International Journal of Pharmaceutics 420 (2011) 111–117

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Synthesis and evaluation of poly(styrene-*co*-maleic acid) micellar nanocarriers for the delivery of tanespimycin

Nate Larson[a,b], Khaled Greish[a,b,*,1], Hillevi Bauer[a,b], Hiroshi Maeda[d], Hamidreza Ghandehari[a,b,c,**]

[a] Department of Pharmaceutics and Pharmaceutical Chemistry, Salt Lake City, UT 84108, USA
[b] Center for Nanomedicine, Nano Institute of Utah, Salt Lake City, UT 84108, USA
[c] Department of Bioengineering, University of Utah, Salt Lake City, UT 84108, USA
[d] Research Institution of DDS, Sojo University, Kumamoto 860-0082, Japan

ARTICLE INFO

Article history:
Received 9 May 2011
Received in revised form 28 July 2011
Accepted 8 August 2011
Available online 12 August 2011

Keywords:
Tanespimycin
17-AAG
Polymeric micelle
Prostate cancer

ABSTRACT

Polymeric micelles carrying the heat shock protein 90 inhibitor tanespimycin (17-*N*-allylamino-17-demethoxygeldanamycin) were synthesized using poly(styrene-*co*-maleic acid) (SMA) copolymers and evaluated *in vitro* and *in vivo*. SMA-tanespimycin micelles were prepared with a loading efficiency of 93%. The micelles incorporated 25.6% tanespimycin by weight, exhibited a mean diameter of 74 ± 7 nm by dynamic light scattering and a zeta potential of -35 ± 3 mV. Tanespimycin was released from the micelles in a controlled manner *in vitro*, with 62% released in 24 h from a pH 7.4 buffer containing bovine serum albumin. The micellar drug delivery systems for tanespimycin showed potent activity against DU145 human prostate cancer cells, with an IC_{50} of 230 nM. They further exhibited potent anti-cancer activity *in vivo* in nu/nu mice bearing subcutaneous DU145 human prostate cancer tumor xenografts, with significantly higher anticancer efficacy as measured by tumor regression when compared to free tanespimycin at an equivalent single dose of 10 mg/kg. These data suggest further investigation of SMA-tanespimycin as a promising agent in the treatment of prostate cancer.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Heat shock protein 90 (Hsp90) is a 90 kDa chaperone protein that facilitates the cellular response to stress by regulating the folding and activity of many client proteins, which include critical growth-stimulating proteins involved in the malignant transformation of various cancers (Sharp and Workman, 2006). Hsp90 expression is elevated during cellular stress conditions such as heat, pH, and glucose deprivation (Buchner, 1999) and in a variety of cancers including melanoma, leukemia, colon, lung, breast, and prostate (Fukuyo et al., 2010).

Geldanamycin (GDM), a benzoquinone ansamycin derived from *Streptomyces hygrocopicus*, is a naturally occurring inhibitor of Hsp90 and has been studied extensively as an anticancer agent (Porter et al., 2009). GDM binds to the N-terminal ATP-binding site of Hsp90 and induces degradation of its client proteins (Whitesell and Lindquist, 2005). This ability of GDM to alter multiple oncogenic pathways makes GDM an attractive therapeutic compound. However, the clinical use of GDM has been limited by multiple factors. It exhibits high hepatotoxicity at therapeutic doses in animal models (Supko et al., 1994), is poorly soluble in water, and is metabolically unstable (Fukuyo et al., 2010). A GDM derivative 17-*N*-allylamino-17-demethoxygeldanamycin (tanespimycin, 17-AAG) has been widely investigated as an alternative to GDM, and has shown less toxicity and comparable activity compared to GDM in mouse models (Kelland et al., 1999; Burger et al., 2004). This drug was the first-in-class Hsp90 inhibitor to enter clinical trials (Banerji et al., 2005). Although the therapeutic index for tanespimycin is increased as compared to GDM, dose limiting toxicity is still due to hepatic and gastrointestinal symptoms (Sausville et al., 2003). Delivery of tanespimycin is difficult due a poor aqueous solubility of 0.02–0.05 mg/mL (Ge et al., 2006), requiring the use of surfactants such as Cremophor® EL, which are known to induce histamine release, resulting in hypersensitivity reactions and anaphylaxis (Rowinsky and Donehower, 1995), and are further associated with hyperlipidaemia, abnormal lipoprotein patters, aggregation of erythrocytes, and peripheral neuropathy (Gelderblom et al., 2001).

* Corresponding author at: Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 383 Colorow Road, Room 339, Salt Lake City, UT 84108, USA. Tel.: +1 801 587 1558.
** Corresponding author at: Department of Pharmaceutics and Pharmaceutical Chemistry and Bioengineering, University of Utah, 383 Colorow Road, Room 343, Salt Lake City, UT 84108, USA. Tel.: +1 801 587 1566.
E-mail addresses: khaled.gerish@utah.edu, khaled.gerish@otago.ac.nz (K. Greish), hamid.ghandehari@pharm.utah.edu (H. Ghandehari).
[1] *Present address*: Department of Pharmacology & Toxicology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand,
Tel.: + 64 3 479 4095; + 64 21 310 335.

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.08.011

Polymeric carriers can increase the solubility of poorly water soluble drugs and can accumulate in tumor tissues via the "enhanced permeability and retention" (EPR) effect (Matsumura et al., 1987; Greish et al., 2003), thereby increasing the therapeutic index for a given chemotherapeutic agent. Polymeric micelles are characterized by a hydrophilic shell which interacts with an external aqueous environment and a hydrophobic core which acts as a depot for hydrophobic drugs. Polymeric micelles were first reported as potential carriers for use in cancer treatment in the early 1980s (Gros et al., 1981) and the field has matured to include a number of candidates currently under clinical investigation (Blanco et al., 2009).

