in vivo *28*: 1021-1032 (2014)

Tolerated Doses in Zebrafish of Cytochalasins and Jasplakinolide for Comparison with Tolerated Doses in Mice in the Evaluation of Pre-clinical Activity of Microfilament-directed Agents in Tumor Model Systems *In Vivo*

MATTHEW TRENDOWSKI, VICTORIA WONG, KARIE WELLINGTON, SUZANNE HATFIELD and THOMAS P. FONDY

Department of Biology, Syracuse University, Syracuse, NY, U.S.A.

Abstract. *Background/Aim: Chemotherapeutic approaches involving microtubule-directed agents such as the vinca alkaloids and taxanes are used extensively and effectively in clinical cancer therapy. There is abundant evidence of critical cytoskeletal differences involving microfilaments between normal and neoplastic cells, and a variety of natural products and semi-synthetic derivatives are available to exploit these differences in vitro. In spite of the availability of such potential anti-neoplastic agents, there has yet to be an effective microfilament-directed agent approved for clinical use. Cytochalasins are mycogenic toxins derived from a variety of fungal sources that have shown promising in vitro efficacy in disrupting microfilaments and producing remarkable cell enlargement and multi-nucleation in cancer cells without producing enlargement and multi-nucleation in normal blood cells. Jasplakinolide is a sponge toxin that stabilizes and rigidifies microfilaments. Insufficient in vivo data has been acquired to determine whether any of the microfilament-directed agents have valuable preferential anticancer activity in pre-clinical tumor model systems. This is partly because the limited availability of these agents precludes their initial use in large-scale mammalian pre-clinical studies. Therefore, the present study sought to determine the tolerated in vivo doses of cytochalasins and jasplakinolide in zebrafish (Danio rerio), a well-studied fish cancer model that is 1.5% the size of mice. We also determined the tolerated levels of a variety of clinically active anti-neoplastic agents in zebrafish for comparison with tolerated murine doses as a means to allow comparison of toxicities in zebrafish expressed as μM concentrations with toxicities in mice expressed in mg/kg. Materials and Methods: Tolerated doses in zebrafish with various cytochalasins or jasplakinolide were determined by adding the solubilized test agent to water in which the fish were maintained for 24 h, then restored to their normal tanks and monitored for a total of 96 h. Results: Cytochalasin D at 0.2 μM gave an approximate LD_{50} in zebrafish, while cytochalasin B was fully-tolerated at 5 μM, and gave an LD_{50} of 10 μM. 21,22-dihydrocytochalasin B was fully-tolerated at 10 μM. Cytochalasin C was tolerated fully at 1 μM, ten-fold higher than the level for cytochalasin D that was tolerated. Jasplakinolide at 0.5 μM did not exhibit any apparent acute toxicity or affect fish behavior for four days, but delayed toxicity was evident at days 4 and 6 when the fish died. Further, the addition of 5 μM glutathione (GSH) at the time of treatment substantially decreased the toxicity of 10 μM cytochalasin B, a level of cytochalasin B that not otherwise tolerated in vivo. Such observations were likely due to GSH-mediated alkylation of C-20 in cytochalasin B, thereby reducing the rate of oxidation to the highly toxic congener, cytochalasin A, and reacting with any cytochalazin A formed. The protective effects of GSH are further supported by its ability to react with α, β-unsaturated ketone moieties, as is found in cytochalasin A. GSH at 0.8 uM was able to reduce the toxicity of 0.8 μM cytochalasin D, but it took 20 μM GSH to fully protect against the toxicity of 0.8 μM cytochalasin D. Conclusion: Pre-clinical evaluation of rare natural products such as microfilamented-directed agents for efficacy in vivo in tumor-bearing zebrafish is a feasible prospect. Dose-limiting toxicities in zebrafish expressed as μM concentrations in water can be used to estimate in vivo toxicities in mice expressed as mg/kg.*

Correspondence to: Matthew Trendowski, Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, U.S.A. Tel: +1 3158078991, e-mail: mrtrendo@syr.edu

Key Words: Cytochalasins, Cytochalasin B, 21,22-dihydro-Cytochalasin B, Cytochalasin D, Cytochalasin C, Jasplakinolide, chemotherapy, glutathione, toxicity, microfilament-directed agents, zebrafish.

