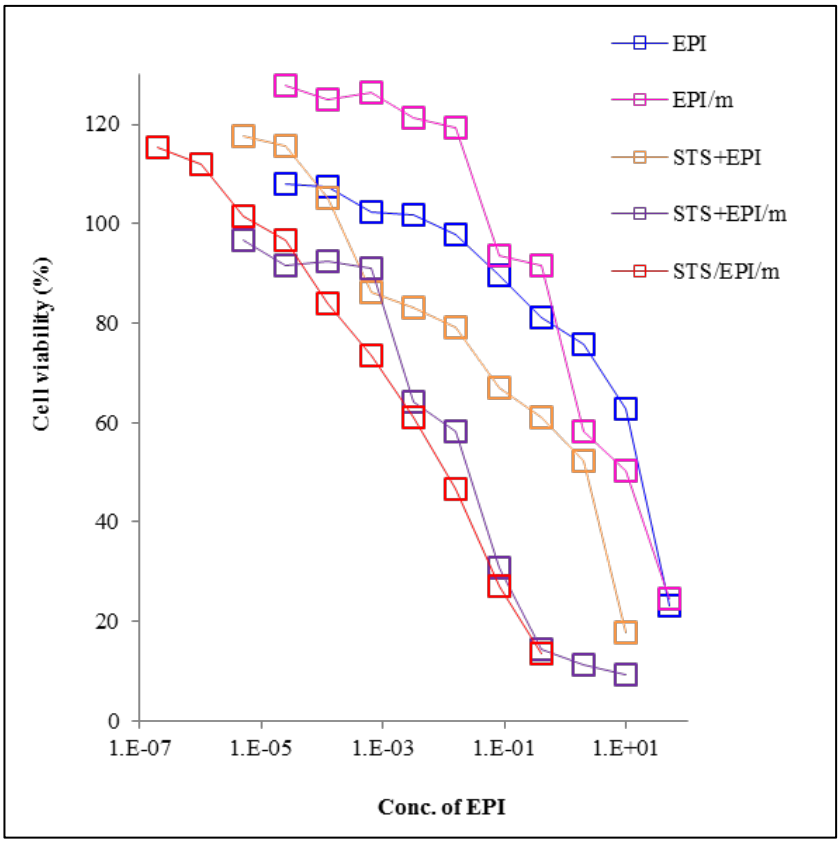


Figure 5. Cell viability for EPI-resistant 4T1 tumor cells exposed to various treatments for 48 h, including free drug of EPI (blue), micelles of EPI/m (pink), STS+EPI (orange), STS plus EPI/m (purple) and STS/EPI/m (red). Data are expressed as the mean ± SD.

Table 3. *In vitro* cytotoxicity of including EPI, STS+EPI, EPI/m, STS+EPI/m and STS/EPI/m against epirubicin-resistant 4T1 tumor cells for 48 h.

IC_{50} of various treatment against EPI-resistant 4T1 cells based on EPI

Treatment	EPI	EPI/m	EPI+STS	EPI/m+STS	STS/EPI/m
μg/ml	23.2	25.8	2.4	0.04	0.01

The STS/EPI/m presented super cytotoxicity against cells collected from naïve 4T1-luc tumors compared with other treatments. The IC50 value of combination of free STS and free EPI was almost close to the IC50 value of combination of free STS plus EPI/m against naïve 4T1-luc cells, which demonstrated that the potent cytotoxicity of free drug of STS. However, the IC50 value of combination of free STS and free EPI against cells collected from EPI-pretreated 4T1-luc tumors was sharply increased due to the drug resistance developed in the EPI-pretreated 4T1-luc tumors which is the main barrier for relapsed tumors treatment (Table 3). Even though cells collected from EPI-pretreated 4T1-luc tumors presented strong drug resistance, the STS/EPI/m still effectively inhibited the activity of these cells and was most potent treatment among the various treatments probably due to synergistic effect based on the synchronized release in cells (Figure 5). These data supported that the super antitumor activity of STS/EPI/m *in vitro* to eradicate CSCs and cancer cells.

3.5 Cancer stem cells elimination of STS/EPI/micelle

To further evaluate the antitumor activity of STS/EPI/m for cancer cell and cancer stem cells eradication, immunostaining was conducted. As the CD44+/CD24- phenotype has been used as a reliable phenotype for breast CSCs, we used the anti CD44 and anti CD24 antibodies for breast tumor tissue staining. For

immunofluorescence studies, tumor tissue slides were added and incubated with CD44/CD24 antibodies after dealing with blocking one buffer, and then stained with IgG. The immunostaining results showed that the 4T1-luc tumors present discrete areas of CD44+/CD24- cells (Figure 6A; HEPES), indicating the amount of CSC in these tumors approximately around 10%.

