Micelle-Encapsulated Thiostrepton as an Effective Nanomedicine for Inhibiting Tumor Growth and for Suppressing FOXM1 in Human Xenografts

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Abstract

The thiazole antibiotic, thiostrepton, has been found to induce cell death in cancer cells through proteasome inhibition. As a proteasome inhibitor, thiostrepton has also been shown to suppress the expression of FOXM1, the oncogenic forkhead transcription factor overexpressed in cancer cells. In this study, we explored the potential in vivo anticancer properties of thiostrepton, delivered through nanoparticle encapsulation to xenograft models of breast and liver cancer. We encapsulated thiostrepton into micelles assembled from amphiphilic lipid-PEG (polyethylene glycol) molecules, where thiostrepton is solubilized within the inner lipid compartment of the micelle. Upon assembly, hydrophobic thiostrepton molecules are solubilized into the lipid component of the micelle shell, formed through the self-assembly of amphiphilic lipid-PEG molecules. Maximum accumulation of micelle-thiostrepton nanoparticles (100 nm in diameter, +16 mV in zeta potential) into tumors was found at 4 hours postadministration and was retained for at least 24 hours. Upon continuous treatment, we found that nanoparticle-encapsulated thiostrepton reduced tumor growth rates of MDA-MB-231 and HepG2 cancer xenografts. Furthermore, we show for the first time the in vivo suppression of the oncogenic FOXM1 after treatment with proteasome inhibitors. Immunoblotting and immunohistochemical staining also showed increased apoptosis in the treated tumors, as indicated by cleaved caspase-3 expression. Our data suggest that the thiazole antibiotic/proteasome inhibitor thiostrepton, when formulated into nanoparticles, may be highly suited as a nanomedicine for treating human cancer. Mol Cancer Ther; 10(12); 2287–97. ©2011 AACR.

Introduction

As the second most common cause of mortality in the United States, cancer poses as a disease that requires urgent improvement of therapeutic strategies. Originally adopted for the topical treatment of veterinary dermatologic infections, thiostrepton, a thiazole antibiotic, has also been shown to inhibit cell growth in a variety of human cancer cell lines (1–3). The apoptotic activity of thiostrepton has been deciphered to be through proteasome inhibition (3–5), resulting in the stabilization of certain proteins that proves fatal to cancer cells. However, one target observed to be suppressed rather than stabilized is that of the forkhead box M1 transcription factor, FOXM1 (3, 6), a protein found to be overexpressed in a variety of human cancers (7–9). It has been suggested that the inhibition of FOXM1 by proteasome inhibitors may contribute to their anticancer activity (6).

Upon discovery, major issues associated with current anticancer drugs such as paclitaxel and tamoxifen were their insolubilities in aqueous solutions (10, 11). Methods to overcome such obstacles included the encapsulation of the drugs into amphiphilic nanoparticle systems, amphiphilic in that they possess a hydrophilic outer shell and a hydrophobic inner core in which the hydrophobic drugs can be solubilized (12–14). Encapsulation into nanoparticles acts not only as a means to solubilize hydrophobic drugs, but as importantly, the association of cancer drugs into nanomeric structures aids their specific accumulation into tumor sites. Compared with those of healthy, non-cancerous tissues, the neovasculature that supplies blood flow to tumors is highly irregular and punctuated with fenestrations. These junctions provide openings with sizes of 700 nm across, increasing their permeability to particles smaller than 700 nm in diameter (15). Unlike small molecules, nano-sized macromolecules are less toxic to healthy organs and noncancerous tissues, as they leave circulation only via diffusion through the fenestrated gaps of tumor blood vessels (16–18). The clinical application of proteasome inhibitors as chemotherapeutic agents has also been associated with levels of high...
toxicity, and is likely to benefit from nanoparticle-protected delivery (19–21).

Like the case of other hydrophobic cancer drugs, the first limitation in showcasing thiostrepton as a chemotherapeutic solution is its insolubility in aqueous solutions. As a lucid and simple method, the use of PEGylated lipids has been described as a means to solubilize hydrophobic drugs into macromolecular delivery vehicles (12, 22, 23). As the encapsulation of proteasome inhibitors into nanocarriers delivery systems for cancer treatment has yet to be described, we carried out this study to investigate the possibility of inducing an enhanced antitumor effect using micelle-encapsulated thiostrepton.

