Synthesis and therapeutic effect of styrene–maleic acid copolymer-conjugated pirarubicin

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Pirarubicin (4′-O-tetrahydroxypropyl)doxorubicin, THP), a semisynthetic derivative of doxorubicin,(1) is one of the anthracycline antitumor drugs used clinically for treatment of various cancers. Compare to doxorubicin, THP shows rapid intracellular uptake,(2) little cardiac toxicity,(3) as well as applicability to doxorubicin-resistant cell lines.(4) However, like many other low molecular weight (MW) antitumor drugs, THP has poor tumor targeting properties, which leads to adverse effects and limits its dosage. Development of drugs with better tumor targeting capability is vital to overcome this drawback.

We have reported that macromolecular drugs (>40 kDa) showed much longer blood circulation and selective tumor accumulation by the enhanced permeability and retention (EPR) effect.(5,6) For this strategy, in our laboratory, styrene–maleic acid copolymer (SMA), an amphiphilic polymer, was used that can encapsulate low MW drugs to spontaneously form micelles.(7,8) Previously we prepared THP-encapsulated SMA micelles by a non-covalent interaction between styrene residues of SMA and THP.(9)

It was reported that the stability of drug-encapsulated micelles by non-covalent interactions was not adequate, and bursting of micelles and the release of low MW drugs in circulation was a problem.(10,11) However, covalently drug-conjugated polymeric drugs showed prolonged blood circulation and higher tumor accumulation than non-covalent micellar drugs.(12–14) Thus, one can achieve both high plasma stability and high tumor delivery by using polymer-conjugated drugs.

In this context, we developed a THP-SMA conjugate with covalent bond for further development. This study explains the preparation of the SMA-THP conjugate, its in vivo pharmacokinetic advantages, and its antitumor effects. Most strikingly, the antimetastatic effects of this micelle were also observed.

Materials and Methods

Materials. Pirarubicin was kindly provided by MicroBiopharm Japan (Tokyo, Japan), and SMA anhydride (Mr 1600) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Trinitrobenzenesulfonic acid (TNBS), BSA, RPMI-1640 medium, DMEM, Evans blue, and other reagents and solvents were purchased from Wako Pure Chemical (Osaka, Japan). Fetal calf serum was obtained from Gibco (Grand Island, NY, USA); MTT was purchased from Dojindo Chemical Laboratories (Kumamoto, Japan). All chemicals of reagent grade were used without purification.

Animals. Male ddY mice (6 weeks of age) and male Sprague–Dawley (SD) rats (6 weeks of age) were purchased...
Chromatography with Sephacryl S-200 (GE Healthcare, Tokyo, Japan) was carried out with 0.01 M phosphate buffered 0.15 M saline (PBS, pH 7.4) (column, $\varphi = 15 \text{ mm}, L = 700 \text{ mm})$.

**Dynamic light scattering and zeta potential.** The SMA-THP conjugate was dissolved in PBS (pH 7.4) at 2 mg/mL and filtered through a 0.2-μm filter. Particle size and surface charge (zeta potential) were measured by light scattering (ELS-22; Photol Otsuka Electronics, Osaka, Japan).

**Fluorescence spectroscopy.** The SMA-THP conjugate was dissolved in PBS at 10 μg/mL containing various concentrations of SDS (0.05–5%) or urea (5–9 M), and fluorescence spectra were recorded with a fluorescence spectrophotometer (FP-6600; JASCO) with excitation at 480 nm and emissions between 500 and 700 nm.

**In vitro cytotoxicity assay.** HeLa cells (human cervical cancer) were maintained in DMEM supplemented with 10% FCS. Colon 26 cells (mouse colorectal cancer) were maintained similarly but in RPMI-1640 medium.

Cells (3000 cells/well) were plated in 96-well plates (Corning, Corning, NY, USA). After overnight incubation, free THP or SMA-THP conjugate was added. After a further 72 h of culture, MTT assay was carried out to quantify the viable cells.

**Intracellular uptake and release of free THP.** HeLa cells (5 × 10^6 cells/well) were placed in a 12-well plate (Corning). After overnight preincubation, cells were treated with free THP or SMA-THP conjugate at 300 μM THP equivalent for indicated times. Cells were then washed twice with PBS, trypsinized, and resuspended in 500 μL methanol (60%) containing 2 M HCl, after which cells were sonicated (30 W for 30 s; Hielscher, Teltow, Germany) and incubated at 50°C for 1 h to hydrolyze THP derivatives, which were extracted by chloroform and quantified by fluorescence intensity (excitation, 480 nm; emission, 590 nm).