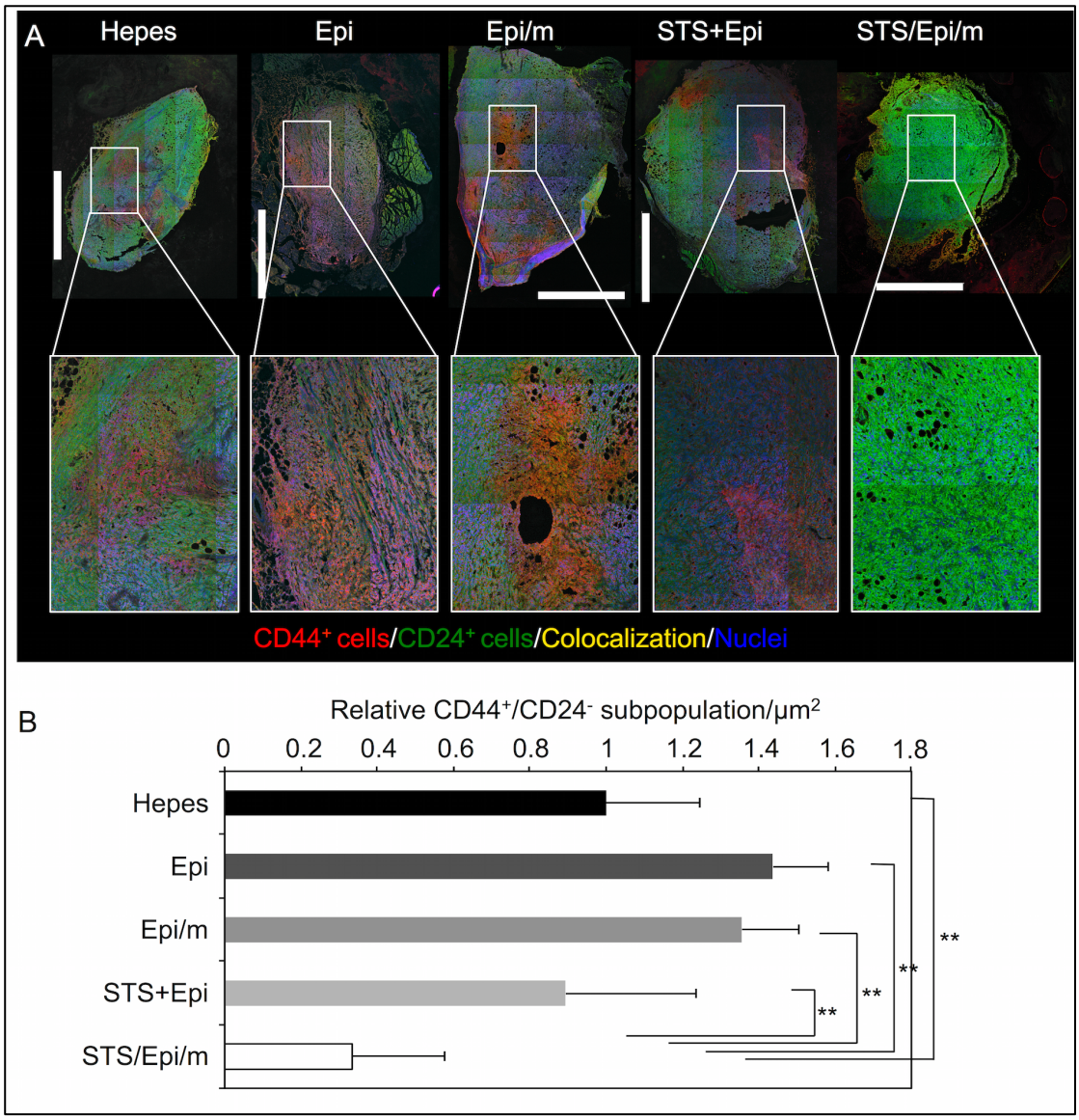


Figure 6. Immunofluorescent histological study of the CD44+ and CD24+ cells in the orthotopic 4T1-luc tumors after drug treatment. A. Microdistribution of CD44+ cells

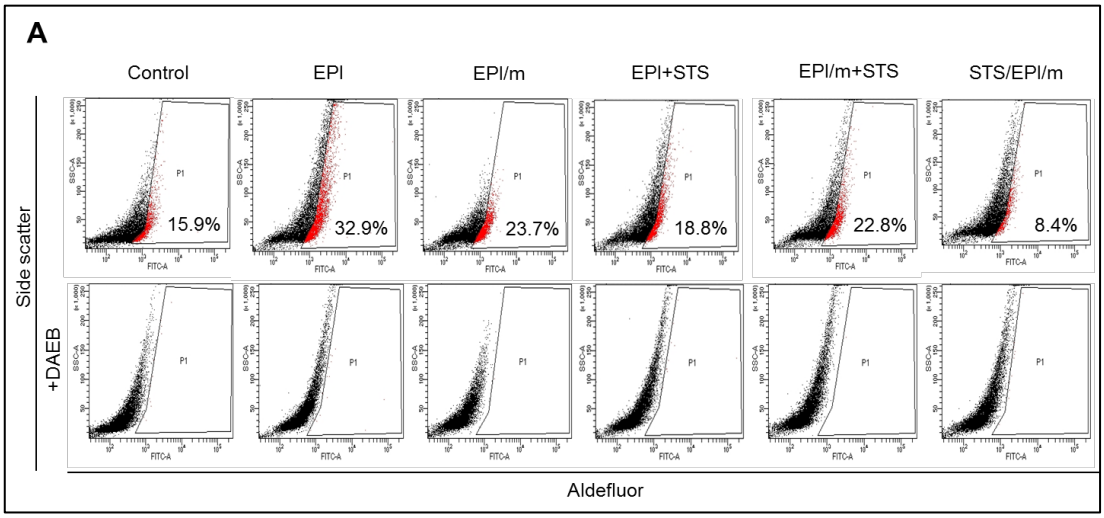
(red) and CD24+ (green) cells in tumor tissues 48 h after treatment with HEPES, Epi, Epi/m, STS plus Epi, and STS/Epi/m. The concentration of Epi was fixed at 6 mg/kg and the dose of STS was 1.2 mg/kg. Scale bars are 2 mm. B. Quantification of the CD44+/CD24- fraction per μm2 in tumor tissue sections. Data are expressed as the mean ± S.D. from 4 pictures obtained from 2 different tumor sections. **P < 0.01 by Student's t-test.

After treatment with various treatments, the levels CD44+/CD24- in the tumors increased (Figure 6), except STS/Epi/m treatment group significantly reduced the CD44+/CD24- fraction in 4T1-luc tumors (Figure 6B), supported that their enhanced activity against CSCs and their strong antitumor effect against naïve tumors. Thus, these results support cooperatively delivering STS and Epi within a single micelle platform to establish dual drug loaded micelles with super antitumor effect on inhibition of both cancer cells and the CSC fraction.

3.6 Effect of drugs on ALDH+ population in Epi-resistant cancer cells

The enhancement in the cytotoxic effects of STS/Epi/m was also assessed by evaluating the remaining CSC fraction in the cells collected from Epi-pretreated tumors after drug exposure. Here, we focused on the ALDH-positive subpopulation, as ALDH-positive breast cancer cells display properties of CSC (13). The cells of Epi-pretreated 4T1-luc tumors were collected and incubated with free Epi, free Epi plus free STS, Epi/m, and STS/Epi/m at the 90% inhibitory concentration of each treatment for 48 h. Thus, except for the cells treated with STS/Epi/m, the levels of ALDH-positive subpopulation were increased after the various *in vitro* treatments (Figure 7).

These results further indicate that STS/Epi/m effectively reduced the CSC population in the Epi-pretreated cells, which could lead to improve therapeutic outcomes even after tumor relapse following Epi treatment.



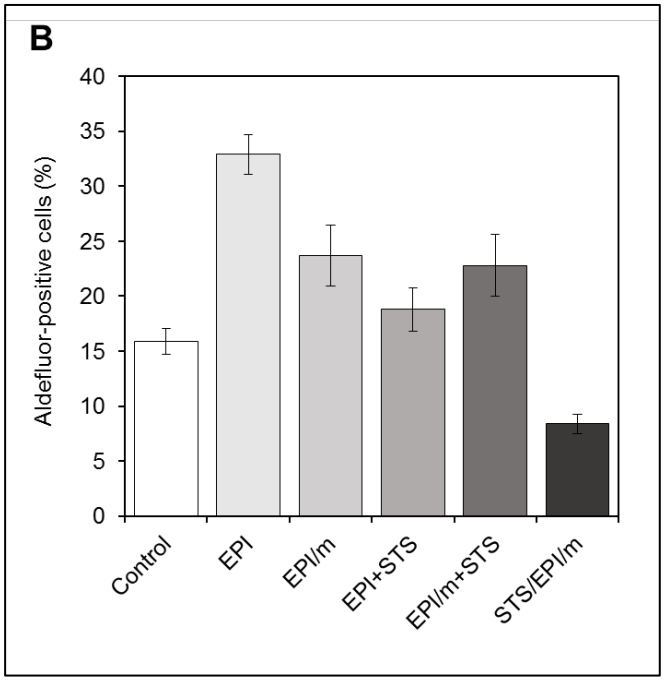


Figure 7. Effect of drug treatment on the ALDH levels in cells from Epi-treated tumors. A. Flow cytometry study of Aldefluor stained cells incubated with HEPES

(control) or 90% inhibitory concentrations of free Epi, free Epi plus free STS, Epi/m, and STS/Epi/m for 48h. The Aldefluor-positive cell population was determined by setting the gate sorting with a negative control (+DEAB; lower panel). B. Quantification of the ALDH+ positive population. Each bar represents mean ± S.E. (n = 4).