**Materials and Methods**

**Cell lines and chemical compounds**

MDA-MB-231-luc-D3H2-LN, human lymph node-derived metastatic mammary gland adenocarcinoma (Caliper Lifescience); HepG2-luc, human hepatocellular carcinoma stably transfected with luciferase-expressing gene (retrovirus). MDA-MB-231 cells were maintained in MEM media (Mediatech) supplemented with 10% FBS (Atlanta Biological), 1% 100× nonessential amino acids (Gibco), 1% 200 mmol/L Na pyruvate (Gibco) and 75 μg/mL Zeocin (Invitrogen). HepG2-luc cells (American Type Culture Collection) were kept in Dulbecco’s Modified Eagle’s Medium media supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco). Cells had not been authenticated by authors. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000-MeO) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); rhodamine-DPPE (Wang and Gartel) was purchased from Avanti Polar Lipids. Thiostrepton (from Streptomyces azureus, 90% purity) was obtained from Sigma.

**Preparation of polymeric micelle-encapsulated thiostrepton**

A thin lipid film of DSPE-PEG2000-MeO and thiostrepton was prepared by mixing the 2 components in chloroform in a 10 mL round-bottomed flask. The chloroform was removed in vacuo and by air-drying, after which the lipid film was hydrated (for final thiostrepton concentration of 1 or 2 mmol/L) with PBS (pH 7) or H2O and vortexed at room temperature for 10 minutes. The lipid-to-drug ratio was varied by keeping the amount of thiostrepton constant (at 1 mmol/L) and increasing the amount of PEG-lipid from 1 to 4 mmol/L. The percentage of encapsulated thiostrepton was measured by centrifuging solutions of micelle-thiostrepton (varying lipid-to-drug ratios) at 8RCF for 2 minutes, after which the supernatant was separated from any precipitated matter (insoluble, unencapsulated thiostrepton). The supernatant was measured for UV absorbance at λ = 300 nm, from which the amount of remaining encapsulated thiostrepton was calculated. To measure the amount of nonencapsulated thiostrepton, the precipitates were dissolved in CHCl3 and examined for UV absorbance at λ = 300 nm. For micelle-only preparations, the thiostrepton component was omitted and only the lipid component was hydrated in aqueous solution to 3 or 6 mmol/L, corresponding to either 1 mmol/L thiostrepton or 2 mmol/L thiostrepton. For fluorescence labeling, DOPE-rhodamine was incorporated at the chloroform stage at a 1:100 molar ratio to DSPE-PEG2000-MeO.

**Physical characterization of micelle-encapsulated thiostrepton**

For drug release studies, micelle-thiostrepton suspensions (3:1 or 4:1 lipid/drug ratios m/m) were incubated in 50% FBS (in PBS, final concentrations of 0.05 mmol/L thiostrepton) over time at 37°C. Amount of remaining encapsulated thiostrepton was measured by centrifuging incubated samples (8RCF, 2 minutes) to remove the non-encapsulated and precipitated thiostrepton from the micelle-encapsulated thiostrepton in the supernatant. The supernatant was then measured for UV absorbance at λ = 300 nm and the concentration of remaining thiostrepton was calculated by extrapolation with calibration curves. Sizes and zeta potentials of nanoparticles (in PBS) were analyzed by dynamic light scattering, using a 5 mW 633 nm laser angled at 90° to the sample. Measurements are presented as volume-weighted multimodal distributions. For in vitro and in vivo applications, micelle-encapsulated thiostrepton solutions were prepared in PBS (sterile) at 1 or 2 mmol/L, respectively. For transmission electron microscopy, 20 μL of micelle-thiostrepton solutions (1 mmol/L thiostrepton/3 mmol/L lipid) or micelle-only solutions (3 mmol/L lipid) in H2O were dispensed onto a 200-mesh Cu grid supported by holey-carbon film and air-dried overnight. TEM images were collected using the JEOL JEM-3010, equipped with a LaB6 electron gun operated at 300 kV. Images were captured using a 1 K × 1 K Peltier-cooled Gatan multiscan CCD camera.