The use of poly(styrene-*co*-maleic acid) (SMA) micelles as drug carriers is currently under investigation (Iyer et al., 2007; Daruwalla et al., 2010). SMA has been proven to be biologically safe and is used clinically in SMANCS, a conjugate of half-butyl SMA bound to the antitumor protein neocarzinostatin (Maeda, 2001). Previous studies have demonstrated immunopotentiating activity associated with SMA moieties, in contrast to immunosuppression that is typically induced by conventional chemotherapeutics (Suzuki et al., 1988, 1990). The styrenic core of SMA micelles has been characterized by a high glass transition temperature (Rodriguez et al., 2008) and a large microviscosity (Claracq et al., 2002), which may help facilitate higher stability and more controlled release rates of drugs from the micelle core. In addition, the hydrophilic surface of SMA micelles is comprised of carboxyl terminated maleic acid groups, allowing easy surface modification or conjugation with targeting moieties.

Previous work by our group (Borgman et al., 2009; Larson et al., 2010) and others (Kasuya et al., 2002) has described the use of HPMA copolymers as drug carriers for geldanamycin derivatives. In these systems, geldanamycin derivatives are covalently bound to the polymer backbone via the lysosomally degradable Gly-Phe-Leu-Gly linker (Subr et al., 1988), resulting in highly stable conjugates with drug release occurring via lysosomal degradation following endocytosis. The use of such systems however requires chemical modification of geldanamycin to facilitate conjugation, and such modifications result in a decrease in the activity of geldanamycin. Self-assembled drug delivery systems do not suffer from this limitation as the drug is most often bound to the carrier through non-covalent hydrophobic interactions.

In the present study, SMA was used to prepare polymeric micelles containing the Hsp90 inhibitor tanespimycin. The micelles were characterized for drug loading efficiency, drug content, size, and zeta potential. The release rate of tanespimycin from the micelles and the ability of the micelles to inhibit the growth of DU145 human prostate cancer cells *in vitro* were evaluated. An *in vivo* preliminary single dose study evaluating the efficacy of the micelles was performed in nu/nu mice bearing DU145 human prostate cancer xenografts.

2. Materials and methods

2.1. Materials

Geldanamycin (NSC 122750) was kindly supplied by the National Cancer Institute Developmental Therapeutics Program (NCI DTP). Allylamine was supplied by Alfa Aesar (Ward Hill, MA, USA). Cumene terminated poly(styrene-*co*-maleic anhydride) was obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA) and supplied with a 1.3:1 mole ratio of styrene:maleic anhydride, an average M_n of approximately 1600 as determined by GPC, and an acid number of 465–495 mg KOH/g. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) was obtained from Sigma–Aldrich Corp. Bovine serum albumin (BSA) fraction V was obtained from MP Biomedicals (Solon, OH, USA). Polyoxyl castor oil (Cremophor® EL) was obtained from BASF Corp. (Florham Park, NJ, USA). Poly(ethylene glycol) 400 was obtained from Dow Chemical Corp. (Petaluma, CA, USA).

2.2. Cell lines and culture

The human prostate cancer cell line DU145 (ATCC, Rockville, MD, USA) was maintained in Eagle's minimum essential medium (ATCC) supplemented with 10% heat inactivated fetal bovine serum. Cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For all procedures, cells were harvested using TrypLE™ Express (Invitrogen, Carlsbad, CA) and cell lines were maintained in a logarithmic growth phase during all studies.

2.3. Synthesis of tanespimycin

200 mg (0.357 mmol) of GDM was dissolved in 10 mL anhydrous dimethylformamide (DMF) at ambient temperature. 80.1 μL (1.07 mmol) of allylamine was added and the solution was kept under nitrogen, protected from exposure to light, and allowed to stir overnight at ambient temperature. The color of the solution changed from bright yellow-orange to dark purple and completion of the reaction was monitored by TLC on silica gel with chloroform:MeOH [9:1] as mobile phase by the disappearance of GDM. DMF was removed by rotary evaporator, and the resulting crude product was recrystallized from H_2O:EtOH [4:1]. The precipitate was analyzed by electrospray ionization mass spectrometry (ESI-MS).

2.4. Preparation of SMA-tanespimycin

Preparation of SMA micelles was similar to the method previously reported with modifications (Greish et al., 2004). First, poly(styrene-*co*-maleic anhydride) was hydrolyzed under aqueous alkaline conditions. Deionized water was adjusted to pH 14 with 4 N NaOH and heated to 70 °C. Poly(styrene-*co*-maleic anhydride) was added under stirring and the solution was maintained at pH 14 and 70 °C. The resulting hydrolyzed SMA solution was adjusted to pH 7.0 with 1 N HCl, diluted to a final concentration of 50 mg/mL, and allowed to cool to ambient temperature. 12 mL (600 mg SMA) was removed and diluted to approximately 60 mL with deionized water. 200 mg of tanespimycin was dissolved in minimal DMSO, and added drop wise while stirring, resulting in a cloudy solution. The solution was then adjusted to a pH of 5.0 and 600 mg EDAC in 5 mL deionized water was added drop wise at pH 5.0 and allowed to stir for 30 min. Next, the solution was adjusted to pH 10.5 by the addition of 1 N NaOH, the pH was subsequently adjusted to 7.0 with 1 N HCl, and the resulting solution was filtered to remove undissolved tanespimycin. 1 N HCl was then added dropwise to the filtrate to precipitate the micelles. The precipitated micelles were then centrifuged and purified by washing repeatedly with cold 0.01 N HCl in deionized water. Residual water/HCl was removed by lyophilization to obtain the final SMA-tanespimycin product.

2.5. Loading efficiency of SMA-tanespimycin

For the purpose of this study, loading efficiency is defined as the total weight of the drug in the final SMA-tanespimycin product divided by the initial weight of the drug introduced for micellar preparation. A standard curve was prepared by serial dilution of tanespimycin in DMSO and quantification of the drug was by UV spectrometry at 335 nm. Loading efficiency and drug content were then obtained by dissolving SMA-tanespimycin in DMSO and measuring absorbance at 335 nm in comparison with the standard curve.

To ensure that the drug was unmodified during micelle preparation, SMA-tanespimycin was further analyzed by reversed phase high-performance liquid chromatography (RP-HPLC). Analysis was performed with an Agilent 1100 LC system equipped with an Alltech Alltima C18 5 μm 150 × 4.6 mm column and a photo diode array detector scanning at 200–500 nm. The mobile phase consisted of deionized water and acetonitrile (ACN), at the following gradient: analysis time 0 min, 35% ACN; 15 min, 65% ACN; 25 min, 75% ACN; 30 min 95% ACN; 39 min, 100% ACN; 40 min, 65% ACN. A post time of 5 min was used to allow column equilibration between samples. The flow rate was maintained at 1.0 mL/min throughout and the sample injection volume was 20 μL. Samples of tanespimycin and SMA-tanespimycin were prepared in deionized water:ACN [65:35] and injected for analysis. The λ_{max} of tanespimycin at 335 nm was used for final quantitative analysis.

