学 位 論 文

Doctor's Thesis

Polymeric SMA-miceller anticancer drugs: enhanced antitumor activity and marked safety via targeted delivery to solid tumors

（スチレンマレイン酸コポリマー（SMA）ミセル型抗癌剤：固形癌を標的としたデリバリーによる抗癌作用と安全性の増強）

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Polymeric SMA-Miceller Anticancer Drugs: Enhanced Antitumor Activity and Marked Safety via Targeted Delivery To Solid Tumors

Doctor's Thesis By

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Chapter 4 Copoly(Styrene-Maleic Acid)-Oxaliplatin Micelles: Better Formulation for Enhanced Tumor Targeting and Lower Toxycity

学位論文抄録

[目的・方法] 癌化学療法の歴史が始まって以来50年間、ネオカルチノスタチンやスマンクスなどの例外を除く抗癌剤の殆ど全ては低分子量の薬剤であった。また抗癌剤の投与量は、その全身的に非選択的な分布ならびに非特異的な毒性のために制限されている。一方、腫瘍選択的抗癌剤のデリバリーに関連して固型腫瘍にユニークな脈管学上の性状として、高分子や脂質の示す enhanced permeability & retention (EPR)効果が知られている。EPR 効果は、固型腫瘍における(1)複数の血管透過性亢進因子の過剰生成、(例、ブラジニキニン、NO、プロスタグランジン、コラゲナーゼ等)、(2)腫瘍血管の解剖学的構築の異常、さらに、(3)高分子物質や脂質のリンパ行性の回収不全が知られている。本研究では平均分子量 1200 のスチレンマレイン酸コポリマー(SMA)を用いて各種抗癌剤(ドキソルビシン、ピラルビシン、亜鉛プロトポルフィリン IX、オキサリプラチン、タクソール、ローズ・ベンガル)含有の新型ミセルを作成し、そのミセル型抗癌剤の性状の解析、各種動物モデルによる抗癌効果、薬理学的動態解析、および安全性の検討等を行うことである。マウス腫瘍としては、S-180 肉腫/ddY マウス、colon 38 腺癌/C57BL/6、Meth A 線維肉腫/Balb C、Lewis 肺癌/C57BL/6、ウサギ VX2 パピローマなどである。ミセルの一般的な調製法として上記 SMA1.0gに対し同量の各種薬剤を溶かし、pH を5～6とし、水溶性カルボジイミドを 1g 加え撹拌下反応ののち、0.1M HCl を加え、pH を4以下とし、ミセルを沈殿物として分離する。次に 0.5M $NaHCO_3$ を加え pH 8 以上で可溶化し、このものを分子量1万の限外膜フィルターにかけ、非通過分画を集める。精製の目的で、このあと 10 倍量の蒸留水を加え限外ろ過操作による 10 倍濃縮操作を3回繰返す。

[結果・考察] 得られた標品をセファデックス G-100 等のカラムクロマトグラフィーにて、分子量約3.5万にシンメトリーなピークをもつ SMA-ピラルビシンミセルを得た。そのものは凍結乾燥可能で、ミセルの溶液状態では蛍光(480 nm ex. 560 nm em)を示さないが、5%SDS あるいは 70%エタノールでミセルが崩解すると、もとの薬剤の 80～90%蛍光を示した。静注後の組織内分布は組織のホモゲネートに酸性アルコールを9倍量加え、上清にピラルビシンを遊離・抽出し、定量した。ピラルビシン、ドキソルビシン、Zn-プロトポルフィリンなどのミセルは何れももとの薬剤と比べ 10～15 倍多く腫瘍内に蓄積し、血中滞留性(AUC)はもとのピラルビシンに比べると 70～100 倍延長した。また、マウスと同様の血中動態の延長がヒト(エジプト)でも確認された。ピラルビシンの例ではもとの薬剤の致死量の 15 倍も投与可能となった。ミセル 1g に対するピラルビシンの包含量は約 0.5g であった。その分子量はもとの薬剤の 627 に対し、約 3.5 万となった。この SMA-ミセルはアルブミンと結合し、その複合体の見かけ上の分子量は約 11 万であった。ミセルから free の薬剤の放出は約3%／日であった。SMA-ピラルビシンミセルは S180 および colon 38 腫瘍に対し i.v. 3 回投与後、300 日目の生存率は各々100%であった。2 年後の S180 の 380 日生存率は 100%であった。

[結論] レシチンなどの乳化成分を用いることなく得られた SMA-単独のミセル化製剤は低分子抗癌剤と比べ大幅(10 倍以上)な毒性の軽減、さらに腫瘍集積性(＞×10)と血中動態の著明な改善をもたらし、強力な抗腫瘍効果を得ることが可能になることがわかった。

Abstract

Conventional anticancer chemotherapy, mainly based on low molecular weight cytotoxic drugs, has been the mainstay of cancer treatments for about one century. Doses that can be afforded to cancer patients are limited by the maximum tolerability of the host due to lack of tumor specific toxicity. This limitation leads to the failure of many chemotherapeutic treatment schedules in curing cancer as well as inflicting sever agonizing side effects on cancer patients. In this context the unique characteristic for solid tumor tissue coined "*enhanced permeability and retention* (EPR) *effect* of macromolecules and lipids" was our strategy to selectively target high molecular weigh micellar anticancer drugs to tumor tissue. This EPR effect is attributed to anatomical alternations such as increased vascular density, impaired lymphatic recovery, and lack of smooth muscle layer in solid tumor vessels. More predominantly, augmentation of vascular permeability by extensive production of vascular mediators such as bradykinin, nitric oxide, prostaglandins, matrix metalloprotinases, VEGF / VPF and others.

In this study we have used the amphyphilic copolymer of styrene-maleic acid (SMA) to construct various anticancer micelles i.e. (doxorubicin, pirarubicin, zinc protoporphyrin ix, oxaliplatin, taxol and rose Bengal) as a new anticancer drug formulations through simple and efficient method of preparation that usually resulted in high recovery of more than 90 % of the original constituent as well as high loading of active compound into the micelle of about 35- 60 % wt/wt. Further more, this method can enable the control of the loading ratio of the parent active constituent into the micelle.

The micelles obtained showed discreetly high molecular weight as well as high solubility in water. SMA micelles exhibited great tumor concentration compared to parent drugs (10-15 times). Remarkable antitumor effect as well as safety was proven for micelles in various tumor models (S-180, colon -38, Meth A, Lewis lung, VX- II, AH 136 B) as well as in a limited compassionate phase 0 clinical trial in Egypt for SMA- Pirarubicin. Doses of SMA-Pirarubicn containing as much as high 15 x the maximum tolerable dose of free pirarubicin were well tolerated in tested mice. SMA has proven to confer immunopotentiating effects such as activation of macrophages, T-cells. and NK cells and induction of interferon in animal models. This advantage in addition to saving the bone marrow from the insult of the cytotoxic effect of the SMA-micelle due to its altered bio-distribution properties, results in active immune system taking part in elimination of cancer cells in synergism with the selective cytotoxic effect of the micelle in tumor tissue.

In effect, the new micelles drugs proved safe, effective through selective tumor targeting and improved pharmacokinetics. Clinical study concerning SMA-Pirarubicin is expected to commence soon.

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LIST OF ABBREVIATIONS

EPR effect; enhanced permeability and retention effect in solid tumor

AUC; area under the concentration-time curve

SMANCS; poly(styrene-co-maleic acid-half butylate) conjugated neocarzinostatin,

HPMA copolymer; N-(2-hydroxypropyl) methacrylamide copolymer

PEG; poly(ethylene glycol)

SMA; poly(styrene-co-maleic acid/anhydride)

IFN; interferon

NCS; neocarzinostatin

PVA;poly(vinyl alcohol),

ROS; reactive oxygen species

PEG; polyethylene glycol

LC_{50}; median lethal concentration

ZnPP; zinc protoporphyrin IX

HO; heme oxygenase

DCDHF-DA; 2', 7'-dichlodihydrofluorecin diacetate

MDR; multidrug resistance

THP; 4'-O-tetrahydropyranyladriamycin, which is also known as pirarubicin

SDS; sodium dodecyl sulfate

EDAC; ethyldimethylaminopropylcarbodiimide

MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MALDI-TOF; matrix-assisted laser desorption-ionization/time of flight

H&E; haematoxylin & eosin

Ox; Oxaliplatin (Trans-l-diaminocyclohexane oxalatoplatinum)

OPD; O-phenylenediamine

CPT; Camptothecin

CHAPTER 1

INTRODUCTION

In spite of the great efforts in the field of oncology, cancer is still the first or second killer of human being in industrialized countries (1). This means search for new and effective drugs for treatment of solid tumors is needed. One of the most crucial problems is lack of selective toxicity against cancer tissues as seen in antibacterial agents because most of the molecular machinery are common for both normal and tumor tissues. Thus, unique tumor selective characters are key issues to undertake tumortropic anticancer drugs. In this context the unique characters for solid tumor tissue known as the phenomenon of *"enhanced permeability and retention* (EPR) *effect* of macromolecules and lipids", can be a corner stone in designing new effective anticancer agents (2). This EPR effect, is attributed to anatomical and pathophysiological alternations, such as increased vascular density, angiogenesis, impaired lymphatic recovery and lack of smooth muscle layer in solid tumor vessels, More predominantly, functional augmentation of vascular permeability by extensive production of vascular mediators such as bradykinin, nitric oxide, prostaglandins, matrix metalloprotinases, VEGF / VPF and others(2-14). These vascular factors cross talk each other and facilitate extravasations of macromolecular drugs. More importantly, drug concentration in tumor tissues far exceeds that of the blood plasma, usually 5-10 folds that would be otherwise very difficult for low molecular weight drugs.

Thus, many efforts have been paid to design macromolecular drugs from low molecular weight anticancer drugs to form biocompatible water-soluble polymers either by

chemical conjugation or by forming liposomal / polymeric micelle encapsulating the drug in the core of it. Such biocompatible polymeric drugs acquire new physiochemical, biochemical and pharmacological characteristics, which grant them full utilization of EPR effect.

1. Consideration in designing macromolecular drugs

Various types of anticancer agents are the targets of polymer conjugation, including antimetabolites, anthracyclin antibiotics, platinum analogues, alkylating agents, as well as peptides, proteins and enzymes. Regarding the carrier polymers both naturally occurring and synthetic origins such as polysaccharides (starch amylose, pullulans, chitins/chitosan), chemically modified gelatin, poly amino acids, polyethylenglycol(PEG),various polyacrylate including hydroxypropylmethacrylamide polymers (HPMA), polyvinylalcohol (PVA), poly(styrene-co-malate), polyvinylpyrrolidone (PVP), and divinyl ether maleic anhydride/acid copolymer (DIVEMA), have been reported (16-49) .

In choosing the candidate drug for conjugation the main factor needs to be considered is the minimum effective molar concentration as it would be impractical to administer milimolar dose range with molecular size about 50 KDa (i.e. 1.0 mmole will be more than 50g).

Candidate polymers for therapeutic use must be devoid of antigenicity, immunogenicity (if not immunopotentiation), hemolytic, procoagulant or cytotoxic activity. Namely, they must be biocompatible in nature. Next important factor is the surface charge of the polymers, as the luminal surface of the blood vessels is covered with the negatively charged chondroitin sulfate, sialayl residues and heparan sulfate. Therefore, macromolecular drugs must be neutral or slightly negatively charged. On the other hand

cationic polymers have gained popularity as gene delivery carriers in part due to their capability of electrostatic interaction between the positively charged amino or imino groups on polymeric carrier and the negatively charged phosphate group of DNA molecules, giving stability to the complex. This result in more neutral complex, and the remaining positive charge of the cationic complex would facilitate internalization into the cytoplasm due to the electrostatic interaction between the cationic charge of polymers and negative charge of plasma membrane. This type of interaction can be more clearly seen *in vitro* setting. In *in vivo* settings, it would be totally different, where such electrostatic attraction will lead to nonspecific interaction with the endothelial cell lining of the vessels, and the cellular or blood plasma components resulting in short plasma half-life. Among its interaction with plasma proteins, activation of complement system or coagulation cascade, if triggered, would result in host toxicity adding to the limitation for wide usage of cationic polymers (50, 51). Systemic studies using plasma proteins or synthetic polymers such as HPMA copolymer have clearly demonstrated that non-cationic materials larger than 40KDa frequently exhibit extended plasma-circulating time (3, 5, 52).

The constitution of polymeric drugs can be made through covalent linking of drugs to a polymeric carrier (pendant type), whereas micellar entrapment of the drug inside of the micellar core can be achieved either via covalent or non-covalent bonds (ionic, hydrophilic hydrogen bond or hydrophobic interaction) usually involving block polymer or copolymer. Polymer-protein conjugates are usually linked via covalent linkage. In case of pendant type conjugates, usually the active components must be detached from the complex in order to achieve therapeutic effect while some polymer-protein conjugates

are active without the need of cleavage. While micelle and liposome type drugs require release of the active components in order to achieve therapeutic effect, those formulations pose special problems; as the stability in shelves or during storage, would be in conflict to time dependent linear release *in vivo*, thus requiring meticulous adjustment to the formulation development. One method to tackle this problem is lowering temperature during storage. Another problem to be noted is that large particles such as liposomes would be the subject of pinocytosis or endocytosis by leukocytes during circulation unless they are made stealth form, for instance, by use of PEG or HPMA conjugation (53). Various chemical bonds are utilized to prepare macromolecular drugs such as amide, ester, hydrazide, azide, imine, thioether and urethane (Fig.1). The characters of these bonds determine the release velocity of the drugs from the polymeric chains (54, 55). For instance, ester bond of a drug to main polymer carrier chain would insure relatively rapid release of the drug due to the abundant esterase in the body. while the more stable amide bond will show slower release profile. Such heterogeneity allow for a range of freedom to construct macromolecular drugs with various capabilities that meet different pathological conditions.

Release of the free drug moiety from the polymeric carrier chain usually involves either pH dependent release or enzyme dependent release. In case of pH dependent release the macromolecular drugs usually take advantage of the extracellular acidic condition in tumor tissues and also in the lysosomal intercellular compartment after endocytosis. In the case of enzyme dependent release, the active drug moiety being attached to the polymeric carrier through specific linker, it is particularly vulnerable to specific intercellular enzymatic cleavage. In this class of polymeric drugs that is designed to be

liberated by intracellular proteases (e.g. cathepsins) specific peptide spacer with defined cleavable amino acid sequence is incorporated into the polymer. For instance the peptedyl spacer (Gly-Phe-Leu-Gly), is used as a preferred spacer to conjugate HPMA polymer and doxorubicin yielding PK-1 (37).

Figure 1 a) Schematic representation of various structures of polymeric drugs.

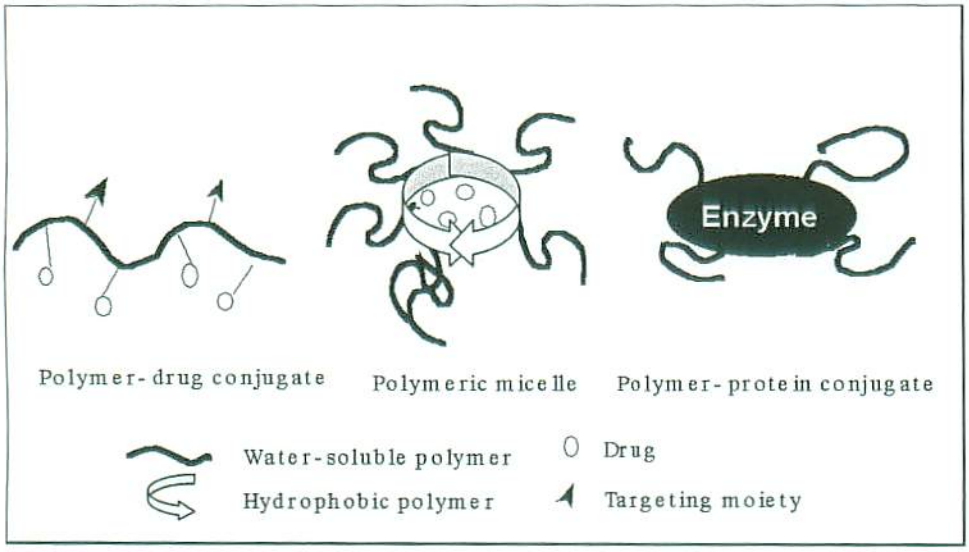
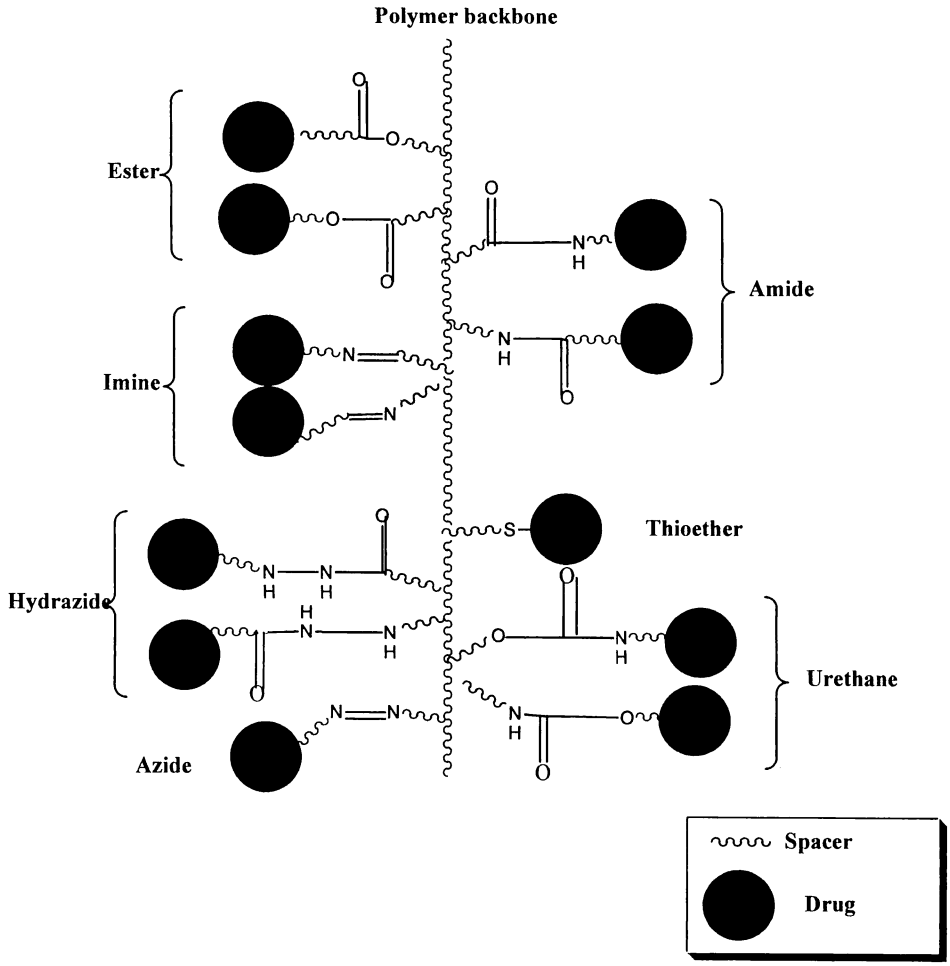


Figure 1 b) commonly used chemical bonds for attachment of drugs to polymeric backbone



3. Enhancement of Plasma half-life of polymeric drugs

It is known that pharmacological effect and the plasma concentration [area under the concentration – time curve (AUC)] are usually parallel. Accordingly, for low molecular weight conventional drugs, slow intravenous infusion is a choice although compliance is poor.

In the past 30 years or so, it was found that molecular size is the most critical parameter to keep drugs in circulating plasma, in addition to the various parameters of biocompatibility as discussed above. Namely, drugs of molecular size less than 40 KDa are cleared rapidly into urine with a plasma half-life of usually less than 5 minutes in mice (e.g. 3 minutes for superoxoide dismatase, 30 KDa; 1.9 minutes for neocarzinostatin, 12 KDa). This is a great contrast to the high molecular weight drugs that usually exhibit extended $t_{1/2}$ of hours. Table 1and table 2 shows the plasma half –life of different compounds in its free and polymeric form.

In view of polymeric drug extended half-life, usually the number of drug infusion becomes less. This will result in great benefit for patients in terms of compliance and quality of life, as well as improved therapeutic efficacy and cost effectiveness of the drugs. The best example of improved therapeutic action due to extended $t_{1/2}$ is PEG-conjugated IFN α-2a .