Cancer chemotherapeutic agents generally exploit cell biological differences between normal and neoplastic cells focusing on nucleic acid and protein synthesis, replication of cell organelles, cell division, and cell movement. Cytoskeletal-directed agents have made an important contribution to the clinical management of cancers because of the crucial role of the cytoskeleton in cell replication and cell movement, but these agents are exclusively directed to the microtubule cytoskeleton. The vinca alkaloids disrupt microtubules while paclitaxel and related natural products rigidify microtubules (1). In both cases these classes of microtubule-directed agents prevent the crucial roles of microtubules from being completed in the target cells. In spite of the impressive effectiveness of microtubule-directed agents in cancer chemotherapy, no microfilament-directed agents have yet been shown to exhibit useful selective cancer chemotherapeutic effects in clinical cancers. Anticancer activity in cell culture systems has been exhibited by various cytochalasin congeners (2-6) and by other microfilament directed agents such as chaetoglobosins (7-9) latrunculins (10, 11), and jasplakinolide (12, 13).

Cytochalasins are mycogenic toxins derived from a variety of fungal sources. The congeners are characterized by a highly substituted perhydro-isoindolone structure that is typically attached to a macrocyclic ring. This macrocycle can vary tremendously among cytochalasins. Carbocycles, lactones or even cyclic carbonates have been identified (2), thereby producing a substantial variety of congeners. Cytochalasins have the ability to bind with microfilaments and block polymerization, subsequently preventing the elongation of actin. As a result of the inhibition of actin polymerization, cytochalasins alter cellular morphology, inhibiting cellular processes such as cell division, and can even induce apoptosis (2-6). Cytochalasin B has shown particular promise in pre-clinical cancer chemotherapy models systems because it appears to preferentially damage malignant cells through multiple mechanisms (14, 15). The cytokinesis inhibitor preferentially enlarges and multinucleates leukemia cells in the presence of normal blood cell populations, making the cells more susceptible to physicochemical therapeutic approaches such as sonodynamic therapy (SDT) and X-irradiation. In effect, malignant cells exposed to cytokinesis inhibitors, such as cytochalasins, have a highly perturbed cytoskeleton due to the disruption of actin polymerization. The cells become polynucleated because nuclear replication continues, but cytokinesis is not possible in the absence of functional microfilaments. In addition, neoplastic cells have exhibited marked increases in mitochondrial activity when exposed to cytochalasins, further amplifying the already excessive metabolic rates observed in tumorigenic growths (3). As such, this opens up the opportunity to use mitochondrial-directed agents that specifically target the organelle. Due to the wide diversity of mechanisms by which cytochalasins damage malignant cells, as well as their ability to preferentially damage leukemia cells in the presence of normal blood cells (3), it appears that microfilament-directed agents should be as valuable in clinical cancer management as are microtubule-directed agents.

However, the efficacy of this broad molecular family has been shown predominantly only in two specific structures (cytochalasins B and D) (2-6, 14-19), and more research is required to determine whether other cytochalasins have clinical potential. Therefore, many *in vivo* studies are needed to accurately assess the potential clinical utility of cytochalasins and other microfilament-directed agents. While there is a limited understanding of the comparative *in vivo* host toxicities in mice of cytochalasins, a suitable model system that permits large-scale examination *in vivo* of these agents which are very rare and expensive natural products has not been developed. Moreover, any approach which proposes to use physicochemical therapeutic approaches such as ultrasound in combination with chemotherapy requires a model system that permits whole-body administration of the physicochemical modality.

In terms of chemotherapeutic efficacy tests *in vivo*, the tolerated doses of each of the agents under investigation must be determined before pre-clinical trials can be carried out. For example, cytochalasin C is virtually identical in structure to cytochalasin D except for the positioning of one carbon-carbon double bond, yet cytochalasin C is 10 times less toxic in mice than is cytochalasin D (20). Even more intriguing, the effects of cytochalasins C and D on cells *in vitro* are essentially identical (2). Understanding the importance of their differences in toxicity, as well as determining whether the aberrant side effects of cytochalasin D can be mitigated, could be crucial for future *in vivo* studies. Further, cytochalasin B and its derivative 21, 22-dihydrocytochalasin B (DiHCB) are both 20-fold less toxic than cytochalasin D in mice and 10-fold less toxic than Cytochalasin A (the C-20-keto-derivative of cytochalasin B) (21). Nevertheless, insufficient *in vivo* data have been acquired to determine whether any of the cytochalasin congeners have profound anticancer activity. Therefore, the entire spectrum of related compounds is of potential chemotherapeutic interest. Modulating the *in vivo* toxicities of cytochalasins would allow higher doses to be tested for pre-clinical and clinical efficacy in the treatment of neoplastic growths.

