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Preparation, *In Vivo* Administration, Dose-Limiting Toxicities, and Antineoplastic Activity of Cytochalasin B

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Abstract

An effective and inexpensive protocol for producing cytochalasins A and B is being disclosed to propose a viable method by which to examine the in vivo antineoplastic activity of these congeners in preclinical tumor-bearing mammalian models. In addition, we determine the maximum tolerated doses of cytochalasin B using multiple routes and formulations, characterize the tissue distribution of intravenous bolus cytochalasin B, and assess the *in vivo* antineoplastic activity of cytochalasin B in comparison in doxorubicin in Balb/c mice challenged intradermally with M109 murine lung carcinoma. We also examine the effects of cytochalasin B against several other murine neoplastic cell lines (Lewis lung, LA4, B16F10, and M5076). Finally, we examine a potential mechanism of the antimetastatic activity of cytochalasin B by observing the effects of the agent on the secretion of *N*-acetylglucosaminidase (GlcNACase) by B16BL6 and B16F10 murine melanomas *in vitro*. The results of the study can be summarized as follows: 1) Cytochalasin B can be safely administered intravenously, intraperitoneally, and subcutaneously in murine models, with the maximum tolerated dose of all routes of administration being increased by liposome encapsulation. 2) Cytochalasin B can significantly inhibit the growth of tumors in mice challenged with M109, Lewis lung, LA4, B16F10, or M5076, producing long-term survival against lung carcinomas and adenocarcinomas (M109, Lewis lung, and LA4) and B16F10 melanoma, but not M5076 sarcoma. These effects were comparable to intraperitoneally administered doxorubicin. 4) Low concentrations of cytochalasin B inhibit the secretion of GlcNACase, indicating that cytochalasin B may inhibit metastatic progression by mechanisms not directly associated with its influence on cell adhesion and motility.

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Introduction

Microtubule-directed agents have long been used in cancer chemotherapy because of their ability to interfere with chromosome congression and the proper segregation of chromosomes, thereby invoking G_2/M arrest and other aberrant characteristics that eventually potentiate apoptosis. In addition to these traditional mitotic inhibitors, cell biology studies have elucidated a tremendous diversity of cytoskeletal-directed agents that affect the two other components of the cytoskeleton (microfilaments and intermediate filaments), many of which exert potent antineoplastic activity [1]. However, despite this diversity of cytoskeletal-directed agents available to oncologists, all currently approved cytoskeletal-directed agents used in the clinic have microtubule-based mechanisms [1]. Although it is true that these compounds act by distinct mechanisms (epothilones and taxanes stabilize microtubules, whereas eribulin and vinca alkaloids disrupt polymerization), they all are microtubule directed and target nuclear DNA replication in dividing cells.

It has been demonstrated that substantial differences exist between the microfilament biochemistry of normal cells and their neoplastic counterparts [1–3] and that these differences affect not only nuclear

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division but also cytokinesis, cell size and shape, cell attachment and detachment, and cell motility. These diverse cellular properties affected by actin polymerization suggest that agents affecting microfilament biochemistry should elicit notable antineoplastic activity as single agents and in combination with other agents and treatment modalities.

Cytochalasins are mycogenic toxins known to disrupt the formation of filamentous (F)-actin and, in some cases, prevent the formation of F-actin in treated cells altogether, thereby precluding the crucial cellular functions of microfilaments [4–6]. More than 60 different cytochalasins from several species of fungi have been classified, and most appear to markedly affect actin polymerization [1,5,7,8]. However, the most studied congener in regard to antineoplastic activity has been cytochalasin B. As a microfilament-disrupting agent, cytochalasin B alters many facets of cell physiology dictated by F-actin, including cell motility, adherence, secretion, drug efflux, deformability, morphology, and size, among many other cellular properties [4,6,9]. Because of the importance of microfilaments in the formation and ingression of the cleavage furrow, cytochalasin B is a potent inhibitor of cytokinesis and has been shown repeatedly to selectively enlarge and multinucleate neoplastic cells [10–14]. Such cells are ideal targets for microtubule-directed and nucleic acid–directed agents, and drug synergy has been noted between cytochalasin B and clinically approved antineoplastic agents that act through these mechanisms [15–17]. Further, we have shown that many cytochalasin congeners inhibit ATP binding cassette transporter-mediated efflux (manuscript under review), tentatively suggesting that cytochalasins and potentially other microfilament-directed agents may be used to treat cancers refractory to normal chemotherapy because of ATP binding cassette transporter-related drug resistance.

In addition to our *in vitro* work, we have extensively characterized the *in vivo* activity of cytochalasin B against solid tumors and disseminated cancers of murine origin [17–19]. Not only does cytochalasin B significantly improve the lifespan of mice challenged with lung carcinoma, melanoma, or leukemia, but long-term survival is frequently observed [17–19]. Interestingly, cytochalasin B appears to have notable efficacy against highly metastatic cell lines, having higher *in vitro* cytotoxicity against cell lines selected for metastatic propensity than for the original parental cancer cell line. In addition, cytochalasin B substantially reduces the extent of metastases found in challenged mice [17–19]. These observations are in accordance to the mechanisms of cytochalasin congeners, as functioning microfilaments are essential for neoplastic cell motility, invasion, and metastasis [20–24].

Due to the potential clinical importance that cytochalasin B and other microfilament-directed agents may have and the large quantities of these highly expensive agents needed for preclinical and clinical study, we are disclosing our method of producing gram quantities of cytochalasin B from *Drechslera dematioidea* so that interested laboratories can reproduce and extend our findings. This purification process also produces a smaller amount of cytochalasin A, which demonstrates marked antineoplastic activity against drug sensitive and resistant SK human ovarian carcinoma cell lines *in vitro* (manuscript under review). In addition, we provide data relevant for characterizing the tissue distribution of cytochalasin B administered as an intravenous (i.v.) bolus dose. We also examine the routes of administration and the maximum tolerated doses (MTDs) of cytochalasin B in multiple murine models. This analysis includes cytochalasin B that has been liposome encapsulated, as we have previously shown that this formulation reduces cytochalasin B-associated immunosuppression while retaining marked antitumor activity [19]. We then extend our previous *in vivo* findings by examining the effects of a well-tolerated i.v. treatment protocol of cytochalasin B in comparison with intraperitoneally (i.p.) administered doxorubicin against M109 murine lung carcinoma growing as an intradermal (i.d.) nodule in Balb/c mice. In addition, we investigate the ability of subcutaneous (s.c.) cytochalasin B to potentiate long-term survival (at least 53 days) in mice challenged with the following murine neoplastic cell lines: M109 lung carcinoma, Lewis lung carcinoma, LA4 lung adenocarcinoma, B16F10 melanoma, and M5076 sarcoma. Finally, we characterize a potential mechanism regarding how cytochalasin B reduces the number of metastases found in treated mice by assessing the *in vitro* effects of the agent on the secretion of *N*-acetylglucosaminidase (GlcNACase) by B16BL6 and B16F10 melanoma cell lines.