Table I. Plasma clearance time of various proteins and their polymer conjugates or modified proteins

Protein	Type of polymer or modification	Molecular mass (KDa)	$t_{1/2}$	$t_{1/10}$	Test animal	Refs.
Neocarzinostatin (NCS)	None	12	1.8 min	15 min	Mouse	56,57
SMANCS	SMA-NCS[a]	16	19 min	5 hr	Mouse	56,57
Ribonuclease	None	13.7	5 min	30 min	Mouse	58
Ribonuclease dimer	Cross-linked	27	18 min	5 hr	Mouse	58
Soybean trypsin inhibitor (SBTI[b])	None	20	< 2 min	3 min	Rabbit	59
Dextran-SBTI	Dextran	127	20 min	> 80 min	Rabbit	59,60
Ovomucoid	DTPA/NH_2/^{51}Cr[c]	29	5 min	34 min	Mouse	56
Cu^{2+}, Zn^{2+} superoxide dismutase (SOD)	None	30	4 min	30 min	Rat	56,61
SOD-SMA	SMA conjugate[a]	40	> 300 min	> 10 hr	Rat	61
Bilirubin oxidase	None	50	< 1 min	1.8 min	Rat	62
PEG[d]-biliribin oxidase	PEG	70	5 min	48 hr	Tar	62
Serum albumin, mouse	None	68	3-4 day[e]	-	Mouse	56
Serum albumin, mouse	Evans Blue dye	-	2 hr	30 hr	Mouse	2.56
Serum albumin, human	Formaldehyde/^{125}I	-	25 min	4 hr	Rat	63
L-Asparaginase	None	65 × (2-8)	1.5-3.4 hr	-	Rat	64
L-Asparaginase-PEG	PEG	-	56 hr	11 day	Mouse	64
Immunoglobin G, mouse	DTPA	150	60 hr	-	Rat	56
α_2-Macroglobulin[f]	Iodination/^{125}I	180 × 4	140 hr	22 day	Mouse	65
α_2-Macroglobulin[f]-plasmin complex	Iodination/^{125}I	180 × 2	2.5 min	20 min	Mouse	65

* min, minute; hr, hour; [a]binds to albumin; [b] SBTI, Kunitz type; c DTPA diethylenetriaminepentaacetic acid;[d] PEG, polyethylene glycol:[e] Human albumin (in humans: 19 days) ;[f]Human.

Table II. Pharmacokinetic parameters of native and polymer-conjugated interferons in human (66-68).

Types of Conjugates of Interferon	Molecular Mass (KDa)	Approximate $t_{1/2}$ (hr) Human, s.c[a].
Native IFN-alpha-2a	18	8
PEG-IFN-alpha-2b	30	54
PEG-IFN-alpha-2a	52	80

On the other hand, for targeted anticancer therapy, this property must be carefully adjusted with the rate of free drug release from the polymeric carrier and rate of tumor accumulation. The ideal macromolecular drug should only release free drug in tumor tissue while stable during circulation. Otherwise, though it takes several hours or longer to accumulate in tumor, the rate of tumor uptake needs to be faster than the rate of drug release. This point is of crucial importance for successful macromolecular anticancer therapy. Overall picture in case of faster drug release from the polymeric carrier during circulation before accumulating in tumor tissue would be that of higher and unexpected toxicity profile of the free drug.

4. Cellular uptake of macromolecular drugs and its antitumor action against multidrug resistant cells

Macromolecular drugs described herein are that of molecular weight larger than 40kD that cannot be internalized into cells by simple diffusion. For such group of drugs, endocytosis is the usual way of internalization (69-71). A recent report describes even more active uptake of macromolecules during mitosis, that add to the efficiency of tumor targeting capability of this class of drugs to dividing cancer cells (72).

After internalization into the cytoplasm, the macromolecular drug is transported into lysosymes that are rich in proteolytic enzymes and characterized by low pH, where the active component would be released. It then diffuses into the cytoplasm or the nucleus where they would exert comparable action to the parent drug. This method of intracellular uptake of macromolecular drugs serve to circumvent the problem arises by the P-glycoprotein dependent efflux system in multiple drug resistance (73, 74). This suggests that the use of polymeric anticancer drugs is a rational approach to circumvent MDR in addition to their other advantages such as higher tumor tropism and improved pharmacological properties.

5. EPR effect

5.1 Theory and principle

Tumor vascular permeability is becoming increasingly important in tumor biology because of its critical role in tumor growth and perhaps in metastasis, as well as in the selective delivery of anticancer agents, particularly macromolecular compounds. Tumor cell aggregates of size as small as 150-200 μm, start to become dependent on blood supply carried out by neovasculature for their nutritional and oxygen supply (75). This newly formed tumor vessels are usually abnormal in form and architecture. They are poorly aligned defective endothelial cells with wide fenestration, lacking smooth muscle layer, or inervation with wider lumen, and impaired functional receptors for angiotensin II. Furthermore, tumor tissues usually lack effective lymphatic drainage (3,5,6) . All these factors will lead to abnormal molecular and fluid transport dynamics especially for macromolecular drugs. Namely, this phenomenon was coined *"enhanced permeability and retention* (EPR)-effect" of macromolecules and lipids in solid tumors (2). The EPR-effect is even more enhanced by great many pathophysiological factors involved in enhancement of extravasation of macromolecules in solid tumor tissues. For instance, bradykinin, nitric oxide / peroxynitrite, prostaglandins, vascular permeability factor (also known as vascular endothelial growth factor VEGF), tumor necrosis factor and others (3-14) .

Table III. Factors affecting the EPR effect of macromolecular drugs in solid tumor (3-14, 76-78)

(i)	Active angiogenesis and high vascular density
(ii)	Extensive production of vascular mediators that facilitate extravasation a) Bradykinin, b) Nitric oxide (NO), c) VPF/VEGF, d) Prostaglandins, e) Collagenase (matrix metalloproteinases, MMPs), f) Peroxynitrite.
(iii)	Defective vascular architecture: for example, lack of smooth muscle layer cells, lack of or reduced receptors for angiotensin II, large gap in endothelial cell-cell junctions, anomalous conformation of tumor vasculature (branching or stretching etc.)
(iv)	Impaired lymphatic clearance of macromolecules and lipids from interstitial tissue (→retention)

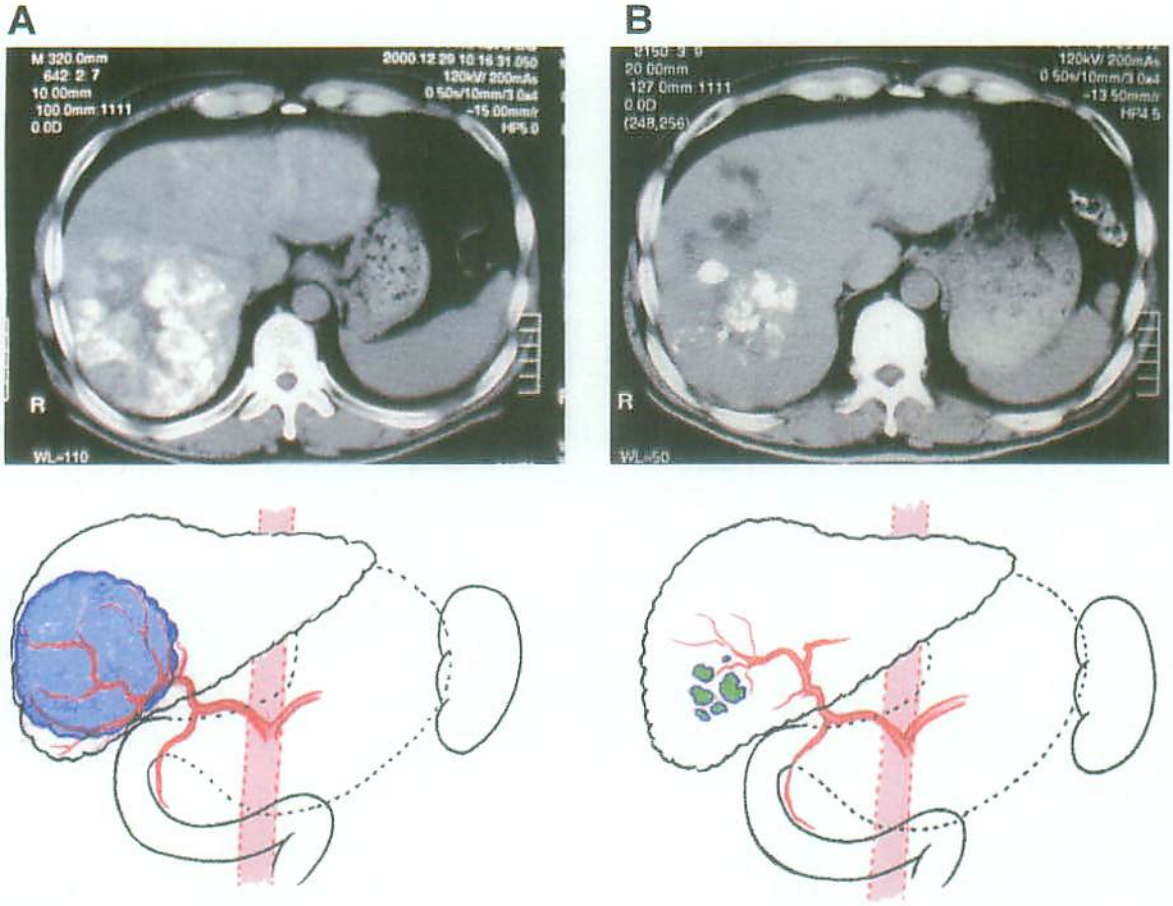
The EPR-effect can be clearly demonstrated in rodent tumors by the intravenous injection of Evans blue dye which complexes with albumin and produces intense blue color in tumor tissues to a great contrast to normal color surrounding tissues (2, 7-9).

On the contrary a hypothetical dogma stated that, the high interstitial pressure in tumor tissue would impede any macromolecular drug from getting into the tumor, thus it is formidable to deliver macromolecular drugs into tumor tissue (79). However, many experimental facts described by us and other investigators clearly showed that tumor interstitial pressure did not prevent macromolecular transport and accumulation into tumor tissue. For instance Sat and Duncan have used HPMA copolymer-doxorubicin conjugate (PK1) as a probe to investigate the extent of the EPR effect in different tumor models, and found that many mouse and human xenograft tumors displayed clear tumor size-dependant EPR-mediated targeting (from ~20% dose/g of tumor tissue in small tumors to 1-5 % dose /g in large tumors). This result was consistent with their previous reports describing the accumulation and retention of ^{125}I-labelled HPMA copolymer in B16F10 and sarcoma 180 tumors (80, 81). Yuan F et al ; (82) measured the size of tumor vessels pores in LS 147 human colon adenocarcinoma implanted in dorsal skin fold chamber in SCID mice and its relation to macromolecular transport into tumor tissues. They demonstrated that the tumor vascular pore could be as large as 0.4 μm in diameter. In another study by Hashizume H et al, using electron microscopy, they were able to identify structural abnormalities in the endothelium of tumor blood vessels due to intercellular openings having a mean diameter of 1.7 μm (range, 0.3–4.7 μm) and

transcellular holes with a mean diameter 0.6 μm in mouse mammary carcinomas. In contrast, the effective diameter of serum albumin with 67 KDa, is 7.2 nm (83). Ohkouchi K et al, used a system composed of Walker 256 solid tumor with a supplying artery and a draining vein to study the extravasation characteristics of mitomycin C-dextran conjugates, where they were able to show the enhanced vascular permeability of macromolecular drugs in solid tumors (84).

To accomplish the EPR effect based cancer drug targeting, the plasma concentration of the drug, generally measured by AUC, must remain high preferably for more than 6 hours (2, 3, 5). Consequently, extravasations into tumor tissue increase progressively with time in a matter of several hours or days, whereas clearance from tumor does not proceed as quickly. Subsequently, the release of the active component or principle from polymeric conjugates (or liposomal drugs) would proceed in tumor tissue to attain therapeutic concentration. For example, we found that SMANCS in Lipiodol (SMANCS /Lipiodol) when administered interarterialy, is cleared very slowly from tumor (clearance takes several weeks), whereas it is delivered and deposited in tumor very quickly. The activity of SMANCS was detected at 20-30 μg/g tumor tissues even 2-3 months after arterial injection of 1 mg/ml (SMANCS /Lipiodol) (85). This remaining activity was more than 100 times of the minimal inhibitory concentration against tumor cells in culture (i.e. SMANCS exhibited a minimum effective concentration below 0.05 μg/ml). This remarkable tumor selective accumulation had resulted in unprecedented antitumor effect in patients with hepatoma (Fig.2).

Figure 2



A typical case of hepatocellular carcinoma treated with SMANCS/Lipiodol. Antitumor effect of intraarterial injection of SMANCS /Lipiodol to a 54 years old patient with clinical diagnosis of unrespectable hapatocellular carcinoma. A) on October 5, 2001 immediately after SMANCS/Lipiodol intraarterial infusion. B) Nineteen months later, after 9 injections of SMANCS/Lipiodol into the hepatic artery, a remarkable reduction of tumor size is seen. Now, the patient is healthy, back to work and has no recurrence or distant metastasis.

Further manipulation of the various factors and mechanisms affecting the EPR effect will enable us to improve targeting efficacy of those drugs (2-14).. Following are the strategies, which have enhanced the therapeutic value of macromolecular drugs.

5.2 Augmentation of drug delivery by modulating the EPR effect

a) Arterial administration using Lipiodol as carrier of polymeric drug

The rationale behind this therapeutic maneuver (arterial administration) is related to the greatest tumor-targeting efficacy, which is attributed to the first-pass effect in addition to the EPR effect. Namely, lipid formulations of SMANCS administered via the tumor-feeding artery result in drug concentrations in tumor of more than 2000 times the concentrations in blood with targeting accuracy of missile(4, 76, 85-87).* [1] Of course, sustained drug retention and slow release from the lipid milieu at tumor site was confirmed *in vivo* not to mention the prominent clinical effect. This highly selective tumor targeting and prolonged retention and drug release makes it possible to have a long interval of a month or longer, before the next administration of drug.

b) Angiotensin II-induced hypertension

As discussed briefly above, the architecture of blood vessels of tumor is quite different from those of normal tissues (89, 90). The response of tumor vessels to the above described vascular mediators, i.e., vasoconstriction by angiotensin II, is impaired, reflecting the anatomical architectural defect as observed under the electron microscope

[1]* This targeting efficacy is far better those of monoclonal antibodies. Critical evaluation of antibody (IgG) and non-antibody targeting delivery to tumor, showed no difference between the two, except for the advantage of the EPR effect which is applicable for both [88].

such as lack of smooth muscle layer. For instance, a slow intravenous infusion of angiotensin II can generate hypertensive state. Under such hypertensive state, e.g., elevation of the systemic blood pressure from 100 mmHg to 160-mmHg, macromolecular drugs such as SMANCS can be delivered more extensively. Namely, if this high blood pressure is maintained for about 15-20 min (at 160 mmHg), about two to three fold increase of drug delivery to tumor is achieved, as evidenced by bioassay of SMANCS even in aqueous formulation, or by radiolabeled albumin as putative polymeric drug (91) . This angiotensin II-induced systemic hypertension opens the vascular endothelial junction in passive manner, thus facilitating transvascular leakage of the macromolecular components into tumor tissue. In contrast, angiotensin II causes blood vessels to constrict in normal tissues and organs, and thus endothelial junctions will contract tighter as smooth muscle cells surrounding the vessels constrict; vasoconstriction (77, 91-95). Consequently, less delivery of unwanted drugs to normal tissues (e.g., bone marrow) would result, i.e. lesser side effects will be seen under angiotensin II induced hypertensive state

c) Angiotensin I converting enzyme (ACE) inhibitor

This enzyme inhibitor is a class of antihypertensive agents that are widely used and very safe. It not only inhibits conversion of angiotensin I to II (thus blocking hypertension), it also inhibits the degradation of bradykinin (BK) due to the similar amino acid sequence at C-terminus of BK to angiotensin I. BK is one of the most potent pain as well as vascular permeability inducers and is generated at inflammatory sites. We reported active generation of BK and apparent presence of extensive BK receptor (B2) recently in various human and rodent solid tumors (8-10, 76, 77, 92-97). Therefore, with the use of

ACE inhibitors such as enalapril or temocapril, one can suppress degradation of BK; thereby activate endothelial nitric oxide synthase to yield nitric oxide (NO). Thus, the EPR effect can be enhanced by modulating both BK and NO (7-9, 77, 92, 95). ACE inhibitors thus increase delivery of macromolecular drugs to the tumor even under normotension. Namely, enalapril and temocapril were shown to enhance the EPR effect (7, 77, 92).

d) Prostacyclin agonists as enhancer of drug delivery based on EPR effect

Prostaglandins I_2 (PG-I_2) and prostaglandins G_1 (PG-G_1) are well known vascular mediators being involved in suppressing platelet aggregation, leukocyte adhesion and enhancing vascular permeability; all common to nitric oxide (7). Injection of this PGI_2 analogue beraprost, which has much longer $t_{1/2}$ *in vivo* than PGI_2, resulted in much enhanced EPR effect similar to ACE inhibitors (98). We believe that this mechanism involves the opening of the tight junction of the endothelium at the postcapillary venule so that plasma component does leak out more effectively into the extravascular space before reaching to the venous side. As an incidental finding, these vasoactive mediators reduced downstream blood flow to an almost negligible amount (to about 10%) (98). Thus, one may be able to enhance selective accumulation of macromolecular anticancer agents in tumor. Likewise, macromolecular tumor imaging agents may be concentrated in tumor tissues, though we need to demonstrate in clinical setting.

CHAPTER 2

SMA-Doxorubicin, A New Polymeric Micellar Drug for Effective Targeting to Solid Tumours

1. Introduction

Doxorubicin quickly became one of the most potent commonly used anticancer agents after its discovery in 1969 (99). However doxorubicin's multiple mechanisms for cell killing were found rather later: Efficient killing of cancer cells is ensured via reactive oxygen species involving quinone group in the anthracycline ring, which is critical for generating intracellular oxidative stress; a DNA intercalating property; and inhibition of topoisomerase (100). Unfortunately, this cytotoxic effect of doxorubicin and many other anticancer agents lacks specificity to cancer cells and hence leads to severe side effects. Acute toxicity affects rapidly dividing cells such as bone marrow cells and intestinal epithelial cells; chronic toxicity affects more stable tissues such as cardiac and hepatic tissues. This nonselective cytotoxicity is the main reason that limits the use of higher doses in most conventional chemotherapeutic drug regimens.

Drug distribution plays a crucial role in toxicity in any tissue, because drugs of small molecular size, i.e., less than 40 kDa (as are almost all conventional chemotherapeutic drugs), are readily distributed via simple diffusion to different organs and tissues, almost indiscriminately, not to mention of unique affinity of individual drugs to certain tissues. Doxorubicin with a molecular weight of 543 Da is known to accumulate preferentially in the heart, and thus cardiac toxicity is the dose- limiting toxicity. This situation is in great

contrast to that for drugs of larger molecular size, over 40 kDa, which accumulate preferentially in tumor tissue because of the unique characteristics of tumor vasculature. Maeda coined the term enhanced permeability and retention (EPR) effect of macromolecular drugs in solid tumors as descriptive of the effect of these larger drugs (2). This phenomenon is attributed to the tumor's anatomical and pathophysiological alterations, such as increased vascular density caused by angiogenesis, impaired lymphatic recovery, and lack of a smooth muscle layer in solid tumor vessels. More important, functional augmentation of this enhanced vascular permeability in tumor tissues has been recognized and includes extensive production of vascular mediators such as bradykinin, nitric oxide, prostaglandins, matrix metalloproteinases, and vascular endothelial growth factor/vascular permeability factor, among others (5, 7, 13). Among normal organs, spleen and liver tend to accumulate polymeric drugs, but the drugs are eventually cleared via the lymphatic system, in contrast to the situation in tumor tissues.

Previously Maeda et al developed SMA (styrene-co-maleic acid copolymer) conjugated neocarzinostatin (NCS), or SMANCS, as a representative and prototypical polymeric drug (101). SMANCS exhibits the EPR effect, and thus tumor-selective drug delivery and retention can be achieved. Indeed in SMANCS with Lipiodol used as a carrier, the tumor/blood ratio of this lipid formulated drug was greater than 2500 when the drug was given intraarterially (4). Consequently, SMANCS therapy, given by arterial injection to patients with advanced hepatocellular carcinoma, produced few side effects but a remarkable therapeutic outcome (102).

One of the advantages of using SMA for polymeric drug development lies in noncovalent

binding to albumin, so that host's albumin, which is the most biocompatible macromolecule, serves as a secondary carrier(103), which aids the EPR effect. The amphiphilic nature of SMA enables a range of formulation possibilities, including an aqueous formulation for iv injection and an oily formulation for interaarterial or oral administration (71, 104, 105). In addition, Maeda et al, showed that SMA confers immunopotentiation in host animals as discussed later, which is in clear contrast to the immunosuppression caused by conventional low molecular weight anticancer drugs.

In this Chapter, the preparation and characterization of the SMA-doxorubicin (SMA-Dox) micelles and its anticancer properties are described.

2. Materials and Methods

2.1. Materials

Doxorubicin was a generous gift from Kyowa Hakko Ltd, Tokyo, Japan. SMA with a mean molecular size of 1280 Da (Mw/Mn: 1.1) was generously supplied by Kuraray Ltd, Kurashiki, Japan. Other reagents were of commercial reagent grade and were used without further purification.

2.2. Synthesis of SMA-Dox micelles

SMA micelles containing doxorubicin was prepared in three steps: First, SMA obtained as alternative copolymer of styrene and maleic anhydride was hydrolyzed in water at pH 14 adjusted by 1 M NaOH and was kept at 50 °C for 4 h. One molecular chain of the resulting styrene maleic acid copolymer has average chain length of 6.4 repeating units of monomer. After hydrolysis, each monomeric unit has a mean molecular size of 221 Da.