Contrary to cytochalasins, jasplakinolide does not inhibit actin polymerization. Rather, it induces polymerization, and then rigidifies the formed microfilaments to prevent actin depolymerization (22, 23). The differences between cytochalasin congeners and jasplakinolide are akin to the differences between vinca alkaloids and taxanes. With the microtubule-directed agents the vinca alkaloids inhibit polymerization, while taxanes stabilize the polymers (24). As

exemplified with microtubules, stabilizing formed polymers rather than disrupting them can also have a deleterious effect on a target cell, suggesting that jasplakinolide is also a likely candidate for chemotherapeutic evaluation.

Unlike cytochalasins, jasplakinolide is derived from marine sponges (25). The compound is a cyclo-depsipeptide containing a tripeptide moiety linked to a polypeptide chain. When cells are treated with jasplakinolide at nontoxic dosages, recovery is marked by a misshapen cytoskeleton, and protrusions on the cell surface become readily apparent (26-28). When applied during mitosis, the compound can also induce the formation of multinucleated cells (27). Interestingly enough, jasplakinolide can induce bundling of Filaments (F)-actin in organisms that hardly ever exhibit this process (28), demonstrating that the compound substantially stimulates microfilament formation.

Current pre-clinical evaluation of potential anticancer agents, combinations and protocols for administration depend extensively on small mammalian models, particularly murine. In fact, current Food and Drug Administration (FDA) directives require pre-clinical data in two different mammalian systems before approval can be sought for clinical trials (29, 30). However, such animal models may not always be appropriate for initial pre-clinical evaluation. Mice must be physically handled to administer the test agents and many mouse tumor models require that the tumor challenge be directly injected into the mouse. Once a prospective chemotherapeutic agent has been administered to a mouse, it is unable to be removed. If rescue agents or other follow-up treatments are part of the protocol, these compounds must be directly administered to the mice.

To circumvent such issues in the initial pre-clinical evaluation of chemotherapeutic agents, small tropical fish models are often used to address and to alleviate some of the limitations cited for mouse models. Fish show avoidance responses and agitation when disturbed, but they do not show the anticipatory anxiety that is apparent when handling mice. In other words, fish do not show evidence that they recognize distress and trauma in another fish in a different tank in the same room, or even in the same tank (31). Fish can be easily and comfortably sedated with anesthesia in their water with no evidence of distress and they can be painlessly terminated by prolonged immersion in water containing anesthetics. Potential chemotherapeutic agents are added to the fishes' water rather than directly to fish (32). Consequently, hydrophobic agents concentrate in the fish, while hydrophilic agents partition more evenly between the fish and the water (31). Fish can be removed from the exogenous source of the test agents whenever this is desired. While the concentration of the chemotherapeutic agent already absorbed by the fish may continue to act, no additional agent will be taken up. Transfer of treated fish to a larger volume of water, possibly with a dissolved hydrophobic agent, should permit the *in vivo* lowering of the lipophilic agents initially partitioning into the fish. This allows for a type of control that is simply not feasible after direct injection into a mouse. Further, rescue agents or combination agents can be added to and removed from the tank water as desired.

Fish models present economic benefits as well. Zebrafish drug screening costs are low due to manageable acquisition, maintenance and disposal fees. In addition, zebrafish have a rapid gestation period, as well as a small body size, allowing smaller doses to be administered in comparison to mammalian models (31, 32). This is particularly important for *in vivo* drug screenings, as many experimental chemotherapeutic agents are expensive, making preliminary large scale studies in mice fairly impractical. Sixty to 100 zebrafish can be treated with the amount of a rare natural product such as jasplaklinolide that would be needed for treating one 20 g mouse.

While *in vivo* fish models have been used substantially in recent chemotherapeutic agent development (33, 34), the apparent limitations of using a non-mammalian system prevent the acquired data from being directly translated in regards to potential clinical evaluation. Nevertheless, the convenience and sample sizes potentiated by fish models provide an initial assessment of whether the prospective chemotherapeutic agent has substantial anticancer activity. *In vivo* host toxicity can be evaluated allowing for the establishing of a treatment protocol that does not exceed the maximum tolerated dose. Therefore, this study seeks to determine the *in vivo* dose-limiting concentrations of cytochalasin congeners and jasplakinolide using zebrafish (*Danio rerio*), and comparing those tolerated doses in zebrafish expressed as μM drug concentration for a given exposure period to tolerated doses in mice expressed as mg/kg.

Materials and Methods

Zebrafish acquisition and maintenance. Zebrafish were bred in the Department of Biology and provided by Dr. Katharine Lewis (Department of Biology, Syracuse University, Syracuse, NY, USA). Additional zebrafish were acquired from a commercial source (Pet Solutions, Beavercreek, OH, USA). Fish were 300 to 400 mg. Fish were maintained in aerated deionized distilled water at 25˚C with 60 μg/ml Instant Ocean® aquarium salt (Instant Ocean United Pet Group, Blacksburg, VA, USA), 6 to 8 fish per 1500 ml.