**Treatment of cells in vitro**

MDA-MB-231 cells were seeded at 1 × 10⁵ cells/10 cm plate and HepG2-luc cells were seeded at 5 × 10⁵ cells/6 cm plate and incubated overnight before treatment. Nonencapsulated thiostrepton [dissolved in dimethyl sulfoxide (DMSO), 10 mmol/L] and micelle-encapsulated thiostrepton (1 mmol/L) were administered to cells at concentrations of 5, 7.5, and 10 μmol/L. Controls for in vitro experiments were cells treated with either DMSO only (6 μL) or micelle-only (30 μmol/L). Cells were treated for 24 hours before harvesting.

**Western blot analysis of cell lysates**

Cells were harvested with IP lysis buffer (20 mmol/L HEPES, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L Na3PO4, 1 mmol/L Na2VO4, 0.2 mmol/L PMSF). For analysis of tissues, liquid N2-frozen sections of
xenograft tumors were homogenized in 1 mL IP lysis buffer. Protein concentration was measured by the Bio-Rad Protein Assay and protein separation was done on 8% or 12% SDS-PAGE gels. Separated proteins were then transferred onto polyvinylidene difluoride membranes (Millipore) and immunoblotted with specific antibodies against FOXM1 (c-20, Santa Cruz), cleaved-caspase-3 (Cell signaling) and β-actin (Sigma; ref. 24). Quantification of cleaved caspase-3 and FOXM1 protein expression levels was done by the "Gel" analysis function in ImageJ.

Cell viability assay
Cells were seeded at a density of 1,000 cells/well into 96-well plates. After incubation overnight, growth media was replaced with media containing free (in DMSO) or micelle-encapsulated thiostrepton at increasing concentrations. DMSO-treated and micelle-only-treated cells were also examined and used to compare with nontreated (media only) cells. Cells were treated for 72 hours before media was spiked with MTT assay reagent (Sigma, prepared in 5 mg/mL in PBS) to render a final 10% concentration. After incubation for 3 hours, MTT/media was removed and purple precipitates were dissolved with 150 μL of DMSO. Wells were measured for absorbance at λ = 550 nm and the % cell viability was calculated as a percentage of the UV absorbance of nontreated cells.

Animal maintenance and tumor xenograft experiments
Animals were maintained and treated in accordance with the guidelines established by the Animal Care and Use Committee of the University of Illinois at Chicago. Tumor models were prepared by implanting cancer cell lines (1 × 10⁶ of MDA-MB-231 and 2 × 10⁶ of HepG2-luc), suspended in 50 μL of 1:1 PBS/Matrixgel into each flank of 4-week-old male athymic mice (Taconic). Treatment of xenograft models with micelle-encapsulated thiostrepton at increasing concentrations. DMSO-treated and micelle-only-treated cells were also examined and used to compare with nontreated (media only) cells. Cells were treated for 72 hours before media was spiked with MTT assay reagent (Sigma, prepared in 5 mg/mL in PBS) to render a final 10% concentration. After incubation for 3 hours, MTT/media was removed and purple precipitates were dissolved with 150 μL of DMSO. Wells were measured for absorbance at λ = 550 nm and the % cell viability was calculated as a percentage of the UV absorbance of nontreated cells.