Materials and Methods

Preparation of Agar and Media Used to Subculture D. dematioidea for Inoculation of Large Mattes

Stocks of *D. dematioidea* (strain: IMI 74812) were acquired commercially (ATCC 24346). Stocks were grown on slants of potato dextrose agar and yeast extract (PDY). PDY was prepared in 250-ml aliquots using 7.5 g potato dextrose agar, 1.25 g yeast extract, and 250 ml deionized, distilled water. The mixture was heated to near boiling on a water bath while being magnetically stirred until all of the solute dissolved. Five milliliters of PDY was added to 15-mm screw top test tubes (8 ml for 18-mm tubes). The tubes were then autoclaved for 30 minutes at a slow cooling rate (liquid cool) before the tubes were slanted so that the media did not reach more than halfway up the tube. The slants were then cooled, placed in an upright position, and inoculated with *D. dematioidea.*

Growth of Large Mold Mattes of D. dematioidea

To prepare the growth media (Tanenbaum medium), 10 g bacto soytone, 40 g dextrose, 20 g yeast extract, 150 g sucrose, and 1 l tap water were added to a 2-l Erlenmeyer flask and then magnetically stirred. Five-hundred-milliliter aliquots of media were then transferred to a large Pyrex dish. Media were then supplemented with 5 crumbled Shredded Wheat biscuits spread evenly across the dish. Each dish was then covered with a double layer of aluminum foil and autoclaved for 90 minutes at a slow cooling rate. This process was then repeated to acquire 2-l Tanenbaum medium.

Mattes of *D. dematioidea* used for cytochalasin preparation were grown in Tanenbaum medium using mold stocks that were the lightest in color as they were the healthiest. Two to three PDY slants were used to inoculate every 500 ml of Tanenbaum medium (2 l total). Mattes were allowed to grow for 15 days on tissue culture plates (20 mm × 245 mm × 245 mm) before being harvested for cytochalasin preparation.

D. dematioidea is a Level 1 Biosafety agent and can be handled with routine sterile procedures and protection from inhalation and exposure to the eyes. Potential allergic responses to high levels of mold spores encountered should be considered, and procedures should be in place to manage any such occurrences.

MeOH Extraction of D. dematioidea

A total of 1.35 kg (wet weight) of healthy mold mattes were lyophilized to acquire 420 g of dry mold. The mold was then blended

with 5 ml MeOH per gram of mold under N_2 gas for 1 minute before being vacuum filtered. The fraction was collected and then set aside. The process was then repeated but only with enough MeOH to put the mold into suspension. The first and second filtrates were then combined to give a total volume of 3 to 4 l. Regular-phase thin layer chromatography (85:15 toluene:MeOH eluent) was used to determine the composition and purity of this crude extract against a small amount of commercially purchased cytochalasin B (Sigma-Aldrich Corp., St. Louis, MO). Water was then added to the filtrates to obtain an 80:20 H_2O:MeOH mixture, bringing the total volume to 12 to 16 l.

Reversed-Phase Thin Layer Chromatography to Acquire Purified Cytochalasin Extract

The 80:20 H_2O:MeOH eluate was subjected to reversed-phase thin layer chromatography with 50 g octadecyl carbon chain (C18)–bonded silica gel mixed with 100 ml MeOH. The column was then air dried for 2 minutes under water aspiration before the column was placed in a dessicator with P_2O_5 and vacuum pumped overnight. The column was then washed with 100 ml hexane before a 200-ml hexane/200-ml 60:40 THF:hexane mixture was used to elute cytochalasin B from the column; eluate was collected as 10-ml fractions. Commercially purchased cytochalasin B was again used to assess the purity of the product using regular-phase thin layer chromatography. Filtrates were then vacuum filtered to acquire precipitant, which was then stored in a dessicator with P_2O_5. Precipitates containing yellow color were purified in hot THF (2-3 ml hot THF per 200 mg precipitate) and eluted through a column containing a 100-g silica, 100-ml 1:1 EtOAc:hexane slurry. The plug filtrate was then evaporated until a precipitate formed. This precipitate was then dissolved in heated chloroform, resulting in the formation of crystalline cytochalasin.

Purification of Cytochalasin Crystals Using Flash Chromatography

Cytochalasin B crystals were dissolved in THF and subjected to flash chromatography using a 150-g silica, 150-ml 1:1 EtOAc:hexane slurry and 350 ml 1:1 EtOAc:hexane as the eluent. Thirty 10-ml fractions were collected and stored at 4°C overnight before being vacuum filtered to collect precipitates. Cytochalasins A and B were then separated using Whatman preparative thin layer chromatography plates (Fisher Scientific International Inc., Hampton, NH). Two distinct bands were then observed, indicating that cytochalasin A (top band) had separated from cytochalasin B (bottom band). These bands were then eluted from the silica using 2 to 3 ml MeOH at a time. The purity of cytochalasins A and B was analyzed using ^1H NMR spectroscopy (spectra not shown). The steps necessary for successful extraction and purification of cytochalasins A and B from mattes of *D. dematioidea* are succinctly highlighted in Figure 1.

Cytochalasin B and Doxorubicin Formulation and Administration

Cytochalasin B was prepared in suspension form in 2% carboxymethyl cellulose 1% Tween 20 (CMC/Tw) and in solution in dimethyl sulfoxide (DMSO) and 33% ethanol:0.9% NaCl solution as previously described [17–19]. In addition, cytochalasin B was liposome encapsulated in unilamellar vesicles of egg phosphatidylcholine:dimyristoylphosphatidylglycerol using methods previously described [19]. Doxorubicin was prepared in solution using the 33% ethanol:0.9% NaCl solution.

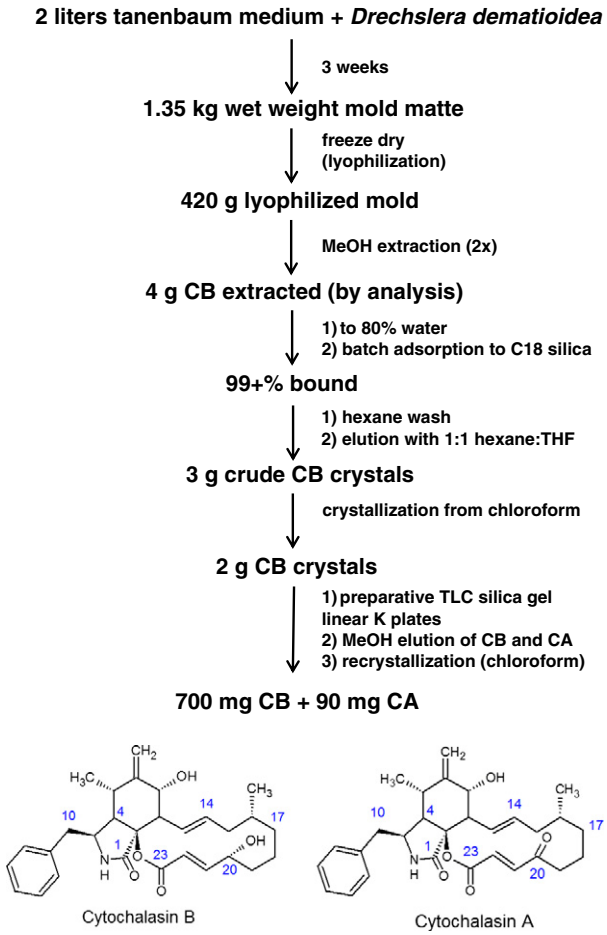

Figure 1. Preparation of cytochalasin B from *D. dematioidea*.