Cytochalasin synthesis and preparation. Our laboratory has previously produced high performance liquid chromatography-(HPLC) pure crystalline cytochalasin B from *Helminthosporium dematioidia* and HPLC-pure crystalline cytochalasin D from *Zygosporium masonii*. The isomerization reaction that converts cytochalasin D to cytochalasin C (Figure 1A) was carried out using a Pd/charcoal catalyst at 25˚C. After filtration of the charcoal catalyst, cytochalasin C was isolated from any remaining cytochalasin D in the reaction product using C-18 reverse-phase thin layer chromatography (RP-TLC) plates with methanol:water, 75:25

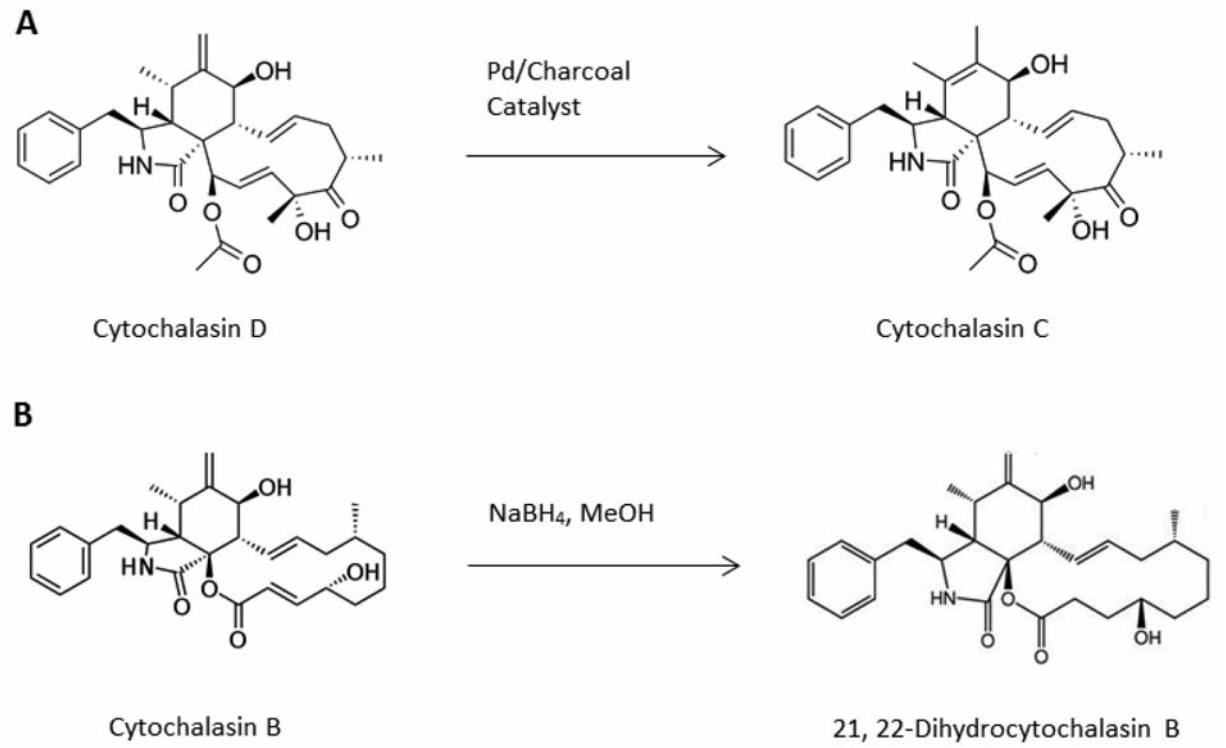

Figure 1. *Preparation of cytochalasin congeners. A) Catalytic isomerization reaction converts cytochalasin D to cytochalasin C. Note that the reaction merely moves the double bond to an adjacent carbon. B) Reduction reaction converts cytochalasin B to 21, 22-dihydrocytochalasin B. Both reactions were carried out at 25˚C.*

v/v as mobile phase, followed by fluorescence quenching. Since cytochalasin D has a markedly higher R_f value than cytochalasin C, the absence of cytochalasin D in the final cytochalasin C product can be established. A small amount of commercial cytochalasin C (Sigma-Aldrich Corp., St. Louis, MO, USA) was characterized by RP-TLC and recrystallized from acetone:hexane for comparison with the purified product. In addition, purified cytochalasin C was examined for purity by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (spectrum not shown). DiHCB was prepared by sodium-borohydride reduction of cytochalasin B in MeOH at 25˚C (Figure 1B). The product was recovered as a chloroform-soluble fraction and crystallized from benzene:hexane. As with cytochalasin C, DiHCB was compared to a commercially purchased sample of DiHCB (Sigma-Aldrich Corp.) and cytochalasin B (Poniard Pharmaceuticals, San Francisco, CA, USA) using RP-TLC. The product was also characterized with ^{1}H NMR spectroscopy. All cytochalasin congeners were solubilized in 95% ethanol (EtOH) to give a final ethanol concentration less than 0.14%. Stock solutions were maintained tightly sealed at –20˚C in an anhydrous environment.