Under these conditions, SMA becomes soluble in weak acid and in alkaline water. Second, for preparation of the micelles, SMA solution was prepared to give a concentration of 10 mg/ml, and then the pH was adjusted to 5. To this solution, doxorubicin at a concentration of 10 mg/ml was added. Instantaneous association between SMA and doxorubicin resulted in precipitation of the micelles. Ethyl dimethyl aminopropyl carbodiimide (EDAC) (10mg/ml) was then added in 10 aliquots over 30 min, and pH was kept at 5 by using 0.01 M HCl with stirring. Precipitates were centrifuged at 8,000 rpm for 30 min. Water was added to the original volume, and the suspension was titrated with 1 M NaOH to pH 10.45. During this step, the suspension becomes a clear solution; the pH then was then adjusted back to 7 by addition of 0.1 M HCl. The third and final step involved purification and concentration of the micelles by using ultrafiltration by means of the Amicon ultrafiltration system (YM-10 membrane; cut-off molecular size 10 kDa). The ultrafiltration system allowed washing of the SMA-Dox micelles against distilled water three times, each time with a ten fold greater volume of water than the volume of SMA-Dox, and finally concentrated to 5 ml. The solution was then subject to gel filtration chromatography with Sephadex G-50 Fine column, as described below.

2.3. Characterization of SMA-Dox micelles

2.3.1. Size-exclusion chromatography

Size-exclusion chromatography with Sephadex G-50 Fine column (Pharmacia LKB, Uppsala, Sweden) was performed to determine the change in apparent molecular size of

the new micelles in 0.2 M $NaHCO_3$, pH 8.2 as eluate and using a column size of Ø 3 x 52 cm. To define the apparent molecular size of the micelles more accurately, various known globular proteins were used as molecular size standards, together with Sephadex G-150 column of Ø 2.5 x 64 cm, with 0.25 M $NaHCO_3$, pH 8.24, as the mobile phase, and 2.75 ml fraction each, was collected.

2.3.2 Fluorescence spectrum

Fluorescence spectrophotometer (F-4500, Hitachi,Tokyo,Japan) was used to compare the fluorescence spectrum of the SMA-Dox micelles with that of free doxorubicin. A 10 μM doxorubicin equivalent of either the micelles or the free drug was prepared in distilled water, 10% sodium dodecyl sulfate (SDS) in water, or 100% ethanol. The sample solutions were excited at 480 nm, and emissions at 450-750 nm were recorded.

2.3.3. Release of free drug from the SMA-Dox complex

To verify release of free drug from SMA-Dox micelles in vitro, 20 mg of the micelles preparation was dissolved in 5 ml of distilled water and placed in sealed dialysis tubes (Mr cut-off 1000; Spectrapor, Spectrum Laboratories Inc., San Diego, CA). The dialysis tubes were submerged in three different solution systems containing 25 ml of (1) 100% ethanol; (2) distilled water, pH 7.4; or (3) distilled water, pH 5.5. The dialysis bags were then incubated for several days at 37 °C in the dark with reciprocal shaking at 1 Hz. The doxorubicin released from the dialysis bags was collected at predetermined times and its amount was quantified by measurement at an absorbance of 480 nm.

2.3.4. Elemental analysis

Analyses of SMA–Dox micelles after purification (fractional precipitation, ultrafiltration,

and column chromatography) were carried out for hydrogen, carbon, nitrogen, and oxygen in the micelles. As SMA contains no nitrogen, the nitrogen present in SMA-Dox reflects the amount derived from incorporated doxorubicin.

2.4. In vitro cytotoxicity

In vitro cytotoxicity of SMA-Dox micelles was determined by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (106) with human colon cancer SW480 cells, which were plated in 96-well culture plates (3000 cells/well). Cells were cultured overnight in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The cells were then incubated in the presence of free doxorubicin or SMA- Dox micelles. Toxicity was quantified as the fraction of cells surviving relative to untreated controls.

2.5. Animal model and evaluation of in vivo treatment

Male ddY mice, 6 weeks old and each weighing 30-35 g, were from SLC, Inc. (Shizuoka, Japan). Mouse sarcoma S-180 cells (2×10^6 cells), maintained as ascitic form tumor in ddY mice, were implanted subcutaneously (sc.) in the dorsal skin of the ddY mice. On day 7 after tumor injection when tumors had reached a diameter of 5-7 mm but no necrotic areas were apparent, free doxorubicin or SMA-Dox in distilled water at the desired concentration was administered intravenously (iv) via the tail vein according to the treatment protocol. Solution concentrations were confirmed by UV/visible spectroscopy. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Kumamoto University, and Graduate School of Medical Sciences.

Growth of the tumors was monitored every 2 days by measuring tumor volume with a digital caliper, which was estimated by measuring longitudinal cross section (L) and transverse section (W) and by calculating according to the formula:

$$V = (L \times W^2)/2.$$

The day of tumor implantation was set as day 0, the tumor volume was expressed in cm^3, and means ± SD were calculated.

2.6. Pharmacokinetics evaluation

(^{14}C) Doxorubicin hydrochloride (Adriamycin; Amersham, Buckinghamshire, UK), 91.9 % radiochemically pure with a specific radioactivity of 3.5 MBq/ mg, was used to synthesize radioactive SMA-Dox micelles as described above. The animal tumor model just described was used for evaluation of tissue distribution of drug after 7 days of tumor implantation. The animals were divided into tow groups: The control group received radiolabeled free doxorubicin and the test group received radiolabeled SMA-Dox micelles. Each animal was given 5 mg/kg of doxorubicin equivalent, in which a fraction containing the radioactive doxorubicin, via the tail vein in a 0.2 ml volume.

At scheduled time points, mice were killed and blood samples were drawn from the inferior vena cava. Mice were then subjected to reperfusion with 20 ml of saline to remove blood components from the blood vessels in the tissues. Then, tumor tissues as well as normal organs and tissues, including liver, kidney, gastrointestinal tract (GIT), heart, lung, brain, and muscles, were collected and weighed. Each 100 mg sample of tissue was mixed with 1 ml of Soluene-350 (Packard Instruments, Groningen, The Netherlands), and the mixtures were solubilized by incubation at 60 °C for 4 h, after which

they were added to 10 ml of Hionic Fluor ® LSC cocktail (Packard Instruments). The radioactivity of plasma and various tissues thus solubilized was measured by using a beta liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan).

2.7. Side effects of SMA-Dox micelles

Mice with S-180 tumors of about 5-7 mm in diameter were used for this study. Either doxorubicin or SMA-Dox micelles were administered as described above and after 1, 2 and 3 weeks of drug administration, 20 μl aliquots of blood from medial canthus were collected for full blood counts via an automated blood counter (F-800 Microcell Counter, Toa Medical Electronics, Kobe, Japan). For measurements of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and total creatine phosphokinase, mice were killed 36 h after administration of either free or micellar doxorubicin, 1 ml samples of blood were obtained from the inferior vena cava. Blood was kept at 0 °C until centrifugation as usual at 4 °C, and plasma was separated and stored at –80 °C until analysis by the sequential multiple Auto Analyzer system (Hitachi Ltd., Tokyo, Japan). At the same time of blood collection, the heart, spleen, liver and kidney were collected after reperfusion with 20 ml of physiological saline containing heparin (5 units/ml) to remove blood components from the blood vessels in tissues. Tissues were fixed with 10% buffered neutral formalin solution and then were embedded in paraffin as usual. Prepared sections were stained with hematoxylin-eosin (H&E).

2.8. Statistical analyses

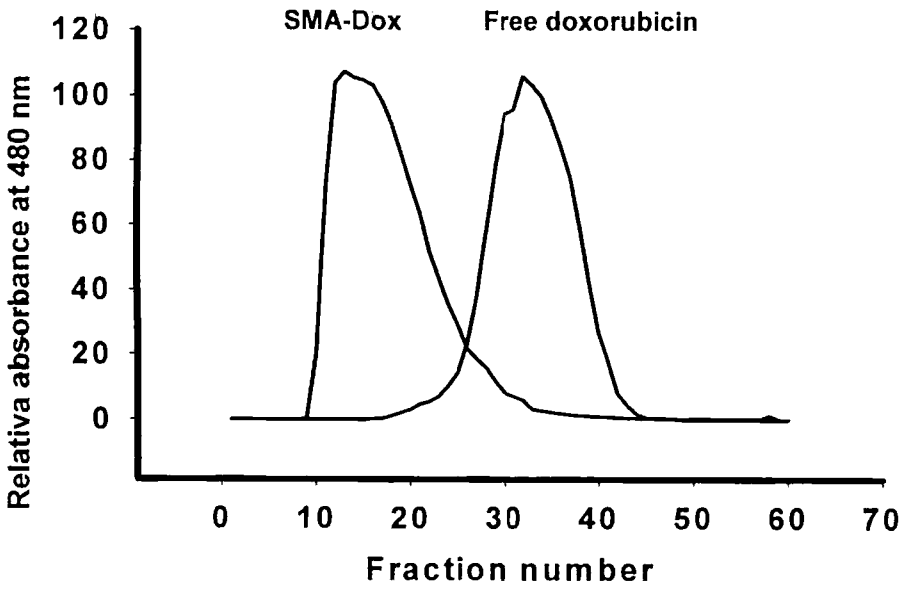
All data were expressed as means ± SD. Student's *t*-test was used to compare differences between experimental groups, and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Synthesis of SMA- Dox micelles

In the present study, we found that the styrene moiety of the amphiphilic polymer SMA formed a complex with the hydrophobic doxorubicin to yield SMA-Dox micelles. The loading efficiency of doxorubicin in the micelles was relatively high: prepared micelles contained 40% (w/w) of doxorubicin, as determined by both UV/ visible spectroscopy and elemental analysis. The yield of the micelles was 80% based on doxorubicin by weight.

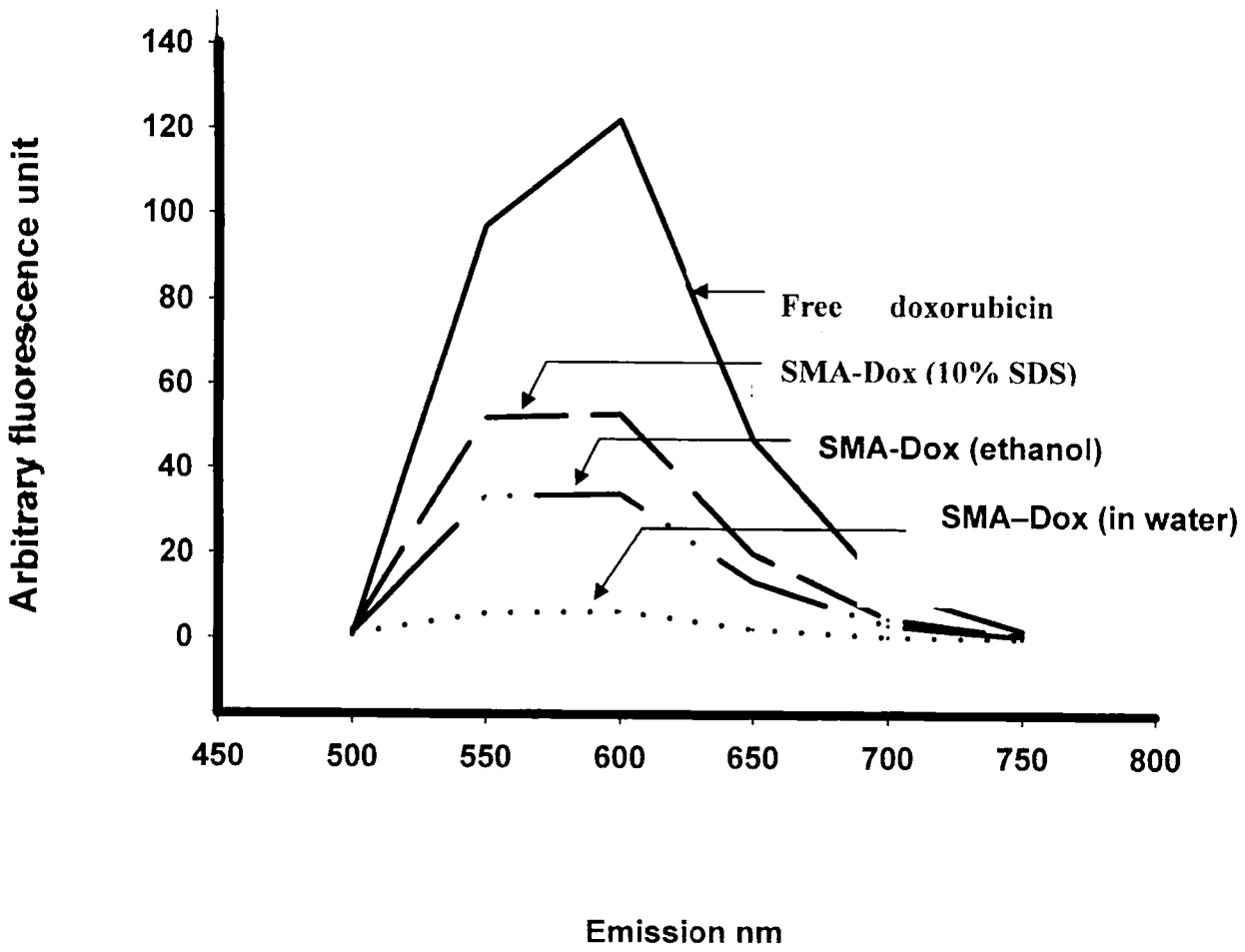
Figure 1



As shown in Fig.1 the SMA-Dox micelles had a clearly increased molecular size compared with free doxorubicin. The average molecular size of the micelles were approximately 34 kDa as determined by Sephadex G-150 chromatographic comparison with the reference molecular markers; bovine serum albumin BSA (67 kDa), ovalbumin

(43 kDa), and NCS (12 kDa). The molecular size of the micelles increased to about 94 kDa when BSA was added. This indicates possible protein- binding of SMA-Dox to albumin, which is plausible in vivo.

Figure 2



3.2. Fluorescence spectrum

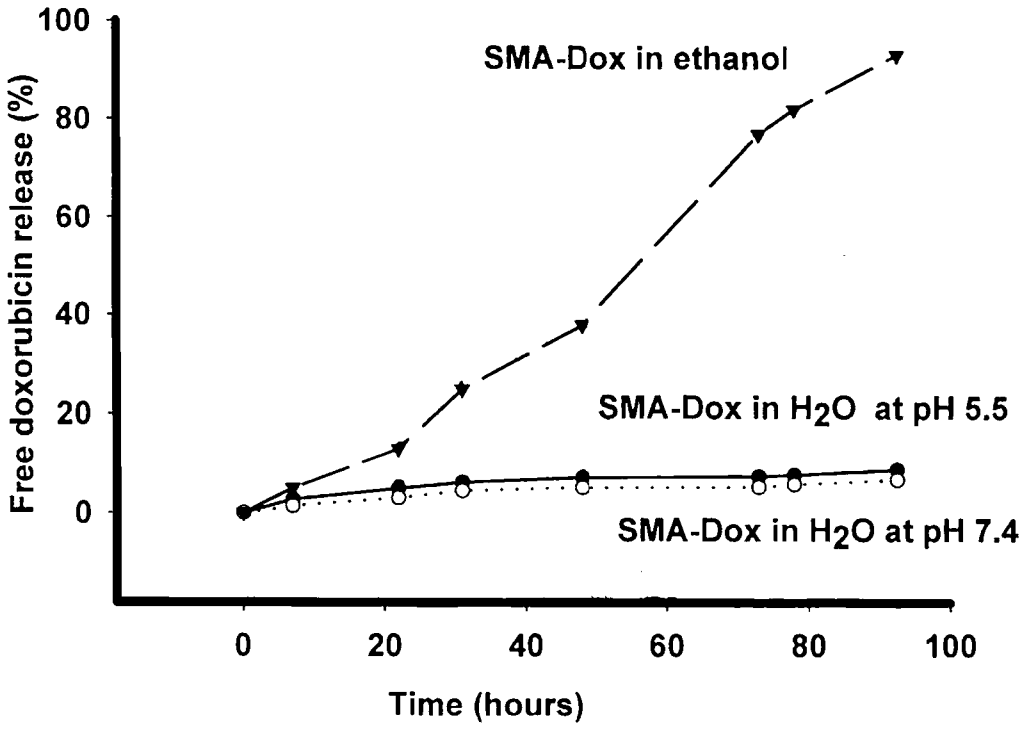
Free doxorubicin when excited at 480 nm shows intense fluorescence emission peaks at both 550 and 590 nm. This fluorescence is greatly quenched when the doxorubicin molecules are close to and interacting with large molecules (107); this was also the case for SMA-Dox micelles, because of energy transfer to aromatic residues in the micelles, which resulted in efficient quenching and suppression of fluorescence. As shown in Fig. 2, the fluorescence spectrum of the SMA-Dox complex was markedly quenched when compared with that of the free drugs, perhaps because the doxorubicin molecules are encased in the SMA micelles or are in very close contact with the aromatic residue of SMA (Fig.3B). In support of this suggestion, the fluorescence intensity was somewhat restored when the micelles were disrupted by exposing to 10% SDS, which would disintegrate the micellar association between the anthracycline and the hydrophobic styrene residue of SMA. Similarly, exposing the SMA-drug micelles to ethanol, which breaks hydrophobic noncovalent bonds of the SMA-drug micelles, made the fluorescence intensity in ethanol increased and approaches that of the drug exposed to SDS solution (Fig.2).

3.3. Free drug release from the SMA-Dox complex in organic media as measured by dialysis

As shown in Fig. 4, free doxorubicin was released very slowly from the SMA-Dox complex in aqueous media: about 3% per day at pH 7.4, with a relatively higher rate at pH 5.5, which indicated poorer stability of the micelles at the lower pH. When the solution outside the dialysis tubes was replaced by ethanol, the release rate increased markedly,

which indicated disruption of the micelles, as described above.

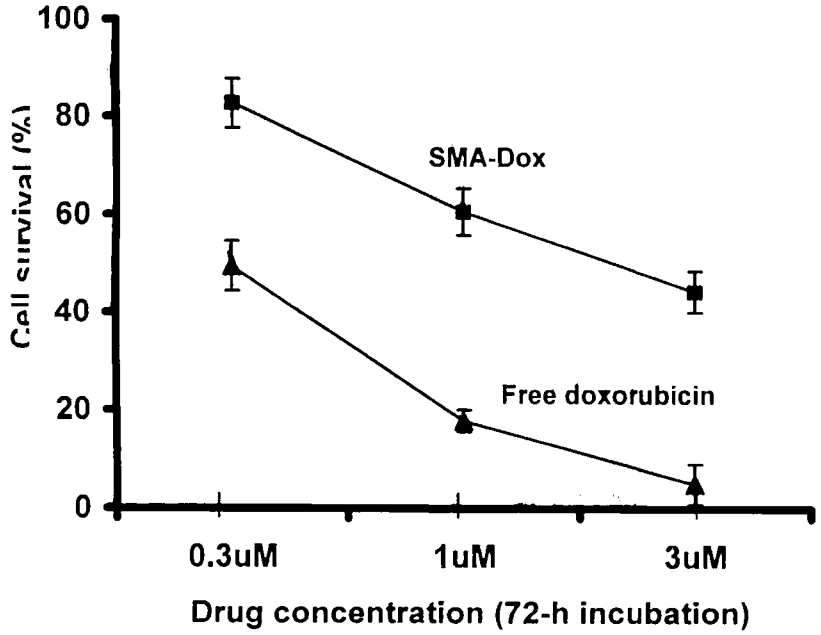
Figure 4



3.4. Biological activity of SMA-Dox against SW480 cells

As shown in Fig.5, the cytotoxic effect of SMA-Dox micelles was considerably lower (about 40%) than that of free doxorubicin in SW480 cells after 72 h incubation. This result can be attributed to a slow uptake into the cells and a strong hydrophobic interaction between doxorubicin and SMA, with a resultant slow drug release rate and thus a delay in bioavailability of free drug to cells in the culture medium.

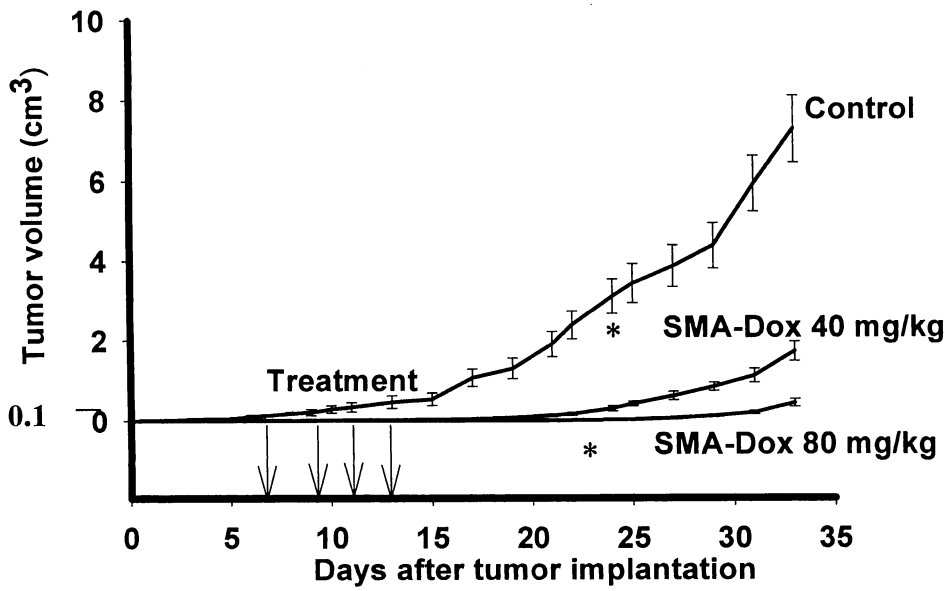
Figure 5



3.5. Antitumor activity of SMA-Dox micelles in vivo

Measurement of tumor volume in animals bearing S-180 tumors for more than 30 days is plotted in Fig. 6, which demonstrates significant suppression of tumor growth by SMA-Dox at total of 40 mg/kg and 80 mg/kg (given in four injections). These doses resulted in 77% and 89% suppression of tumor growth, respectively. Mice receiving the same dose of free doxorubicin equivalent died within 1 week because of drug toxicity.