2.6. Size and zeta potential of SMA-tanespimycin

SMA-tanespimycin was prepared in 50 mM sodium phosphate buffer pH 7.4 at a concentration of 1.0 mg/mL for analysis. All measurements were performed at 25 °C. A Malvern Zeta Sizer ZEN3600 (Malvern Instruments Inc., Westborough, MA) was used to determine mean *Z*-average size, size distribution and zeta-potential. All measurements were performed on three separately prepared samples.

2.7. Drug release from SMA-tanespimycin

The release of tanespimycin from the micellar preparations was evaluated using a dialysis method and compared to the release in a standard vehicle formulation of EtOH:Cremophor® EL:PEG 400 [2:1:1] (EtOH:CrEL:PEG) (Zhong and Licari, 2005). SMA-tanespimycin was prepared at a concentration of 2.5 mg/mL (0.625 mg/mL tanespimycin) and drug-EtOH:CrEL:PEG was prepared by dissolving tanespimycin in EtOH:CrEL:PEG followed by a 10× dilution to yield a final concentration of 0.625 mg/mL. Samples were prepared in a 50 mM sodium phosphate buffer pH 7.4 or phosphate buffered saline (PBS) pH 7.4 with 40 mg/mL BSA. 4 mL of each sample was placed in a dialysis tube with a molecular weight cutoff of 3500 Da and dialyzed against 5 L of either 50 mM sodium phosphate buffer pH 7.4 or PBS pH 7.4. Media outside the dialysis membrane was changed periodically to ensure a constant sink condition. At each predetermined time point, 200 μL of the sample inside the dialysis membrane was removed and analyzed spectrophotometrically at 335 nm. Quantification of percent release was performed by comparison of sample absorbance with calibration curves prepared for SMA-tanespimycin and tanespimycin-EtOH-CrEL:PEG for each test condition. All experiments were performed in triplicate. Percent release is reported as mean ± standard deviation.

2.8. In vitro growth inhibition against human prostate cancer cells

The ability of the SMA-tanespimycin to inhibit the growth of DU145 human prostate cancer cells was evaluated using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt (WST-8) cell viability assay. 3000 DU145 cells per well were plated in 96-well plates for 24 h. Cell culture medium was then removed and cells were treated with SMA-tanespimycin, tanespimycin dissolved in EtOH:CrEL:PEG, or controls for 72 h. Following treatment, medium was removed and wells were washed with 200 μL PBS. 100 μL of 10% (v/v) WST-8 reagent in complete growth medium was added to each well, cells were incubated at 37 °C/5% CO_2 for 120 min and absorbance at 450 nm minus 630 nm was determined by UV spectrophotometry. Relative viability was calculated by normalization of the absorbance of untreated cells. All experiments were performed in triplicate, with $n = 3$ wells per replicate. Non-linear least-squares regression analysis and calculation of IC_{50} was performed using GraphPad Prism.

2.9. In vivo efficacy

Six-week-old athymic (*nu*/*nu*) mice were obtained from Charles River Laboratories (Davis, CA, USA) and used in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. Mice were anesthetized using 4% isoflurane mixed with oxygen followed by subcutaneous injection of 1×10^7 DU145 cells per flank ($n = 5$ mice per treatment group). When the mean tumor size had reached approximately 50 mm^3 (about 10 days after tumor inoculation), the mice were treated with a single injection of either saline (control), free tanespimycin dissolved in DMSO, or SMA-tanespimycin at a dose of 10 mg/kg drug equivalent. The animals were routinely monitored and tumor growth was measured twice weekly and tumor volume was calculated as length × width^2 × π/6. Tumor volumes at each time point were normalized by the initial tumor volume and are reported as mean ± standard error of the mean. Animal weights were also measured at each time point and normalized to initial weight reported as mean ± standard deviation.

2.10. Statistical analysis

For release studies, tumor regression, and animal weight data, differences between data sets were determined by two-way repeated measures ANOVA using GraphPad Prism. Where differences were detected, a Bonferroni post-test was used to test for significance between groups. The significance level was set to $\alpha = 0.05$ for all statistical tests.

3. Results and discussion

3.1. Synthesis and preparation of SMA-tanespimycin

The development of polymeric micelles in drug delivery has primarily focused on the use of amphiphilic block copolymers with poly(ethylene glycol) (PEG) as the hydrophilic segment and a polyester or a poly(amino acid) derivative as the hydrophobic segment (Croy and Kwon, 2006). The loading of hydrophobic drugs and the assembly of such copolymers into micellar structures is commonly performed using methods such as: (1) basic equilibration, (2) dialysis, (3) oil/water emulsion, (4) solution casting, or (5) freeze drying (Gaucher et al., 2005). The present study describes polymeric micelles prepared by varying the pH of an aqueous solution containing poly(styrene-*co*-maleic acid) copolymers and tanespimycin, as a hydrophobic drug.

Tanespimycin was synthesized from GDM, and the resulting purple solid was collected and identified as tanespimycin by ESI-MS. SMA-tanespimycin was prepared by varying the pH of an aqueous solution of hydrolyzed SMA (Fig. 1).

3.2. Characterization of SMA-tanespimycin

The preparation of polymeric micelles as drug carriers often employ methods that result in either low loading efficiency or low drug loading capacity (Park, 2007). Using the aforementioned process, hydrolyzed SMA incorporated tanespimycin with a loading efficiency of 93% (Table 1), a significant improvement over previous studies describing polymeric micellar formulations of tanespimycin (Xiong et al., 2009; Shin et al., 2009).