To measure the release of free THP, HPLC analysis was carried out. HeLa cells (2 × 10^6 cells) were placed in a cell culture dish (φ = 60 mm; Corning). After overnight preincubation, cells were treated with free THP or SMA-THP conjugate at 300 μM THP equivalent for indicated times. Cells were washed twice with PBS, trypsinized, and resuspended in 300 μL DMF; then sonicated in ice-chilled conditions, followed by centrifugation to remove insoluble debris. The supernatant was then subjected to HPLC as described above.

**Pharmacokinetics and tissue distribution.** All animal experiments were carried out according to the Laboratory Protocol for Animal Handling of Sojo University (Kumamoto, Japan).

Mouse sarcoma S-180 cells (2 × 10^6 cells) were implanted s.c. in the dorsal skin of male ddY mice. When diameters of the tumors reached approximately 10 mm, 10 mg/kg THP equivalent drugs in saline was injected i.v. into the tail vein. At the indicated time after i.v. injection, mice were killed and blood was collected. Blood and tissues, especially in the lymph nodes (mesenteric lymph nodes), were dissected, weighed, and methanol (60%) containing 2 M HCl (1 mL per 100 mg tissue) was added for homogenization. Then THP derivatives in the homogenate were measured following the protocol described above.

Similar experiments were also carried out in SD rats (6 weeks of age) to investigate the drug distribution in normal tissues, especially in the lymph nodes (mesenteric lymph nodes) and the bone marrow (from the femoral bone).

**In vivo antitumor activity of SMA-THP conjugate.** Colon 26 cells (1 × 10^6 cells) were implanted s.c. in the dorsal skin of male BALB/cCrSlc mice. The S-180 tumor model was prepared as described above. When tumors reached a diameter of approximately 4–6 mm, free THP or SMA-THP conjugate at desired concentrations was administered i.v. The tumor volume and body weight of the mice were measured during the study.

**Synthesis of SMA-THP conjugate.** Figure 1 shows a scheme of the synthesis of SMA-THP conjugate. The amino group of THP was used to react with the maleic anhydride residue of SMA to form an amide bond. Briefly, 200 mg SMA anhydride and 100 mg THP were mixed in 10 mL N,N-dimethylformamide (DMF) followed by addition of 20 μL triethylamine. The reaction was carried out under stirring for 24 h at room temperature in the dark. After the reaction, SMA-THP conjugates were precipitated and washed by diethyl ether, which was then removed unreacted THP using DMF as an eluate. Then 0.1 M NaHCO₃ (pH 8.2) was added to SMA-THP conjugate to hydrolyze remaining maleic anhydride residues, followed by dialysis against distilled water and ultrafiltration (Millipore, Bedford, MA, USA) with a 50-kDa cut-off membrane, and then lyophilization.

The TNBS method was used to quantify amino groups in SMA-THP conjugate. The THP content in this conjugate was quantified by the absorbance of THP at 480 nm spectrophotometrically.

**High performance liquid chromatography.** High performance liquid chromatography was carried out by using the LC-2000Plus series HPLC system (JASCO, Tokyo, Japan) equipped with a PU-2080 pump, UV-2075 UV/visible detector, and 807-IT integrator. Multimode size exclusion chromatography was carried out using an Asahipak GF-310 HQ column (7.5 × 300 mm) (Showa Denko, Tokyo, Japan). The mobile phase used DMF at a flow rate of 0.5 mL/min; eluate was monitored at 480 nm for THP.

**Sephacryl S-200 size exclusion chromatography.** Size exclusion chromatography with Sephacryl S-200 (GE Healthcare, Tokyo, Japan) was carried out with 0.01 M phosphate buffered 0.15 M saline (PBS, pH 7.4) (column, $\varphi = 15 \text{ mm}, L = 700 \text{ mm})$.
period. Tumor volume (mm$^3$) was calculated as ($W^2 \times L)/2$ by measuring the width ($W$) and length ($L$) of the tumor.