Biodistribution of micelle-thiostrepton nanoparticles
Rho-labeled micelle-thiostrepton nanoparticles were injected via the tail-vein into nontumor bearing or MDA-MB-231 tumor-bearing animals (1 cm³ per tumor) at a dose of 1.8 mg thiostrepton per animal. At 4, 17, and 24 hours postinjection, animals were anesthetized with isoflurane and imaged using the Xenogen IVIS system for rhodamine-fluorescence distribution. Animals were then sacrificed and organs removed for further fluorescence imaging. Background autofluorescence was eliminated using a spectral unmixing algorithm provided by Living Image software. For comparison with free, nonmicelle-encapsulated drug, thiostrepton was dissolved to concentrations of 36 mg/mL in N,N-dimethylacetamide/polyethylene glycol/Tween 80 formulation (2:7:1; ref. 25) and administered at a dose of 50 μL per animal (2 animals, total 4 tumors) by intraperitoneal injection. Tumors were collected at 4 and 24 hours postinjection and were homogenized (Fisher, Polytron) in 1 mL lysis buffer. Tumor homogenates were treated with 200 μL 10 mmol/L HCl for 24 hours at 4°C. One milliliter of chloroform was then added and the homogenates were vortexed for 10 minutes. Samples were incubated at room temperature for 24 hours before vortexing, after which samples were centrifuged and the chloroform supernatant layer was collected and dried in vacuo. For HPLC/mass spectrometry studies, extracted samples were reconstituted in 50 μL of chloroform and 450 μL of MeOH. Using an Agilent Zorbax 300sb-C8, 2.1 × 50 mm column, the isocratic method of 30% of 100% H₂O/0.1% formic acid and 70% of 100% methanol/0.1% formic acid at 200 μL/mL for 6 minutes was run. The AUC of HPLC traces that correlated to thiostrepton retention times were used to calculate the concentrations of thiostrepton, as compared with a thiostrepton/HPLC calibration curve.

Treatment of xenograft models with micelle-encapsulated thiostrepton
Animals bearing tumors were randomized into groups of 4 to 5, where groups were administered with micelle-only controls at 200 mg DSPE-PEG2000-MeO/kg, (200–250 μL of 6 mmol/L DSPE-PEG2000-MeO in PBS) or 30 mg/kg of thiostrepton encapsulated in micelles (2 mmol/L thiostrepton/6 mmol/L DSPE-PEG2000-MeO in PBS, 200–300 μL). Treatments were done once every 2 days for 12 to 14 treatments, during which tumor volumes were monitored with calipers (V = l × w × h = volume, mm³). In the MDA-MB-231 model, injections were carried out daily for the last 5 days. Animals were also weighed once a week. After 12 to 14 treatments, animals were sacrificed and tumors removed.

Immunohistochemical analysis of paraffin-embedded tissue
Formalin-fixed tumor tissues were embedded in paraffin and sliced to 4-μm-thick sections. Sections were deparaffinized by submerging sample-containing slides into a series of solvents of decreasing hydrophobicity. Heating samples in 10 mmol/L citric acid (pH 6) was used for antigen retrieval. After blocking with 3% H₂O₂ in MeOH, and further blocking in normal goat serum in PBS, slides were treated with primary anti-FOXM1 (Santa Cruz, k-19), anticleaved caspase-3 or control IgG antibodies at concentrations of 1:100, in 1% bovine serum albumin/PBS. Biotinylated secondary antibody treatment and Avidin HPR treatment was carried out according to the Vectastain ABC Kit manual (anti-rabbit, Vector Labs), followed by staining with 3,3′-diaminobenzidine (DAB; Sigma; D-0426). Cleaved caspase-3–stained samples were counterstained with hematoxylin (Vector Labs). Samples were dehydrated in the series of solvents and mounted with Permount (Fisher) mounting agent. Slides were analyzed on a Zeiss Apotome microscope.
Results

Preparation of micelle-encapsulated thiostrepton

The purpose of this study was to formulate thiostrepton, a highly hydrophobic anticancer drug into nanoparticle delivery vehicles and to examine its effect as a nanomedicine against cancer in vivo. Encapsulation of thiostrepton (Fig. 1A) into nanomeric micelles was conducted by using the lipid-hydration method, and encapsulation conditions were optimized by varying the lipid-to-thiostrepton ratio. The lipid considered for this application was DSPE-PEG₂₀₀₀-MeO (Fig. 1A), a polymer-lipid conjugate chosen for its ability to spontaneously form micelles with hydrophobic cores upon dispersion in aqueous solutions (22). PEGylated lipids are amphiphilic lipid-polymer conjugates where the polymer component is a hydrophilic polymer chain such as polyethylene glycol (PEG). Upon hydration, these lipid-PEG conjugates form amphiphilic micellar structures consisting of a hydrophilic polymer shell, and an organic lipidic core (Fig. 1B). It is within this lipid-rich area that thiostrepton can be solubilized and ultimately incorporated into nano-sized macromolecular structures that can aid its accumulation into tumor sites (26–28). At concentrations above reported critical micelle concentrations (23, 29), various drug-to-lipid ratios were examined for their drug encapsulation efficiency (Fig. 1C). By keeping the concentration of