Jasplakinolide acquisition and formulation. Jasplakinolide was acquired from Enzo Life Sciences (Enzo Biochem Inc., Farmingdale, NY, USA) in a 100 μg sample and was dissolved in 140 μl of rigorously anhydrous 100% MeOH to give 0.71 μg/μl (1 nmole/μl) 1 mM jasplakinolide stock solution. The stock solution was maintained tightly sealed at –20˚C in an anhydrous environment.

Administering cytochalasin congeners to assess comparative toxicities and to determine maximum-tolerated doses in vivo. Prior to treatment, zebrafish were removed from holding aquaria and washed in warmed, distilled water to remove thiols from the water in the holding aquaria. Each fish was then transferred to an individual experimental tank with the same concentration of aquarium salt and allowed to acclimatize for one hour before chemotherapeutic agents were administered. Zebrafish behavior and survival were monitored by direct observation and by time-lapse digital video-capture using Connectix® Quick-Cam (30 frames/min capture, playback at 10-frames/s; Logitech International S.A., Newark, CA, USA). Each treatment was administered to 8 individual zebrafish to generate a sample size sufficient to assess toxicity. Survival was monitored up to 72 h and zebrafish were blotted and weighed after death. Surviving zebrafish were returned to holding aquaria, but not used for further toxicity experiments. When necessary to prevent further suffering from drug toxicity, zebrafish were euthanized by prolonged exposure to 20 mg/100 ml tricaine mesylate (MS-222, Sigma Aldrich Corp.), and survival time was recorded at the time of MS-222 administration.

In order to modulate toxicities of cytochalasins *in vivo*, thiol agents were used to react with electron-acceptors generated from cytochalasin metabolism. Figure 2 shows the oxidation of cytochalasin B to cytochalasin A which is a potent thiol acceptor, due to the presence of its highly reactive α, β-unsaturated ketone group (35). Cysteine (CysSH) and ethyl-CysSH (CysEt), glutathione (GSH), 2-mercaptoethanol (BME) and thiocholesterol

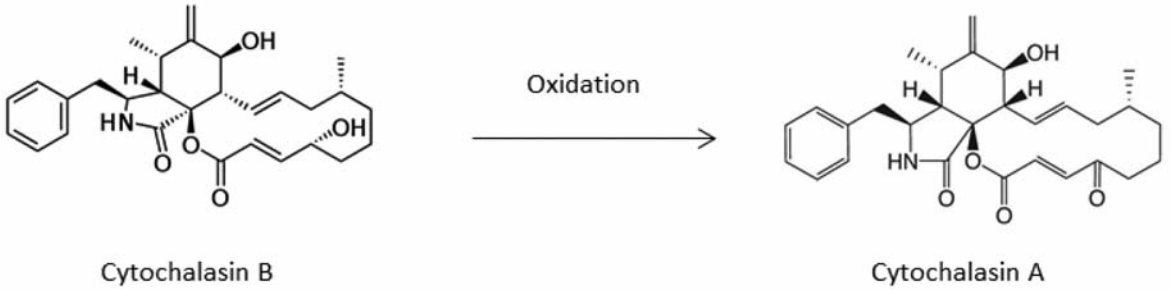

Figure 2. *Conversion of cytochalasin B to cytochalasin A. Although the congeners are extremely similar in structure, different functional groups are noted at C-20. Hydroxyl groups are readily converted into carbonyls through oxidation. Therefore, it is likely that the observed toxicity of cytochalasin B in vivo is due to oxidative conversion into its highly toxic congener, cytochalasin A.*

(TC) were added concurrently with cytochalasin B to act as thiol reagents or to test the importance of free thiols. S-methyl (MeGSH) and S-ethyl GSH (EtGSH) were used to establish the importance of the free thiol group in GSH for protection against toxicities. While cytochalasin D has a sterically hindered methyl group at C-20, it is possible that it too, or another hydroxyl group in the compound, is also oxidized. As such, GSH was also added to cytochalasin D in an attempt to reduce toxicity for chemotherapeutic assessment *in vivo*.