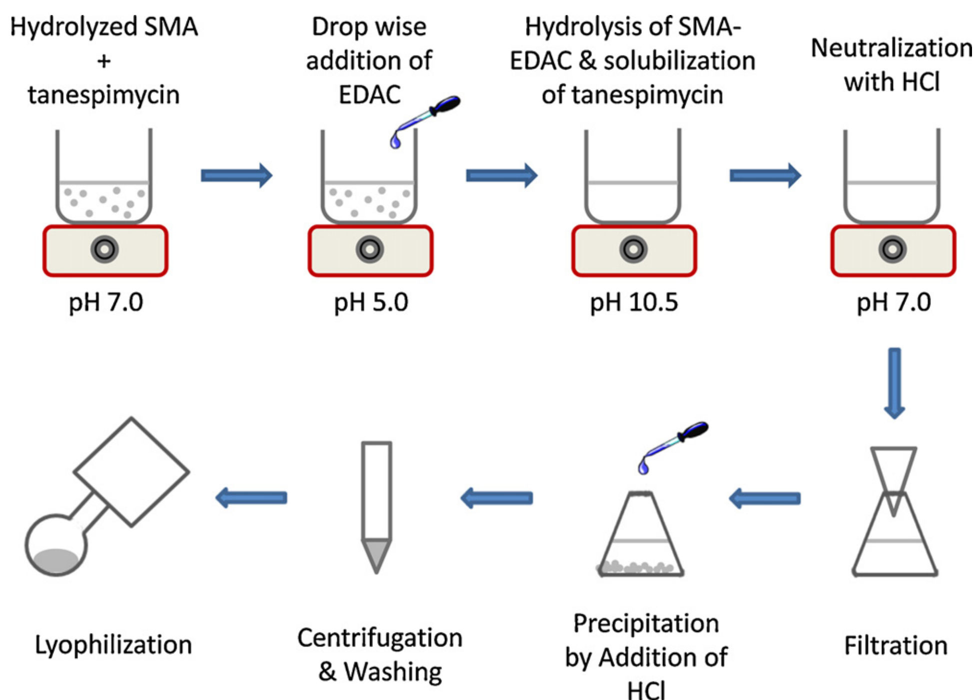

Fig. 1. Preparation of SMA-tanespimycin micelles. SMA-tanespimycin micelles were prepared by first suspending tanespimycin in an aqueous solution of hydrolyzed SMA containing EDAC. The solution was then adjusted to alkaline conditions which resulted in encapsulation of tanespimycin by SMA micelles. The resulting solution was neutralized and SMA-tanespimycin micelles isolated.

The ability of SMA based micelles to achieve high drug loading has previously been demonstrated for the hydrophobic drugs zinc protoporphyrin (Iyer et al., 2007) and doxorubicin (Greish et al., 2004). In the current study, a high drug loading of 25.6% tanespimycin by weight was observed for SMA-tanespimycin as determined by UV spectrophotometry (Table 1). The micelles were highly soluble, with a drug equivalent aqueous solubility of >5.0 mg/mL as measured in pH 7.4 PBS buffer, whereas free tanespimycin was soluble only at 0.021 mg/mL. To ensure that tanespimycin remained unmodified during micelle preparation, SMA-tanespimycin in comparison with free tanespimycin was analyzed by RP-HPLC. Both tanespimycin and SMA-tanespimycin showed a prominent peak at 15.2 min, and both peaks exhibited UV spectra characteristic of tanespimycin (data not shown). Drug loading of tanespimycin as determined by RP-HPLC was 25.2% by weight and in agreement with drug loading determined by UV spectrophotometry.

Size is a critical parameter for macromolecular drug delivery systems designed to escape renal filtration. It has been proposed that carriers with sizes greater than 10 nm accumulate in the tumor tissues via the EPR effect. SMA-tanespimycin micelles had a mean diameter of 74 ± 7 nm and a polydispersity index of 0.31 ± 0.08 as measured by dynamic light scattering (Fig. 2). The micelles had a zeta potential of -35 ± 3 mV as measured in a 50 mM phosphate buffer at pH 7.4. SMA micelles were characterized by carboxyl terminated maleic acid surface groups which impart a negative charge to the micelles.

The high loading efficiency and possibility to tune the loading ratio of tanespimycin into SMA micelles represents significant advantages for industrial scale up, in contrast to many other micellar systems.

Table 1
Characteristics of SMA-tanespimycin micelles.

Property	Mean	SD
Amount of tanespimycin introduced for micelle preparation (mg)	150.0	–
Drug loading efficiency (%)[a]	93.1	–
Drug loading (% wt/wt)	25.6	–
Mean micelle diameter (nm)[b,c]	74	7
Polydispersity index	0.31	0.08
Zeta potential[c] (mV)	−35	3

[a] Drug loading efficiency calculated as mg tanespimycin solubilized by SMA micelles/mg tanespimycin introduced for micelle preparation.
[b] Z-Average size as measured by dynamic light scattering (DLS).
[c] As measured in 50 mM phosphate buffer at pH 7.4.

3.3. Release of tanespimycin from SMA-tanespimycin micelles

The incorporation of tanespimycin into SMA micelles can increase tumor uptake and alter biodistribution, resulting in an increase in the therapeutic index of the drug. For this to occur, it is essential that tanespimycin is retained by the carrier for a period of time to allow drug accumulation in tumor tissue via

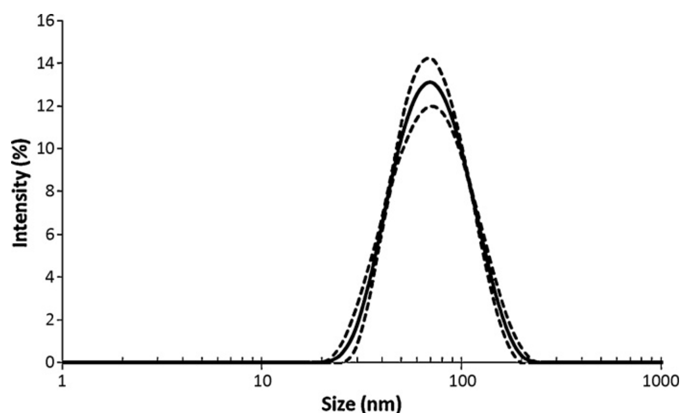

Fig. 2. Size distribution of SMA-tanespimycin micelles. The size distribution of SMA-tanespimycin micelles was determined using a Malvern Zeta Sizer at a concentration of 1.0 mg/mL at pH 7.4. Data are expressed as mean (solid line) ± SD (dashed line) (N = 3).