Suspension formulations of cytochalasin B required that the agent was emulsified in the CMC/Tw vehicle. This was accomplished by dissolving 1 ml Tween 20 into 90 ml pyrogen-free water before adding 2 g carboxymethyl cellulose sodium salt. The mixture was stirred with a magnetic stirrer for ~ 1 hour before being added to 9 ml distilled water to make a 2% carboxymethyl cellulose/1% Tween 20 vehicle. Cytochalasin B was then added to a sterile plastic syringe before CMC/Tw was added to a second syringe. The hubs of a 22-gauge emulsifying needle were attached to each syringe. The vehicle was gently pushed into the cytochalasin B–containing syringe and then slowly shaken. After the suspension passed through the 22-gauge needle, the suspension was passed through a 25-gauge needle with an air bubble of ~ 0.5 ml kept in the storage syringe to resuspend cytochalasin B before the next transfer to the injection syringe. The emulsified cytochalasin B was then kept in the original syringe, lying on its side in a refrigerator at 4°C for 12 hours. Afterward, cytochalasin B–CMC/Tw was lightly shaken to resuspend the agent and then reemulsified through a 25-gauge needle before being capped before use.

Preparation of Cytochalasin B with ^3H-Labeled Phenylmethyl Group

^3H-labeled cytochalasin B was prepared to determine the distribution of the agent in Balb/c mice, as assessed by high-performance liquid chromatography (HPLC). Before the inoculation

of Tanenbaum medium with *D. dematioidea*, the medium was supplemented with 2 mCi [^{3}H] phenylalanine (side chain ^{3}H, 15 Ci/mol, in sterile ethanol:water, from ICN) in 500 ml of medium, along with one crushed wheat biscuit. The purified product was again subjected to preparative thin layer chromatography in reduced light and was eluted with methanol before use to prevent the formation of cytochalasin A over time.

Conversion of M109 to Continuous Culture (M109c)

M109 murine lung carcinoma cells were converted to continuous culture using methods previously described [17]. As in the previous papers of this series [17–19], comparison between animals challenged i.d. with M109c cells and matched groups challenged with *in vivo* passaged M109 cells showed that the challenges were indistinguishable in terms of growth kinetics, invasion, metastasis, and host survival.

Solid Tumors In Vivo

M109 murine lung carcinoma was maintained as a spontaneously invasive and metastatic model by serial passage s.c. in Balb/c mice as previously detailed [19], with the passage challenge being 2×10^5 trypan blue–negative cells. For chemotherapy testing, Balb/c mice under isoflurane anesthesia (Sigma-Aldrich Corp.) were challenged i.d. on the lower right ventral quadrant over the peritoneum after first removing hair with a chemical depilatory agent. The challenge dose was 2×10^5 trypan blue–negative cells in a volume of 200 μl. Tumor cells were obtained from early s.c. tumor *in vivo* passaged donors (M109) or from continuous cultures of M109 lung carcinoma cells (M109c). Tumor growth was monitored by daily caliper measurements, which were facilitated by the localized growth of the i.d. challenge contained entirely in a region initially free of hair. Effects on primary tumor appearance, growth rate, invasion, and metastases to the lungs, liver, kidneys, pancreas, and spleen were determined as previously detailed for s.c. challenges with M109 tumor [18].

The other solid tumor lines (Lewis lung, LA4, B16F10, and M5076) were given as a 1×10^6 cell challenge s.c. in Balb/c, C57BL6, or B6D2F1 mice. The procedures for measuring tumor growth were the same as with M109 lung carcinoma.

Statistics

Survival analysis used the Cox-Mantel test as detailed by Lee [26]. Effects on tumor growth rates were evaluated by repeated-measures analysis of variance using SAS (Cary, NC) software. Tests of hypotheses for between-subject effects were applied, and of time versus group interactions using the Geisser-Greenhouse adjustment in cases where tests of orthogonal components showed absence of sphericity. Differences in lung metastases between treated and control groups were evaluated with a Student-Fisher *t* test.

Analysis of N-Acetylglucosaminidase Activity in B16BL6 and B16F10 Murine Melanomas after Administration of Cytochalasin B

B16BL6 and B16F10 murine melanomas were cultured using methods previously described [17,19].Cells were subcultured when they reached 1×10^6 cells/ml. The confluent monolayer was treated with 0.2% trypsin-EDTA solution 10× (Sigma-Aldrich Corp.) for 1 minute at 37°C, and the suspended cells were seeded at 4×10^4 cells/cm^2 in 25-cm^2 culture flasks. Cells were allowed to attach before being treated with 0.1 or 1 μM cytochalasin B for 48 hours. GlcNACase activity of untreated and cytochalasin B-treated cells was assessed as previously described [27].

Results

Preparation of Cytochalasins A and B

Procedures shown in Figure 1 produced 700 mg of HPLC-pure cytochalasin B and 90 mg of HPLC-pure cytochalasin A. Purity was >99%. R_f values corresponded to commercially obtained congeners, and ^{1}H NMR spectra were in accordance with expected peak values. Crystalline cytochalasin B had a melting point of 216°C to 218°C (commercial cytochalasin B: 217°C to 220°C; literature reference melting points range from 215°C to 223°C) and showed no evidence of fluorescence quenching, iodine vapor positive contaminants, or *p*-anisaldehyde–positive contaminants when 100 μg was analyzed by thin layer chromatography, indicative of a highly pure product. The radioactive labeling procedure produced 30 mg ^{3}H labeled cytochalasin B at the phenylmethyl group that was >99% pure as assessed by analytical thin layer chromatography, radiochromatography, and ^{1}H NMR spectroscopy. Specific activity was 3 μCi/mmol.