Figure 6



3.6. Pharmacokinetics and drug distribution

As shown in Fig. 7 A and B, the t½ α (half-life) of SMA-Dox micelles increased about 400 fold, while the area under concentration (AUC) time curve was 25 times greater than that of free drug when calculated for up to 3 days (72 h). Micellar drug concentration in tumor was 13 times higher than free drug in animals 24 h after iv administration. Micellar drug concentration in tissues was higher in the liver, spleen, and kidney, compared with the concentration of free drug after 24 h. The micellar drug tumor/ heart concentration ratio was 4.2 compared with 0.33 in case of free doxorubicin.

Figure 7 A)

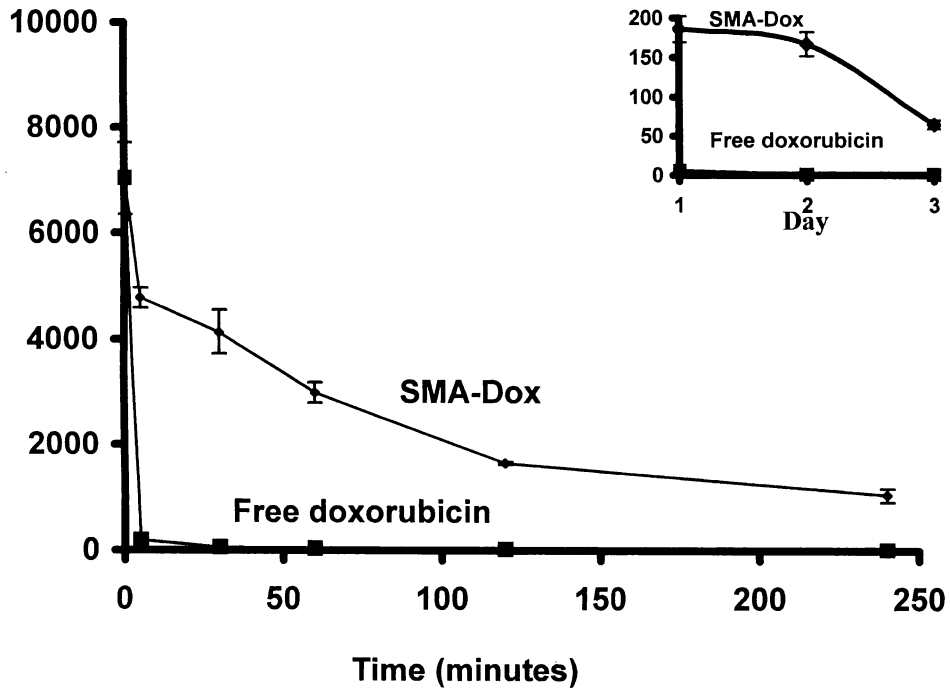
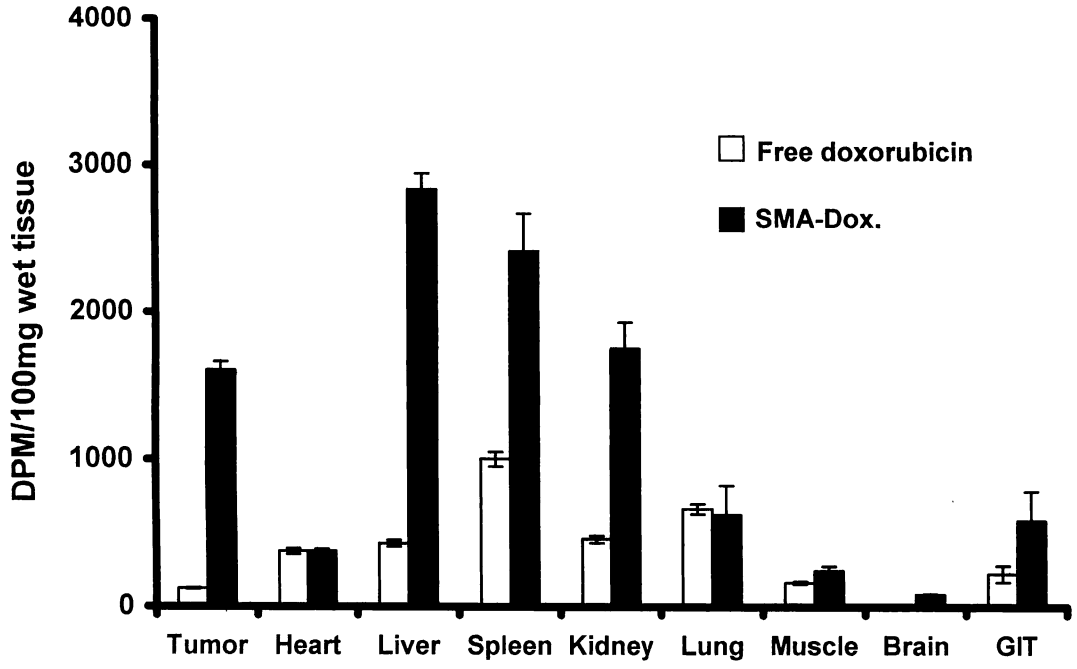

10000
8000
6000
4000
2000
0
0
50
100
150
200
250
Time (minutes)
SMA-Dox
Free doxorubicin
200
150
100
50
0
1
2
3
Day
SMA-Dox
Free doxorubicin

Figure 7 B)



3.7. Potential side effects of SMA- Dox micelles

Tables 1 and 2 show blood counts and cardiac and liver function measures in mice receiving 10 mg/kg free doxorubicin and animals receiving 20 mg/kg SMA-Dox complex (20 mg/kg free doxorubicin equivalent). Values for animals receiving the micellar drug were not statistically different from values for normal healthy mice. In contrast, animals receiving free doxorubicin showed serious side effects, with significant differences from the control group.

Table 1 Blood assays for ddY mice receiving a single dose of 10 mg/kg free doxorubicin or 20 mg/kg equivalent dose of drug as SMA-Dox

Drug treatment	Time when measured	Hemoglobin (g/dl)	White blood cell Count (cells/μl)	Platelet count (/mm^3)
Control		14.9 ± 0.46	8012 ± 565	132.8 ± 5.36
Free doxorubicin	1 week	14.9 ± 0.20	3516 ± 292	86.5 ± 12.70
	2 weeks	12.9 ± 0.05	4450 ± 1028	80.8 ± 10.60
SMA-Dox				
	1 week	15 ± 0.29	6966 ± 554	106.0 ± 3.60
	2 weeks	16 ± 0.72	6366 ± 502	133.0 ± 2.10

Values are means ± SD.

Table 2 Liver function and cardiac enzymes assays for ddY mice receiving a single dose of 10 mg/kg free doxorubicin or 20 mg/kg equivalent dose of drug as SMA-Dox.

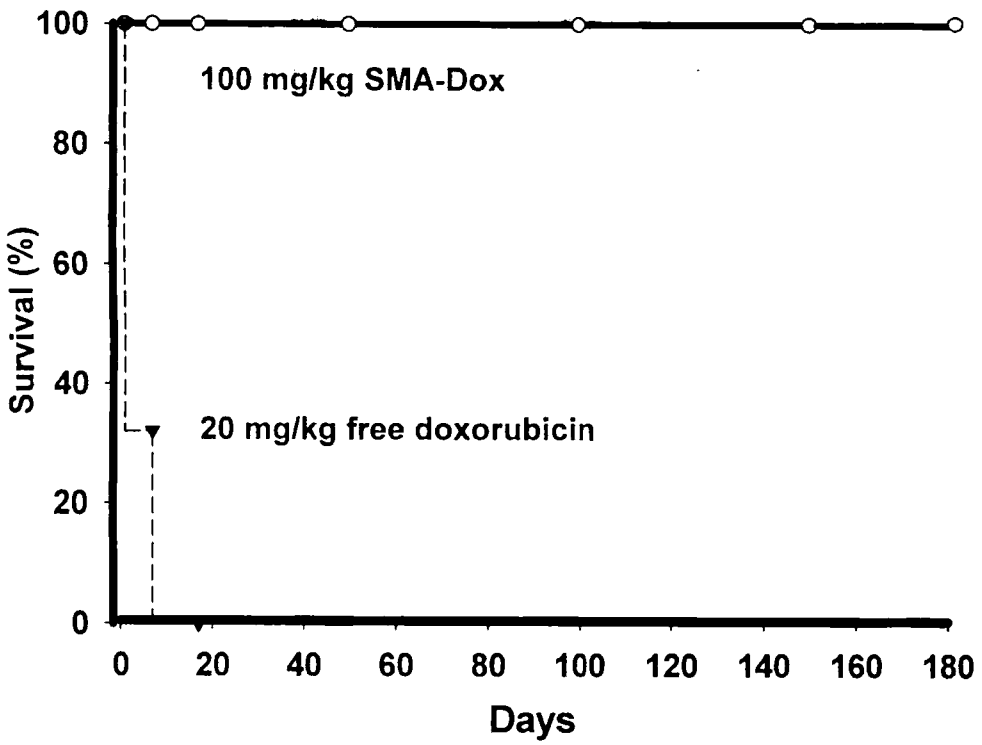
Drug treatment	CPK (U/L)	LDH (IU/L)	ALT (IU/L)	AST (IU/L)
Control	309 ± 42	260 ± 62.4	37 ±7.1	81 ± 7.29
Free doxorubicin 10 mg/kg	1359 ± 169*	606 ± 57*	51 ± 8	232 ± 28*
SMA-Dox 20 mg/kg	344 ± 47	287 ± 26	47 ± 5.6	78 ± 6.1

Values are presented as means ± SE, CPK, creatine phosphokinase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

* Difference from control is statistically significant.

Animals receiving a total dose of up to100 mg/kg SMA-Dox when administered by four injections (25 mg/kg x 4) during 1 week, or 70 mg/kg when administered as a single iv bolus, showed extended survival for more than 6 months compared with animals receiving a total dose of 20 mg/kg free doxorubicin administered by 4 injections (5 mg/kg x 4); all mice receiving free drug of died within 1 week (Fig. 8). Cardiac H&E staining showed extensive inflammation in animals receiving free doxorubicin, whereas animals receiving SMA-Dox showed no abnormalities.

Figure 8



4. Discussion

The main limitation in cancer chemotherapy is the severe toxicity to normal organs and hence a restriction in terms of doses prescribed. The fact that, the more potent the anticancer agents, the greater the toxicity to normal tissues leads to the use of only a fraction of the curable dose of a drug. This is especially true for the anticancer agent doxorubicin, which has notorious cardiotoxic effects. In the present study, we prepared SMA-Dox micelles by a simple procedure, which consists of SMA-copolymer and drug only (Fig. 3A). It is most likely that, the surface of the outer shell of the micelles have polar ionized carboxyl group of the maleic acid with the inner core consisting mainly of the hydrophobic styrene group and doxorubicin (Fig.3 B). In agreement with this model, the prepared micelles exhibited quenched fluorescence after its formation, with the original fluorescence partially restored after disruptive treatment with 10% SDS as well as ethanol, which disrupted micellar structure (Fig. 2, Fig. 3 B). This model of micelles formation was further validated by an experiment that showed very slow release of doxorubicin from SMA-Dox micelles in water, about 3% per day, but an increased release rate of about 25 % per day when the water was replaced by ethanol. This finding indicated the dissociation of the micelles as a result of noncovalent bond disruption in ethanol, and hence liberation of free doxorubicin, (Fig. 4).

The synthetic protocol of this micelle formation was initially intended to cross- link between amino group of doxorubicin and carboxyl group of maleic acid in SMA by the treatment of EDAC; involving dehydration reaction aiming the formation of carboamide linkage (2).

However, this strategy did not proceed as we found that free doxorubicin was easily liberated from the SMA-Dox micelles without harsh hydrolytic conditions. This notion is consistent with the absence of amide bond signals when the complexed polymer was examined by the infrared spectroscopy in contrast to other cases (108). Furthermore, we found that substituting EDAC with other polyamines can serve similar catalytic function for molecular orientation to form SMA-micelles.

This micelles formation resulted in an apparently large molecular size, about 34 kDa (Fig. 1), which become even larger in vivo because of binding to albumin (103), so that the final average size was 94 kDa. This large molecular size allows the EPR effect to operate, by which selective accumulation of macromolecules and lipids occurs in a variety of solid tumors (2, 3, 80,109).

According to the EPR effect, biocompatible macromolecules and lipids preferentially and spontaneously will leak out from tumor vessels into tumor tissues and remain sequestered in the vicinity of tumor as a result of anatomical and pathophysiological abnormalities of tumor tissues (2,3, 8, 9, 81, 86, 88,110-113). This EPR effect is a molecular size-dependent phenomenon: it functions when the molecular size is above Mr 40,000 (2, 3, 80, 86 ,112-113). This size dependency is inversely correlated with renal clearance of the compounds (2, 3, 86, 88, 110, 112-113). Therefore, the EPR effect is seen with any biocompatible macromolecule that has a molecular size larger than the renal excretion threshold, provided that the level remains high in the circulating blood, preferably for more than 6 h.

As expected, free doxorubicin (Mr 543) was rapidly cleared from the circulation after iv

injection (Fig. 7A) and was not retained in tumor tissue (Fig. 7B.). In contrast, SMA-Dox micelles showed an increased in vivo half-life (Fig. 7A), and, more important, greater selective accumulation in tumor tissue based on the EPR mechanism. The relative concentration of SMA-Dox micelles in tumor tissue was about 13 times higher than that after an equivalent dose of free doxorubicin (Fig. 7B). Previously similar beneficial effects of polymeric carriers were found, including pyran copolymer, succinylated keratin, and polyethylene glycol (86,109, 12-115). However, the advantage of using SMA is that it is amphiphilic and can form micelles (101), which may encapsulate the hydrophobic doxorubicin in its core. Thus high drug loading was possible (doxorubicin loading was about 40%), and was also high in total recovery of the doxorubicin (80%)without elaborate technique being required. Another advantage of SMA is that, in animal models it confers immunopotentiating effects such as activation of macrophages, T-cells, and NK cells and induction of interferon (116-118).

The micellar form showed lower cytotoxicity in vitro compared with free doxorubicin (Fig. 5). Lower in vitro cytotoxicity is also known for other polymeric doxorubicin compounds; such as hydroxypropylmethacrylate- copolymer conjugates (38, 81). This finding can be explained by slow intracellular uptake of the polymeric drug by endocytosis, in addition to a strong association between the polymeric carrier and the drug, which results in slow release of free drug from the polymeric micelles.

Results from in vivo studies showed that drug concentration was much higher in tumors than in normal tissues because of the EPR effect, and hence, as expected, a higher antitumor effect was observed. These results, in addition to a higher drug release rate

under relatively acidic conditions in tumor tissue (119), confirmed the more potent antitumor activity of SMA-Dox micelles in vivo.

The remarkable safety of SMA-Dox with doses up to 10 times the maximal tolerable dose of free doxorubicin is evidence of the improvement imposed by stable micelles formation and tight entrapment of this drug in the micelles, as verified in vitro and in vivo (Fig. 5,8 and table 1,2). The stable macromolecular nature prevents leakage of SMA-Dox from vessels in normal tissues during circulation (Fig. 4).

In other words, successful tumor-targeted therapy requires higher rate of drug accumulation in tumor tissue, than the release rate of the drug from the micelles during circulation, as shown for SMA-Dox, to avoid systemic toxicity.

In conclusion, the present study demonstrated the preparation of SMA-Dox micelle, its tumor-targeted property and antitumor effect, plus remarkable safety of SMA-Dox micelles in tested mice.

CHAPTER 3

Copoly(Styrene-Maleic Acid)-Pirarubicin Micelles: High Tumor Targeting Efficiency With Little Toxicity

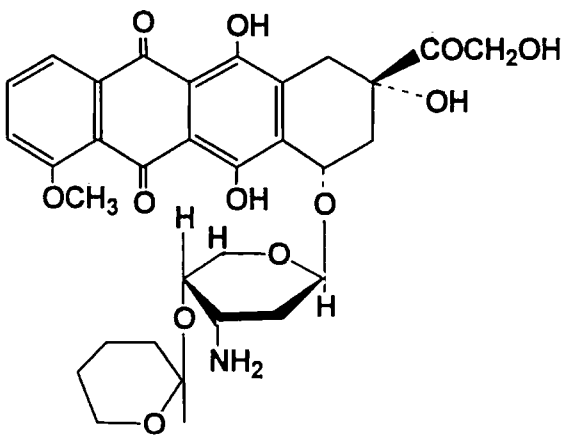
1. Introduction

Pirarubicin, (4′-O-tetrahydropyranyladriamycin, or THP) is an anthracycline antibiotic that was developed during a search for less cardiotoxic agents among the 4′ -O-substituted anthracyclines (120). Pirarubicin proved effective against hematological malignancies and was used for treatment of acute leukemias and malignant lymphomas, as well as breast cancer (121, 122). The drug has both lipophilic and hydrophilic properties, which allows either intravenous (i.v.) or intraarterial injection with Lipiodol used as a carrier for treatment of liver metastasis and hepatocellular carcinoma (123). Unfortunately, THP lacks specificity for cancer cells, and its potent cytotoxicity, like that of many other low-molecular-weight cytotoxic anticancer agents leads to severe side effects. As in most conventional chemotherapeutic drug regimens, this non-selective cytotoxicity is the primary reason for dose limitation.

It was previously found that drugs of large molecular size, i.e., those of more than 40 kDa, preferentially accumulate in tumor tissue because of the unique characteristics of the tumor vasculature (2, 3, 76, 77 86, 124, 125). Solid tumor tissue usually shows increased vascular density caused by angiogenesis, impaired lymphatic recovery system, and lack of a smooth muscle layer in the blood vessels. More important, functional augmentation of enhanced vascular permeability in tumor tissues has been identified

and involves extensive production of vascular mediators such as bradykinin, nitric oxide, prostaglandins, matrix metalloproteinases, and vascular endothelial growth factor/vascular permeability factor, among others (5, 7, 10, 13, 14, 126). In the Chapter, the development of micelles containing copoly(styrene-maleic acid) (SMA) and pirarubicin (THP) (Fig. 1) is described. This micelle with a large molecular size, takes advantage of the EPR effect and achieve tumor-selective drug delivery as well as an excellent therapeutic effect.

Figure 1



One advantage of using SMA for polymeric drug development relates to its noncovalent binding to albumin, which thus permits plasma albumin, one of the most biocompatible macromolecules, to serve as a secondary carrier of the drug (103). This SMA-micelle preparation takes advantage of the EPR effect. The amphiphilic nature of SMA enables a wide range of formulation possibilities, including an aqueous formulation for i.v. injection and an oily formulation for intraarterial or oral administration (4. 85. 87. 103, 104, 127). With higher lipophylic nature, facilitated uptake into cells was demonstrated (105). In addition, it was previously shown that when animals were

treated with SMA conjugates, the SMA moieties conferred immunopotentiating activity (116-118, 128); this result is in clear contrast to the immunosuppression caused by conventional small-molecular-size anticancer drugs.

In the previous chapter we described the preparation and characterization of SMA-doxorubicin micelles, which showed marked tumor accumulation in tumor after i.v. administration compared with that obtained with free doxorubicin, i.e., a 13-fold increase in tumor uptake of drug and a 25-fold increase in the plasma level of drug (AUC) in a 24-h period (129). The SMA-doxorubicin micelle preparation produced a potent antitumor effect with fewer side effects at doses as high as 100 mg/kg of doxorubicin equivalent; in contrast, the parent drug had an LD_{100} of 10 mg/kg (129).

In this chapter , we describe the preparation and characterization of SMA-THP micelles and the anticancer properties of this formulation.

2. Materials and *methods*

2.1. Materials

Pirarubicin (also referred to as THP), was a generous gift from Meiji Seika Kaisha, Ltd, Tokyo, Japan. SMA as anhydride form of maleyl group with a mean molecular size of 1435 Da (Mw/Mn: 1.1), the range being between 901 and 1781 Da, was generously supplied by Kuraray Ltd, Kurashiki, Japan. Other reagents were of commercial reagent grade and were used without further purification.