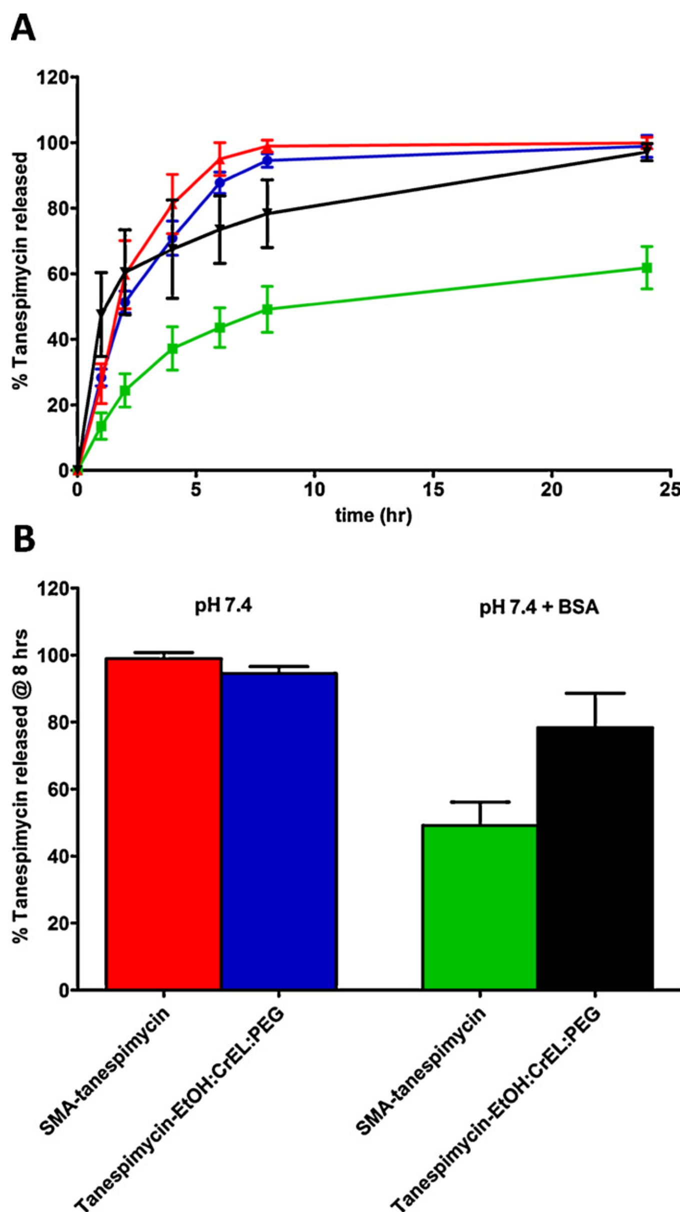

Fig. 3. Release of tanespimycin from SMA-tanespimycin. Release of tanespimycin from SMA micelles was evaluated using a dialysis method and compared to tanespimycin formulated in EtOH:CrEL:PEG as control. (A) Release of tanespimycin from SMA-tanespimycin (red) and tanespimycin-EtOH:CrEL:PEG (blue) in a pH 7.4 buffer and release of tanespimycin from SMA-tanespimycin (green) and tanespimycin-EtOH:CrEL:PEG (black) in a pH 7.4 buffer containing 40 mg/mL BSA. (B) Release of tanespimycin at 8 h. Data expressed as mean ± SD ($N = 3$). *Difference detected at $p < 0.05$ significance level. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

the EPR effect. However, release from the carrier is also essential to allow tanespimycin to elicit its pharmacologic effect. Release rate is therefore a critical parameter in anticancer macromolecular drug delivery, and an ideal anticancer carrier should be able to retain its cargo for approximately 6 h while tumor accumulation occurs (Greish, 2007), followed by complete drug release. To address this issue, the *in vitro* release rate of tanespimycin from SMA-tanespimycin micelles was evaluated using a dialysis method and compared to the release of tanespimycin formulated in EtOH:CrEL:PEG. Release was assessed in a pH 7.4 buffer where 51% and 95% tanespimycin was released from SMA-tanespimycin micelles in 2 and 8 h, respectively. Release from tanespimycin formulated in EtOH:CrEL:PEG was not significantly different, with 60% and 99% released in 2 and 8 h, respectively. To better ascertain release of tanespimycin from the blood plasma compartment *in vivo*, a second test condition was evaluated wherein BSA was included inside the dialysis bag at a physiologically relevant concentration of 40 mg/mL. Release of tanespimycin from SMA-tanespimycin micelles in the presence of BSA was reduced at all time points greater that 1 h ($p < 0.001$) as compared to release from pH 7.4 buffer alone (Fig. 3). Release of tanespimycin from SMA-tanespimycin micelles was also significantly reduced as compared to tanespimycin formulated in EtOH:CrEL:PEG at all time points greater than 1 h ($p < 0.001$), with only 62% release observed in 24 h (Fig. 3). This reduction in release rate can be explained by the well characterized noncovalent binding of SMA to serum albumin (Kobayashi et al., 1988), and it is thus anticipated that serum albumin can serve as a secondary carrier for SMA-tanespimycin micelles *in vivo*.