Tissue Distribution of Intravenously Administered Cytochalasin B in Balb/c Mice

^{3}H-labeled 3.5 mg/kg cytochalasin B was administered as an i.v. bolus dose in Balb/c mice at time 0 and followed over the course of 720 minutes. The total dose per mouse was 70 μg (3.5 μg/g). Tissue distribution revealed that the agent has a marked affinity for the intestines, as 8.75 μg/g tissue was present 30 minutes postinjection and 1.5 μg/g tissue remained after 180 minutes (Figure 2*A*). Furthermore, it appears that cytochalasin B remains in the intestines for some time, as 0.58 μg/g tissue remained after 720 minutes (Figure 2*A*). The 6 tissues out of the 12 analyzed that had the highest levels of cytochalsin B at time 0 or at 15 minutes after i.v. treatment are shown in Figure 2*A*. The five highest concentrations at 15 minutes were in the liver, lungs, intestines, pancreas, and tail (2.8 to 4 μg/g tissue). The kidneys had an initial concentration of 3 μg/g at the time of injection, but showed only 0.4 μg/g at 15 minutes. The six tissues with the lowest distributions at 15 or 30 minutes included the heart, ovaries, spleen, bladder, and stomach. Very little cytochalasin B was detected in the brain initially (0.25 μg/g tissue) or at any time from 15 to 180 minutes (Figure 2*A*). Interestingly, tissues appeared to follow two types of distribution; either cytochalasin B reached peak concentrations shortly after administration, followed by a gradual decrease (liver, tail, lungs, kidneys, and pancreas), or cytochalasin B concentrations spiked at 30 minutes, followed by a gradual decrease (intestines, heart, spleen, ovaries, and bladder).

Analysis of cytochalasin B tissue distribution was then extended to 24 hours using a 5-mg/kg i.v. bolus dose, which is equal to 5 μg/g, or a 100-μg total dose for a 20-g mouse (Table 1). Interestingly, the concentration of cytochalasin B in the lungs was markedly increased with this higher dose at 30 minutes (0.8 μg/g tissue with 3.5 mg/kg and 11.1 μg/g tissue with 5 mg/kg). In addition, the plasma concentration of cytochalasin B steadily declined over the course of 24 hours, and it appears that the $t_{1/2}$ of an i.v. bolus dose in the blood is between 0.5 and 12 hours in Balb/c mice. The highest tissue concentrations at 30 minutes in Table 1 were recorded in the lungs, intestines, and liver (3 μg/g to 11 μg/g), in agreement with observations made in Figure 2*A* for the six highest tissues. The heart, spleen, ovaries, kidney, bladder, and stomach were among the tissues with the lowest distribution at 30 minutes in Table 1, again consistent with observations in these tissues for a lower dose of cytochalasin B shown in Figure 2*A*. Table 1 also shows tissue

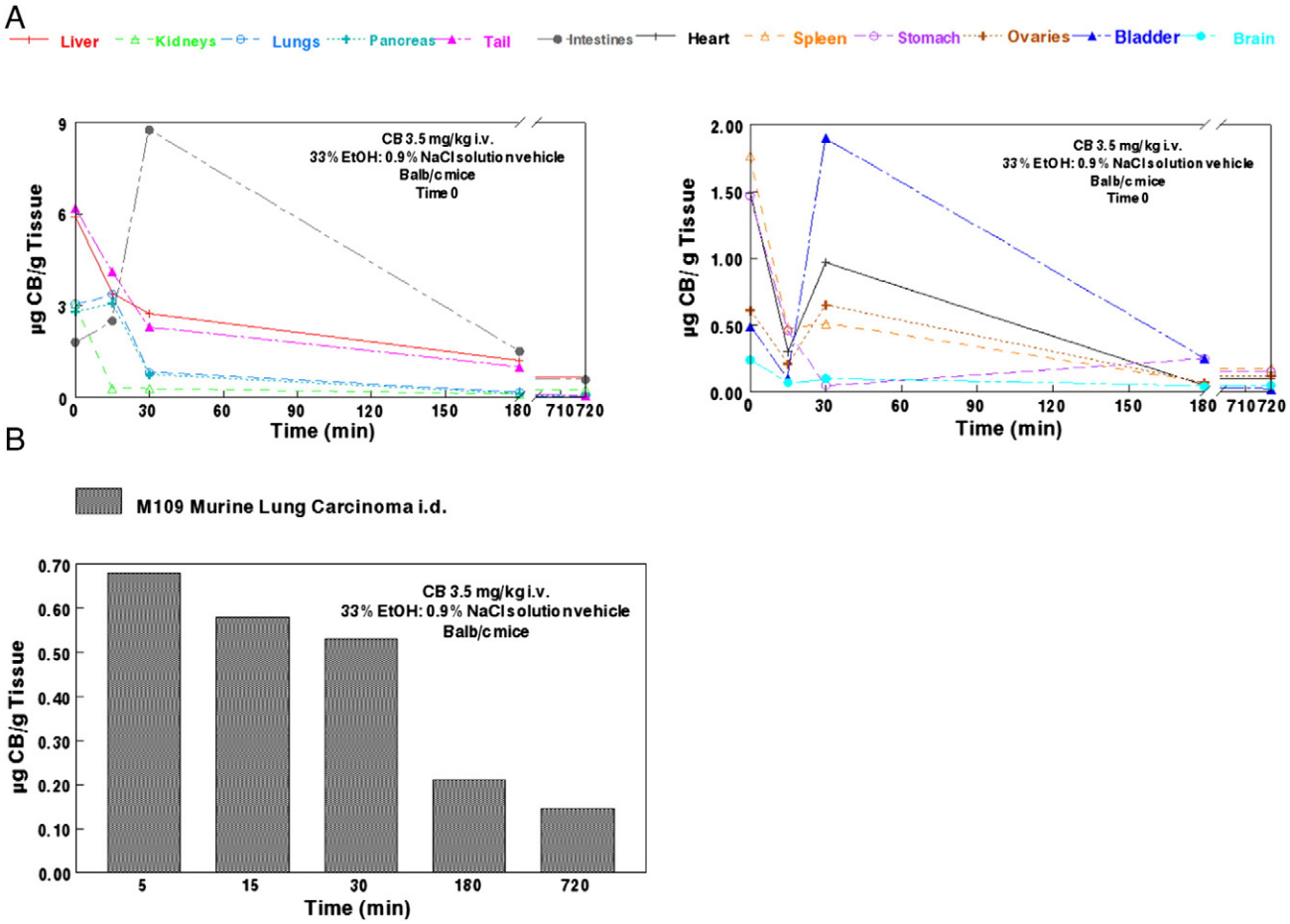

Figure 2. Tissue distribution of ^{3}H-labeled 3.5 mg/kg cytochalasin B administered as an i.v. bolus dose in Balb/c mice over the course of 720 minutes. (A) Distribution in nonneoplastic tissues. (B) Distribution in an i.d. tumor of M109 murine lung carcinoma. Analyses were performed by analytical HPLC.

distributions to renal lipids, lymph nodes, and thymus that were not examined among the 12 tissues shown in Figure 2*A*. Cytochalasin B remaining in the tail was not examined among the tissues shown in Table 1. It is important to note that, even at this higher concentration of cytochalasin B, concentrations in the brain never rose above 0.08 µg/g tissue. The potential therapeutic implications of this observation will be elaborated upon in the discussion.