4. Discussion

Effective therapies against CSCs, in addition to traditional chemotherapies, appear to be critical for attaining long-term patient survival and avoiding recidivition of the disease. Herein, we found that the pan-kinase inhibitor STS and its homologues can effectively eradicate the CSC subpopulation in mesothelioma cells at picomolar concentrations. The incorporation of STS into EPI/m not only overcame the intrinsic issues of STS for clinical translation, such as poor tumor selectivity, low solubility in water, and losing activity by bonding with specific protein in human blood, but also presented as a safe and potent therapeutic compound cooperatively synchronizing the therapeutic effects of STS and EPI at the intracellular level. Thus, the resulting STS/EPI/m eradicated orthotopic xenografts of Epi-resistant mesothelioma by down-regulating drug resistance mechanisms and killing the CSC in the tumors to elongate the median survival of mice for more than 1 year (14). These findings indicate the high potential of targeted therapies of STS for treating relapsed and recalcitrant tumors and the ability of polymeric micelles to develop into multicomponent nanocarriers rationally devised for achieving synchronized and cooperative drug interactions with synergistic therapeutic effects.

Nanomedicine has presented advantages for combining therapeutic agents within single platforms for regulated pharmacokinetics and concentration of drugs in tumors (15-16). Our study constructs the strategy by developing multicomponent polymeric micelles designed to achieve synchronized synergistic drug cooperation from a molecular viewpoint, which affect interconnected signaling pathways in cancer cells and CSCs. Thus, the multicomponent STS/Epi/m rationally considered the interaction of STS and Epi molecules for encapsulation in the core of micelles and simultaneous release inside the cells, which allows corresponding delivery of both drugs, with STS working as a companion for Epi, where the ABC transporters and Akt induced by EPI as well as the intrinsic Epi-resistance mechanisms in CSCs are down-regulated by STS. Additionally, STS/Epi/m establish on the standpoints of practicality and safety, demonstrating that this approach has the potential to launch translational therapies with outstanding efficacy for efficient eradication of tumors and circumvention of recurrence by eliciting synergistic functions through coherent synchronization of drug actions within tumor cells.

5. Conclusion

The STS/EPI/m showed outstanding antitumor activity against breast cancer. The STS/EPI/m significantly inhibited the naïve 4T1 tumor growth with safe profile and decreased the tumor growth rate of EPI-pretreated 4T1-luc tumors at the meantime significant extended the survival. These results demonstrated that STS and EPI were successfully co-delivered and released within cells of tumor tissue allowing STS to eradicate CSCs fractions and EPI to delete cancer cells. The STS/Epi/m presents

superior antitumor activity against breast cancer and have great potential as an attractive strategy for CSCs therapies by decreasing the recurrence of tumor and achieving prolonged survival. Such activity profile could be beneficial in the clinic, where breast cancer patients receiving EPI usually show the recurrence of aggressive tumors.

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Chapter 5.

In vivo Antitumor Activity of STS/EPI/m against Recalcitrant Tumors

Chapter 5. *In vivo* Antitumor Activity of STS/EPI/m against Recalcitrant Tumors

Abstract

In vivo studies were performed to characterize the biological behavior of the STS/EPI/m. Therefore, antitumor activity of STS/EPI/m against a series of intractable tumor models: a subcutaneous BxPC3 pancreatic tumor model and a RenCa tumor model. The results showed that the tumor growth was significantly decreased and the STS/EPI/m extended the survival of RenCa tumor bearing mice. The STS/EPI/m micelles treated mice lived longer than mice with other treatment. These outstanding results obtained in all the tumor models suggest that STS/EPI/m loaded micelles could be a promising treatment for recalcitrant tumors.

1. Introduction

Recalcitrant cancers are defined as malignancies having less than 20% of 5-year relative survival rate. Kidney and pancreatic cancer are among such tumors. Pancreatic cancer is highly malignant with the ability of invading other organs (1). At early stage of pancreatic cancer, no symptoms are detected, until it has reached relatively late stage when the cancer has invaded to other parts of the body (2). Because of this, pancreatic cancer show poor prognosis, and only one quarter of the patients survive one year, and less than 5% live for five years (3), event after the usual treatments of surgery, chemotherapy, radiotherapy or the combination of these depending on the cancer stage (4). Kidney cancer is relatively easier to detect at early stage than pancreatic cancer, so it is possible to remove it before it metastasizes to

other parts of body, which is easier for treatment (6). However, kidney tumors grow fast, rapidly developing metastasis (7). Thus, while the 5-year survival rate for kidney cancer at stage 1 is more than 80%, after the tumor has metastasized to distant organ the 5-year survival rate drops to just 8%. Therefore, the development of effective therapies against such intractable tumors is imperative.

In this chapter, we evaluated the potential of STS/EPI/m for treating kidney and pancreatic tumors. Thus, the antitumor activity of STS/EPI/m was evaluated against an orthotopic kidney tumor mouse model based on murine RenCa cells and in a subcutaneous pancreatic tumor model based on human pancreatic adenocarcinoma cells (BxPC3). The results showed that the volume of these tumors was significantly decreased. Moreover, the STS/EPI/m significantly extended the survival of RenCa tumor bearing mice. The outstanding results obtained in all the tumor models suggest that STS/EPI/m loaded micelles could be promising treatment for recalcitrant tumors.

2. Experiments

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, 0.22μm) were

purchased from Millipore Co. (Massachusetts, USA). Cell culture medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Poly-L-lysine coated glass slides were purchased from Sigma-Aldrich (Sigma-St. Louis, USA). Fetal bovine serum (FBS), Alexa 647 or 488 anti-rat/rabbit secondary antibodies (Invitrogen, Carlsbad, CA). Anti-CD44 antibody (ab112178) and Anti-CD24 antibody (ab202963) were purchased from Abcam (Cambridge, UK). Blocking One Buffer was purchased from Nakalai Tesque Co., Ltd. (Tokyo, Japan).

2.2 Cell lines

BxPC3 cells (Human primary pancreatic adenocarcinoma) and Renca cell line (derived from a tumor that arose spontaneously as a renal cortical adenocarcinoma in Balb/cCr mice) were purchased from Caliper Life Sciences (Hopkinton, Massachusetts). The cells were cultured in RPMI 1640 plus 2mM Glutamine plus 10% FBS and 1% streptomycin/penicillin, and maintained at 37 °C and 5% CO2.

2.3 Animals

All animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated by the University of Tokyo. BALB/c mice at age of 6 weeks were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). For establishing tumor models, tumor cells were subcutaneously inoculated into BALB/c mice. Tumors were allowed to mature until the size of the tumor reached 5 mm in diameter.