3.4. Cytotoxicity of SMA-tanespimycin micelles in vitro

The ability of SMA-tanespimycin micelles to inhibit the growth of DU145 human prostate cancer cells was evaluated *in vitro* using a WST-8 cell viability assay. Incorporation of tanespimycin into SMA-tanespimycin micelles resulted in a decrease in its ability to inhibit the growth of DU145 cells with an IC_{50} of 230 ± 10 nM as compared to an IC_{50} of 15.0 ± 0.3 nM for tanespimycin solubilized in EtOH:CrEL:PEG (Fig. 4). For controls, SMA and EtOH:CrEL:PEG solutions were subjected to the same dilution protocol and evaluated for growth inhibition ability and showed no cytotoxicity over an equivalent concentration range. During the 72 h incubation time of these studies, the release of tanespimycin from SMA-tanespimycin micelles was most likely minimized because of the lack of a perpetual sink condition. Although SMA-tanespimycin micelles were less toxic as compared to free tanespimycin, they remained cytotoxic in the nanomolar concentration range. This reduction in cytotoxicity of SMA-tanespimycin micelles as compared to free tanespimycin can be explained by a number of possible factors. The micelles first need to be taken up by cells via endocytosis, whereas tanespimycin can passively diffuse across cellular membranes. Uptake of the micelles could further be reduced due to the interactions of the negatively charged carboxylic acid rich surface of the micelles with negatively charged membranes. Tanespimycin must also be released from the micelle core in order for it to elicit its pharmacological effect, which occurs over a prolonged time interval, further reducing overall cytotoxic exposure. The reduced toxicity of SMA-tanespimycin micelles can potentially be advantageous in an *in vivo* scenario by minimizing systemic exposure of tanespimycin while allowing time for accumulation in tumor tissues to occur.

3.5. In vivo efficacy of SMA-tanespimycin micelles

The delivery of tanespimycin via SMA-tanespimycin micelles results in an increase in the therapeutic index for tanespimycin. Toward this aim, a preliminary *in vivo* efficacy study was performed in athymic nu/nu mice bearing subcutaneous DU145 human prostate cancer xenografts. As accumulation in tumor tissues via the EPR effect was anticipated for SMA-tanespimycin micelles, a subtherapeutic dose of 10 mg/kg tanespimycin equivalent was used. A single dose of SMA-tanespimycin micelles, free tanespimycin, or saline were injected via tail-vein injection and animals were monitored twice weekly for changes in tumor volume as an indicator of efficacy (Fig. 5). Body weight was also recorded as an indicator of general toxicity (Fig. 5). At a single dose of 10 mg/kg tanespimycin equivalent, SMA-tanepsimycin micelles resulted in a reduction in normalized mean tumor volume that was maintained throughout the duration of the study (23 days), whereas

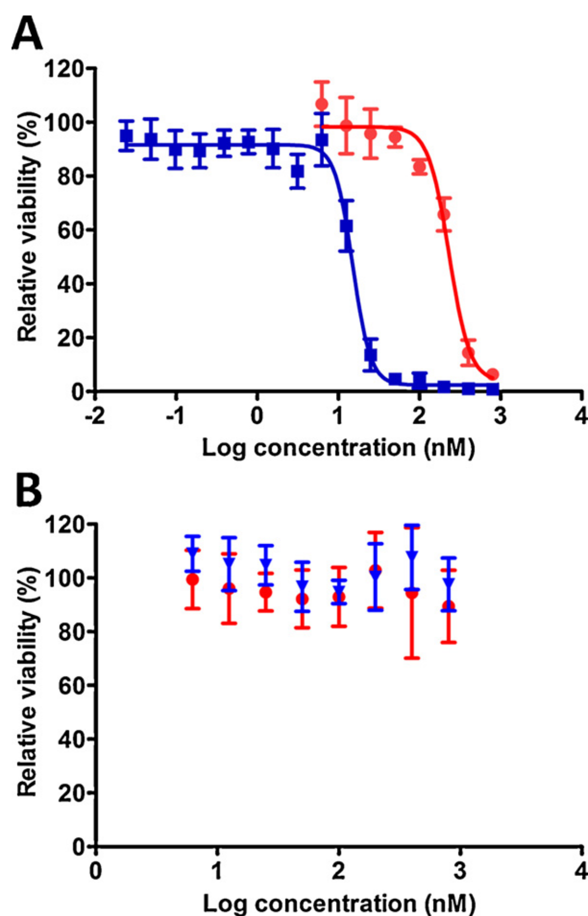

Fig. 4. Cell growth inhibition of SMA-tanespimycin micelles. DU145 human prostate cancer cells were treated for 72 h with increasing drug equivalent concentrations of tanespimycin formulated in EtOH:CrEL:PEG (blue), SMA-tanespimycin micelles (red) (A), EtOH:CreEL:PEG vehicle alone (blue) or hydrolyzed SMA (red) (B). Following treatment, cell viability was assessed by WST-8 assay. Non-linear regression and IC_{50} values were determined by analysis using GraphPad Prism. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

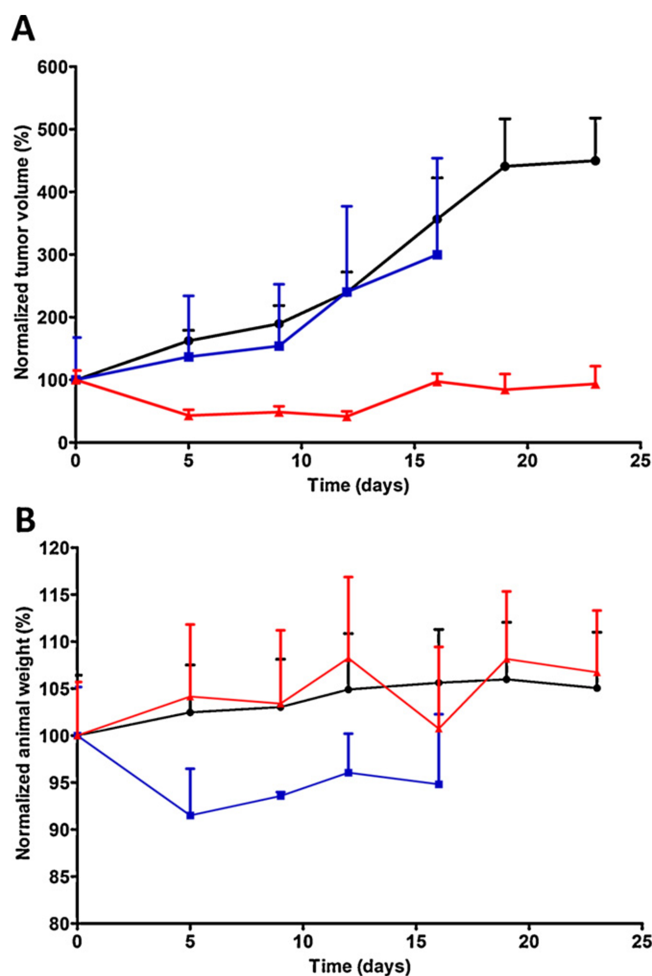

Fig. 5. *In vivo* efficacy of SMA-tanespimycin micelles. Athymic nu/nu mice bearing DU145 tumor xenografts were treated IV with a single dose of 10 mg/kg tanespimycin (blue) or 10 mg/kg tanespimycin equivalent SMA-tanespimycin micelles (red) and compared with a saline injection control group (black). (A) Normalized tumor volume was measured as a function of time for each treatment group. (B) Normalized animal weight was assessed as a measure of general toxicity. Data are expressed as mean ± SEM ($N = 5$ per treatment group). *Difference detected at $p < 0.05$ significance level. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