Table 1. Tissue Distribution of ^{3}H-Labeled cytochalasin B Administered as a 5-mg/kg i.v. Bolus dose, 100 µg/20 g Mouse, in Balb/c Mice

µg CB/g Tissue			
Tissue	0.5 Hour	12 Hours	24 Hours
Lungs	11.1	9.1	0.99
Intestine	8.8	0.73	0.87
Liver	3.04	0.32	0.05
Stomach	1.79	0.26	0.7
Renal lipids	1.3	2.4	0.68
Bladder	0.75	0.82	0.60
Lymph nodes	0.69	0.64	0.36
Kidney	0.64	0.12	0.08
Ovaries	0.64	0.40	0.28
Spleen	0.58	0.42	0.20
Thymus	0.49	0.52	0.41
Heart	0.45	0.24	0.19
Pancreas	0.23	0.21	0.12
Brain	0.06	0.08	0.07
Blood	0.13/ml	0.04/ml	0.02/ml

Tissues are ordered in descending order of concentration at 0.5 hour other than blood, which is measured per ml.

When Balb/c mice were challenged i.d. with M109 lung carcinoma and treated with 3.5 mg/kg i.v. bolus cytochalasin B, the agent appeared to have affinity for the tumor that was in the range exhibited in Figure 2*A* and Table 1 by normal tissues with intermediate cytochalasin B distribution such as the bladder, lymph nodes, thymus, kidney, ovaries, spleen, and heart (Figure 2*B*). Peak cytochalasin B concentration in the tumor nodule was 0.68 µg/g tissue 5 minutes postinjection and remained above 0.55 µg/g for at least 30 minutes. As with normal tissue, the concentration of cytochalasin B appeared to gradually decrease between 180 and 720 minutes, demonstrating that the agent remains in neoplastic tissue for at least 12 hours after i.v. bolus administration. A concentration of 0.2 µg cytochalasin B/g tumor at 3 hours corresponds to a concentration of 50 µM cytochalasin B, assuming a 1-ml volume/g tumor. This is ~25-fold higher than the bioactive concentration of cytochalasin B *in vitro* against M109c lung carcinoma at 3 hours [17].

Maximum Tolerated Dose of Cytochalasin B Using Various Routes of Administration and Formulations in Multiple Murine Models

The MTDs of cytochalasin B in suspension, solution, or liposome encapsulation administered i.p., i.v., or s.c. in Balb/c, B6D2F1,

Table 2. Maximum Tolerated Doses of Nonencapsulated Cytochalasin B and Liposomal Cytochalasin B Administered i.p., i.v., and s.c. in Balb/c, B6D2F1, CD2F1 *, and C57B1/6 Mice

Route	Vehicle	Formulation	Schedule (Days)	mg/kg	Max. Wt. Loss (%)	Drug Deaths
i.p.	CMC/Tw	Suspension	1	50	7	2/34
	CMC/Tw	Suspension	1, 4, 7	50/day	6	0/16
	DMSO	Solution	1	25	2	1/5
	CMC/Tw	Liposome	1	400	2	0/40
s.c.	CMC/Tw	Suspension	1	100	3	0/31
	CMC/Tw	Suspension	1	150	7	4/28
	DMSO	Solution	1	100	7	5/32
	CMC/Tw	Liposome	1	>500	7	0/25
i.v.	CMC/Tw	Suspension	1	20	1	0/10
	EtOH/Sal	Solution	1	10-20	1-2	1/20
	CMC/Tw	Liposome	1	25	1	0/10

Max. Wt. Loss: maximum weight loss experienced after receiving the indicated dose.
* CD2F1 mice could only tolerate 10 mg/kg i.v. as a bolus injection and had a maximum weight loss of 2%.

CD2F1, and C57B1/6 mice are presented in Table 2. When administered i.p. or s.c., a lower MTD of cytochalasin B is reached with the agent in solution (25 mg/kg and 100 mg/kg) rather than in suspension (50 mg/kg and 150 mg/kg). Nevertheless, equal doses of cytochalasin B in suspension or solution can be administered i.v. to mice (20 mg/kg). It should be noted that CD2F1 mice could tolerate only 10 mg/kg i.v. as a bolus injection. The maximum weight loss experienced during i.p. and s.c. administration ranged from 2% to 7%, and for i.v. administration, it ranged from 1% to 2%.

As previously demonstrated in [19], the MTD of cytochalasin B could be dramatically increased through liposome encapsulation. Whereas the i.v. MTD of liposomal cytochalasin B increased only by 5 mg/kg (20 to 25 mg/kg), the i.p. MTD increased by 8-fold (50 to 400 mg/kg), and the s.c. MTD increased by more than 3-fold (150 to >500 mg/kg). Furthermore, the maximum % weight loss after administration of the MTD of liposome encapsulated cytochalasin B was decreased for i.p. administration and remained equal for s.c. and i.v. administration when comparing liposome-encapsulated and nonencapsulated cytochalasin B. A previous study has shown that cytochalasin B–induced immunosuppression is partially reversed when cytochalasin B is administered in liposomal form [19].

Effects of Intravenously Administered Cytochalasin B and Intraperitoneally Administered Doxorubicin on M109c Murine Lung Carcinoma Challenged Mice

Administration of cytochalasin B as a 5-mg/kg/day i.v. bolus dose on days 1 to 5 to Balb/c mice challenged i.d. with 2×10^5 M109c cells appeared to markedly perturb tumor growth (Figure 3*A*). At 30 days postchallenge, cytochalasin B–treated mice had a mean tumor volume of 0.53 cm^3 (standard error of the mean; SEM = 0.04), whereas mice treated with the vehicle had a mean tumor volume of 0.9 cm^3 (SEM = 0.034). Doxorubicin administered as a 5-mg/kg i.p. dose once or twice had a notable antitumor effect, but it was not as pronounced as cytochalasin B (Figure 3*A*). These effects on tumor growth appear to be supported by the long-term survival potentiated by the 5-mg/kg/day i.v. bolus cytochalasin B treatment protocol (Figure 3*B*). There was a 50% survival rate at least up to day 53 for mice challenged with an i.d. tumor nodule in the cohort treated five times with 5-mg/kg i.v. bolus cytochalasin B (n = 16). This is a highly significant prolonging of life expectancy, as all mice treated with the vehicle were dead by day 42 (n = 10). By contrast, mice treated once with 5 mg/kg i.p. doxorubicin were all dead by day 50 (n = 10), and only 1 in 10 mice treated twice with 5 mg/kg i.p. doxorubicin was alive after 53 days. These data are further supported by a Cox-Mantel test that indicated a $P < .05$ for each treatment cohort.