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) and STS in DMF, 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 flask bottom, followed by addition of 10 mL of neutral buffer into the flask containing the dried sample. The mixture was sonicated 30 min. The resulting micelles were then purified by ultrafiltration (MWCO = 30,000 Da). Finally, the micelles were filtered by using a PES filter (0.22 μm). Micelle size was determined by Zetasizer Nano ZS (Malvern, UK.).

2.5 Loading efficiency of STS/Epi/m

pH-Sensitive STS/Epi/m incubating were disrupted by incubation in 1 N HCl of 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* Antitumor activity against BxPC3

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 BxPC3, a series of kidney cells were evaluated. Thus, the various cell lines of different cancers were grown in the RPMI-

1640 medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO2 humidified atmosphere. The confluent monolayer cells were harvested and seeded into 96 wells microplates at a density of 5000 cells per well. The cells were cultured overnight in the incubator for cell attachment and cell recovery after trypsinization before subjected to various treatments in one hundred microliter culture medium and incubation for 48 h. Then, Cell Proliferation Kit I solution was used to deal with cells for 4 h, 100 milliliter of the solubilization solution was directly added to wells containing culture medium and MTT for solubilization of cells. Add 100ml of 20 percent of SDS-Hcl was added to each well. Then the intensity was measured by using a microplate reader (Infinite M1000 PRO, Tecan, Männedorf, Switzerland) at 570 naonometers. Empty well of plate was counted as blank, and cells without adding treatment but with MTT and SDS-Hcl, were used as control. Cell viability was expressed as the percentage of MTT counts of treated cells relative to those without MTT treatment.

2.7 Antitumor activity evaluation against BxPC3 tumor models

BxPC3 cells (1000000 in 100μl PBS buffer) were injected under the skin in the right part of each Balb/c nude mice using a mouse injection needle. Then dividing the tumor-bearing mice into 6 groups (n = 8) when the size of tumor was approximately 50 mm^3. Mice were treated with EPI, EPI + STS, EPI/m, STS/EPI/m and 10Mm HEPES buffer intravenously via tail vein every 4 days for 12 days (6 mg/kg EPI, 1.2 mg/kg STS, total: 3 injections). To evaluate the antitumor activities and systematic toxicities of various formulations, in every two days tumor volume and body weight were determined and the calculation of the tumor volume based on the following

equation V = (Length × Width^2)/2, where L is the longest dimension and W is the shortest dimension. The body weight was measured to evaluate the systemic toxicities (8).

2.8 *In vitro* Antitumor activity against RenCa 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 against RenCa cells were evaluated. Thus, the cells were grown in medium (RPMI-1640) plus 10 percent of FBS at 37 Celsius degree in 5 percent of CO2 humidified atmosphere. The confluent monolayer cells were collected and cultured evenly into 96 wells microplates with the cell concentration of 5000 cells per well. The cells were cultured overnight in the incubator for cell attachment and cell recovery after trypsinization before subjected to various treatments in one hundred microliter culture medium and incubation for 48 h. Then, ten microliter Cell Proliferation Kit I solution which contains 5 microgram MTT per milliliter was added to each well. After cells with MTT were incubated for 4h, 100ml of the solubilization solution was directly added to wells containing culture medium and MTT for solubilization of cells. Add 100 microliter of 20 percentage of SDS-Hcl was added. The intensity was determined by using a microplate reader (Infinite M1000 PRO, Tecan, Männedorf, Switzerland) at 570 nanometers. Empty well of plate was thought as blank, and cells without adding treatment but with MTT and SDS-Hcl, were used as control. Cell survived rate was presented as the percentage based on the ratio of MTT determination of cells with or without MTT treatment, and the experiment was conducted three times.

2.9 Wound healing assay

Renca cells with a density of ten to five times were plated onto 24 wells plates to form a confluent monolayer. The 'scratch wound' then was prepared by using a 0.2-ml pipette tip scratching across monolayer of cells and then washing away cell debris with phosphate-buffered saline (Figure 1). RMPI medium plus 10% FBS was added after finishing scratching, and images captured at two time points of 0h and 48h, respectively. The collected images were analyzed to quantify cell migration. This inhibition of the metastasis related to the decreased mobility of RenCa cells after treatment with STS/EPI/m. The migration rate: the value of previous scratch width minus new scratch width, and then get the varied width to divide the previous scratch width, then time 100%.

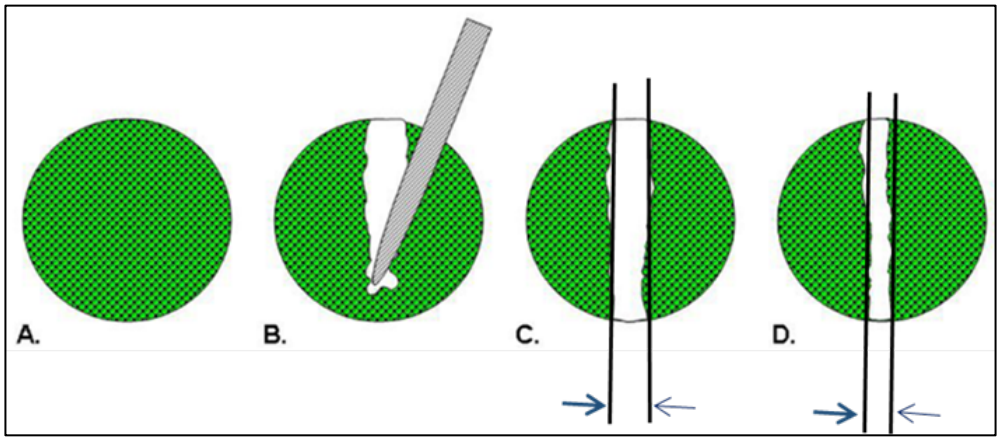

Figure 1. Making an empty space in a single layer of cells (A) by moving a pipet tip across the single layer of cells (B). The empty space is imaged and calculated to quantify the width of the wound before cells migrating (C) and after cells have migrated to close the empty space (D) (9).

3 Results

3.1 Cytotoxicity of various treatments against BxPC3 and Kidney cancer cell lines

In vitro cytotoxicity of STS/EPI/m against BxPC3 human pancreatic adenocarcinoma was studied after cells exposed to various treatments for 48 h, including free drug of EPI, free drug of STS, combination of free drugs of STS plus EPI, micelles of EPI/m, STS+EPI/m and STS/EPI/m. With the combination of STS and EPI, the cytotoxicity was sharply increased compare to free drug of EPI. The STS/EPI co-loaded micelles showed significant cytotoxicity compared with other treatment (Figure 2). The IC50 value was calculated according to the cell viability (table 1).