Lung metastasis is known to occur in the colon 26 tumor model.$^{15,16}$ Thus we also investigated the antimetastatic effect of SMA-THP conjugate in this model. Namely, on day 50 after tumor inoculation in the dorsal skin (40 days after the treatment described above), Evans blue at 10 mg/kg in saline (0.1 mL) was injected i.v. After 24 h, mice were killed, perfused with saline, and the lung was excised. Metastatic lung tumors were observed as blue tumor nodules macroscopically and the number of metastatic nodules was counted.

**Toxicity of SMA-THP conjugate.** Male ddY mice were used to evaluate toxicity of SMA-THP conjugate. Free THP (5 mg/kg, almost maximum tolerable dose [MTD]) was injected i.v. only once, and SMA-THP conjugate was injected i.v. on days 1 and 3 at doses of 10 or 30 mg/kg THP equivalent. The doses were the same as those used in treatment of S-180 tumors. At 36 h and 1 and 2 weeks after the last drug administration, mice were killed and blood samples were obtained to examine hematolgy (white blood cells [WBC], red blood cells [RBC], and hemoglobin levels) and liver, heart, and kidney functions: aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatin kinase, blood urea nitrogen, and total creati
gogenesis was considered statistically significant when $P < 0.05$.

**Results**

**Synthesis of SMA-THP conjugate.** From 200 mg SMA and 100 mg THP, 225 mg SMA-THP conjugate was obtained, with a THP loading of 30% (w/w), suggesting that the ratio of SMA and THP in the conjugate is approximately one THP/SMA chain. The HPLC analyses showed that SMA-THP conjugate was clearly larger than free THP, and neither free THP nor decomposition product was detected (Fig. 2a). The HPLC analysis using the Capcell Pak C18 column (Type MG, 5 μm, 4.6 mm internal diameter [I.D.] × 250 mm; Shiseido Fine Chemicals, Tokyo, Japan) with 1% acetic acid:acetonitrile mixture (1:1) as eluate also showed that SMA-THP conjugate did not contain unconjugated free THP (Fig. S1). The TNBS assay showed there was no amino group left in SMA-THP conjugate (Fig. 2b). Moreover, SMA-THP conjugate showed better water solubility than free THP, that is, more than 150 mg/mL (45 mg/mL THP) in PBS.

**Micelle formation and albumin binding of SMA-THP conjugate in aqueous solution.** Dynamic light scattering analysis of SMA-THP conjugate showed that the hydrodynamic diameter was 18.9 ± 10.0 nm (Fig. 2c), which means the conjugate existed in associated form as a micelle in PBS. The zeta potential of the micelle was −32.38 mV in PBS, in contrast to approximately −50 mV unreacted SMA (data not shown).

In aqueous solution, the fluorescence of SMA-THP conjugate was suppressed to approximately 15% of free THP. It was restored by adding SDS (Fig. 2d) or urea (Fig. 2e) to some extent, suggesting that THP on the SMA chain may be interacting with aromatic molecules of styrene residues, forming π−π interaction, and it may be tightly packed inside the micelle.

Size exclusion chromatography using Sephacryl S-200 showed the apparent size of SMA-conjugated THP in PBS was 62 kDa (Fig. 2f, g), which shows as a discrete single peak in the light scattering profile (cf. Fig. 2c) as a single entity (Fig. 2f, SMA-THP conjugate alone). One noteworthy property of SMA is its albumin binding capacity, which was also supported by the present study. Namely, SMA-THP conjugate behaved like larger molecules in the presence of BSA in a BSA dose-dependent manner, mostly forming a complex of 140 kDa at the BSA concentration of 10 mg/mL (Fig. 2f). In addition, quenching of fluorescence in SMA-THP conjugate was not affected in the presence of BSA (data not shown), which indicates that the conjugate may interact with albumin without disturbing the micellar structure, that is, SMA-THP conjugates bind to albumin as polymeric micelles.

**In vitro cytotoxicity and intracellular uptake of SMA-THP conjugate, and release of free THP.** As shown in Figure 3(a), SMA-THP conjugate showed dose-dependent cytotoxicity against HeLa cells with an IC$_{50}$ of 22 μM. In contrast, free THP showed much potent cytotoxicity (IC$_{50}$, 0.17 μM). Similar results were observed in colon 26 cells: the IC$_{50}$ of free THP was 0.02 μM, compared to SMA-THP of 12.6 μM (Fig. 3b). In both cell lines, cytotoxicity of SMA-THP conjugate was less than 1/100 compared with that of free THP.