In addition, the same dose of cytochalasin B markedly reduced the extent of terminal lung metastases observed in Balb/c mice challenged with a single i.d. tumor inoculation (Figure 3*C*). As previously mentioned [19], mice challenged i.d. with M109c lung carcinoma present significant metastases only in the lungs. The effects of cytochalasin B on reduction in lung metastases appear to be dose dependent, as demonstrated when the 5-mg/kg i.v. bolus dose was administered daily one to five times (Figure 3*C*). Mice bearing an i.d. tumor and treated with 1, 2, 3, 4, or 5 peritumoral doses of cytochalsin B showed a clear dose-dependent response with a 90% reduction in lung metastases at the terminal stages in the mice receiving five doses of cytochalasin B peritumorally. The cohort of mice treated with i.v. cytochalasin B 5 mg/kg/day for 5 days had a mean of seven nodules (SEM = 2.8), whereas the cohort treated with the vehicle only had a mean of 62 nodules (SEM = 5.4). These effects on terminal lung metastases were significantly different compared with the two doxorubicin cohorts, as mice treated with 5 mg/kg i.p. doxorubicin on day 1 had a mean of 57 nodules (SEM = 3.2), whereas those treated with the same dose on days 1 and 3 had a mean of 50 nodules (SEM = 4.9).

Effects of Cytochalasin B on Prolonging the Survival of Mice Challenged with Various Solid Tumors

Cytochalasin B administered s.c. as a 100-mg/kg dose on day 1 appeared to have marked antineoplastic activity against most of the solid tumor challenges (Figure 4), as a significant proportion of the mice lived at least 53 days after the initial tumor challenge: M109 lung carcinoma (4/10 mice), Lewis lung carcinoma (5/10 mice), LA4 lung adenocarcinoma (6/10 mice), and B16F10 melanoma (4/10 mice). Although 100 mg/kg cytochalasin B s.c. significantly prolonged the survival of mice challenged with M5076 sarcoma in comparison with the vehicle-only treated mice, all mice in this cohort were dead by day 30; the treatment was unable to produce any long-term survivors at day 53 (0/10 mice), and all mice were dead by day 44 (Figure 4).

Effects of Cytochalasin B on N-Acetylglucosaminidase Activity in B16BL6 and B16F10 Murine Melanomas

Before administration of cytochalasin B, B16BL6 and B16F10 melanomas had an extracellular percentage of GlcNACase compared with total enzyme activity ranging from 27% to 41% (Table 3). A total of 0.1 μM cytochalasin B administered for 48 hours decreased this range to 23% to 28%. One micromolar of cytochalasin B administered for the same length of time further reduced this range to 12% to 27%. Interestingly, cytochalasin B appeared to have no influence on the total amount of GlcNACase in either cell line, suggesting that cytochalasin B may inhibit the secretion of GlcNA-Case and potentially other glucosaminidases. The importance of inhibiting the secretion of glucosaminidases in neoplastic cells will be elaborated upon in the discussion.

Discussion

In the present study, we have demonstrated that cytochalasin B can be safely given to murine models through multiple routes of administration. As previously shown [17,18], cytochalasin B can be administered i.p., i.v., and s.c. in Balb/c mice, with the MTD being higher using a suspension (CMC/Tw) rather than solution vehicle