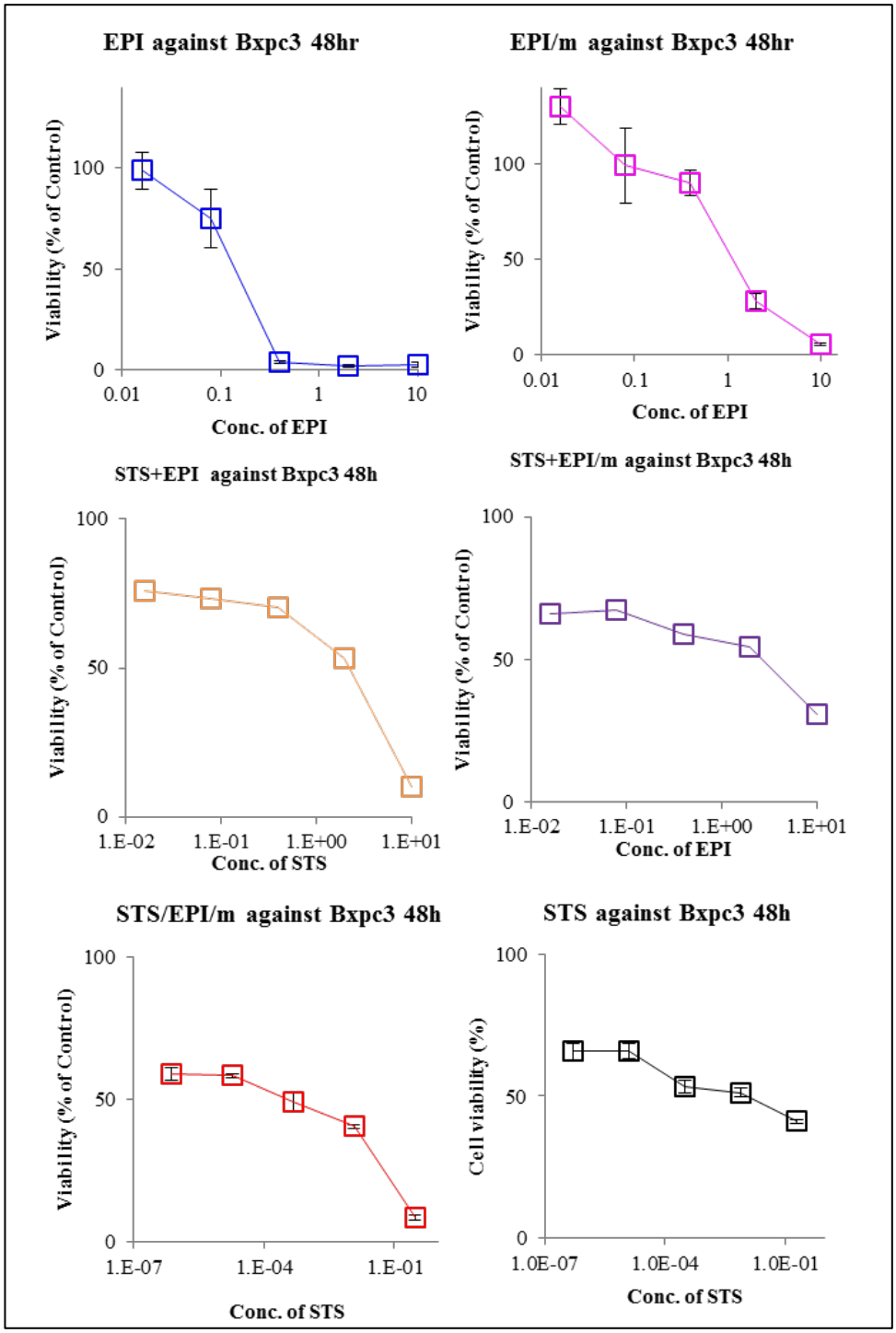

Figure 2. Cell viability for BxPC3 cancer cells exposed to various treatments for 48 h, including free drug of EPI (blue), free drug of STS (black), combination of free drugs

of STS plus EPI (orange), micelles of EPI/m (pink), STS+EPI/m (purple) and STS/EPI/m (red). Data represent the means ± SE.

Table 1. *In vitro* cytotoxicity of including free drug of EPI free drug of STS, combination of free drugs of STS plus EPI, EPI/m, EPI/m plus STS and STS/EPI/m against BxPC3 cells for 48 h. IC50 value obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

BxPC3 cells treated with various treatments for 48 h		
Various treatments	IC50 of EPI (μg/ml)	IC50 of STS (μg/ml)
EPI	0.9	—
EPI/m	5	—
STS+EPI	0.07	0.01
STS+EPI/m	0.4	0.08
STS/EPI/m	0.05	0.001
STS	—	0.0002

The cytotoxicity ability based on IC50 of EPI/m is almost five folds of the free EPI based on the concentration of EPI (Table 1), the difference of this cell inhibition activity may mainly be caused by the uptake methods of each treatment. Free EPI is relatively easier and quicker to diffuse through the cellular membrane, and the EPI/m have to get released gradually inside of endocytosis under proper acid environment to allow Epi molecules to take its function. It may also lead to different cytotoxicity of

the combination of free Epi plus STS compared with the combination of free STS plus Epi/m. The combination of free drugs presented stronger cytotoxicity. Interestingly, STS conducted the most powerful cytotoxicity against BxPC3 cells compared with free Epi plus STS or Epi/m plus free STS (Table 1).

3.2 *In vivo* antitumor activity of various treatments against BxPC3 tumor

To evaluate the antitumor activity of STS/EPI co-loaded micelles, mice were separated into 6 groups in random (n = 8). Mice then were treated with EPI, EPI/m, STS/EPI/m and 10Mm HEPES buffer intravenously via tail vein every 4 days for 12 days (6 mg/kg EPI, 1.2 mg/kg STS, total: 3 injections). Tumor volume and body weight were measured every two days to evaluate the antitumor activities and systematic toxicities of various formulations. The body weight after the treatment was also monitored and shown in Figure 3.

The tumor growth inhibition ability was evaluated based on the tumor growth. The STS/EPI/m strongly suppressed the BxPC3 tumor growth, on the other hand, the EPI/m treated group failed to significantly inhibited the tumor growth compared with HEPES buffer injected group. The enhanced antitumor activity of STS/EPI/m probably due to the synergistic effect of STS and EPI which were synchronized delivered into tumor tissues and released into cellular endosomes to exert their toxic ability for achieving goal of deleting both cancer cells and CSCs in the BxPC3 tumor tissue.