Figure 1. A, molecular structures of the thiazole antibiotic, thiostrepton, and DSPE-PEG₂₀₀₀-MeO, the PEGylated amphiphilic lipid used to encapsulate thiostrepton into micellar nanoparticles. B, representation of micelle-thiostrepton nanoparticles, depicting the solubilization of hydrophobic thiostrepton molecules in the inner hydrophobic compartment of the micelle nanoparticle. The nanoparticle is formed by the self-assembly of DSPE-PEG₂₀₀₀-MeO and thiostrepton in aqueous solutions. C, formulation studies to identify the lowest lipid-to-drug ratio required to completely encapsulate 1 mmol/L of thiostrepton. A 3:1 lipid:drug ratio m/m was identified as the most acceptable ratio for further study. All measurements represent the average of 3 separate experiments, and error bars represent SD.
thiostrepton constant at 1 mmol/L and increasing the concentration of DSPE-PEG2000-MeO, it was found that highest encapsulation efficiencies were obtained after lipids outnumbered thiostrepton molecules by 3-fold (3:1 PEG-lipid/thiostrepton, m/m), after which there was no significant increase in amount of thiostrepton encapsulated. The optimal formulation of 3:1 DSPE-PEG2000-MeO/thiostrepton (m/m) was chosen to be used for studying the effect of nanoparticle-encapsulated thiostrepton on cancer cells. Assembled micelle-thiostrepton structures were found to be in the form of nanoparticulate structures with hydrodynamic dimensions of 100 nm in diameter and −16 mV in zeta potential (Fig. 2A).

An inherent negative charge (negative zeta potential) on the surfaces of nanoparticles is desirable for delivery to tumor sites, as to prevent opsonin recognition that could lead to macrophage-assisted clearance from circulation (30, 31). Opsonization, the immune response action of tagging positively charged alien particles with negatively charged opsonin proteins should be minimized as to avoid clearance by macrophage recognition (32). Transmission electron microscopy images illustrated the difference in micelles before and after drug

![Figure 2. A, final nanoparticle-thiostrepton formulations (3:1 DSPE-PEG2000-MeO/thiostrepton, m/m) were found to be approximately 100 nm in diameter and −16 mV in zeta potential. B, electron microscopy images of micelles only and micelle-thiostrepton confirm the dimensions of the micelle-encapsulated complexes. C, release profile of thiostrepton from micellar vehicles (3:1 lipid/drug ratio, m/m) in serum solutions at 37°C that mimic in vivo environments (blood circulation). In high serum conditions, thiostrepton can remain encapsulated within micelle nanoparticles for at least 24 hours at 37°C. Each measurement represents the average of 3 separate experiments, and error bars represent SD.](image-url)
encapsulation and confirm the presence of 100 nm diameter micelle-thiostrepton species (Fig. 2B). The release profile of the optimal formulation were examined by incubating micelle-thiostrepton nanoparticles (3:1 lipid/drug, m/m) in PBS solutions containing 50% FBS at 37°C, to mimic the plasma serum conditions of in vivo blood circulation (Fig. 2C). In 50% FBS, the integrity of the micelle-thiostrepton structure was maintained for long periods of time, where 90% of thiostrepton was retained within nanoparticle structures after 24 hours of incubation. This finding is particularly significant for the in vivo application of such nanoparticles, where the maintenance of drug encapsulation in circulation for longer periods is required for accumulation into tumor sites.