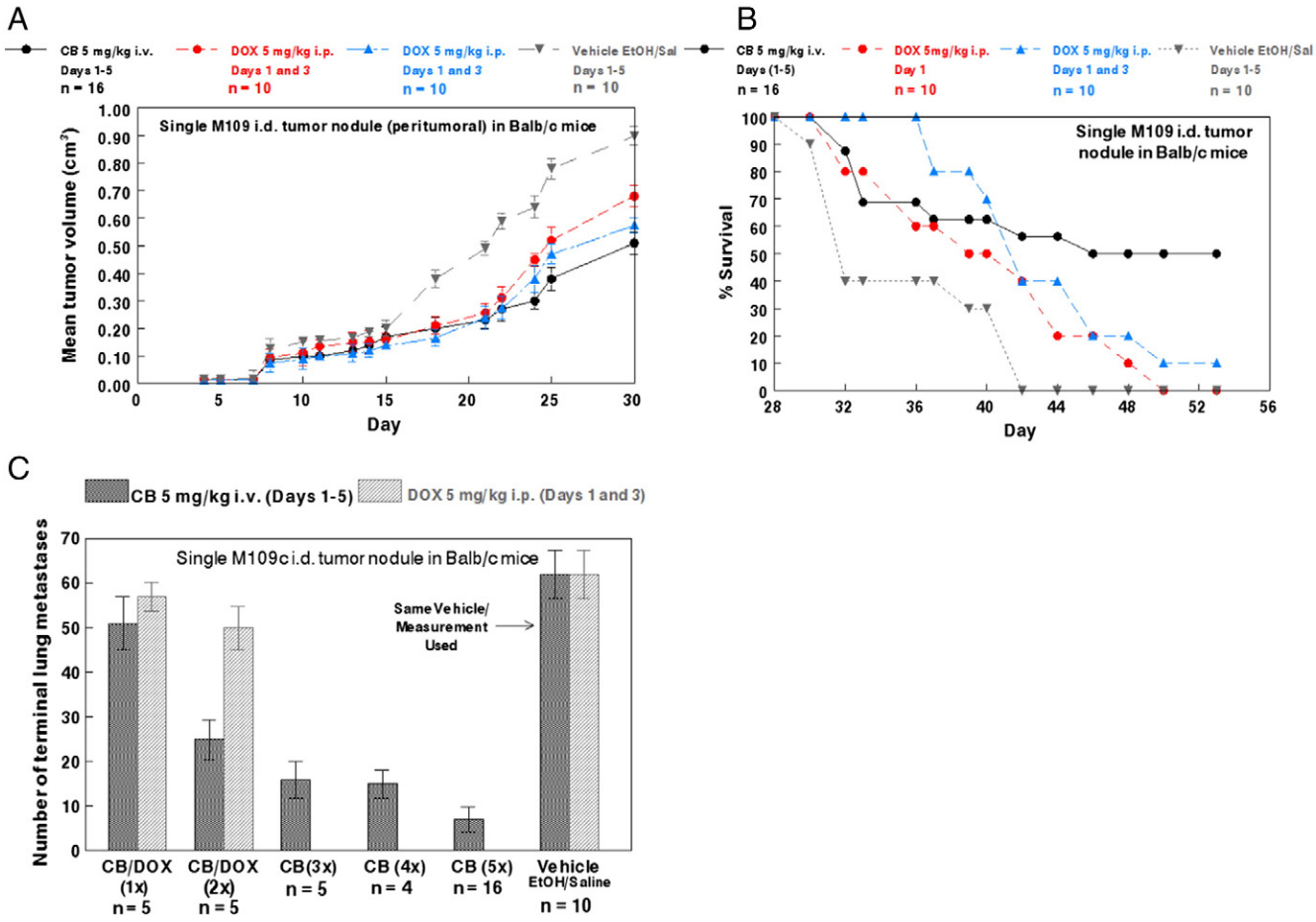

Figure 3. Effects of cytochalasin B administered five times as a 5-mg/kg i.v. bolus dose and doxorubicin administered once or twice as a 5-mg/kg i.p. dose on M109 murine lung carcinoma. (A) Effects on the long-term growth of established M109c i.d. tumor nodules. Cytochalasin B was administered on days 1 to 5 following tumor challenge. Doxorubicin was administered on day 1 and on days 1 and 3 following tumor challenge. There were 16 mice in the CB-treated cohort, 10 mice in both doxorubicin-treated groups, and 10 mice in the cohort that received only the vehicle. The CB-treated group was significantly different from the control and doxorubicin-treated groups; $P < .01$ for between-subject effects, $P < .05$ for time versus group interactions. Bars reflect SEM of the treatment groups. (B) Effects of cytochalasin B administered as a 5-mg/kg i.v. bolus dose and doxorubicin as a 5-mg/kg i.p. dose on the survival of Balb/c mice challenged with M109c lung carcinoma. Survival of mice treated with cytochalasin B ($n = 16$) was significantly different from that of mice receiving the doxorubicin regimens ($n = 10$ for both groups) or only the vehicle ($n = 10$); $P < .05$, as assessed by a Cox-Mantel test. (C) *In vivo* activity of cytochalasin B administered as a 5-mg/kg i.v. bolus dose or doxorubicin administered as a 5-mg/kg i.p. dose against the metastatic spread of M109c lung carcinoma. Size of all treatment groups is indicated in the figure. The lungs were the only tissue that contained substantial metastases. Bars reflect SEM of each treatment group.

(DMSO) for i.p. and s.c. administration (Table 2). However, the EtOH/saline solution produced as high an MTD as did CMC/Tw suspension when administered as an i.v. bolus dose (20 mg/kg), with the exception of CD2F1 mice that could tolerate only 10 mg/kg cytochalasin B. In [19], we demonstrated that liposome-encapsulated cytochalasin B could dramatically increase the MTD of the agent when administered i.p. We have now demonstrated that liposome-encapsulated cytochalasin B increases the MTD of i.v. and s.c. administered cytochalasin B (20 to 25 mg/kg i.v. and 100 to >500 mg/kg s.c.). In addition, we demonstrated that liposome-encapsulated cytochalasin B can be safely administered i.p. at 400 mg/kg, an eight-fold higher dose than can be used with non–liposome-encapsulated cytochalasin B.

We have previously characterized the pharmacokinetics and tissue distribution of nonencapsulated and liposome-encapsulated cytochalasin B administered i.p [19,25]. The present study extended these analyses to i.v. nonencapsulated cytochalasin B. Although there has been concern that cytochalasin B could have potential cardiotoxicity *in vivo*[28,29], the relatively low tissue concentration of cytochalasin B found in this organ after i.v. bolus administration 0 to 24 hours postinjection (Figure 2 and Table 1), combined with the low rate of drug-related deaths at notably high drug concentrations of cytochalasin B using multiple routes of administration (Table 2), suggests that cytochalasin B does not elicit cardiotoxicity to any significant degree when administered as an i.v. bolus dose. This is important, as cytochalasin B has shown marked drug synergy with multiple nucleic acid–directed agents including doxorubicin [17], a member of the anthracycline drug class that is noted for substantial cardiotoxicity [30]. Therefore, the concomitant administration of cytochalasin B and anthracyclines remains a feasible combination for evaluating antineoplastic activity in murine models.

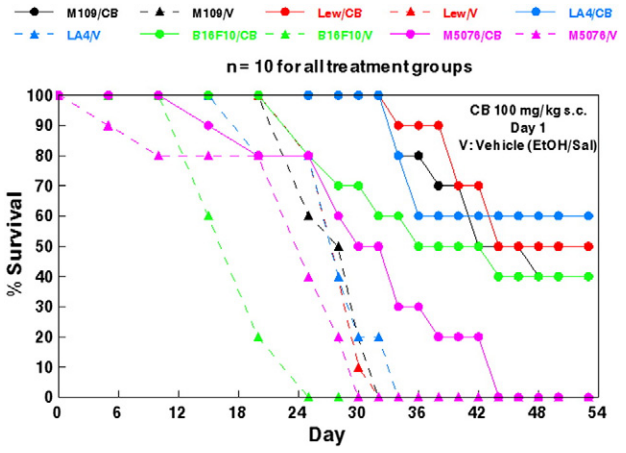

Figure 4. Effects of cytochalasin B administered once as a 100-mg/kg s.c. dose on potentiating long-term survival in mice challenged with various solid tumors. Mice were challenged i.d. with M109 lung carcinoma and s.c. with Lewis lung carcinoma, LA4 lung adenocarcinoma, B16F10 melanoma, or M5076 sarcoma. In each challenge, the survival of tumor-bearing mice treated with cytochalasin B (denoted by CB) was significantly different than the vehicle-only–treated group (denoted by V); $P < .05$, as assessed by a Cox-Mantel test. There were 10 mice for all cohorts.

Table 3. Effects of Cytochalasin B on Secretion of *N*-Acetylglucosaminidase by B16BL6 and B16F10 Murine Melanomas *In Vitro*

A	B	C	D	E	F	G
CB 48 h (μM)	Ex-Cell GlcNACase (Units/mg)	In-Cell GlcNACase (Units/mg)	Total GlcNACase (B + C)	Ex-Cell % of Total GlcNACase	In-Cell % of Total GlcNACase	Total GlcNACase (% of Ctrl)
B16BL6						
0	315	840	1155	27	73	100
0.1	288	969	1257	23	77	109
1	175	1260	1435	12	88	124
0	748	1084	1832	41	59	100
1	565	1486	2051	27	73	112
B16F10						
0	1065	2273	3338	32	68	100
0.1	951	2406	3357	28	72	101
0	932	2004	2936	32	68	100
1	420	2635	3055	14	86	104

Although it appeared that mice were able to tolerate five doses of 5.0-mg/kg cytochalasin B bolus i.v. administered once a day for 5 consecutive days, there was a noticeable increase in the concentration of cytochalasin B in the lungs when the concentration was increased from 3.5 to 5 mg/kg [0.04 μg/g tissue after 12 hours (720 minutes) vs 9.1 μg/g tissue after 12 hours]. Such a marked increase in tissue concentration of cytochalasin B in the lungs after only a small dosage increase is notable and may raise concern for potential pulmonary toxicity. Nevertheless, i.v. cytochalasin B was tolerated in suspension at 20 mg/kg as a bolus dose and increased to 25 mg/kg after liposome encapsulation. Therefore, it may be possible to combine cytochalasin B with nucleic acid–directed agents that exhibit marked pulmonary toxicity, such as bleomycin [30], without subjecting tumor-bearing mice to intolerable side effects.