2.2. Chemical analysis

Visible/UV absorption was determined by use of a spectrophotometer (U-200, Hitachi Ltd., Tokyo, Japan). THP content was quantified via analysis of UV absorption at 487 nm. Fluorescence spectra were obtained by use of a fluorescence spectrophotometer (F-4500, Hitachi), excitation at 480 nm and emission at 450-750 nm. Infrared (IR)

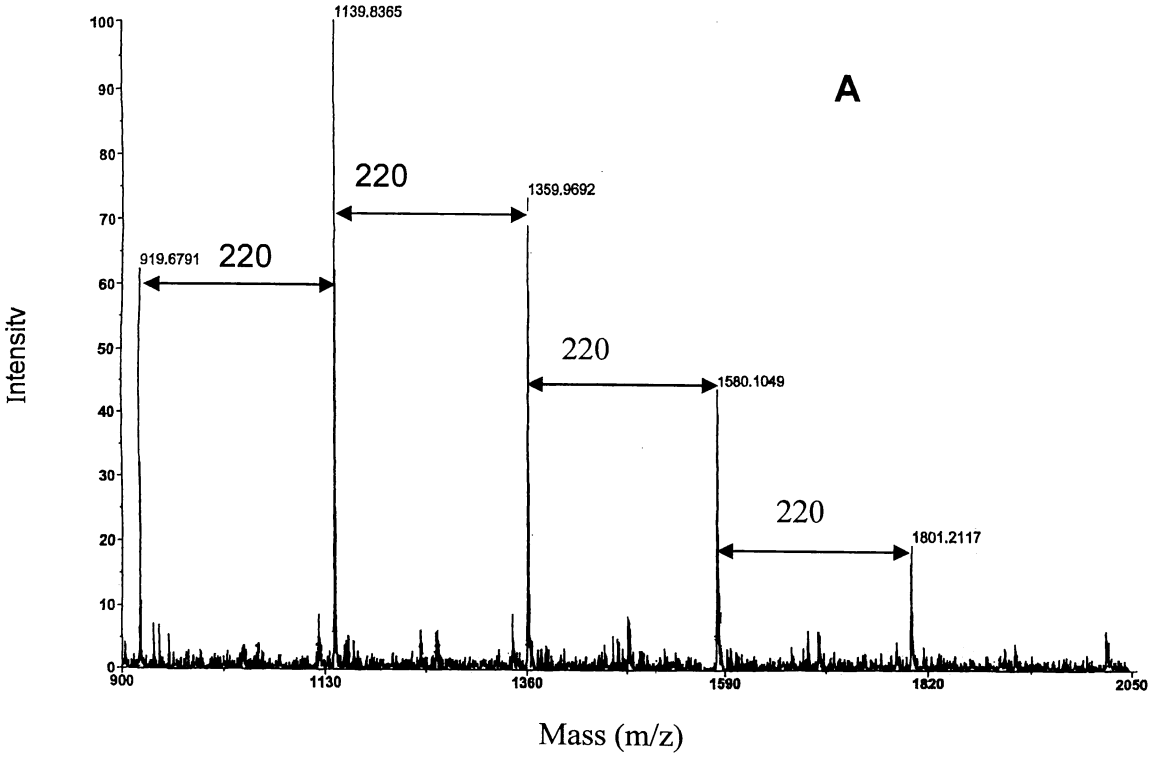
spectra were recorded with a JIR-6500w spectrometer (JEOL, Tokyo, Japan) using KBr disc. Mass spectra were obtained by means of matrix-assisted laser desorption-ionization/time-of-flight (MALDI-TOF) mass spectrometry (Voyager System 6192, ABI, Foster City, CA).

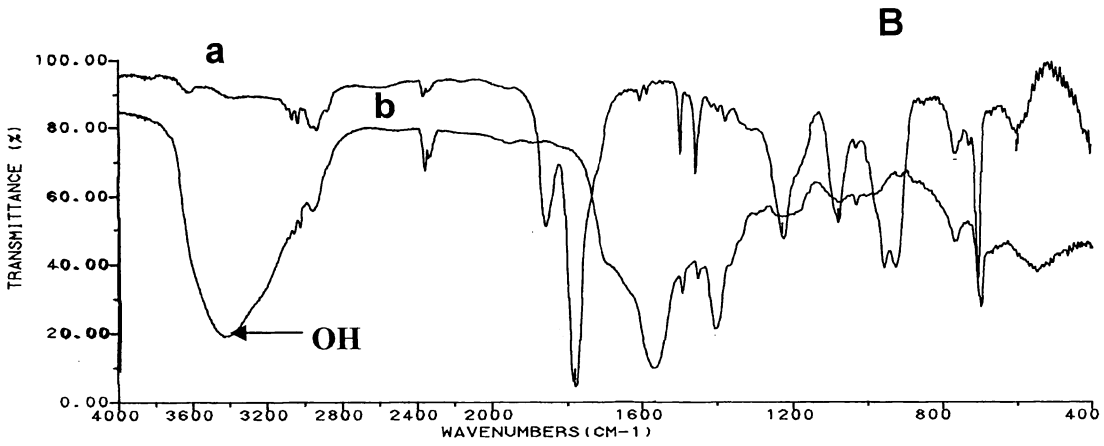
2.3. Preparation of SMA-THP micelles

Micelles were prepared as previously described in SMA-doxorubicin micelles preparation (129). Briefly SMA, (anhydrous) copolymer was hydrolyzed in 0.05 M NaOH. Hydrolysis produced an increase of 18 Da. Consequently, the mean molecular size of each repeating unit become 220 Da (Fig. 2A). IR spectra showed the appearance of an OH signal at about 3400 cm^{-1} (Fig. 2B a, b).

< Fig.2 A, B >

Figure 2





To form the micelles, SMA powder, was dissolved in water at pH 5.0 at 10 mg/mL, then THP solution at a concentration of 10 mg/mL was added to this solution, Followed by addition of the water soluble carbodiimide, ethyldimethylaminopropylcarbodiimide (EDAC) to 10 mg/mL. Resulting precipitates were washed and collected by centrifugation at 8000 rpm and solubilized then reconistuted in pH 7.0. The micelle solution (about 10 mg of THP/mL) was washed and concentrated to one tenth of the original volume by means of the ultrafiltration method with the Amicon ultrafiltration system (YM-10 membrane; cut-off molecular size of 10 kDa), which was repeated 3 times.

2.4. Sephadex G-50 and G-150 Gel filtration

Size-exclusion chromatography with Sephadex G-50 Fine column (Pharmacia LKB, Uppsala, Sweden) was performed to separate the large-molecular-size SMA-THP micelles (with 0.2 M $NaHCO_3$, pH 8.2, as eluant, and a column size of Ø 3 x 52 cm), and the peak fraction was lyophilized. The powder thus obtained was subjected to IR and UV/visible spectroscopy.

To define the apparent molecular size of the micelles more accurately, various known

globular proteins were used as standards. For this analysis, a Sephadex G-150 chromatography column of Ø 2.5 x 64 cm, with 0.25 M $NaHCO_3$, pH 8.2, as eluant, was used.

2.5. Dynamic Light Scattering (DLS measurement)

The light scattering (DLS) measurements were performed on photal DLS-7000 HLs instrument (Otsuka Electronics, Osaka, Japan); equipped with a He-Ne laser(10mW) at a wavelength of 632.8 nm. The scattering angle was fixed at 90^0 and the temperature of the sample was maintained at 25^0 C. Particle size determination was carried out by using 2mg/ml sample prepared in PBS (pH 7.4). The sample was filtered through 0.2m filter (Millex, Millipore, USA) prior to measurement.

2.6. Release of free drug from the SMA-THP micelles

To verify the release of free drug from SMA-THP micelles in vitro, 20 mg of the micellar preparation was dissolved in 5 mL of distilled water and placed in sealed dialysis tubes (Mr cut-off of 1000 Da; Spectrapor, Spectrum Laboratories Inc., San Diego, CA). The dialysis tubes were submerged in one of three different solutions: (a) 100% ethanol; (b) aqueous solution, pH 7.4; or (c) aqueous solution, pH 5.5. The dialysis tubes, with 25 mL of each solution above, were reciprocally shaken at 1 Hz for several days at 37 °C in the dark. Aliquots of dialysate solution were taken at predetermined time points, and the amount of THP in the dialysate was quantified by absorption at 487 nm.

2.7. In vitro cytotoxicity

In vitro cytotoxicity of SMA-THP micelles was determined by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (106) with human breast cancer MCF-7 cells and colon cancer SW480 cells, which were plated in

96-well culture plates (3000 cells/well). Cells were cultured overnight in Dulbecco's modified Eagle's medium .with 10% fetal calf serum. The cells were then incubated in the presence of free THP or SMA-THP micelles for 3 days. Toxicity was quantified as the fraction of cells surviving relative to untreated controls.

2.8. Evaluation of intercellular uptake of SMA-THP compared with free THP

Intercellular uptake of both SMA-THP micelle and the free THP were evaluated through fluorescence emission. 5000 SW-480 cells per plate were incubated overnight in 12 well plate in DMEM 10% FCS at 5% CO_2. Either SMA or SMA-THP was added at 10 uM to the culture media. At specific time points, the media with drug were removed; cells were then homogenized by 1% triton x 100 (250 uL) , then THP was extracted by using in 4 N HCl 67% ethanol 1 ml. The amount of internalized intercellular THP in case of SMA micelle or free drug was measured after excitation at 480 nm by relative fluorescence intensity at 550 nm.

2.9. Evaluation of the antitumor effect of SMA-THP micelles in animal tumor models

Various mouse tumor models including sarcoma S-180, Meth A, Lewis Lung Carcinoma, and Colon 38 were tested. Briefly 2 x 10^6 cells were implanted subcutaneously in the dorsal skin of 6-week-old mice each. On day 7 after tumor cell inoculation, when tumor size was 5-7 mm in diameter but contained no necrotic areas, i.v. injections of free THP or SMA-THP micelles, in distilled water at the desired concentration, were administrated into the tail vein. Concentrations of the solutions were standardized via visible absorbance at 487 nm. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Kumamoto University.

Tumor growth was measured every 2 days by size of the tumors with a digital caliper, the tumor volume (V) being estimated by using the measured longitudinal cross section (L) and transverse section (W) according to the formula,

$V = (L \times W^2)/2$

The day of tumor implantation was set as day 0; the tumor volume was expressed in cm^3.

2.10. Pharmacokinetics and drug distribution

The animal tumor model just described was used for evaluation of tissue distribution of drug after 7 days of tumor implantation. The animals were divided into two groups: The control group received free THP and the test group received SMA-THP micelles. Each animal was given 5 mg/kg of THP equivalent, via the tail vein in a 0.2 ml volume.

At scheduled time points, mice were killed and blood samples were drawn from the inferior vena cava. Mice were then subjected to reperfusion with 20 ml of saline to remove blood components from the blood vessels in the tissues. Then, tumor tissues as well as normal organs and tissues, including liver, kidney, gastrointestinal tract (GIT), heart, lung, brain, and muscles, were collected and weighed. Each 1 gm sample of tissue was mixed with 10 ml of 4 N HCl 67% ethanol, and then heated for 70° C for 5 minutes then homogenized. This solution was centrifuged for 5 minutes at 7000 RPM then the resulting supernatant was diluted 100 X in 4 N HCl 67% ethanol. The amount of THP in case of SMA micelle or free drug was measured after excitation at 480 nm by relative fluorescence intensity at 550 nm.

2.11. Side effects of treatment with SMA-THP micelles

Mice with S-180 tumors of about 5-7 mm in diameter were used for this study. Either free THP or SMA-THP micelles were administered as described above. One, 2 and 3

weeks after drug administration, 20 μL samples of blood were collected from the medial canthus for complete blood counts by means of an automated blood counter (F-800 Microcell Counter, Toa Medical Electronics, Kobe, Japan). For measurements of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and total creatine kinase, mice were killed 36 h after administration of either free drug or micellar THP, and 1 mL samples of blood were obtained from the inferior vena cava. Blood was kept at 0 °C until centrifugation at 4 °C, 3000 rpm for 15 min; and plasma was separated and stored at −80 °C until analyses by use of the Auto Analyzer system (Hitachi Ltd., Tokyo, Japan). At the time of blood collection, tissue specimens (heart, spleen, liver, and kidney) were also obtained; reperfusion with 20 mL of physiological saline containing heparin (5 units/mL) had been performed to remove blood components in blood vessels of the tissues. These tissue specimens were fixed in formalin and then embedded in paraffin and stained with hematoxylin-eosin (H&E).

2.12. Statistical analyses

All data were expressed as means ± SD. Student's *t*-test was used to compare differences between experimental groups.

3. Results

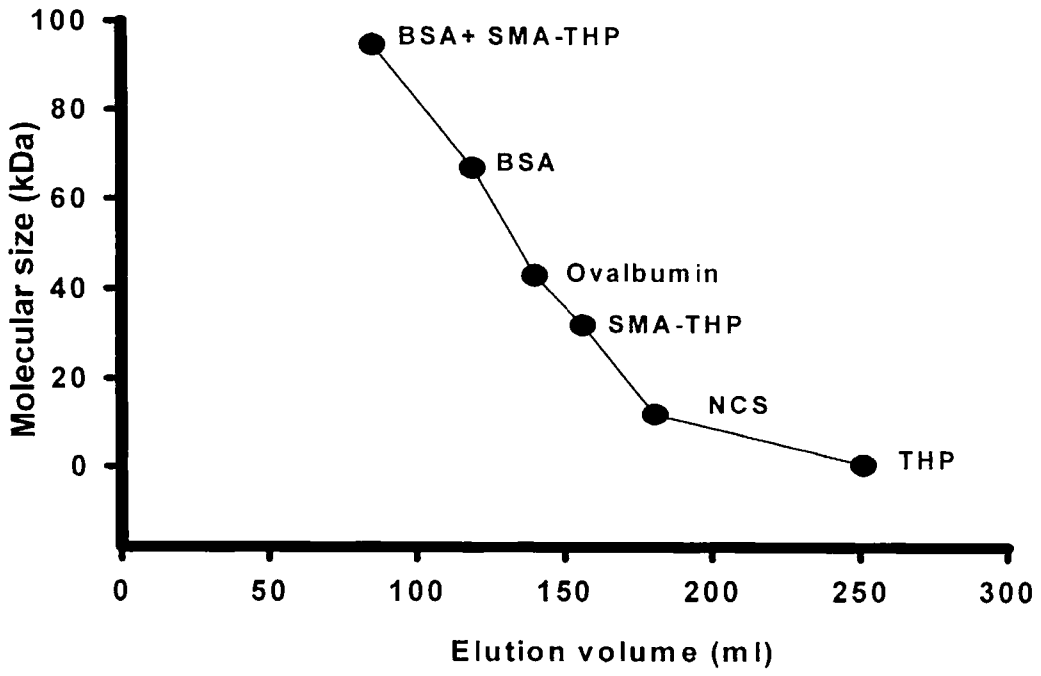
3.1. Synthesis of SMA-THP micelles

The loading efficiency of THP in the micelles was rather high: the micelles contained 60% (w/w) THP, as determined by spectroscopy and elemental analysis (i.e., N, C, and H content). The yield of drug from the micelles was more than 80% (by weight). It is most likely that the surface of the micellar outer shell has the polar, ionized carboxyl group of the maleic acid and the inner core consists mainly of the hydrophobic styrene

group and pirarubicin chromophore.

As shown in Fig. 3, the SMA-THP micelles had a clearly increased molecular size compared with free pirarubicin. The average molecular size of the micelles was approximately 34 kDa (Fig. 3): as determined by Sephadex G-150 column chromatography. The molecular size of the micelles increased to about 94 kDa when bovine serum albumin (40 mg/mL) was added, which indicates binding of SMA-THP to albumin .

Figure 3

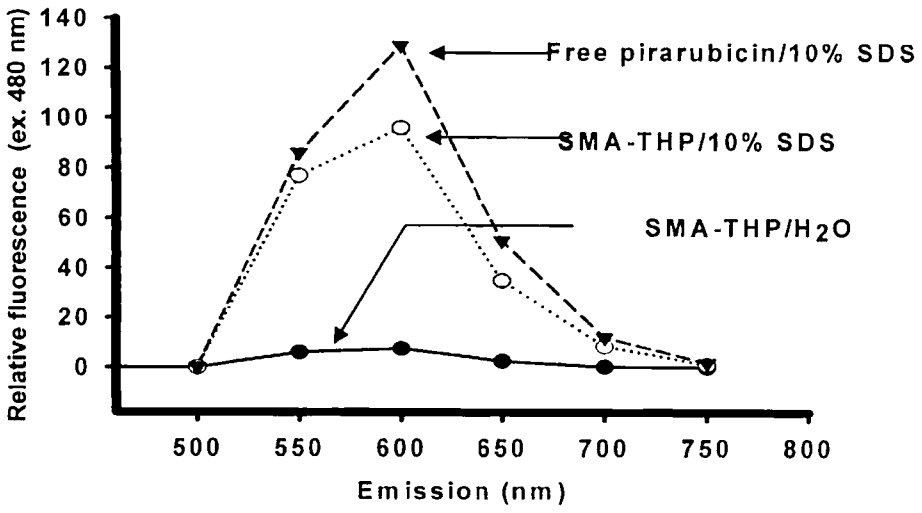


3.2. Studies on miceller formation

When excited at 480 nm, free THP showed intense fluorescence emission with a maximum peak between 550 and 600 nm (maximum at about 590 nm) (Fig. 4). Figure 4 shows that, in fact, the fluorescence spectrum of the SMA-THP complex was

markedly quenched when compared with that of the free drug, perhaps because the THP molecules form part of the core of the SMA-THP micelles, or are in very close contact with SMA (or styrene). The fluorescence intensity was restored when the micelles were disrupted by exposure to 10% SDS, which destroyed the micellar association between the anthracycline ring and the hydrophobic styrene residue of SMA

Figure 4



Consequantly, Sephadex G-50 chromatography showed that SMA-THP exposed to 10% SDS had a distinctly smaller molecular weight equivalent to free THP, compared to the untreated micelles (data not shown).

3.3. Free drug release from SMA-THP micelles

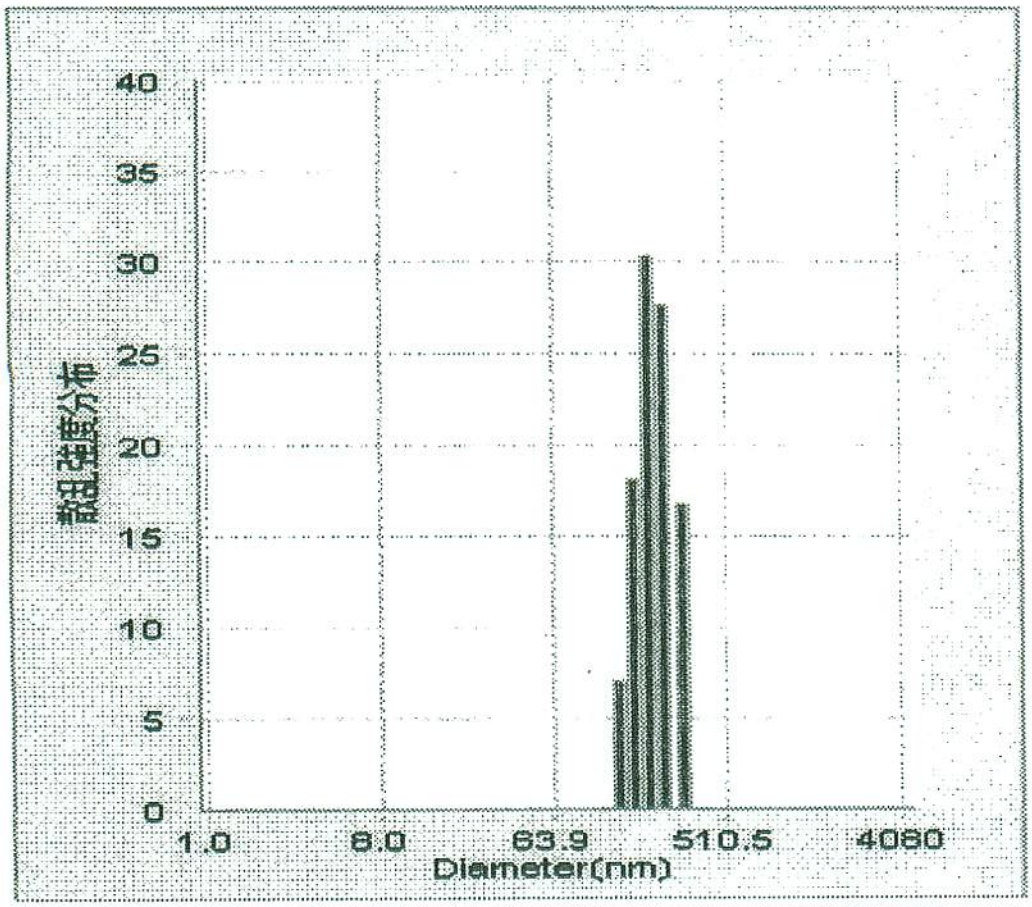
Free pirarubicin was released very slowly from the SMA-THP complex in aqueous media: about 3% per day at pH 7.4 and 4% per day at pH 5.5, which indicated higher

stability of the micelles at neutral pH. When the solution outside the dialysis tubes was replaced by ethanol, the release rate increased markedly, which indicates that disruption of the micelles occurred, as described above.

3.4. Dynamic Light Scattering

As shown in figure 5, the Mean particle size of SMA-THP was found to be 248 nm. This large molecular size can be as larger as 315 nm in vivo due to albumin binding.

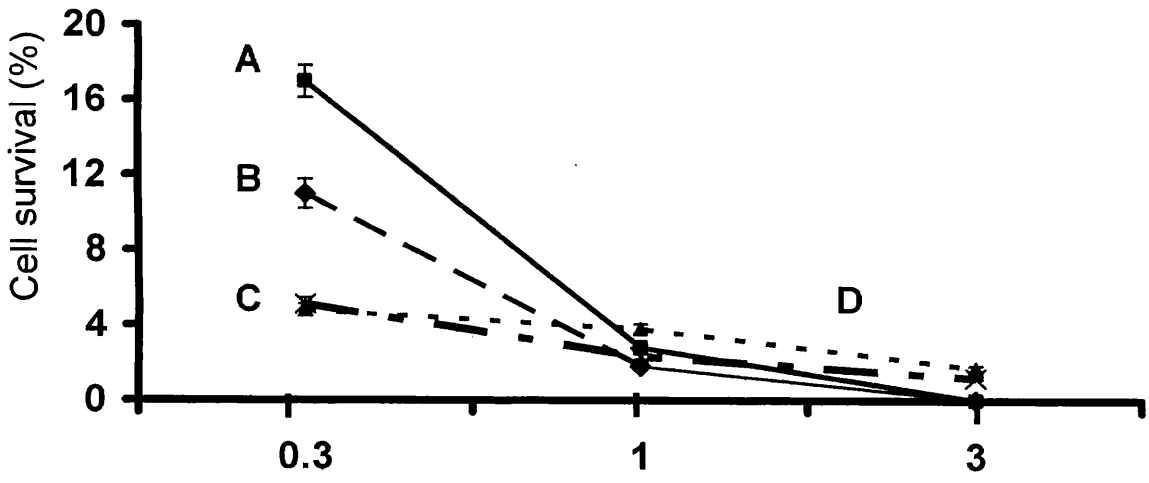
Figure 5



3.5. In vitro biological activity of SMA-THP micelles

As shown in Fig. 6, the cytotoxic effect of SMA-THP micelles on MCF-7 and SW480 cells was quite similar to that of free drug (93-101%) after 72 h of incubation.