**Micelle encapsulation enhances apoptotic effect of thiostrepton on breast cancer and liver cancer cells in vitro**

Treatment of MDA-MB-231 breast cancer and HepG2-luc (luciferase-expressing) liver cancer cells with micelle-encapsulated thiostrepton resulted in an enhancement of cleaved caspase-3 expression (a marker of apoptosis), compared with those treated with nonencapsulated thiostrepton (Fig. 3A and B). Also observed is the further suppression of FOXM1, in cells treated with micelle-encapsulated thiostrepton, compared with free thiostrepton. As thiostrepton is highly insoluble (precipitates of DMSO-dissolved thiostrepton are highly visible when added to cell media), it is likely that its full encapsulation into micelles increases its availability to cancer cells in vitro. Cell viability assays also confirmed the enhanced effect of thiostrepton, when delivered to cells through micelle-encapsulation (Fig. 3C and D). In terms of mechanism of drug internalization within the cancer cells, it is likely that thiostrepton is released into the cell media before it is internalized into cancer cells, as incubation with fluorescently-labeled micelle-thiostrepton did not show cell-associated fluorescence over time (Supplementary Data S1). As for empty micelles, they alone did not have an effect on cell viability, suggesting their low toxicity for in vivo applications.

**Accumulation of micelle-encapsulated thiostrepton into MDA-MB-231 breast cancer xenografts and its effect on tumor growth**

Nano-sized particles can localize specifically into tumor sites by bypassing healthy tissue and diffusing through the leaky fenestrations in the tumor blood vessels, a vascular characteristic shared with no other organ apart from the liver (33). Further to their specific localization, nano-sized structures are retained in tumor sites due to the impaired lymphatic drainage system of tumor tissue (16). The ability of administered micelle-thiostrepton to localize into tumors was investigated using nude mice bearing MBA-MB-231 xenografts. The tumor retention and biodistribution of micelle-thiostrepton was studied by LC/MS of the tumor homogenates and by live animal fluorescence imaging.

![Figure 3](http://example.com/figure3.png)

**Figure 3.** HepG2-luc (A) and MDA-MB-231 (B) cells were treated with micelle-encapsulated thiostrepton and nonencapsulated thiostrepton in vitro. After 24 hours, cell lysates were examined for expression of the oncogenic transcription factor FOXM1 and for cleaved caspase-3. The effect of thiostrepton (micelle-encapsulated or nonencapsulated) on cell viability was examined by the MTT assay on HepG2-luc cells (C) and MDA-MB-231 cells (D). In all cases, micelle-encapsulated thiostrepton induced greater levels of cell death and inhibited cell viability more efficiently in cancer cells compared with nonencapsulated thiostrepton. Values represent averages of 4 individual experiments and error bars represent SD.
Upon establishment of subcutaneous tumors, fluorescently-labeled micelle-thiostrepton was administered through the tail vein and animals were monitored for rhodamine-associated fluorescence by live whole body imaging (Xenogen IVIS). Accumulation of fluorescence into tumor sites (live and \textit{ex vivo} imaging) was observed to occur to a maximum at 4 hours postadministration (Fig. 4A). Localization into the liver was also prominent at 4 hours postadministration, likely an event of diffusion through the sinusoidal capillaries (33, 34), which are also punctuated with fenestrations as it is in angiogenic tumor vessels. By 17 hours, micelle-associated fluorescence had greatly diminished in tumor regions, although a small amount appeared retained. As fluorescence labeling only monitors the movement and retention of the lipid moiety of the micelle-thiostrepton complex, tumor homogenates were further extracted with chloroform (in which thiostrepton is soluble) and the amount of tumor-accumulated thiostrepton was examined using LC/MS. It was found that tumor-associated thiostrepton was in fact in higher concentrations at 24 hours postinjection, compared with that at 4 hours postinjection (Fig. 4B). In conjunction with the fluorescence-biodistribution data, this is indicating that micelle-thiostrepton complexes arrive at tumor sites intact, and that it is also likely that thiostrepton continues to accumulate into tumors 4 hours postadministration (up until 17 hours), after which dissociation of the micelle-thiostrepton occurs and the lipid component is cleared from the region. Furthermore, the percentage injected dose of micelle-thiostrepton to arrive at tumors was approximately 30% per tumor, and considering there were 2 xenograft tumors per animal, 60% of the injected dose was tumor-localized (Fig. 4B). We compared the tumor accumulation of micelle-thiostrepton with that of its solubilization into the previously reported \textit{N,N}-dimethylacetamide/polyethylene glycol/Tween 80 formulation (25) and found that micelle-encapsulated thiostrepton accumulated into tumors with greater efficiency, where an increase in approximately 10-fold of thiostrepton concentrations were detected in each.
tumor (Fig. 4B). In addition, pilot studies also showed the effect on xenograft growth of thiostrepton delivered through the dimethylacetamine-based formulation was much weaker in MDA-MB-231 tumors compared with delivery through micelle-encapsulation (data not shown).