normalized mean tumor volume in saline injected animals increased 450%. Relative to saline injected animals, SMA-tanespimycin resulted in a statistically significant decrease in normalized mean tumor volume at all time points following treatment ($p < 0.05$). A single dose of free tanespimycin showed no evidence of efficacy at 10 mg/kg, and normalized mean tumor volume was not different from saline injected animals at all time points. Animals in the free tanespimycin group were euthanized on day 16 as one animal showed excessive tumor burden. SMA-tanespimycin micelles in comparison with free tanespimycin showed a significant reduction in normalized mean tumor volume on day 16 ($p < 0.05$). These results coupled with the *in vitro* cytotoxicity results suggest that SMA-tanespimycin micelles have the ability to accumulate in tumor tissues and inhibit tumor growth *in vivo*.

Animal weights were measured twice a week as an indicator of general toxicity during the efficacy study. Relative to saline injected animals, animals treated with SMA-tanespimycin micelles showed no difference in normalized mean animal weight during the study. An 11% reduction in normalized mean animal weight was observed for free tanespimycin as compared to saline injected animals at day 5 ($p < 0.05$); differences detected at subsequent time points were not statistically significant. However, it is unclear whether the solvent used to solubilize tanespimycin has contributed to the overt tanespimycin toxicity in this study, or the manifested toxicity was totally due to the free drug. These results demonstrate that SMA-tanespimycin micelles were well tolerated.

This efficacy study was preliminary in its nature as parameters such as maximum tolerated dose and dosing schedule were not optimized. However, the results of the study demonstrate that SMA-tanespimycin micelles were well tolerated and effective in reducing DU145 tumor growth, and suggest that the therapeutic index of tanespimycin is increased by encapsulation in SMA micelles.

Overall, the data from this study demonstrates that delivery of tanespimycin using SMA micelles has several distinct advantages. First, the aqueous solubility of tanespimycin is increased by encapsulation into SMA micelles, and because these interactions are non-covalent, the potent activity of tanespimycin is maintained. Second, the preparation of SMA-tanespimycin micelles is simple, straightforward, and efficient in loading tanespimycin, thus allowing cost efficient scale up at later stages of development. Third, the macromolecular nature of SMA-tanespimycin micelles allows accumulation into tumor tissues, resulting in an increase in the therapeutic index of tanespimycin. These features combine to suggest that SMA-tanespimycin micelles have the potential to increase the efficacy and safety profile of tanespimycin.

4. Conclusion

SMA-tanespimycin micelles were prepared in a simple manner with a high loading drug efficiency of 93%. The micelles incorporated 25.6% tanespimycin by weight and exhibited properties that allow for increased blood circulation and tumor accumulation *in vivo*. Tanespimycin was released from the micelles in a controlled manner and showed potent activity against DU145 human prostate cancer cells *in vitro*. The micelles also were well tolerated and exhibited potent anti-cancer activity in nu/nu mice bearing subcutaneous DU145 human prostate cancer tumor xenografts, with a significant increase in efficacy as measured by tumor regression as compared to free tanespimycin at an equivalent single dose of 10 mg/kg. These data suggest that the therapeutic index of tanespimycin is increased by incorporation into SMA micelles.

Acknowledgements

This research was supported by the National Institutes of Health grant R01 EB007171 and the Utah Science Technology and Research (USTAR) initiative.

References

Banerji, U., O'donnell, A., Scurr, M., Pacey, S., Stapleton, S., Asad, Y., Simmons, L., Maloney, A., Raynaud, F., Campbell, M., Walton, M., Lakhani, S., Kaye, S., Workman, P., Judson, I., 2005. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J. Clin. Oncol. 23, 4152–4161.

Blanco, E., Kessinger, C.W., Sumer, B.D., Gao, J., 2009. Multifunctional micellar nanomedicine for cancer therapy. Exp. Biol. Med. 234, 123–131.

Borgman, M.P., Aras, O., Geyser-Stoops, S., Sausville, E.A., Ghandehari, H., 2009. Biodistribution of HPMA copolymer-aminohexylgeldanamycin-RGDfK conjugates for prostate cancer drug delivery. Mol. Pharm. 6, 1836–1847.

Buchner, J., 1999. Hsp90 & Co.—a holding for folding. Trends Biochem. Sci. 24, 136–141.

Burger, A.M., Fiebig, H.H., Stinson, S.F., Sausville, E.A., 2004. 17-(Allylamino)-17-demethoxygeldanamycin activity in human melanoma models. Anticancer Drugs 15, 377–387.

Claracq, J., Santos, S.F.C.R., Duhamel, J., Dumousseaux, C., Corpart, J.-M., 2002. Rigid interior of styrene–maleic anhydride copolymer aggregates probed by fluorescence spectroscopy. Langmuir 18, 3829–3835.

Croy, S.R., Kwon, G.S., 2006. Polymeric micelles for drug delivery. Curr. Pharm. Des. 12, 4669–4684.

Daruwalla, J., Nikfarjam, M., Greish, K., Malcontenti-Wilson, C., Muralidharan, V., Christophi, C., Maeda, H., 2010. In vitro and in vivo evaluation of tumor targeting styrene–maleic acid copolymer-pirarubicin micelles: survival improvement and inhibition of liver metastases. Cancer Sci. 101, 1866–1874.

Fukuyo, Y., Hunt, C.R., Horikoshi, N., 2010. Geldanamycin and its anti-cancer activities. Cancer Lett. 290, 24–35.