In parallel with these findings, SMA-THP conjugate showed much slower intracellular uptake than free THP, that is, uptake of SMA-THP conjugate at 120 min after treatment was approximately 1/40 that of free THP (Fig. 3c). Figure 3(d-i) shows the HPLC analysis of free THP taken up into HeLa cells at 6 h after treatment. When cells were treated with SMA-THP conjugate, no free THP but only SMA-THP conjugate was detected in cells (Fig. 3d-ii). Even after 40 h of incubation, release of free THP was not observed in cells (Fig. 3d-iii). At 40 h, death of some HeLa cells was observed.

**Pharmacokinetics and tissue distribution of SMA-THP conjugate after i.v. injection.** As shown in Figure 4(a), free THP was cleared rapidly from blood circulation after i.v. injection, whereas SMA-THP conjugate remained high in blood circulation for a much longer time, with a 24.5-fold higher concentration curve than free THP. At 24 h after i.v. injection (Fig. 4b), free THP showed the highest concentration in spleen, followed by liver; the accumulation in tumor was very low, at approximately 13% of that in spleen. Accumulation of SMA-THP conjugate was highest in liver, while drug levels in tumor were higher than most other normal tissues. The high accumulation in liver is similar to previous SMA-conjugated drugs (e.g. SMANCS) as well as other macromolecular drugs, mostly through the reticuloendothelial system (e.g. macrophages). In addition, high accumulation of SMA-THP conjugate in kidney was also observed, which is probably due to the disintegrated chemical unit of SMA-THP conjugate from micelles whose molecular size (approximately 2200) is smaller than the renal threshold (40 kDa).

It should be noted that the tumor accumulation of SMA-THP conjugate increased to 8.4 times higher than that of free THP. Moreover, the concentration of SMA-THP conjugate in liver, spleen, and kidney decreased gradually and the concentrations were 24.5%, 27%, and 19% at 72 h compared with those at 24 h, respectively (Fig. 4c). In contrast, the concentration of SMA-THP conjugate in tumor at 72 h remained at 75% of that at 24 h, which was the highest concentration, followed by liver, spleen, and kidney (Fig. 4c).

Tissue distribution was also examined using healthy male SD rats. At 24 h after i.v. injection, levels of SMA-THP conjugate in plasma and liver were significantly higher than that of free THP (Fig. 4d). However, no or minimal accumulation of SMA-THP was found in brain, lung, and stomach compared
with free THP. In other tissues, no significant differences were observed between free THP and SMA-THP conjugate. Similar to findings in S-180 bearing mice, accumulation of this conjugate in normal tissues including liver, kidney, and bone marrow decreased very rapidly from 24 to 72 h (Fig. 4e).

**In vivo therapeutic effects against primary tumor and metastasis.** As shown in Figure 5(a), SMA-THP conjugate suppressed tumor growth in a dose-dependent manner, but no decreases in body weight of mice were observed. Free THP at 5 mg/kg, which is close to the MTD, also showed antitumor effects, but body weight loss was significant (Fig. 5b).

More importantly, reduction of metastasis in the lung by SMA-THP conjugate (Fig. 5c,d) was remarkable. As shown in Figure 5(c), in untreated control mice numerous metastatic lung tumor nodules were observed that were stained by Evans blue albumin according to the EPR effect. Treatment with SMA-THP conjugate (30 mg/kg THP equivalent) virtually abolished these lung metastasis (Fig. 5d). In contrast, treatment with free THP (5 mg/kg) showed no apparent effect on lung metastasis. In addition, at 10 days after tumor inoculation and the start of treatment, no obviously visible tumor nodules were found in lungs of mice (data not shown).

Similar therapeutic effects were also seen in the S-180 tumor model (Fig. 6a,b). We observed apparent cure of tumors in three out of five mice by both doses of the conjugate without recurrence longer than 120 days after tumor inoculation (Fig. 6c).