The biological effect of micelle-encapsulated thiostrepton on tumor growth was monitored in MDA-MB-231 subcutaneous tumor models. The specific dose for administration (40 mg/kg) was selected after pilot studies showed it to be the minimal concentration to exhibit anticancer effects. Injections were administered 3 times a week, which after 14 treatments, reduced tumor growth by up to 4-fold, compared with nontreated tumors (Fig. 4C). As determined from the release studies, it is likely that thiostrepton is not released from the micellar vehicle before it reaches the tumor sites, as thiostrepton can remain encapsulated for at least 24 hours in high serum conditions. Internalization of whole rhodamine-labeled micelle-thiostrepton complexes were shown to be possible at higher serum conditions, as indicated by increase in cell-associated fluorescence over time (Supplementary Data S1). Therefore, it is likely that micelle-thiostrepton complexes arrive intact at tumor vicinities and are internalized into the tumor cells by mechanisms such as endocytosis. As with nontreated tumors, tumors treated with an equivalent dose of empty micelles (without thiostrepton) increased steadily in size over time, and a reduction in tumor growth rate was not observed (Fig. 4C). Final tumor weights of harvested tumors also correlated to tumor volume data, where nontreated tumors were on average 4 times heavier than micelle-thiostrepton–treated tumors (Fig. 4D and E).

**Micelle-encapsulated thiostrepton inhibits tumor growth in a HepG2-luc liver cancer subcutaneous xenograft model**

Subcutaneous HepG2-luc liver cancer cells were left to proliferate until the size of the liver cancer xenografts reached 200 mm$^3$, as to examine the effect of thiostrepton treatment on already larger tumors. As in the previous tumor model, the accumulation of micelle-thiostrepton into HepG2-luc xenografts was proven using rhodamine-associated whole body imaging (Supplementary Data S2). Doses were then administered at 30 mg/kg, 3 times a week for 4 weeks and tumor progression was monitored by both caliper measurements and luciferase imaging. Again, the specific dose was chosen after pilot studies suggested it to be the minimum at which an effect can be observed in this model tumor. After completion of the dosing schedule, micelle-thiostrepton–treated tumors were found to be half the volume of the nontreated groups (Fig. 5A). The implanted HepG2-luc tumors also express luciferase proteins, therefore, their change in luciferase expression during the dosing gave insight into tumor cell viability. Compared with that of day 0 (day of beginning of treatment), tumor-associated luciferase in micelle-thiostrepton–treated tumors was much less than that of nontreated tumors (Supplementary Data S3). After the treatment schedule, tumors were also removed and weighed, and treated tumors were found to be half the weight of nontreated tumors (Fig. 5B and C).

Removed tumors from xenograft models were then analyzed for markers of apoptosis by Western blot and immunohistochemistry. Homogenized tumors showed overall an evident increase in the expression of cleaved-caspase-3, a marker of apoptosis (Fig. 6A). Furthermore, for the first time, it is shown that the treatment of *in vivo*
cancers with thiostrepton, or any proteasome inhibitor leads to suppression of the cancer-associated transcription factor, FOXM1. Immunohistochemistry of tumor samples reinforce the effect found in homogenized tumors, where the expression of cleaved-caspase-3 is higher and FOXM1 levels are lower in micelle-treated tumors, compared with nontreated tumors (Fig. 6B).

Discussion

In this article, we aimed to highlight 2 main findings related to thiostrepton-based cancer therapy. First, we showed that the encapsulation of thiostrepton into nanomicelles greatly enhances its solubility and particularly optimizes its tumor-associated biodistributional profile upon in vivo administration. Second, we showed that the treatment of xenograft tumors with nanoparticle-thiostrepton results in reduced tumor growth rate, accompanied by molecular changes such as the suppression of the FOXM1 protein and induction of cell death indicated by caspase-3 cleavage.