Gaucher, G., Dufresne, M.H., Sant, V.P., Kang, N., Maysinger, D., Leroux, J.C., 2005. Block copolymer micelles: preparation, characterization and application in drug delivery. J. Control. Release 109, 169–188.

Ge, J., Normant, E., Porter, J.R., Ali, J.A., Dembski, M.S., Gao, Y., Georges, A.T., Grenier, L., Pak, R.H., Patterson, J., Sydor, J.R., Tibbitts, T.T., Tong, J.K., Adams, J., Palombella, V.J., 2006. Design, synthesis, and biological evaluation of hydroquinone derivatives of 17-amino-17-demethoxygeldanamycin as potent, water-soluble inhibitors of Hsp90. J. Med. Chem. 49, 4606–4615.

Gelderblom, H., Verweij, J., Nooter, K., Sparreboom, A., 2001. Cremophor® EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur. J. Cancer 37, 1590–1598.

Greish, K., 2007. Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines. J. Drug Target 15, 457–464.

Greish, K., Fang, J., Inutsuka, T., Nagamitsu, A., Maeda, H., 2003. Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting. Clin. Pharmacokinet. 42, 1089–1105.

Greish, K., Sawa, T., Fang, J., Akaike, T., Maeda, H., 2004. SMA-doxorubicin, a new polymeric micellar drug for effective targeting to solid tumours. J. Control. Release 97, 219–230.

Gros, L., Ringsdorf, H., Schupp, H., 1981. Polymeric antitumor agents on a molecular and on a cellular level? Angew. Chem. Int. Ed. Engl. 20, 305–325.

Iyer, A.K., Greish, K., Fang, J., Murakami, R., Maeda, H., 2007. High-loading nanosized micelles of copoly(styrene-maleic acid)-zinc protoporphyrin for targeted delivery of a potent heme oxygenase inhibitor. Biomaterials 28, 1871–1881.

Kasuya, Y., Lu, Z.R., Kopeckova, P., Tabibi, S.E., Kopecek, J., 2002. Influence of the structure of drug moieties on the in vitro efficacy of HPMA copolymer-geldanamycin derivative conjugates. Pharm. Res. 19, 115–123.

Kelland, L.R., Sharp, S.Y., Rogers, P.M., Myers, T.G., Workman, P., 1999. DT-diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. J. Natl. Cancer Inst. 91, 1940–1949.

Kobayashi, A., Oda, T., Maeda, H., 1988. Protein binding of macromolecular anticancer agent SMANCS: characterization of poly(styrene-co-maleic acid) derivatives as an albumin binding ligand. J. Bioact. Compat. Polym. 3, 319–333.

Larson, N., Ray, A., Malugin, A., Pike, D.B., Ghandehari, H., 2010. HPMA copolymer-aminohexylgeldanamycin conjugates targeting cell surface expressed GRP78 in prostate cancer. Pharm. Res. 27, 2683–2693.

Maeda, H., 2001. SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv. Drug Deliv. Rev. 46, 169–185.

Matsumura, Y., Oda, T., Maeda, H., 1987. General mechanism of intratumor accumulation of macromolecules: advantage of macromolecular therapeutics. Gan To Kagaku Ryoho 14, 821–829.

Park, K., 2007. Nanotechnology: what it can do for drug delivery. J. Control. Release 120, 1–3.

Porter, J.R., Ge, J., Lee, J., Normant, E., West, K., 2009. Ansamycin inhibitors of Hsp90: nature's prototype for anti-chaperone therapy. Curr. Top. Med. Chem. 9, 1386–1418.

Rodriguez, V.B., Henry, S.M., Hoffman, A.S., Stayton, P.S., Li, X., Pun, S.H., 2008. Encapsulation and stabilization of indocyanine green within poly(styrene-alt-maleic anhydride) block-poly(styrene) micelles for near-infrared imaging. J. Biomed. Opt. 13, 014025.

Rowinsky, E.K., Donehower, R.C., 1995. Paclitaxel (taxol). N. Engl. J. Med. 332, 1004–1014.

Sausville, E.A., Tomaszewski, J.E., Ivy, P., 2003. Clinical development of 17-allylamino, 17-demethoxygeldanamycin. Curr. Cancer Drug Targets 3, 377–383.

Sharp, S., Workman, P., 2006. Inhibitors of the HSP90 molecular chaperone: current status. Adv. Cancer Res. 95, 323–348.

Shin, H.-C., Alani, A.W.G., Rao, D.A., Rockich, N.C., Kwon, G.S., 2009. Multi-drug loaded polymeric micelles for simultaneous delivery of poorly soluble anticancer drugs. J. Control. Release 140, 294–300.

Subr, V., Kopecek, J., Pohl, J., Baudys, M., Kostka, V., 1988. Cleavage of oligopeptide side-chains in N-(2-hydroxypropyl)methacrylamide copolymers by mixtures of lysosomal enzymes. J. Control. Release 8, 133–140.

Supko, J.G., Hickman, R.L., Grever, M.R., Malspeis, L., 1994. Preclinical pharmacologic evaulation of geldanamycin as an antitumor agent. Cancer Chemother. Pharmacol. 36, 305–315.

Suzuki, F., Munakata, T., Maeda, H., 1988. Interferon induction by SMANCS: a polymer-conjugated derivative of neocarzinostatin. Anticancer Res. 8, 97–103.

Suzuki, F., Pollard, R.B., Uchimura, S., Munakata, T., Maeda, H., 1990. Role of natural killer cells and macrophages in the nonspecific resistance to tumors in mice stimulated with SMANCS, a polymer-conjugated derivative of neocarzinostatin. Cancer Res. 50, 3897–3904.

Whitesell, L., Lindquist, S.L., 2005. HSP90 and the chaperoning of cancer. Nat. Rev. Cancer 5, 761–772.

Xiong, M.P., Yáñez, J.A., Kwon, G.S., Davies, N.M., Forrest, M.L., 2009. A cremophor-free formulation for tanespimycin (17-AAG) using PEO-*b*-PDLLA micelles: characterization and pharmacokinetics in rats. J. Pharm. Sci. 98, 1577–1586.

Zhong, Z., Licari, P.J., 2005. Pharmaceutical solution formulations containing 17-AAG. US Patent Application, 20,050,256,097.