**Toxicity of SMA-THP conjugate.** After single injections of the conjugate at different doses (50, 100, or 150 mg/kg THP equivalent ×1), body weight loss was only observed at the dose of 150 mg/kg several days after injection (Fig. 6d). That loss was restored later, however, suggesting that the MTD may be higher than 150 mg/kg, 30 times higher than that of free THP. When free THP was injected twice into S-180 tumor-bearing ddY mice at 10 mg/kg, all mice died within 1 week of the last injection. In colon 26 tumor-bearing BALB/cCrSlc mice, free THP injected once at 5 mg/kg induced continuous decrease of body weight for several days (cf. Fig. 5b).
In addition, as shown in Table 1, no apparent changes in WBC and RBC counts, hemoglobin levels, or kidney function were found after i.v. injection of 10 and 30 mg/kg SMA-THP conjugate. However, a significant suppression of RBC and hemoglobin levels was observed 1 and 2 weeks after free THP treatment (5 mg/kg\(^9\)). There was slight increase in aspartate aminotransferase after injection of SMA-THP conjugate (30 mg/kg\(^9\)), which might be caused by high accumulation of the conjugate in liver, as revealed by the distribution study (cf. Fig. 3b). Another point to note was that the higher dose of SMA-THP conjugate (30 mg/kg THP equivalent \(\times\)2) induced a weak increase of creatine kinase at 36 h after injection. The increases were dose-dependent but lower than those induced by free THP.

**Discussion**

In the present study, we prepared an SMA-THP conjugate in which all THP residues were covalently conjugated to the SMA chain, for achieving better circulation stability and tumor-targeting ability (Fig. 1). During preparation, SMA-THP conjugate was purified by use of column chromatography with
DMF to remove non-covalently bound THP, whereas such low MW drugs encapsulated in micelles cannot be removed in an aqueous column system.

Results of dynamic light scattering (Fig. 2c) and Sephacryl S-200 chromatography (Fig. 2f) suggested that SMA-THP conjugate behaved more similarly to much larger macromolecules in aqueous solution (φ = approximately 19 nm; 62 kDa) than its chemical unit (SMA [1600] + THP [627]). These findings indicate that several SMA-THP conjugates are associated tightly to form a micelle structure, also evidenced by fluorescence quenching of SMA-THP conjugate (Fig. 2d,e). This emergence of fluorescence phenomenon was similarly observed in drug-encapsulated SMA micelles previously developed in our laboratory.(8,9) Moreover, SMA-THP conjugate was found to bind with BSA, thus behaving as a much larger molecule (Fig. 2f), with an apparent size of 140 kDa (Fig. 2g). The albumin binding capacity of SMA is important for prolonged blood circulation of SMA-modified drugs, which also confers higher biocompatibility.(17,19) Because albumin is abundant in blood, it is expected that most SMA-THP conjugate exists as a complex with albumin in vivo, which consequently led to remarkably prolonged circulation time and much higher tumor accumulation by the EPR effect, compared to free THP (Fig. 4a,b). In addition, although SMA-THP conjugate in normal tissues such as liver, kidney, and bone marrow was cleared time-dependently (Fig. 4c,e); it showed superior accumulation and retention in tumor tissue. These properties then resulted in the remarkable in vivo antitumor effect of SMA-THP conjugate without apparent toxicity (Figs 5,6), which was also supported by the results of hematology examination and liver, heart, and kidney functions (Table 1).

The in vitro cytotoxicity of SMA-THP conjugate was lower than 1/100 of free THP (Fig. 3a,b), which may be partly related to the lower intracellular uptake of SMA-THP conjugate compared to free THP (Fig. 3c).
Table 1. Effect of free plasmalbumin (THP) and tyrosine-maleic acid copolymer (SMA)-conjugated THP (SMA-THP conjugates) on hematology and liver, heart, and kidney functions

| Control (no drug) | Free-THP (30 mg/kg) | SMA-THP (15 mg/kg, THP equivalent) | SMA-THP (30 mg/kg, THP equivalent) |
|------------------|---------------------|-----------------------------------|------------------------------------|
| Hb (g/dL) | 13.4 ± 0.7 | 12.0 ± 0.3 | 9.5 ± 0.4 |
| WBC (10⁶/μL) | 6.9 ± 0.7 | 9.5 ± 0.3 | 9.9 ± 0.6 |
| AST (U/L) | 22.0 ± 0.7 | 45.1 ± 0.3 | 43.7 ± 0.4 |
| ALT (U/L) | 43.7 ± 0.7 | 14.4 ± 0.3 | 15.3 ± 0.4 |
| LDH (U/L) | 126.3 ± 0.7 | 70.0 ± 0.3 | 67.3 ± 0.6 |
| CRE (mg/dL) | 1.8 ± 0.7 | 14.9 ± 0.3 | 26.1 ± 0.4 |