In addition to its concentration in normal tissue, it appears that i.v. cytochalasin B readily infiltrates neoplastic tissue, as demonstrated with M109 lung carcinoma (Figure 2*B*). The concentration of cytochalasin B in M109 tumor nodules leveled off between 180 and 720 minutes, indicating that sustained levels of the agent can be found in malignant growths 12 hours after a single i.v. bolus administration of 3.5 mg/kg. These data indicate that cytochalasin B can reach its intended target and remain in significant concentration hours after administration. Nevertheless, we have demonstrated that cytochalasin B can exert antitumor activity in M109 lung carcinoma without having to be in direct contact with the tumor nodule [19], and direct cytotoxicity may not be necessary for significant cytochalasin B–mediated tumor regression.

Cytochalasin B is a notably lipophilic compound, suggesting that it might be considered for use as an i.v. administered agent to cross the lipophilic blood brain barrier (BBB) in brain cancer chemotherapy. However, HPLC analysis of ^{3}H-labeled cytochalasin B revealed that the agent does not reach high tissue concentrations in the brain when administered i.v. (0.06 μg/g tissue 30 minutes after a 5.0-mg/kg dose). This is likely due to the relatively high molecular weight of cytochalasin B (MW = 479.6), as bulky compounds have difficulty traversing the BBB [30]. For comparison, the lipophilic alkylating agent temozolomide, which is known to readily cross the BBB, has an MW = 194.151. Nevertheless, these low concentrations of cytochalasin B may still exhibit notable *in vivo* antineoplastic activity against brain tumors, or it may be possible to administer cytochalasin B intrathecally into mammalian preclinical models to assess its efficacy against brain tumors. These studies are currently under way in our laboratory.

In [17–19], we demonstrated that cytochalasin B has marked *in vivo* antineoplastic activity in murine models challenged with solid localized tumors, as well as against disseminated cancers. In this study, we confirmed that cytochalasin B markedly affects the progression of M109 lung carcinoma (Figure 3*A*) and can produce long-term survival when administered i.v. or s.c. (8/16 mice treated i.v. were alive at 53 days, whereas 4/10 mice treated s.c. were alive at 53 days; Figures 3*B* and 4, respectively). These effects were comparable to doxorubicin administered once or twice as a 5-mg/kg i.p. dose (Figure 3), indicative of the marked antitumor activity elicited by cytochalasin B in the M109 tumor model system. In addition, it appears that cytochalasin B has marked cytotoxicity for metastatic cells, as the agent reduced the number of M109c lung metastases from a mean of 62 nodules when treated only with an EtOH/saline vehicle to a mean of 7 nodules when treated five times with 5-mg/kg cytochalasin B i.v. bolus (Figure 3*C*). The reduction of metastases was greater than 5 mg/kg doxorubicin i.p., which had a mean of 57 nodules with only one injection, and a mean of 50 nodules after two injections (Figure 3*C*). Further, the reduction in lung metastases potentiated by cytochalasin B appeared to be dose dependent, further confirming the antitumor activity the agent has toward metastatic cells. These effects on reduction in metastatic nodules are very similar to our observations in [17,18], indicating that cytochalasin B can inhibit metastatic progression in tumor-bearing murine models.

Although we have previously demonstrated the antitumor activity of cytochalasin B *in vivo* against M109 lung carcinoma, B16F10 melanoma, and P388/S and P388/ADR leukemia [17–19], we have now extended these observations to Lewis lung carcinoma and LA4 adenocarcinoma. These data suggest that cytochalasin B is notably active against murine lung cancers and that investigating its potential in human lung tumor xenograft models is warranted. It is also important to note that although cytochalasin B was able to prolong

the life of mice challenged with M5076 sarcoma, the agent was unable to produce any long-term survivors at day 53. This is an initial indication of the type of malignancies that cytochalasin B may not be efficacious for, but certainly does not preclude its examination in other sarcoma models.

Previously, we have demonstrated that cytochalasin B has a marked increase in cytotoxicity *in vitro* against murine melanoma cells selected by *in vivo* serial passage for an increased propensity to metastasize [17]. When combined with our data on *in vivo* metastases reduction, it appears that metastatic cells are particularly sensitive to cytochalasin B. These observations are in accordance with the importance of microfilaments in motility and invasion [31,24,23,32,33]. Significantly inhibiting actin polymerization would likely inhibit the rate at which neoplastic cells metastasize and therefore highlights the potential utility of microfilament-disrupting agents in the clinical setting. The utility of cytochalasin B in antimetastatic therapy may extend further than inhibiting the motility of highly malignant cells, as demonstrated by the ability of the agent to disrupt the secretion of GlcNACase in B16BL6 and B16F10 melanoma cells (Table 3). GlcNACase and other glycosidases are used by metastatic cells to degrade the carbohydrate polymers that mask the protein backbone of the extracellular matrix, enabling nearby proteases to provide the physical space needed for malignant cell migration [34–37]. Inhibiting the secretion of glycosidases by metastatic cells would reduce the extent of carbohydrate degradation, thereby reducing the efficacy of proteases to clear a navigable path to the vasculature necessary for intravasation.

Conclusion

Cytochalasin B, its congeners, and other microfilament-directed agents comprise a potentially novel class of chemotherapeutic agents, as they have shown *in vitro* and *in vivo* efficacy against many forms of malignancy [1]. The method by which our laboratory extracts cytochalasins A and B from *D. dematioidea* is provided so that other interested laboratories can evaluate and extend our findings and contribute additional preclinical evidence to warrant clinical examination. This method is cost effective and substantially decreases the expense of *in vivo* work involving cytochalasins A and B. To compare, a matte of *D. dematioidea* can produce the gram quantities of cytochalasins A and B needed for extensive mammalian study for less than $400 (420 g dry mold produces ~700 mg cytochalasin B and 90 mg cytochalasin A). By contrast, 10 mg cytochalasin A and 50 mg cytochalasin B from a commercial supplier can cost around $633.00 and $1245.00, respectively. In addition, partial or total syntheses of cytochalasins have been described [38–40] and are another alternative to commercial purchases. Although it is true that most promising drug leads fail to exhibit clinical efficacy, microfilament-directed agents have yet to be critically evaluated in controlled clinical trials. Critical assessment of these novel antineoplastic agents is indeed warranted and hopefully will establish a new avenue of chemotherapy.

Acknowledgements

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