Determining jasplakinolide toxicity in vivo. Cytotoxicity of Jasplakinolide *in vitro* with U937 leukemia cells was determined with 2-fold serially-increasing concentrations from 8 nM to 1 μM. IC_{50} after 2 days was determined to be 0.5 μM. Cell size distribution determined with a TC20 cell counter and with a Z2 Coulter Counter gave a size range of 6 to 20 μm with no evidence of extreme cell enlargement that is exhibited by cytochalasin B and other cytochalasin congeners.

For determination of limiting doses of jasplakinolide *in vivo*, two adult 300 mg zebrafish were maintained in 50 ml of aerated water in a glass beaker suspended in a fish tank to maintain 25˚C. Jasplakinolide in MeOH (1 mM) was added to 0.5 μM (25 μl MeOH in 50 ml; 0.05% MeOH). Fish were observed continuously for 12 h and maintained overnight for 24 h, followed by a 96 hour observational period following cessation of treatment.

Results

Comparative toxicities of cytochalasin congeners in zebrafish. Figure 3 shows the comparative toxicities of Cytochalasins B, D, C and DiHCB. Cytochalasins C and D are compared at 1 μM. Cytochalasin B is evaluated at 5, 10, or 20 μM. DiHCB is shown at 10 μM. Cytochalasin D was clearly the most toxic congener with 80% lethality within 8 hours, and 100% lethality after 12 h. Cytochalasin C was far less toxic than its progenitor isomer, cytochalasin D. Cytochalasin C was more toxic than cytochalasin B or DiHCB. Cytochalasin B was not toxic at 5 μM, but demonstrated an increasing toxicity at 10 μM and lethality at 20 μM. Interestingly, DiHCB was not toxic at 10 μM, indicating that it was more tolerable than its oxidized congener.

Toxicity of jasplakinolide in zebrafish. No deleterious effects were observed during the 24 h drug exposure. However, one fish died during the 96 h follow-up assessment, and the other died shortly after. The determination of dose-limiting toxicity *in vivo* will require for testing at lower concentrations of jasplakinolide. Approval for such a test is being sought from the Institutional Animal Care and Use Committee (IACUC).

Mitigation of cytochalasin B and D toxicities with glutathione: Effect of thiol and alkyl-thiol agents. GSH at 10 μM (Figure 4, pink line) and 5 μM (Figure 4, blue line) protected against toxicity by 10 μM cytochalasin B (Figure 4, black line), while MeGSH (Figure 4 green line) and EtGSH (Figure 4, orange line), as expected, had no protective effect. Neither 10 μM CysSH (Figure 4, royal blue line), nor 10 μM BME (Figure 4, brown line) protected against 10 μM cytochalasin B toxicity. These thiol or alkyl-thiol agents, unlike glutathione, did not protect and in fact may have enhanced the toxicity of 10 μM cytochalasin B. The very high toxicity of 20 μM cytochalasin B (100% lethal within 8 hours) was delayed, but not mitigated with 20 μM or 50 μM GSH (Figure 4, red lines). Equimolar concentrations of GSH with 0.8 μM cytochalasin D delayed and reduced the toxicity of cytochalasin D in treated zebrafish while 20 μM GSH completely protected again 0.8 μM cytochalasin B which was otherwise 100% lethal after 23 h (Figure 5). TC actually decreased the viability of cytochalasin B-treated zebrafish as all were dead by 24 h, indicating that lipophilic thiol containing compounds may not be effective in reducing cytochalasin toxicity. As with TC, CysSH and BME did not produce a reduction in cytochalasin B toxicity, further indicating that GSH has special properties allowing it to reduce the *in vivo* toxicities of both cytochalasin B (Figure 4, rose and blue lines) and cytochalasin D at low concentration (Figure 5, rose line) or at high concentration (Figure 6, orange line).

Comparison of drug toxicity between zebrafish and mice. The tolerated doses of cytochalasins and jasplakinolide in zebrafish parallel those that are found in mice (Table 1). In addition,