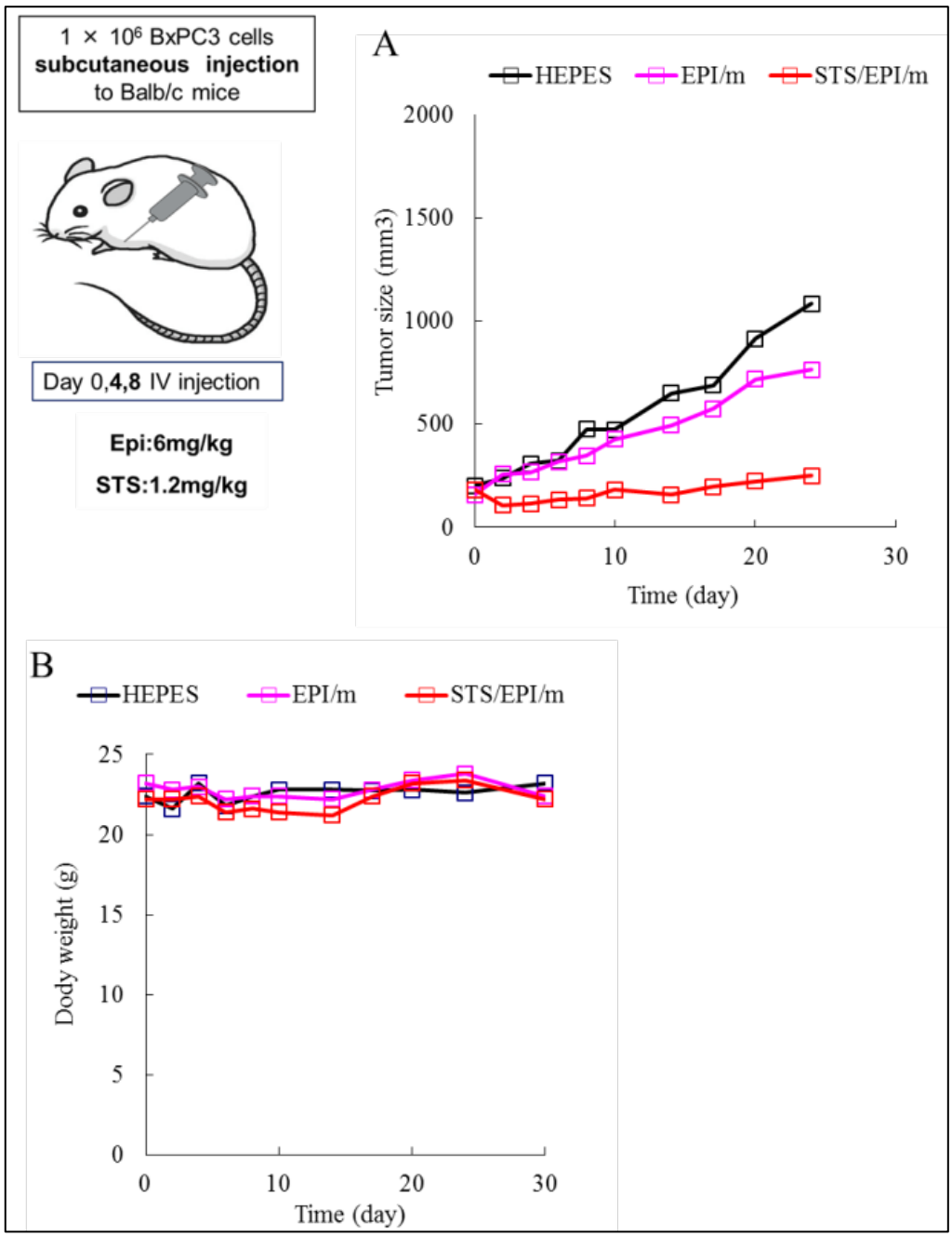

Figure 3. Antitumor activity of EPI/m and STS/EPI/m against BxPC3 tumor model. HEPES (black); EPI/m (pink); STS/EPI/m (red). A. Tumor volume (mm^3); B. Body weight of mice for HEPES, EPI/m and STS/EPI/m treatment. Data are expressed as the mean ± SD.

3.3 *In vitro* antitumor activity of various treatments against RenCa cells

In vitro cytotoxicity of STS/EPI/m against RenCa kidney cancer cells was studied after cells exposed to various treatments for 48 h, including free drug of EPI, free drug of STS, combination of free drugs of STS plus EPI, micelles of EPI/m, STS+EPI/m and STS/EPI/m.

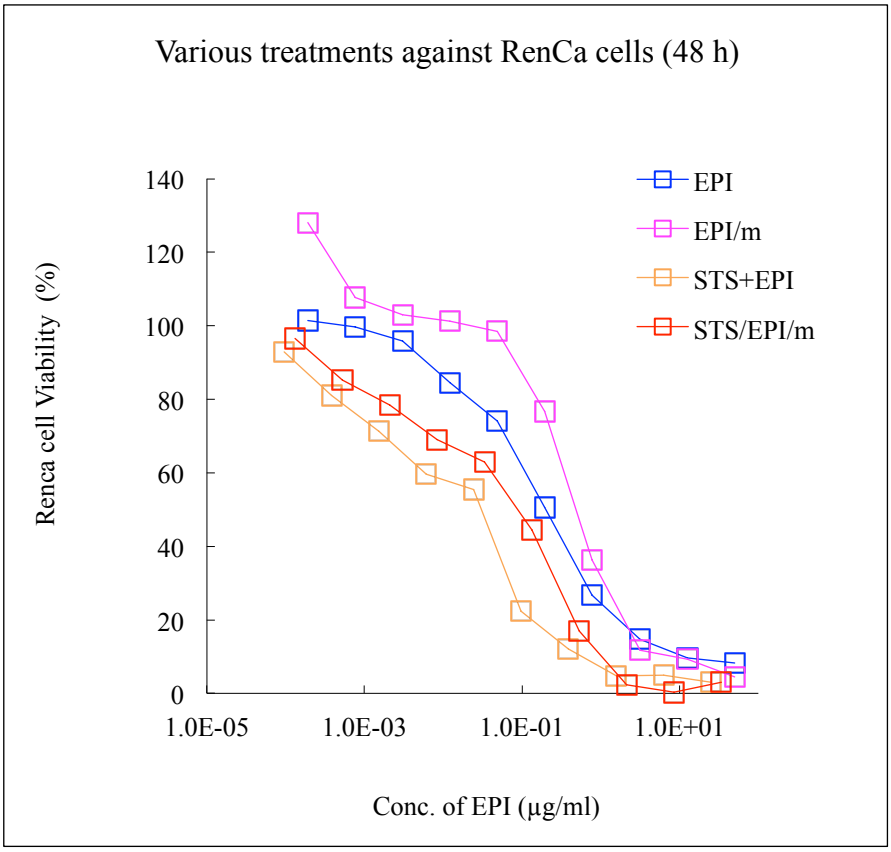

Figure 4. Cell viability for RenCa cells exposed to free drug of EPI (blue), combination of free drug of STS and free drug of EPI, EPI/m (pink) and STS/EPI/m (red) for 48 h. Data represent the means ± SE.

The free drug combination of STS and EPI showed the most potent cytotoxicity compared with other treatments. The STS/EPI/m demonstrated significant inhibition

of cell growth and proliferation compare to EPI/m (Figure 4). The IC50 value was calculated according to the cell viability (table 2).

Table 2. *In vitro* cytotoxicity of RenCa cells exposed to free drug of EPI (blue), combination of free drug of STS and free drug of EPI, EPI/m (pink) and STS/EPI/m (red) for 48 h.

IC50 (μg/ml)[a]			
EPI	EPI/m	STS+EPI	STS/EPI/m
0.2	0.6	0.04	0.1

[a] IC50 value obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

3.4 Inhibition migration ability of STS/EPI/m

Cancer metastasis has great relationship with treatment efficacy. To evaluation the cell migration inhibition ability of STS/Epi/m, the wound-healing experiment was conducted. The result indicates that the inhibition ability of STS/Epi/m is significant compared with the inhibition ability of EPI/m on cell migration of RenCa cell line indicating the potential of STS/Epi/m for inhibiting metastasis formation (Figure 5).