Figure 6

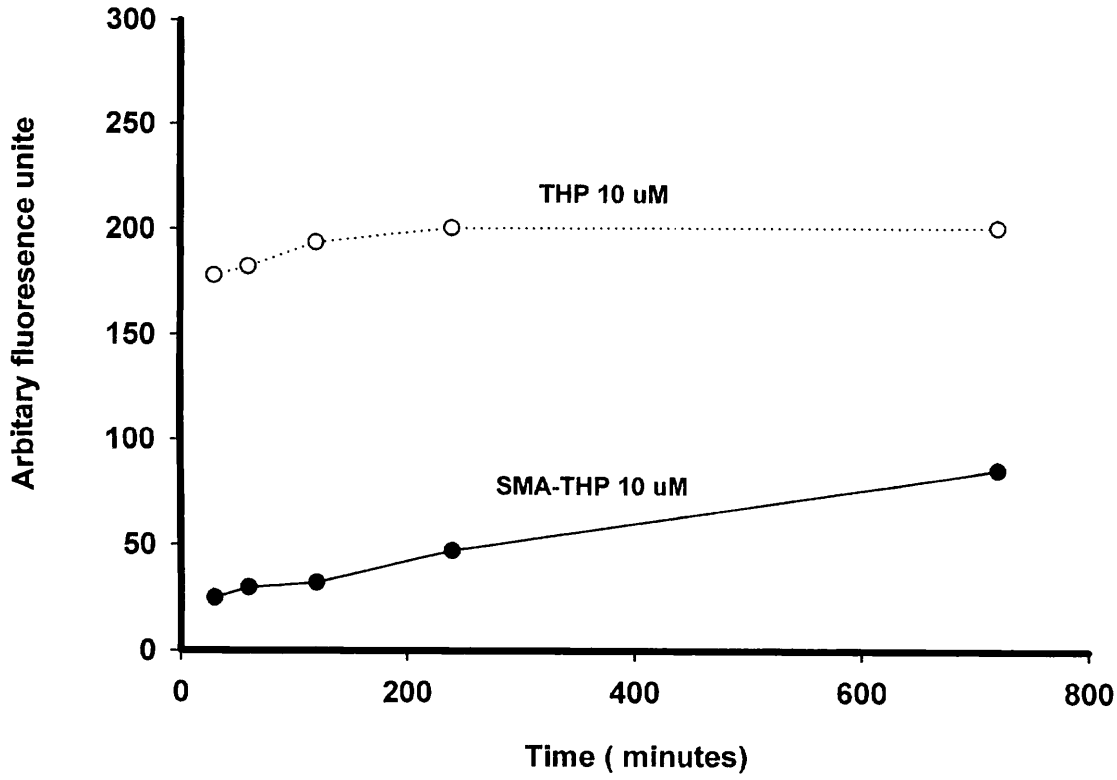


In vitro cytotoxicity of SMA-THP compared with that of free THP in MCF-7 and SW480 cells. A) SMA-THP in MCF-7 cells. B) Free THP in MCF-7 cells. C) SMA-THP in SW480 cells. D) Free THP in SW480 cells. Cells were exposed to free drug or to SMA-THP for 3 days. Cell viability was determined by the MTT assay. Values are means ± SD; n = 12.

3.6. intercellular uptake of SMA-THP compared with free THP

SMA-THP micelle showed delayed intracellular internalization compared to free THP. The amount of the drug detected by fluorescence intracellular was time dependent. The slow intercellular uptake combined with the prolonged circulation time of the drug can be of advantage in covering long duration with considerable antitumor activity.

Figure 7



3.7. Antitumor activity of SMA-THP micelles in vivo

For evaluation of antitumor activity tumor volume was measured in various mice tumor bearing models for more than 30 days and survival of these mice was followed for more than 300 days as (table 1). Treatment with SMA-THP micelles at two dosages, 20 and 40 mg/kg of free THP equivalent (given in four equal injections), completely suppressed tumor growth in S-180 tumor bearing ddY mice (Fig. 7). All mice treated with SMA-THP micelles were tumor free in case of S-180 and 80% in colon 38 and considerable reduction of tumor size in case of LLC and Meth A models. This finding is in great contrast to treatment with free THP, at 10 mg/kg total dose, which caused all mice to die within 1 week because of toxicity

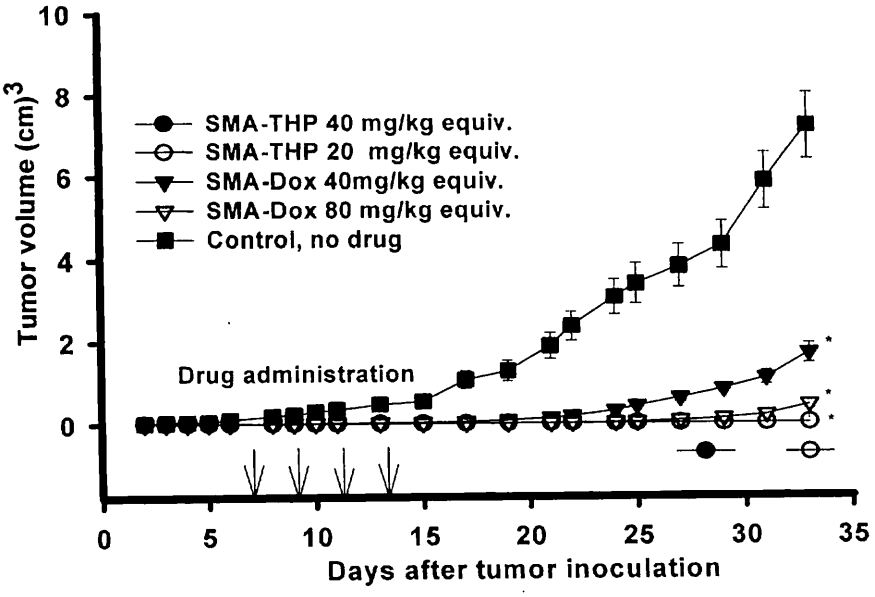
Table 1 Effect of SMA-pira micelle treatment on animal survival in different murine tumor models

Type of tumors	Dose, total (mg/kg)*	Mean survival days (T/C)	120 days survrival T/C (%)	ILS (%)
S-180	20 (5mg x 4)	600/43	6/0 (100)	1395
Colon-38	60 (30 mg x 2)	308/35	4/0 (80%)	880
Meth A	25 (12.5 mg x 2)	70/27	1/0 (20%)	259
Lewis lung	25 (12.5 mg x 2)	32/26	0/0 (0%)	123

- *Maximal tolerable dose (MTD) in mice average 80 mg/kg free pira equivalent;

  MTD of free pirarubicin is 7-10 mg/kg (both bolus, i.v.).

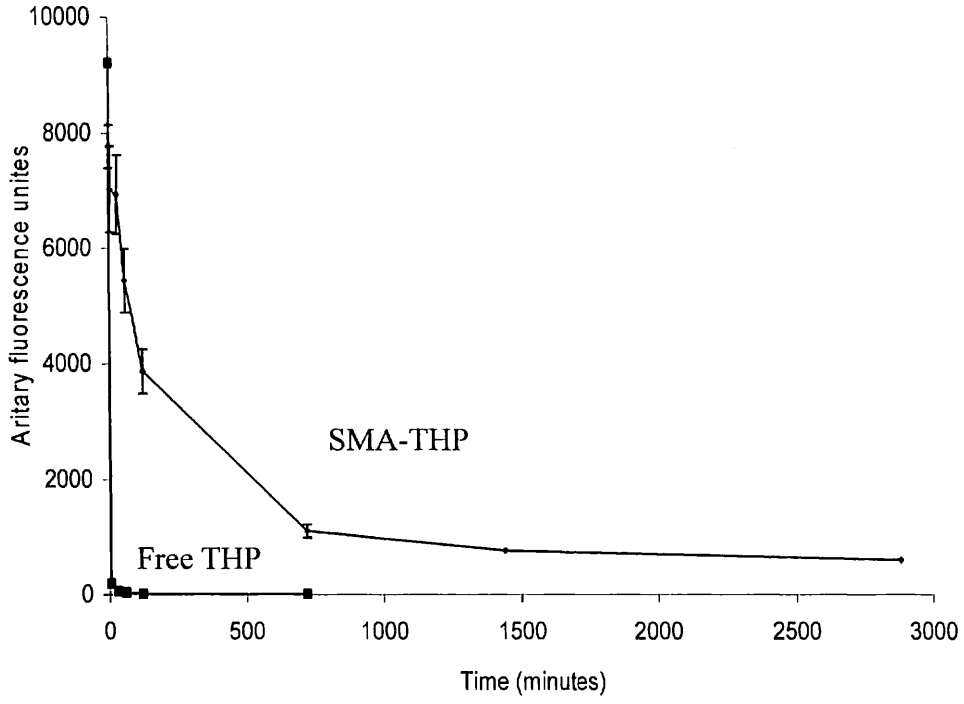
Figure 8



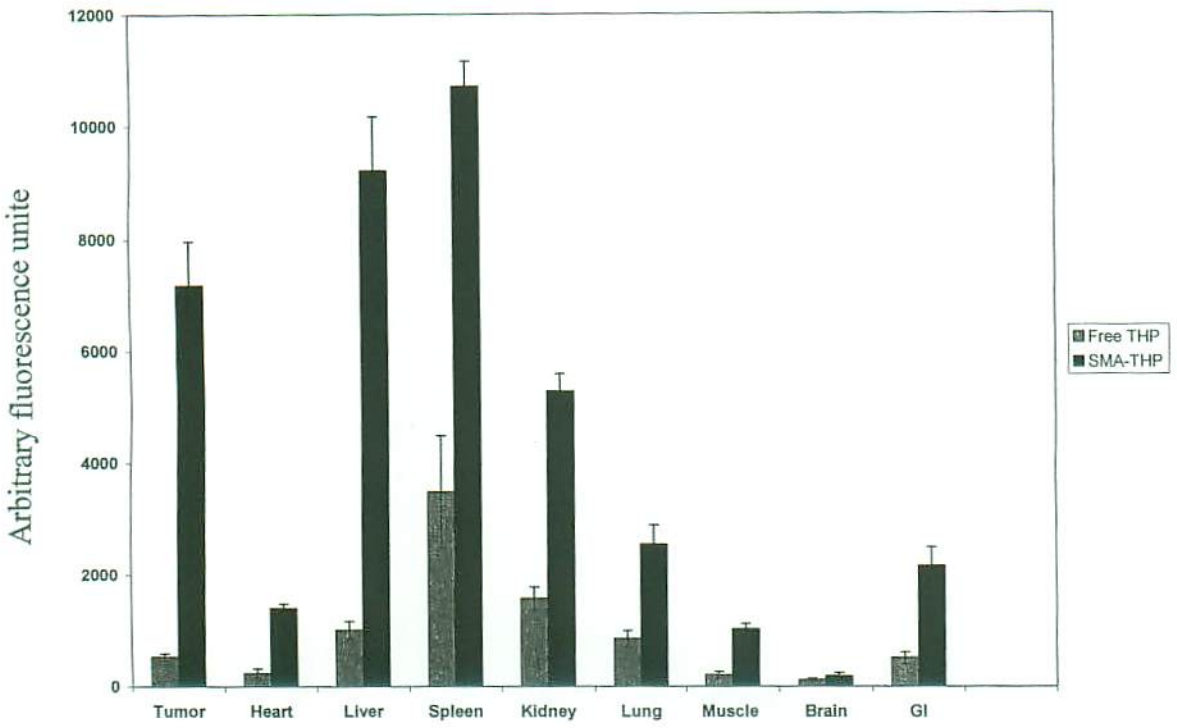
3.8. Pharmacokinetics and drug distribution

As shown in Fig. 8 A and B, the t½ α (half-life) of SMA-THP micelles increased about 1000 fold, while the area under concentration (AUC) time curve was 200 times greater than that of free drug when calculated for up to 3 days (72 h). Micellar drug concentration in tumor was 13.38 times higher than free drug in animals 24 h after iv administration. SMA-THP micelles showed enhanced concentration in both liver and spleen which makes it possibly applicable for liver tumors and hematological malignancies involving the spleen.

Figure 9 A



B



3.9. Potential toxicity of SMA-THP micelles

Tables 2 and 3 show results of blood assays and cardiac and liver function tests for mice receiving 33 mg/kg SMA-THP micelles (20 mg/kg free pirarubicin equivalent) and for mice receiving 10 mg/kg free pirarubicin. The values obtained for mice receiving the micellar drug (33 mg/kg) were not statistically different from those of normal healthy mice. In contrast, animals receiving free pirarubicin showed serious side effects, with significant differences from the control group without drug on all measurements.

Table 2. **Blood analysis for ddY mice receiving a single dose of 10 mg/kg free pirarubicin or 20 mg/kg pirarubicin equivalent as SMA-THP[a]**

Drug treatment	Time when measured	Hemoglobin (g/dL)	White blood cell count (cells/□L)	Platelet count (/mm^3)
Control		14.9 ± 1.3	8012 ± 1559	132.8 ± 15.3
Free pirarubicin	1 week [b]	11.1 ± 2.6*	2533 ± 305*	67.6 ± 2.5*
SMA-THP	1 week	15.0 ± 2.5	6016 ± 1622	141.3 ± 16.1
	2 weeks	14.1 ± 1.6	5900 ± 2128	130.3 ± 10.9
	3 weeks	15.9 ± 0.4	6366 ± 1903	120.0 ± 32.5

[a]Values are means ± SD; n= 4, * the difference from the control was statistically significant ($p< 0.05$). [b] Only one animal survived after the first week.

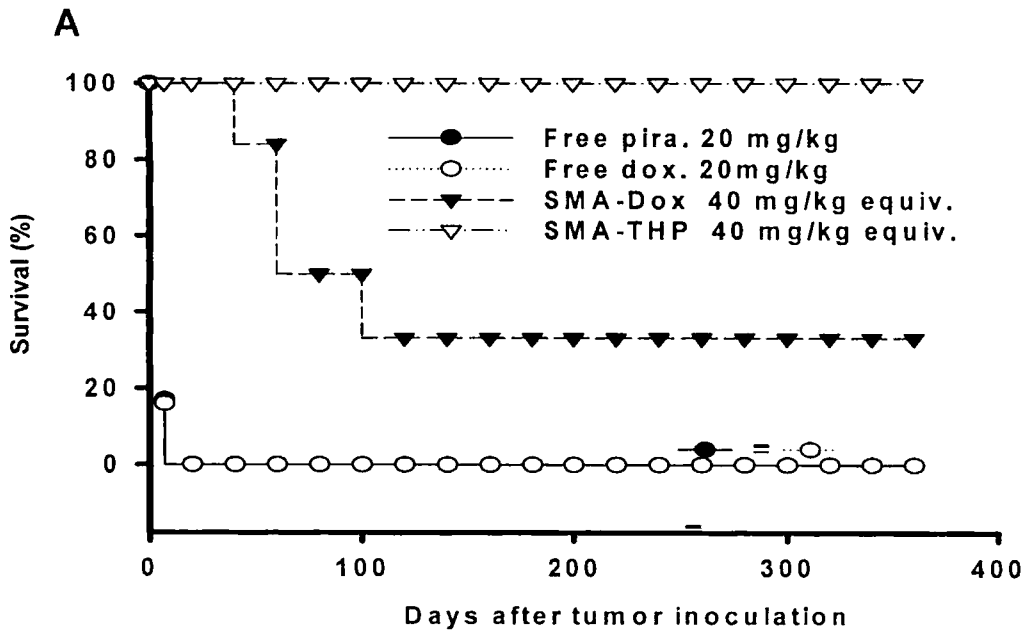
Table 3. **Liver function and cardiac enzyme assays for ddY mice receiving a single dose of 10 mg/kg free pirarubicin or 20 mg/kg pirarubicin equivalent dose as SMA-THP[a]**

Drug treatment	CK (U/L)	LDH (IU/L)	ALT (IU/L)	AST (IU/L)
Control	309 ± 42	260 ± 62.4	37 ± 12	81 ± 7.3
Free pirarubicin	1704 ± 102*	634 ± 58*	53 ± 8.2	239 ± 18*
SMA-THP	381 ± 35	318 ± 42	58 ± 11	73.5 ± 6

[a]Values are means ± SE. CK, creatine kinase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.* The difference from the control was statistically significant ($p< 0.05$).

All S-180 tumor-bearing mice receiving a total dose of 166 mg/kg of the micellar preparation (100 mg/kg free pirarubicin equivalent), by four i.v. injections (41.5 mg/kg x 4) in 1 week, or 133 mg/kg as a single i.v. bolus, survived for more than 12 months. In contrast, mice receiving a total dose of 20 mg/kg free THP by four i.v. injections (5 mg/kg x 4) had a much reduced survival: 83% died within 1 week and the remaining 17% died within 3 weeks (Fig. 10).

Figure 10



Pathological examination of cardiac tissue by H&E staining showed extensive inflammation in animals receiving injections of free THP (5 mg/kg x 4), whereas tumor-bearing mice receiving SMA-THP (41.5 mg/kg x 4) showed no abnormalities.

4. Discussion

In the present study, we found that SMA, the styrene moiety of the amphiphilic polymer, formed a complex with pirarubicin (THP) to yield SMA-THP micelles. We also found that hydrolysis of SMA was essential for this complex formation: the hydrolyzed SMA was highly water-soluble and could form micelles rapidly. Treatment of anhydride acid of SMA with alkali resulted in the opening of the maleic anhydride ring in SMA via hydrolysis and the addition of an OH group, as evidenced by a 3400 cm^{-1} signal in the IR spectrum (Fig.2 B, a,b) and an increase in the unit molecular size of SMA from 202 Da (styrene-maleic anhydride) to 220 Da (styrene maleate) because of the addition of 18 Da of H_2O (Fig. 2A).

Our initial intent regarding the SMA-THP complex was to induce covalent cross-links between the amino group of pirarubicin and the carboxyl group of maleic acid in SMA by treatment with EDAC, facilitated by a dehydration reaction to produce a carboamide linkage (2). However, this reaction did not proceed. In contrast, as described above, SMA and THP formed micelles, as evidenced, in part, by the ease with which free THP was liberated from the SMA-THP micelles without the use of harsh hydrolytic conditions, that is, by either ethanol or 10% sodium dodecyl sulfate (SDS) (Fig. 4). IR spectroscopy of SMA-THP micelles showed the absence of signals corresponding to amide I and amide II, in contrast to other cases in our study (108). Furthermore, we found that other polyamines used instead of EDAC could serve a similar catalytic function for molecular orientation during formation of SMA micelles (not shown).

In case of SMA-THP the high fluorescence of THP, was almost completely quenched when the THP molecules were closely attached to or interacting with other aromatic molecules, the styrene moiety of SMA, in the binding pocket of albumin (or other

proteins), or other high-affinity species (107). We thus anticipated that the energy transfer from the THP chromophore to aromatic residues of styrene in the micelles would result in efficient quenching and suppression of fluorescence. The SMA-THP micelles showed little release in vitro, this stability of the micelles at physiological pH would thus prevent rapid release of free THP from micelles during circulation. The free drug would not be able to gain access to critical organs and tissues, which would be protected from the toxic effects of THP. Such stable micelles or macromolecular drugs will also benefit the functioning of the EPR effect for SMA-THP: the slower drug release rate during circulation than the rate of drug uptake into tumor ensured a greater antitumor effect without systemic toxicity.

While lower in vitro cytotoxicity is known for other polymeric anthracycline conjugates or complexes such as hydroxypropylmethacrylate copolymer-conjugated doxorubicin, (38, 81). In this study we found that free THP and miceller THP have almost similar in vitro activity. To explain the cause of the similar in vitro cytotoxicity of the free drug and the micellar drug, we are currently investigating the glucose transporter system to be partially responsible for receptor-mediated uptake, which may account for the high activity of the SMA-THP micelles. In addition to the endocytic mechanism by which most polymeric drugs operate, receptor-mediated uptake may occur: the one extra pyranyl residue in pirarubicin, compared with doxorubicin, may be involved in a receptor-mediated transport (130).

The reduced toxicity of the micelles can be attributed to the EPR effect and tumor-selective drug targeting. This EPR effect is a molecular size-dependent phenomenon, which is seen for molecular sizes above Mr 40,000 (3, 125). For example, molecular size of THP with biocompatible SMA, which binds with albumin,

becomes larger (Fig. 3). Such large macromolecules leak out of tumor vessels into tumor tissues (interstitium) and remain sequestered in the vicinity of the tumor as a result of anatomical and pathophysiological abnormalities of tumor tissues i.e. by EPR effect (2, 3, 77, 80, 102, 110, 111, 113, 131). The dependence on molecular size of drug uptake into tumor is inversely correlated with the renal clearance of small molecular compounds (2, 3, 76, 77, 125). Therefore, the SMA-THP micelles, with an apparent molecular size in plasma of 94 kDa, will remain in circulation and, as a result of the EPR effect, lead to more selective targeting of drug to tumor. This selective tumor concentration was evidently demonstrated with SMA-THP micelle with tumor concentration of the miceller drug 13 times higher than that of equivalent dose of free drug.