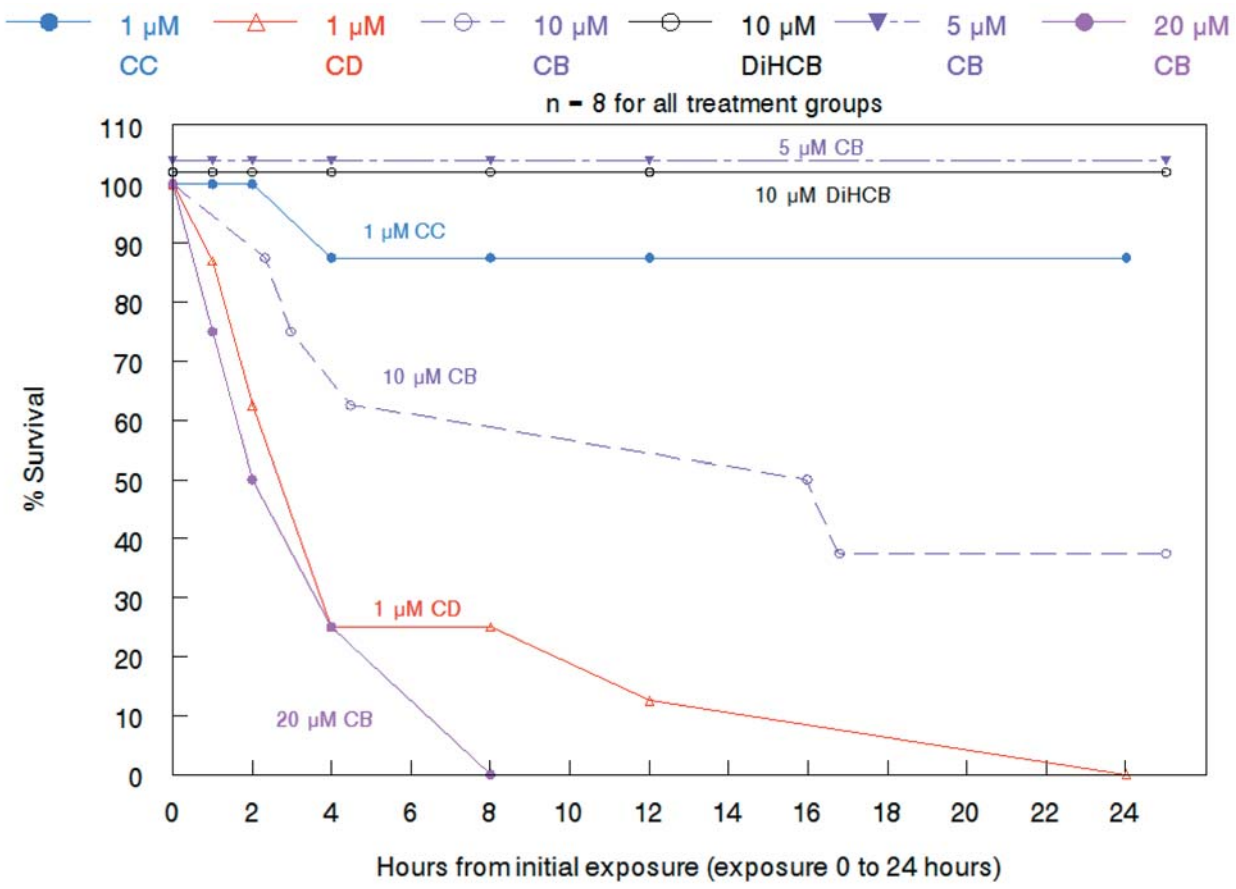

Figure 3. *Comparison of toxicities of cytochalasins congerers in zebrafish. Abbreviations used are as follows: CB (cytochalasin B), CD (cytochalasin D), DiHCB (21, 22-dihydrocytochalasin B), CC (cytochalasin C). All DiHCB-treated zebrafish remained viable beyond 96 h after the experimental period, indicating it was significantly less toxic than other cytochalasin congeners. Fish treated with 1 µM CC or 10 µM CB alive after 24 h also survived past the observational period.*

drug toxicities of clinically approved chemotherapeutic agents were found to establish a reference point of cytochalasin and jasplakinolide toxicity *in vivo*.

Discussion

The pre-clinical evaluation in tumor-bearing zebrafish of cytochalasins and other rare microfilament-directed natural products is an attractive prospect both from the point-of-view of the very limited availability of the agents and to make possible the development of whole-body physico-chemical treatment approaches amplifying the effects of the cytoskeletal-directed agents. Although cytochalasins B and D exhibited significant toxicity in treated zebrafish, the effects could be readily ameliorated with the addition of glutathione. Further, DiHCB was markedly less toxic in zebrafish than was cytochalasin B, and cytochalasin C synthesized from cytochalasin D was significantly less toxic than the progenitor compound. The toxicities of cytochalasin congeners *in vivo* can be mitigated through the choice, or chemical modification of the cytochalasin, or through the use of protective agents such as GSH.