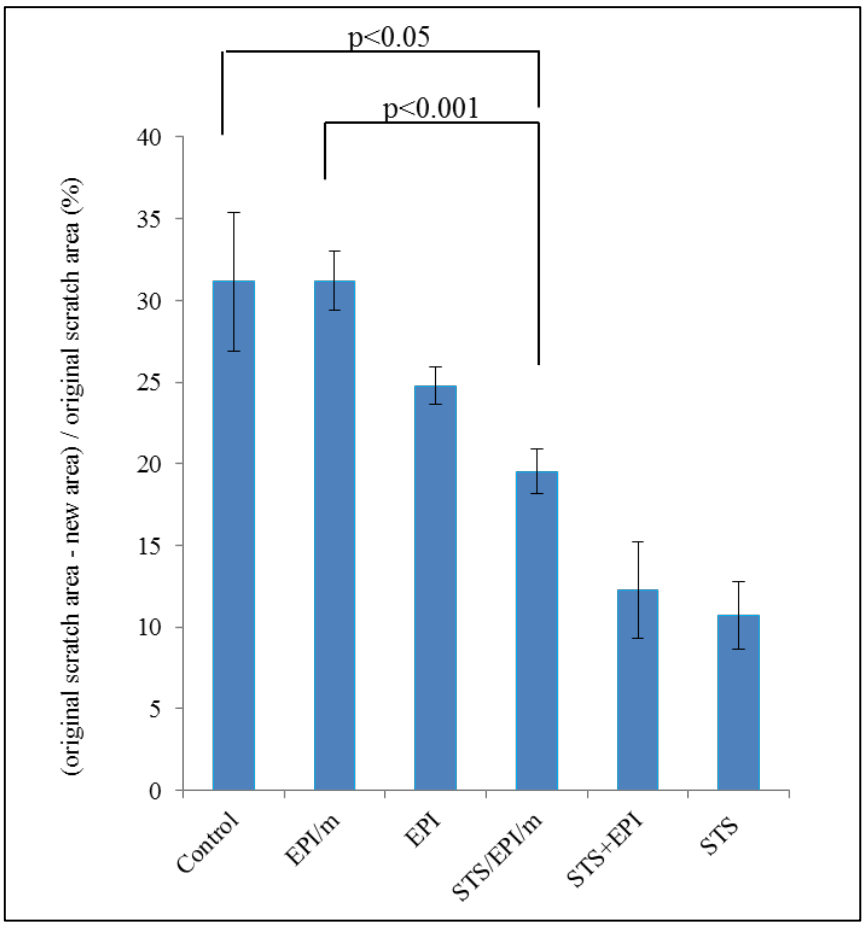

Figure 5. The inhibition ability of various treatment against cell migration of RenCa cell line. Concentration of IC10 was used for incubation. ($p<0.05$; $p<0.001$)

4. Discussion

In this chapter, the cytotoxicity of STS/EPI/m was evaluted against a series of cell lines. Staurosporine (STS) has been conformed to be a broad spectrum inhibitor of protein kinases (10). The apoptosis induced by STS was reported to be involved with the damage of mitochondria with involved the release of cytochrome c from mitochondria (11). The results of STS/EPI/m against a series of cells may be connected with the synergistic effect of STS and EPI encapsulated in the core structure, also related to the synchronized release inside of cells. Metastatic tumors are very

common in the late stages of cancer. The STS/EPI/m also presented the inhibition ability of cancer cells migration.

5. Conclusion

The STS/EPI-loaded micelles showed enhanced antitumor activity against pancreatic and kidney tumor models. The enhanced ability to inhibit tumors of STS/EPI-loaded micelles is probably due to the synergistic interaction between STS and EPI and synchronized release inside of tumor cells. Unfortunately, despite our efforts, the CSC population in these tumors has not been identified so far. Therefore, to confirm the anti-CSCs properties of STS/EPI/m, further efforts are necessary. Nevertheless, the potent antitumor effects displayed by STS/EPI/m in such intractable tumor models support the use of these micelles for achieving superior therapeutic responses in malignancies that immediately require effective treatments.

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Chapter 6.

General Conclusion and Future Perspective

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General conclusion

Our work on the STS/EPI/m has demonstrated novel and powerful advantages that nanomedicine can have over conventional small molecule therapies. First, and foremost, the extreme cytotoxicity of staurosporine, along with its relatively fast inactivation upon administration makes this drug a poor candidate for anticancer therapy. The decision to combine STS and Epi into the same micelle for synergistic effect demonstrates the versatility and potential of nanomedicine for treating drug resistant tumors. It is reasonable to conclude that such strategies can be employed to combine other drug systems to treat drug resistant tumors as well. Our work demonstrates that there is great potential for nanomedicines coloaded with multiple drugs to treat drug resistant cancers.

As a pan kinase inhibitor, STS has an extremely high binding affinity to kinase enzymes, which prevents ATP from binding to these enzymes. This action begins a cascade of intracellular pathways that result in apoptosis in concentrations as low as 4 nM. Since staurosporine does not have any particular affinity to cancer cells or tumors, there is most likely no dosage of free staurosporine that can treat the cancer without damaging the patient. Indeed, our *in vivo* data demonstrates that even at a dosage that kills half of the mice, there is no effect of free staurosporine on tumor size, whereas our micelles show significant anticancer activity at the same concentration without any body weight loss.

Another reason for STS weak effect on tumors lies in its fast inactivation. Staurosporine is immediately recognized in the patient's body as a foreign substance, and is quickly degraded by enzymes present in the serum, purged *via* the reticulo endothelial system, or simply removed by the kidneys. By encapsulating STS in the micelles, the PEG shell shields staurosporine from serum proteins and macrophages, while the large size of the micelle prevents filtration by the kidneys. The large size of the micelle also contributes the endothelial permeability and retention (EPR) effect, allowing for accumulation of micelle into the tumor for higher efficacy.

The concept to load two drugs into a micelle is a simple idea, but there is a considerable challenge when deciding which drugs to load, why to load them, and how to load them. We decided on STS and EPI because of their synergistic effects, and because of their aromatic structures.

When deciding which drugs to administer, it should be noted that drugs can have various interactions that can either enhance or weaken the effect of the co-administered drug. In brief, drug interactions can be described as synergistic, additive, or antagonistic. Synergistic interactions work together to enhance the other drugs effect, additive drugs simple work together, and antagonistic drugs weaken the effect of the co-administered drug. Naturally, we selected our drugs for synergistic interaction, such that when co-administered, each drug would enhance the other drug's effects.

Although synergy is important, the ability to load both drugs into one micelle is equally as important. In order to load both drugs into the same micelle, they should both have chemical properties that allow them to self assemble with the polymer block copolymer to form micelles. Some advantageous properties may include, but are not

limited to hydrophobic groups, aromatic rings, primary amines, and carboxylate groups. By taking advantage of the modifiable chemistry of our polymer block copolymers, we can incorporate a variety of drugs. Finally, the co-loaded micelle must remain stable in circulation and release in the tumor. The stability and release of both drugs from the micelles must be considered, which increases the difficulty of self assembling and characterizing such a system.