The high safety profile of these SMA-THP micelles can be attributed to the nature of the distribution of polymeric drugs (i.e. the EPR effect). In our previous work, we showed that SMA-doxorubicin micelles accumulated in tumors 13-fold more than that did free doxorubicin. Further more, doxorubicin delivered to cardiac tissue after administration of these micelles was only 8%. However, as is evident from results presented in Fig. 8 and table 1, the new SMA-THP micelles had a far better antitumor effect, with even lower doses of the micellar drug, and produced longer survival; these effects may be attributed to higher activity of the drug compared with doxorubicin (129). The EPR effect-dependent distribution profile (high drug concentration in tumor but low drug level in normal tissues) ensures a higher margin of safety for normal organs and tissues, the end result being safer dose escalation of the SMA-THP micelle preparation, up to 15 times higher than the maximum tolerable dose of free pirarubicin.

In addition to its pharmacokinetic and pharmacodynamic advantages, SMA is known to

confer immunopotentiating effects, such as activation of macrophages, T-cells, and natural killer cells, and induction of interferon (116-118, 128). These immune system modifications can be considered another benefit of SMA-based micelles.

In conclusion, the present study described the preparation and characterization of SMA-THP micelles, the antitumor effect of this formulation, and preliminary safety data in mice. Further study of this micellar preparation is now commencing with our collaborators in Australia and Egypt.

CHAPTER 4

Copoly(Styrene-Maleic Acid)-Oxaliplatin Micelles: Better Formulation For enhanced Tumour Targeting Efficiency and lower Toxicity

1. Introduction

Colorectal cancer is one of the leading causes of death from malignant diseases in the Western world. Worldwide, 50% of patients who present with colorectal cancer will develop metastatic disease and eventually die from this malignancy (132). Recently, significant advances have been made in the medical treatment of advanced colorectal cancer with the introduction of novel cytotoxic drugs, such as irinotecan and oxaliplatin. Oxaliplatin is a novel cytotoxic platinum compound that differs both structurally and in its spectrum of activity from the related and widely used chemotherapeutic agents cisplatin and carboplatin. Platinum compounds exert their cytotoxic effects through the formation of DNA adducts that block both DNA replication and transcription, resulting in cell death in actively dividing cells as well as through the induction of apoptosis. Unlike these *cis*-diamine platinums, oxaliplatin contains a 1,2 diaminocyclohexane carrier ligand. This structural alteration results in formation of bulkier platinum-DNA adducts that may be more difficult to repair, leading to increased inhibition of DNA synthesis and induction of apoptosis (133).

Oxaliplatin and it is currently licensed as treatment for patients with colorectal cancer. Oxaliplatin has also shown activity in various other cancers, including ovarian, breast, and lung (134-136). Neurotoxicity is the principal and dose-limiting toxicity of oxaliplatin(137). As described in previous chapters, SMA miceller association with oxaliplatin could prevent such side effect through protecting the drug in circulation which prevents the release of the drug. On the other hand once delivered into tumor tissues drug can be released upon intercellular internalization leading to the desired cytotoxic effect inside the tumor tissue.

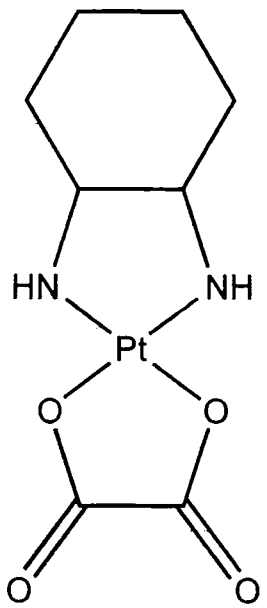
Furthermore, the SMA-oxaliplatin micelle can attain high molecular weight; this will enable the new formulation to take advantage of the EPR effect resulting in higher drug concentration in tumor tissue with enhanced efficacy as well as less side effects as demonstrated for other SMA-Micelles.

2 Materials and Methods

2.1 Materials

Oxaliplatin was a generous gift from Debiopharm, Lausanne, Switzerland. SMA with a mean molecular size of 1280 Da (Mw/Mn: 1.1) was generously supplied by Kuraray Ltd, Kurashiki, Japan. Other reagents were of commercial reagent grade and were used without further purification.

Figure 1 Oxaliplatin



Oxaliplatin; Trans-l-diaminocyclohexane oxalatoplatinum;

2.2 Preparation of SMA-Oxaliplatin Micelle

Two grams of SMA and 2.25 gm Ox powder were added to 1 L of 10 mM Na_2CO_3, pH 11.1. The mixture of SMA and Ox suspension was briefly sonicated with intermittent 0.5 min sonication cycle for 4 min, using rod type φ 7 mm sonifier (Dr.Hielscher gmbh, Germany). The mixture was then incubated for 7 days at 45 °C, under150 rpm in a shaking incubator. The mixture was mostly soluble, but not totally soluble, it was centrifuged at 4000 rpm for 15 min. The resultant supernatant [preparation A] was then concentrated to 40 ml using Amicon ultrafiltration membrane with cut-off of 3 kDa under N_2 pressure. The concentrate was subjected to gel chromatography (Sephadex G-50 F, column size; 56 x 2.4 cm), eluting by deionized water, each tube contained 9 ml. Fraction 1 [referred SO-1] and fraction [referred SO-2] were obtained. Each fraction was monitored by UV absorption at 250 nm and 280 nm, and peak fractions were pooled and concentrated to 20 ml, then washed 3 times with 10 times-excess volume of deionized water using Amicon ultrafiltration system (YM-3 membrane; cut-off molecular size of 3 kDa), finally each concentrate (SO-1 and SO-2), was freeze-dried. The insoluble fraction was designated fraction [B], it was then resuspended in 500 ml of 10 mM Na_2CO_3, and further incubated for 12 h at 45 °C, 150 rpm in shaking incubator. This reaction mixture was further subjected to G-50 column chromatography, and similar to fraction [A], fraction 1 [SO-1B] and fraction 2 [SO-2B] were obtained. Each peak fraction was pooled and concentrated to 20 ml, then washed 3 times with 10 times-excess volume of deionized water using 3 kDa cut-off Amicon ultrafiltration membrane, similar to fraction [A]. Finally the resulting concentrates were freeze dried.

2.3. Characterization of SMA-Ox micelles

2.3.1 Size-exclusion chromatography

Size-exclusion chromatography with Sephadex G-50 Fine column (Pharmacia LKB, Uppsala, Sweden) was performed to determine the change in apparent molecular size of the new micelles in DW and using a column size of Ø 56 x 2.4 cm. To examine effect of

albumin binding, Sephadex G-50 column of Ø 46 x 0.6 cm, with 0.25 DW as the mobile phase, and 3 ml fraction each, was collected.

2.3.2 Determination of the platinate content in SMA-Ox micelles

Two methods were used to quantify the platinate content. First method through using Visible/UV absorption as determined by use of a spectrophotometer (U-200, Hitachi Ltd., Tokyo, Japan). SMA has negligible reading at 280 nm, thus the reading at this wave length was used to quantify the intact Ox in the SMA-Ox micelle. The second method is a colorimetric using o-phenylenediamine (OPD) in brief , 1.2mg of OPD is dissolve in 1ml of DMF to this a sample solution 1ml (containing platinate/Pt) is added. The mixture is boiled at 100°C for 30min. Color (brownish) appears. when cool to room temperature , absorbance at 420nm is measured. It is interesting that absorbance of oxaliplatin or SMA-oxaliplatin per se [before decomposition] of 1mg/ml at 420nm is negligible.

2.3.3 Infrared (IR) spectra

IR spetrum were recorded with a JIR-6500w spectrometer (JEOL, Tokyo, Japan) using KBr disc.

2.3.4 Elemental analyses of SMA–Ox micelles

After column chromatography, elemental analysis was carried out for hydrogen, carbon, nitrogen, and oxygen in the micelles. As SMA contains no nitrogen, the nitrogen present in SMA-Ox reflects the amount derived from incorporated oxaliplatin.

2.3.5 Stability of SMA-oxaliplatin micelles

To examine the stability of oxalipaltin compared to SMA-Ox micelles, 1 mg/ml of free Ox, Free Ox mixed with aqueous SMA, or SMA-Ox micelle were incubated at 37° C in various solutions, namely 0.1M $NaHCO_3$, DW, PBS, 0.1M NaCl and 0.1M Na_2CO_3.
After given time UV reading at both 250 nm and 280 nm were recorded.

2.3.6 Release of free drug from the SMA-Ox complex

To verify release of free drug from SMA-Dox micelles in vitro, 20 mg of the micelles preparation was dissolved in 5 ml in various solutions and placed in sealed dialysis tubes (Mr cut-off 1000; Spectrapor, Spectrum Laboratories Inc., San Diego, CA). The dialysis tubes were submerged in three different solution systems containing 25 ml of (1) 0.1M NaCl (2) distilled water and (3) 0.1M Na_2CO_3. The dialysis bags were then incubated for

several days at 37°C in the dark with reciprocal shaking at 1 Hz. The free Ox released from the dialysis bags was collected at predetermined times and its amount was quantified by measurement by UV absorbance as well as colorimetric method as described above.

2.4. In vitro cytotoxicity

In vitro cytotoxicity of SMA-Ox micelles was determined by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (106) with human colon cancer SW480 cells, which were plated in 96-well culture plates (3000 cells/well). Cells were cultured overnight in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The cells were then incubated in the presence of free oxaliplatin or SMA- Ox micelles. Toxicity was quantified as the fraction of cells surviving relative to untreated controls.

2.5 In vivo testing of SMA-oxaliplatin micelles

A limited pilot study for evalution of in vivo activity of SMA-oxaliplatin were carried out. For this purpose, male ddY mice, 6 weeks old and each weighing 30-35 g, were from SLC, Inc. (Shizuoka, Japan). Mouse sarcoma S-180 cells (2×10^6 cells), maintained as ascitic form tumor in ddY mice, were implanted subcutaneously (sc.) in the dorsal skin of the ddY mice, then animals were given various concentrations of Ox or SMA-Ox micelles. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Kumamoto University, and Graduate School of Medical Sciences.

2.6 Statistical analyses

All data were expressed as means ± SD. Student's t-test was used to compare differences between experimental groups, and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Micelle formation

Both SMA-oxaliplatin micelles (SO) [preparation A and B] formed two discrete peaks of relatively homogeneous molecular species that designated Fraction 1 and Fraction 2. which will be referred as SO-1 and SO-2, respectively. SO-1 exhibited molecular weight above 100 kDa, while SO-2 is about 9 kDa, as proved by both gel chromatography and various ultra-filtration membranes. SO-2 instantly binds to albumin resulting in size approximately 76 kDa in the presence of serum. High molecular weight nature of both preparations will enable selective tumor targeting and accumulation in tumor through the EPR effect.

Fig. 2 A

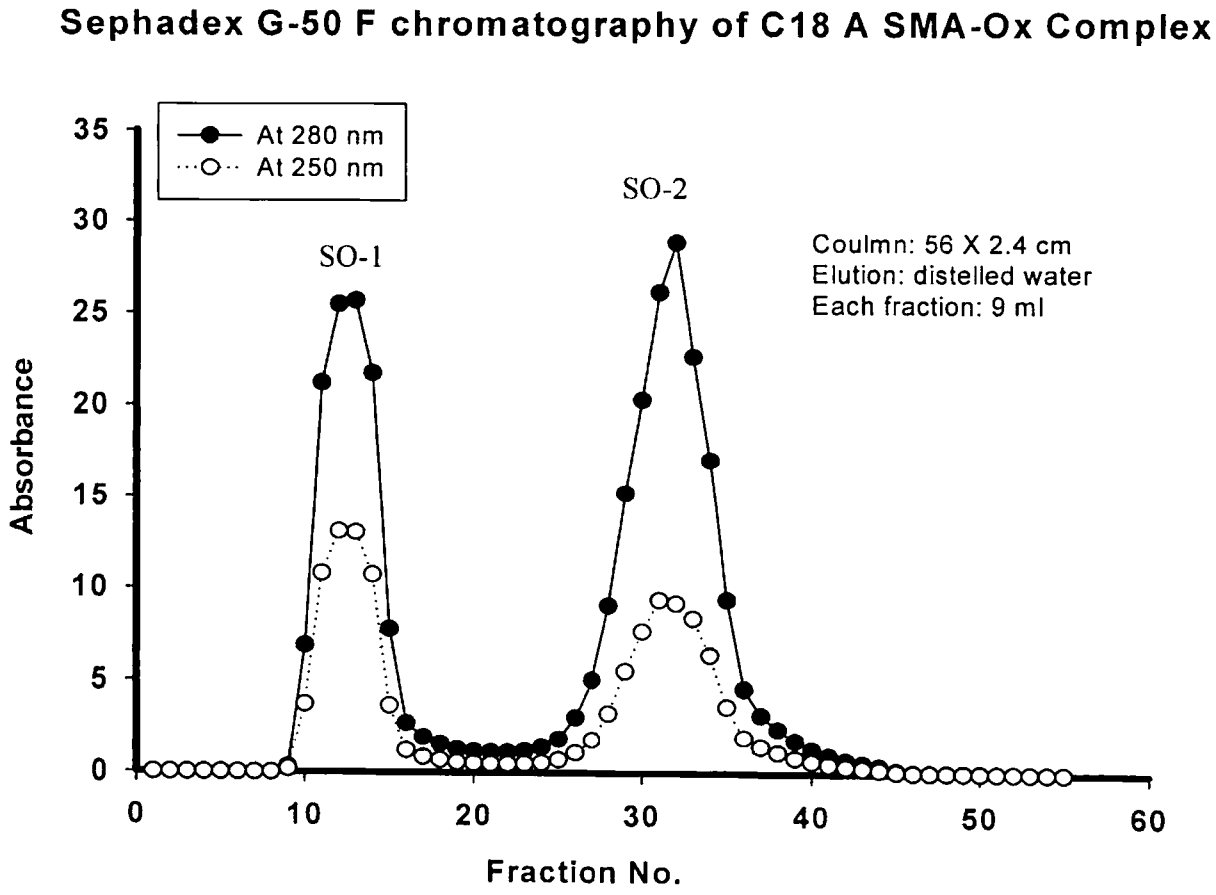
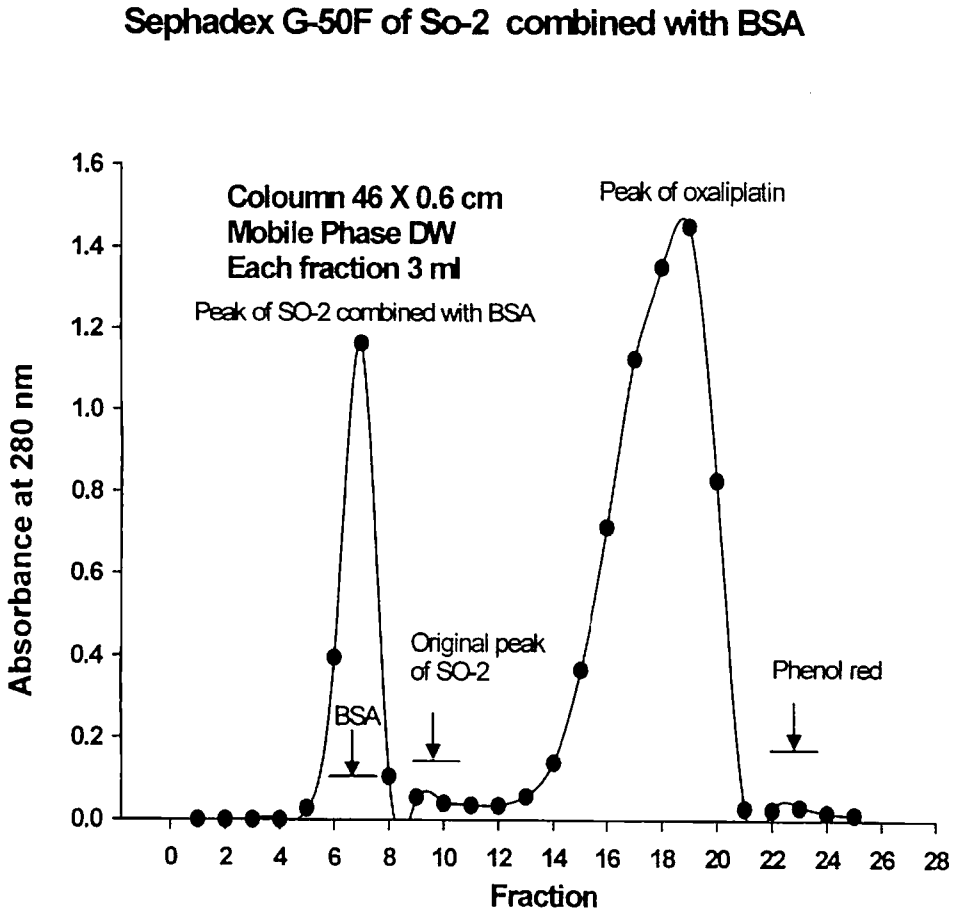


Fig. 2 B



Aliquot samples of BSA, So-2, and free oxaliplatin were mixed and applied in 2 ml volume to the coulmr The loss of So-2 (frn 2) peak indicates albumn binding, furthermore, Platinate content in first peak were varified by the modified OPD method

3.2. Palatinate content

Palatinum content in SMA-ox micelles varies from one preparation formula to the other, depending on the feed ratio of both SMA and oxaliplatin. The higher feeding ratio of oxaliplatin to SMA tends to yeild the higher oxaliplatin content in SMA-Ox micelle. This is also associated with higher yield of SO-1. In a representative SO-1, the oxaliplatin content is about 51 % gm/gm ratio, while in SO-2, the oxaliplatin content is about 32.1% per miceller weight.

3.3. IR spectrum

The obtained micelles showed strong OH signals for both SO-1 and SO-2 around 3400 cm^{-1}, that is more stronger in case of SO-1.

Fig. 3 A IR Spectrum of SO-1

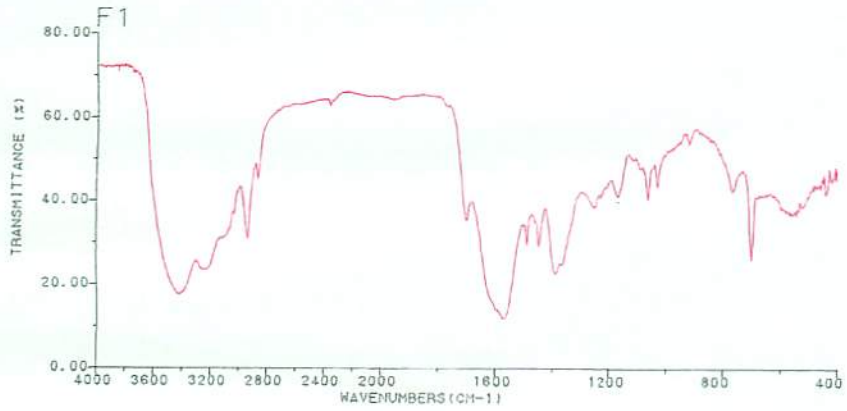


Fig. 3 B **IR Spectrum of SO-2**

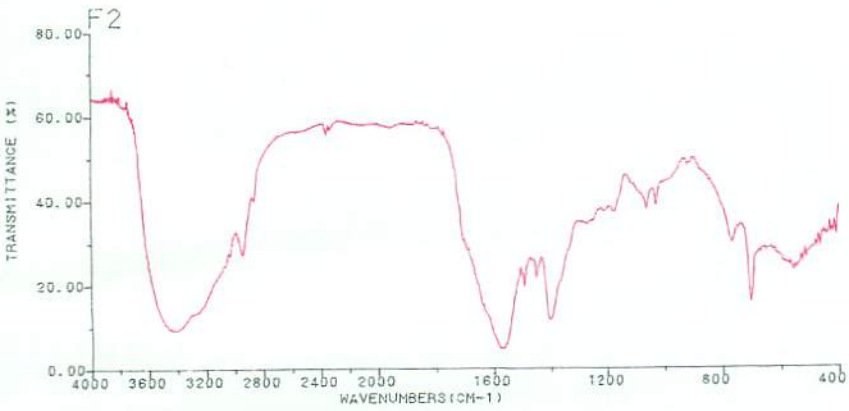
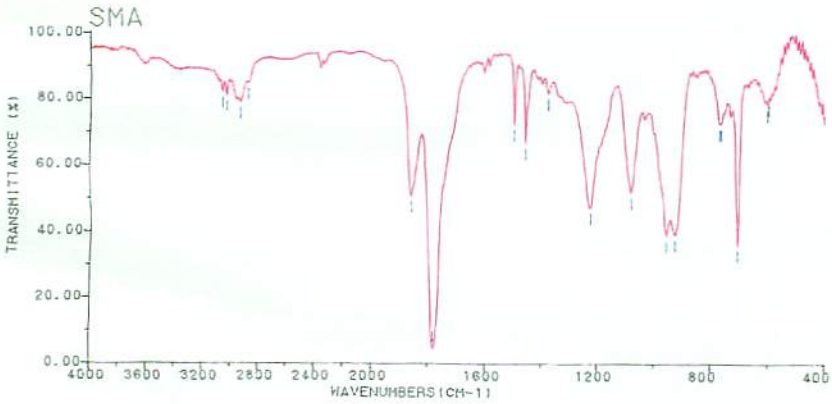


Fig. 3 C **IR Spectrum of the reactant anhydrous SMA**



3.4. Elemental analysis

Elemental analysis of both SO-1and SO-2 reflects small difference in deduced palatinate content as seen by different N ratio between the two preparations.