Thiostrepton, the FOXM1/proteasome inhibitor is highly insoluble, therefore we used a noncovalent solubilization method, using the polymer-lipid conjugate DSPE-PEG2000-MeO to encapsulate thiostrepton within its lipophilic compartment (12, 23, 27, 35). The essential feature of using the DSPE-PEG2000-MeO PEG-lipid conjugate to solubilize hydrophobic drugs lies in the conjugate’s ability to spontaneously self-assemble into nanomicellar structures upon suspension in aqueous solutions (36, 37). Particles of nanometer dimensions have abilities to selectively target tumors, as opposed to the nonselectivity of nonencapsulated small molecule drugs. Once in circulation, nanoparticles bypass healthy tissue and organs to localize specifically in cancers (and the liver) due to their diffusion through the fenestrations within tumor-associated vasculatures. Blood vessels supplying noncancerous tissues are usually highly regular, whereas those of tumors are irregular and leaky, a result of rapid angiogenesis evolved for tumor growth.

Our final micelle-thiostrepton nanoparticles were found to be around 100 nm in diameter and ~16 mV in zeta potential. These 2 characteristics are advantageous for cancer-specific delivery, as illustrated by the biodistributional and tumor-accumulation data in breast and
liver cancer xenograft models (Fig. 4A and B and Supplementary Fig. S2). Small diameters are required for diffusion through tumor vasculature, and negative surface charges can prolong the circulation times of nanoparticles by preventing their adsorption of Kupffer cell-recognizable protein tags, hence enhancing their accumulation into tumor sites (36, 38). The tumor specificity of nanoparticles also increases the concentration of tumor-localized thiostrepton, where a 6-fold increase in thiostrepton was detected in tumors treated with micelle-encapsulated thiostrepton, compared with those treated with nonmicelle-encapsulated thiostrepton (Fig. 4B). Prolonged retention of nanoparticulate structures in tumors, compared with livers, is likely due to the lack of lymphatic drainage associated with tumors (39–41). It seems that accumulation of nanoparticles into the liver cannot be avoided, as liver-associated vasculature is also leaky in nature and punctuated with fenestrations (33). However, liver-associated accumulation is not an entirely negative consequence, as this nanoparticle characteristic could be exploited for treatment of liver-associated diseases. In response, we had also shown that micelle-thiostrepton nanoparticles were able to accumulate solely in the liver in normal nude mice, when xenograft tumors were absent (Supplementary Data S4). As it has been described that the suppression of FOXM1 in hepatocellular carcinomas leads to reduced cancer growth (42), the liver-targeting effect of our nanomedicine system makes it a promising candidate for treatment of liver cancers.

In terms of anticancer effects, micelle-encapsulated thiostrepton was further found to reduce the growth rates of MDA-MB-231 breast cancer xenografts and HepG2-luc liver cancer xenografts, compared with that of micelle only and nontreated controls (Fig. 4 and Fig. 5). The reduction in tumor growth rate is likely a result of increased cell death in tumor cells, as indicated by the increase in cleaved caspase-3 expression in tumors treated with micelle-thiostrepton (Fig. 6). The change in tumor growth rate after thiostrepton treatment is also associated with the suppression of FOXM1 (Fig. 6), although at present, exact mechanisms are unknown. FOXM1, as an oncogenic transcription factor, has been shown to be suppressed upon treatment with thiostrepton in human cancer cell lines in vitro (1, 2, 5, 43). Here for the first time, we show that thiostrepton inhibits FOXM1 expression in vivo, in xenograft tumors, confirming the notion that FOXM1 is general target of proteasome inhibitors (Fig. 6). The anticancer effect of micelle-thiostrepton nanoparticles, coupled with their liver-targeting effect, advocates the development of micelle-encapsulated thiostrepton as a novel potential nanomedicine treatment for liver cancer patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Micelle-Encapsulated Thiostrepton against Cancer


Molecular Cancer Therapeutics

Micelle-Encapsulated Thiostrepton as an Effective Nanomedicine for Inhibiting Tumor Growth and for Suppressing FOXM1 in Human Xenografts

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