After taking into account these considerations, we determined that STS and EPI were particular candidates for combination in a single micelle platform. We first selected epirubicin because it is a conventional chemotherapeutic drug with a long history in the market, and immense human patient data. We then looked for another potential drug that could enhance EPI's effect that could be coloaded into our micelles using the same chemistry. STS was selected to be our candidate drug because it has been demonstrated that STS can suppress EPI resistance. STS, like EPI, has a flat planar aromatic structure, ideal for loading into a micelle via hydrophobic and π-π interactions. Thus, by these two reasons alone, STS could be considered a good candidate for co-delivery with epirubicin.

When these two drugs are co-loaded into a micelle, a more unique synergy comes into play, as STS has been demonstrated to have the ability to kill CSCs, while epirubicin is a DNA intercalator that mainly kills dividing cells. At first glance, it may appear that there is no particular reason why this is important. However, cancer tumors are known to be very heterogenous, with subpopulations of both CSCs and rapidly dividing cells. Furthermore, it is believed that the rapidly dividing cells can change phenotype and revert to CSCs, and vice versa. This presents a particular challenge: if a cancer cell can change its phenotype to protect itself from a cytotoxic drug, it may be

possible to completely eradicate the tumor? We believe that by co-loading both a CSC killing drug with another drug that can kill rapidly proliferating cells, we can effectively "trap" a cancer cell and force apoptosis. If the cell is rapidly proliferating, epirubicin will kill it, while if the cell tries to evade epirubicin by changing phenotype to a CSC, then STS will kill it.

This effect can only take place if both drugs are simultaneously internalized by a cancer cells. The ABC transporter, an efflux pump that is upregulated in cancer cells, imparts drug resistance to a cancer cell by providing it with the ability to actively remove drugs from the cell. Thus, if both drugs are administered separately, it possible for cancer cells to evade cell death through a combination of phenotype switching and efflux pumping. For example, a CSC exposed to epirubicin could upregulate its ABC transporters, and then switch phenotype to a rapidly proliferating cell. Afterwards, it may be exposed to STS, but as a rapidly proliferating cell, STS's effect will be significantly weakened. However, by loading both drugs into the same carrier, the cancer cell has no options to evade the drugs.

Our delivery platform demonstrates proof of concept that combining two therapeutic drugs into one particle has significant benefits over simply delivering two drugs freely. Previous work from our group has already demonstrated that micelle technology can allow drugs to accumulate into tumors, as well as reducing many of the adverse effects that free drugs can induce. However, this is the first time our group has demonstrated that by combining two synergistic drugs into the same micelle, there is a significantly enhanced anticancer effect over simply delivering the same two drugs. This work builds upon the extensive existing scientific literature on by introducing a new application of nanoparticle drug delivery systems.

Future Perspectives

Although we have demonstrated our micelle has potent anticancer efficacy and can kill CSCs, we have to demonstrate the ability for the micelle to target the CSC niche within the tumor. As cancer tumors are heterogenous, the microenvironment around the CSC subpopulations may not be conducive for micelle penetration. Thus, more work will be needed to characterize the CSC niche, and to find how efficient the micelles are accumulating within the CSC niche. Based on our previous work showing that the tumor penetration of sub-50 nm polymeric micelles is high even in hypopermeable tumor tissues, it is likely that a micelle broadly distributing throughout the tumor can also reach the CSC niche. Besides, advanced approaches, such as installing ligands on the surface of the micelles that binds to surface proteins specific to CSCs or altering the tumor microenvironment to enhance the penetration of the micelles into the tumor, could be applied for breaking into the CSC niche and exerting strong tumor inhibitory effects.

Additionally, we are interested in observing the antimetastatic activity of our micelles. Our micelles have demonstrated efficacy against primary tumors, but our micelles are designed to treat late stage drug resistant metastatic cancers. Although we have demonstrated that our micelles can overcome drug resistance and treat relapsed tumors, we have not yet studied the effect of our micelles on metastatic tumor sites. As metastasis is the leading cause of cancer death, the further evaluation of the effect against metastatic tumor models is mandatory. I anticipate that due to the high incidence of CSC in the metastatic disease, the STS/EPi/m would present outstanding efficacy.

The approach of co-loading drugs into micelles open a new door for many possibilities. The efficacy of many conventional chemotherapy drugs can be enhanced by using our method of co-loading micelles. Although our STS and Epi co-loaded micelles have demonstrated significant antitumor activity in breast cancer models, as well as recalcitrant tumors, such as pancreatic and kidney cancer, it is reasonable to expect that our drugs may not be effective in other tumor models. Thus, there are many opportunities to develop new multidrug micelles to treat cancer.

Finally, a key demonstration of our system is that the combination of two drugs being simultaneously internalized by a cell significantly enhances their effect. Because of STS's cytotoxicity and instability, a micelle is required to safely deliver it. However, it is reasonable to expect that other drug systems may be more stable and safe. Thus, it may be possible to integrate multiple synergistic drugs together in a single micelle platform with other stimuli responsive functions besides pH, for example, thiol linkers responsive to redox signals within cells. Such formulations should consider the predominant signals occurring in CSCs and CSC niches for activating the drugs, thereby, gaining in specificity, efficiency and safety.

Publication

Articles

1. Juanjuan Zhang, Hiroaki Kinoh, Louise Hespel, Xueying Liu, John Martin, Tsukasa Chida, Horacio Cabral and Kazunori Kataoka, "Effective Treatment of Drug Resistant Recurrent Breast Tumors Harboring Cancer Stem-like Cells by Staurosporine/Epirubicin Co-loaded Polymeric Micelles. *J. Control. Release*, under revision

2.Hiroaki Kinoh, Juanjuan Zhang, Xueying Liu, Horacio Cabral and Kazunori Kataoka, "Staurosporine/Epirubicin-loaded polymeric micelles improve survival in a orthotopic model of intractable renal cancer" in preparation.

Proceedings

1. Juanjuan Zhang, Xueying Liu, Hiroaki Kinoh, John Martin, Tsukasa Chida, Shigeto Fukushima, Yutaka Miura, Horacio Cabral and Kazunori Kataoka. Development of Staurosporine/Epirubicin-loaded Polymeric Micelles Directed to Achieve Robust Therapies against Differentiated Cells and Cancer Stem-like Cells in Solid Tumors. The 11th SPSJ International Polymer Conference (IPC2016) Fukuoka December, 2016.
2. Juanjuan Zhang, Hiroaki Kinoh, Xueying Liu, John Martin, Tsukasa Chida, Shigeto Fukushima, Yutaka Miura. Horacio Cabral and Kazunori Kataoka Development of Co-drug Delivery Micelles for Cooperative Synergistic Cancer Treatment. The 3rd COINS International Symposium "Towards Smart

Health Society - Challenge of Kawasaki based Medical Innovation " Kawasaki December, 2016.

3. Juanjuan Zhang, Hiroaki Kinoh, Xueying Liu, John Martin, Tsukasa Chida, Shigeto Fukushima, Yutaka Miura. Horacio Cabral and Kazunori Kataoka Development of Co-drug Delivery Micelles for Cooperative Synergistic Cancer Treatment. The 11th Annual Symposium on Nanobiotechnology, Kawasaki February, 2017.