Table 1 Elemental analysis of SO-1 and SO-2 micelles

Compound	H %	C %	N %
Free Ox	3.55	24.18	7.05
SMA-Ox F1 (SO-1)	5.03	41.23	3.5
SMA-Ox F2 (SO-2)	5.17	44.44	2.04

3.5. UV spectroscopy of SMA-Ox micelles

SO micelle shows different ultraviolet spectrum as compared with free oxaliplatin. SMA has negligible reading at 280 nm, thus the reading at this wave length was used to quantify the intact Ox in the SMA-Ox micelle.

Fig. 4 A UV absorbance of SMA-Ox Complex

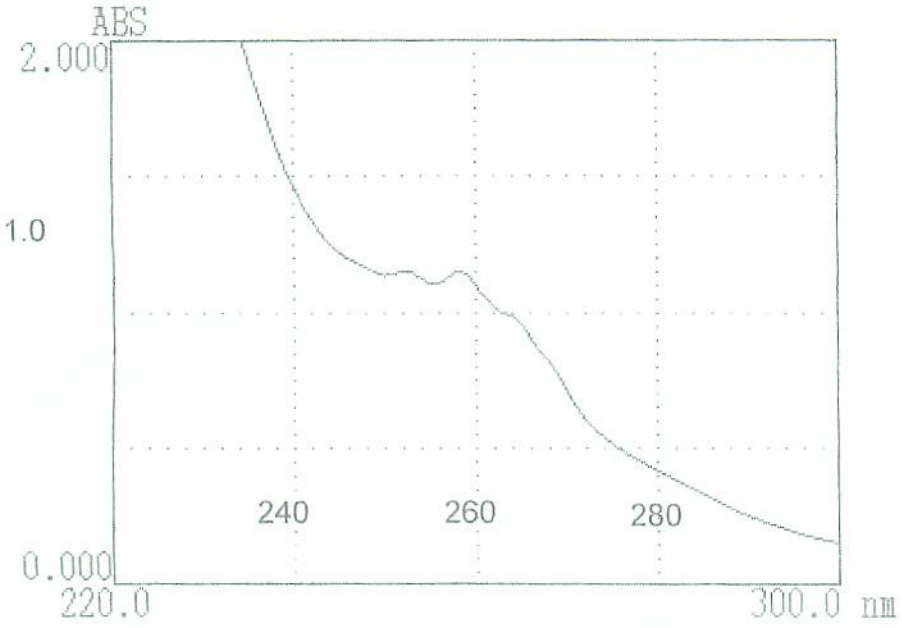


Fig. 4 B UV absorbance of free oxaliplatin

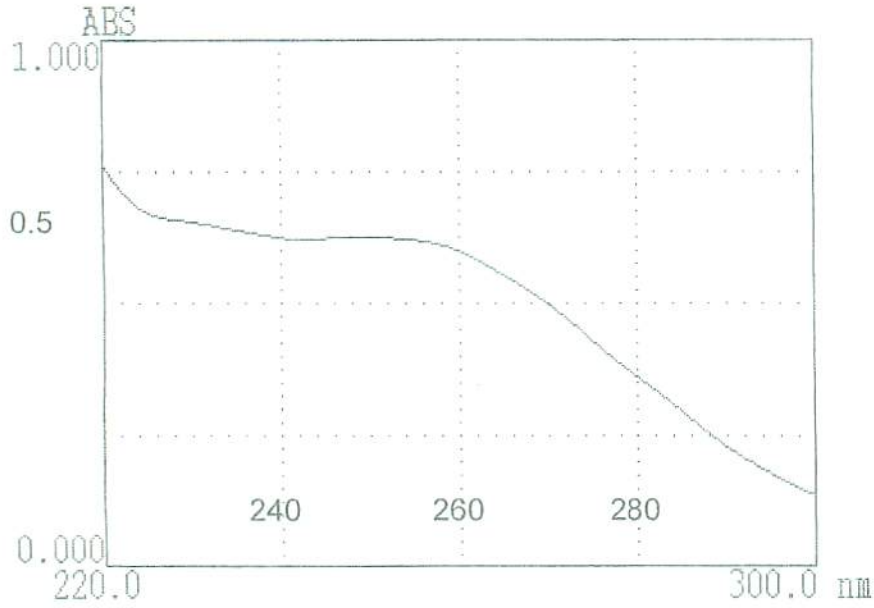
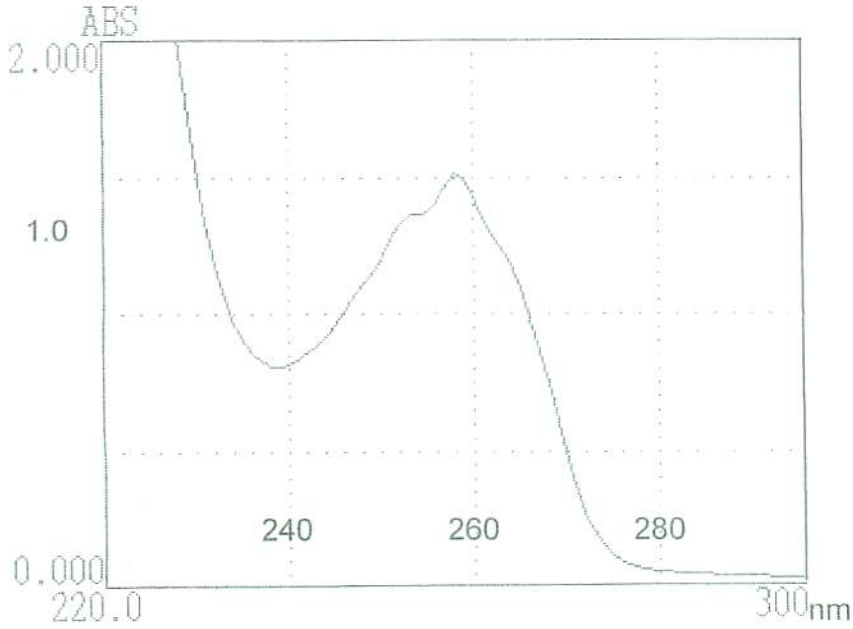


Fig. 4 C UV absorbance of free SMA



3.6. Water solubility

SO-1 shows poor solubility in water, while SO-2 is highly soluble in water, and the result is reverse concerning solubility in oil reflecting more hydrophobic nature of SO-1.

3.7. Stability of SMA-oxaliplatin micelles

Both SO-1 and SO-2 proved to be very stable in different buffer systems and at different pHs. This is a great contrast to free Ox. SO-1 seems very stable under the similar condition (cf. Table 2). Because of its poor solubility, most of stability tests were conducted for SO-2.

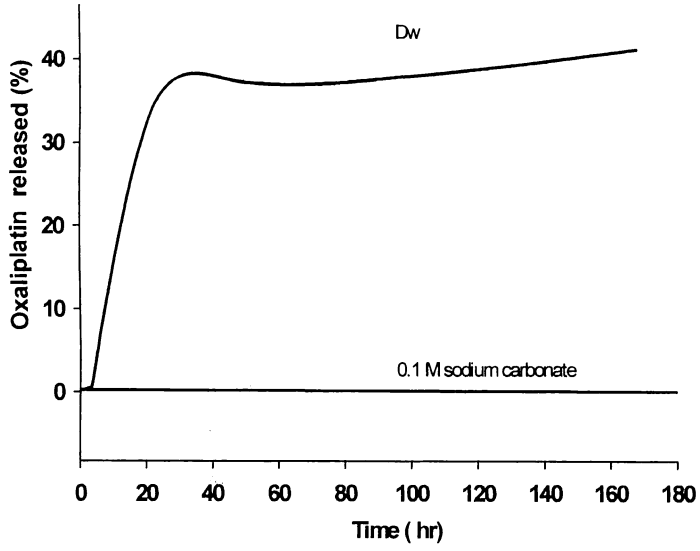
Table 2 Stability of SMA-Ox complex in different buffer systems

Compound	H_2O	PBS	0.1M NaCl	0.1M Na_2CO_3		0.1M $NaHCO_3$	
	Activity (%)	Activity (%)	Activity (%)	Activity (%)	pH after incubation	Activity (%)	pH after incubation
Free Ox 0m	100	100	100	100	11.5	100	9.5
60m	100	97	93	50		76	
360m	100	91	91	35		46	
1 day	100	56	39	22	9.1	46	8.5
2 days	87	26	14	7	8.6	45	8.4
SMA-OX (SO-2)							
0m	100	100	100	100	11.5	100	9.5
3days	100	100	100	100	11.4	100	9.6

3.8. Release rate of oxaliplatin from SMA-Ox. micelles

In contrast to free oxaliplatin, SO-1, and -2 are very stable in the presence of various ions such as NaCl, carbonate and bicarbonate, with negligible release rate for up to 5 days in case of SO-2. In case of water, SO-2 shows initial burst within 1 day of about 40 % then a plateau phase for up to 5 days was observed.

Fig. 5 Release rate of Ox from SO-2 micelle

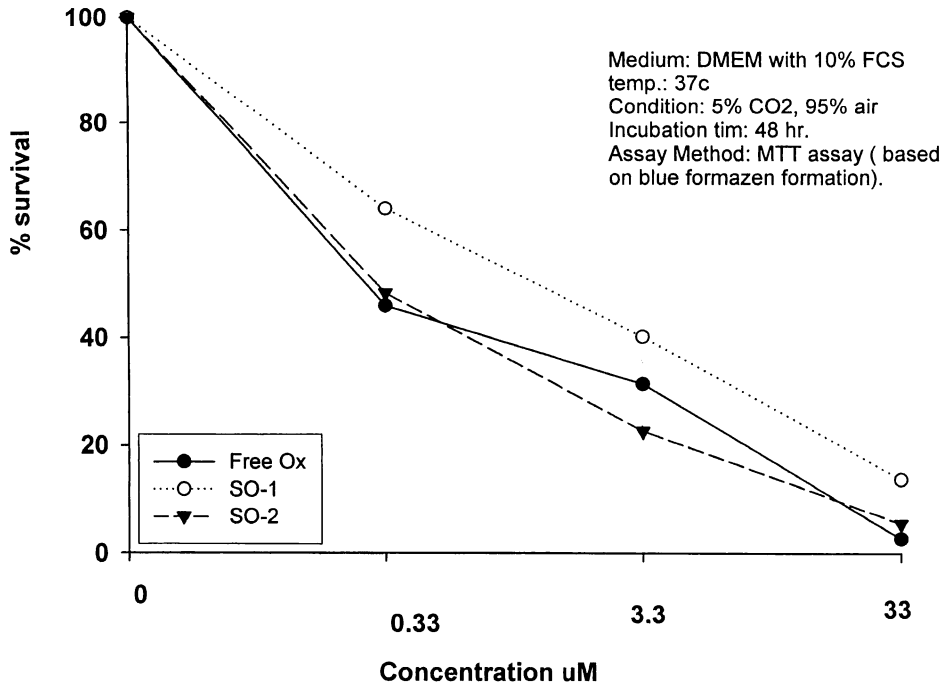


2ml of 4mg/ml SO-2 placed in 0.5 kDa cut -off dialysis bag and immersed in 20 ml water, pH 6.8. The dialysis bags were kept at 37C under 200 rpm shaking and reading at 280 nm was recorded which correspond to oxaliplatin.

3.9. In vitro activity of SMA-Ox

Both SO-1and SO-2 showed considerable cytotoxic effect in MCF-7, SW-480 and KB cancer cell lines. While SO-2 showed almost comparable cytotoxic effect compared to free oxaliplatin, SO-1 is slightly less cytotoxic. The oily formulation of SO-1 is more effective due to more solubility resulting from the hydrophobic nature of SO-1.

Fig.6 In vitro cytotoxic effect of SMA-Ox compounds in SW/480 cells



3.10. In vivo cytotoxic effect of SMA-Ox

The results obtained confirmed the cytotoxic effect as well as the safety of the formulated new SMA-oxaliplatin micelles.

According to this limited study, both SO-1 and SO-2 can be used for iv. Injection, though SO-1 will be in the form of suspension that should be well mixed prior to administration into animals.

For further detailed study we intend to use a total dose of 40 and 60 mg/kg of free oxaliplatin equivalent, to be given iv. in 4 divided doses with 2 days interval between doses after 1 week of tumor inoculation. The drug may be dissolved in deionized water, 5 % bicarbonate or 5 % glucose, and maximum of 0.2 ml / dose should be injected slowly into the tail vein of the mice.

4. Discussion

As discussed in the previous chapters SMA due to its amphiphilic nature has the ability to instantly form micelles in aqueous media (129, 138). The proposed micelle structure would hypothetically include in the center the hydrophobic styrene residue and pointing outward is the carboxyl group of maleic acid. We expect the new SMA-Ox micelle to have the similar structure. In IR spectrum both SO-1 and SO-2 there is no unique bond characteristic peak such as amide I/II bond generated in this miceller structure(108).

Both IR spectra of SO-1 and –2 were slightly different from SMA per se. Namely, small peaks at 1700 cm^{-1} and 2900 cm^{-1} in SO-1 not seen clearly in SO-2. One possible explanation of such result is the different miceller association of SMA and oxaliplatin and molecular structure between SO-1 and SO-2.

In accordance with this postulated miceller structure, is the different release patterns seen in different solutions which might reflect this chemical structure of the complex where the surface of the SO-2 micelle may be mainly ionic, partially disrupted in water, but maintained in the presence of electrolites. This slow release rate is encouraging for further in vivo study of these micelles because it allows time for tumor concentration (> several hours), without disintegration. Further examinations are needed to verify the advantageous release profile of SMA-ox micelles in various physiologic milieus. This chemical structure may also be confirmed through the results of stability study of SMA-Ox at various electrolytes. Oxalate and platinate groups of oxaliplatin seem to be fixed inside the SMA polymer micelles, thus sealed from outside ions or buffer system for inactivation, thus resulting in much higher stability than free oxaliplatin. This advantage of the miceller formulation can prove higher effectiveness in vivo, where the free Ox will be otherwise undergo (in part) to decomposition due to various ions in the blood plasma before reaching to the tumor tissues, not to mention of rapid renal clearance (131).

In the current work, we have modified the colorometric method used for detection of cis-platinum (139) in order to measure the palatinate content in SMA-Ox micelle. The modification we made includes; reaction at 100°C for 30min instead of 10min, absorbance reading at 420nm instead of 703nm, Both SMA-oxaliplatin-micelle and

oxaliplatin is required to be broken down by treating the sample at 100°C, in 0.1MNaCl for 1hr to make platinate, then it will yield the color when mixed with OPD as above. The new method were able to detect the presence of 1 μ gm of palatinate efficiently. However, when we compared our tow quantification methods (UV and colomotry using OPD), we found some discrepancy with UV method tends to underestimate the amount of palatinate.this difference can be indicative of decomposed oxaliplatin fraction which has no UV absorbance. The reduced UV absorbance after micelle formation might be indicative of structural integrity of oxaliplatin, where oxalate could have been replaced either by carboxalate, or hexanediamine group .

Both So-1 and SO-2 can be administered iv., where SO-1 may be in homogenous suspension, there is still may be possibility to make clear aqueous solution of SO-1. However, this property suits well for oily formulation for oral route administration for SO-1. Advantage of oily formulation is that it can be administerd through the oral route. Upon intestinal absorption of oily formulation and it will most likely carried into the blood stream by chylomicron based transcytosis, where M cells and lymphatic system will play an important role. In case of SMANCS blood concentration after oral administration of oily formulation was 11 times better than aqueous one (104). Furthermore, oily formulation with Lipiodol can enable i.a injection for arterially accessible bulky tumors.

This is in great contrast to free Ox which shows no solubility in Lipiodol, medium-chain triglycerides, camellia oil or other edible oils.

In conclusion we report here the formulation, characterization and activity of new polymeric micelle of SMA-Ox.

CHAPTER 5

Future Prospects

We have shown in the previous chapter the great potentiality for SMA to form various micelles through a simple and efficient method of preparation. Due to acquiring high molecular weight, SMA micelles were able to take advantage of the EPR effect resulting in higher tumor accumulation. . This, in turn, resulted in higher dose administration with enhanced anticancer effect meanwhile less or no side effects.

In addition to the previously mentioned SMA- micelle, we had prepared and characterize many other micelles including:

A- SMA-ZnPP

Metal protoporphyrins (PP), e.g. FePP , showed many interesting aspects in terms of cancer treatment. One aspect is their ability to kill cells through photosensitization (140). Other aspect is their inhibition of the enzymatic function of heme oxygenase -1 (HO-1). HO-1 is an inducible enzyme by various insults such as oxystress, irradiation and nitric oxide, and it is found in the inflammatory tissues (141). Finally, they have been found to have a role in signal transduction regulation affecting oncogenic gene expression in human myelogenous leukaemia cell line in vitro (142).

ZnPP is substrate analogue for HO-1, by which heme is metabolized to iron, carbon monoxide and biliverdin which then ultimately converted to bilirubin. Bilirubin usually serves to reduce intracellular oxidative stress via its potent antioxidant potential . Over expression of HO-1 in many human and murine tumour tissues suggested a role for this enzyme in promotion of both tumour survival and growth (12). Since bilirubin is the very potent antioxidative agent, suppression and depletion of HO-1 would make cells valuable to oxystress or radiation (143,144). Thus, such tumor cells treated with inhibitor of HO-1 can be very sensetive to singlet oxygen generated by UV. One major limitation to practical application of PP, in biological and medical purpose, is their poor water solubility. In our earlier work we developed water soluble form of ZnPP by conjugating with polyethyl glycol (PEG), which consequently become macromolecular nature (108) . As one mole of ZnPP become 11,000 dalton, this means the amount needed to be injected will be so large in order to build up enough molar concentration of ZnPP. However, with

advent of our miceller technology using SMA, it becomes possible to encapsulate the drugs at very high loading level as much as 47% in the SMA-micelle, which liberate free ZnPP in time dependent manner. Currently along with our collaborators in both Australia, Austria and Germany we are investigating the effect of this macromolecule on solid and haematological malignancies weather through, HO-1 inhibition, phototoxicity and / or signal transduction modulation.

B- SMA-Camptothecin (CPT)

Camptothecins are a group of potent cytotoxic agents first iolated from Camptotheca accuminata. This group of synthetic agents excerts its cytotoxic effect in deviding celles through inhibiton of topoisomerase I . Besides the common problem of low molecular anticancer drugs of lacking specificity to tumor celles, this group of agents posses two major limitation , the first is weak water solubility and the second is their high affinity to human serum albumin in a reaction that render the drug inactive. 20(S)-camptothecin (CPT) posses a lactone ring that is essential for the cytotoxic effect of the drug on topoisomerase. This lactone group usually exists in equilibrium with an open ended carboxylate form of the drug. The lactone group react with human albumin at physiological pH to form favorably the inactive carboxylate form (145).

We had formulated SMA-CPT micelles to enhance the water solubility of this agents as well as protecting the sensitive lactone group, which is essential to the cytotoxic effect. In addition, improved pharmacokinetics and intratumor concentration due to the EPR-effect can be achieved.

C- SMA-Taxol

Paclitaxel is a natural complex of the taxane diterpenoids family. The drug has been first isolated in 1967, chemically defined in 1971, and in 1981 its anticancer activity was proved in vitro. Taxol was approved by the FDA for the treatment of ovarian cancer in December 1992, and for the treatment of breast cancer in April 1994. The major problem concerning the use of Taxol is its poor water solubility. For this reason taxol was dissolved in Cremophor and ethanol for clinical use. This resulted in allergic reaction and

neurotoxicity in a subset of patients (146). In order to improve water solubility and intratumor concentration of Taxol, we used SMA to form new SMA-Taxol micelles. The results of micelle activity in vitro (SMA-Taxol showed higher cytotoxicity than free Taxol) is encouraging for further future studies of this micelle formulation.

D- SMA-Bradykinin mimetics

Bradykinin (BK) is an autocrine growth factor for lung and prostate cancers. BK also facilitates tumor extension by increasing tissue permeability and stimulating angiogenesis. Peptide BK antagonists are in development as potential new drugs for lung cancer. Newer nonpeptide BK antagonists have even higher potency against lung cancer, in vitro and in vivo. These compounds have now been applied to the study of prostate cancers, and have been found to be effective. Prostate cancer cell line PC3 is derived from a late-stage, hormone-independent, metastatic tumor; its growth is difficult to inhibit. BK antagonists, while are active and have led the way to development of new peptide and nonpeptide agents for prostate cancer. In addition to inhibiting cancer cell growth directly, they inhibit angiogenesis mediated by vascular endothelial growth factor, and inhibit increased tissue permeability mediated by membrane metalloproteases in these tumors (147). In collaboration with Colorado School of Medicine, we successfully have been able to form SMA-BK antagonist micelle that were 10 folds more active than equivalent doses of free drug in growth inhibition of PC3 prostate cell line in nude mice.

In conclusion, the current research work addresses targeted treatment of solid tumor, which is one of the most common causes of morbidity and mortality in global community. Provided that our pilot studies were validated, this regimen of SMA-polymeric anticancer therapy would be one of the most powerful clinical tools for treating cancer patients, with the least side effects and highest targeting capability.

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