Values are means ± SD (n = 4). *p < 0.05, significant differences from untreated control group, according to Student’s t-test. Free-THP was given i.v. on day 1, and SMA-THP conjugate was taken up into HeLa cells as the intact conjugate form and release of free THP from this conjugate did not occur in cells at least 40 h after treatment (Fig. 3d-ii,iii), which may also be partly responsible for the lower cytotoxicity of SMA-THP conjugates. These findings suggested that SMA-THP conjugate per se might show antitumor activity without release of free THP. These results are consistent with previously published reports showing that conjugated drugs with no cleavable bond could also exhibit cytotoxic activity, but the polymer-conjugated drugs usually exhibit lower in vitro cytotoxicity. The low cytotoxicity as well as rapid clearance of SMA-THP conjugate from normal tissues (Fig. 4c,e) may contribute to lower levels of damage to normal tissues in vivo (Figs 5,6, Table 1). However, high accumulation of SMA-THP in tumor could ensure sufficient drug concentration (e.g. higher than in vitro IC₅₀) to fulfill antitumor activity.

Regarding the possible mechanisms of action of SMA-THP conjugate, it was reported that albumin was taken up by tumor cells and degraded to generate amino acids and energy in the cells, by which albumin-conjugated drugs were internalized into tumor cells. The SMA-THP conjugate might also be taken up into tumor cells together with the uptake of albumin, and it may then detach from albumin through degradation of albumin, followed by the breakdown of micelle formation in tumor cells (e.g. in lysosomes). Recently, we also reported that breakdown of micelles could be facilitated by amphiphilic components of cell membrane, that is, lecithin. Amphiphilic cell membrane components may thus also contribute to the breakdown of micelles of SMA-THP conjugate during internalization into tumor cells. However, SMA-THP conjugate itself, but not the released free THP, is probably responsible for the antitumor effect of SMA-THP in this study; no release of free THP from SMA-THP conjugate was observed even after 40 h of incubation (Fig. 3d-ii,iii).

In cancer treatment, control of metastatic tumors is a significant problem and a major cause of therapeutic failure. Tumor metastases are often invisible and emerge at distant sites, and their response to chemotherapy and radiotherapy is rather inefficient. Under such circumstances, an important finding of the present study is that treatment with SMA-THP conjugate against colon 26 tumors showed not only growth suppression of implanted tumor in dorsal skin, but also almost complete simultaneous eradication of metastatic tumor in lung (Fig. 5).

We previously reported that SMA-conjugated neocarcinostatin SMANCS showed an antilymphatic metastatic effect mostly because of the EPR-based tumor targeting. The EPR effect has also been observed in small tumor nodules (e.g. 200 μm in diameter). In this study, we also found that Evans blue-bound albumin selectively accumulated in metastatic lung cancers by the EPR effect (Fig. 5c). Polymer-conjugated drugs or macromolecular drugs showing the EPR effect in metastatic tumor is a very intriguing finding in this report. Although no obviously visible lung metastatic tumor nodules were found at 10 days after tumor inoculation and start of treatment, SMA-THP conjugate may accumulate in the small invisible metastatic tumor nodules by the EPR effect, and exert an antitumor effect. However, the major cause of decreased metastatic lung tumors by treatment with SMA-THP conjugates is considered to be the consequence of growth suppression of the inoculated primary tumor. Further studies of the effect of SMA-THP conjugate on tumor metastases are warranted.

Conjugation of drugs with SMA is known to confer immunopotentiating effects, such as activation of macrophages,
T cells, and natural killer cells, and induction of interferon.\(^{32-35}\) Such an effect of SMA-THP conjugate might thus confer another benefit for development of macromolecular antitumor drugs for clinical application. In conclusion, covalently linked SMA-THP conjugate showed prolonged circulation time and tumor-targeting properties based on the EPR effect. Consequently, marked tumor-inhibitory effect on colon carcinoma 26 metastasis to the lung. \(^{75}\)

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Disclosure Statement
The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** High performance liquid chromatography analyses using a Capcell Pak C18 column of free pirarubicin (THP) and styrene-maleic acid copolymer (SMA)-conjugated THP (SMA-THP conjugate).