The ability to mitigate the toxicities of cytochalasins B and D is of special importance to pre-clinical development as they are the only compounds in the cytochalasin family that have demonstrated repeated anticancer effects *in vitro* and *in vivo* (14-19). While the reduced toxicities of cytochalasin C and DiHCB in comparison with cytochalasin D and cytochalasin B respectively are intriguing, it has not yet been demonstrated that these congeners exhibit broad-spectrum efficacy in malignant cell lines or in tumor model systems *in vivo*. However, DiHCB has demonstrated effects in HeLa human cervical carcinoma cells (39), warranting further investigation of its chemotherapeutic potential.

The critical comparative evaluations of cytochalasin congeners and synthetic derivatives *in vivo* becomes far more

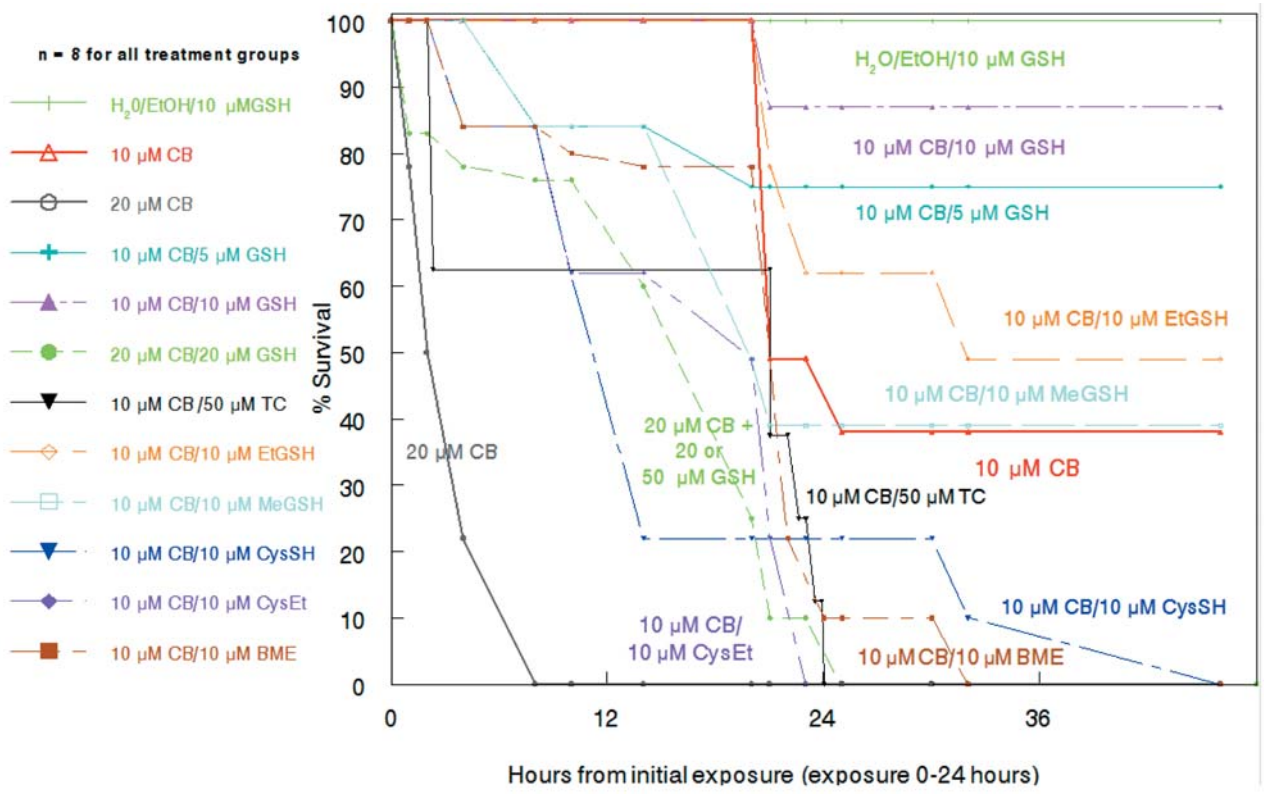

Figure 4. *Cytochalasin B toxicity at 10 µM and 20 µM in Zebrafish and the effect of glutathione and other SH and alkyl-S-R derivatives. GSH=glutathione (γ-L-Glutamyl-L-cysteinylglycine).*

feasible using a zebrafish vertebrate model prior to work with murine model systems because a 300-mg zebrafish is 1.5% the size of a 20-g mouse. Highly expensive congeners and rare synthetic derivatives of natural products affecting the cytoskeleton of normal, as well as neoplastic cells, can be tested in zebrafish, especially in conjunction with a zebrafish cancer model.

Comparisons of *in vivo* murine limiting doses (expressed in mg/kg) and zebrafish limiting doses (expressed as µM concentration in the water) show that the relative toxicities of cytochalasins and of Jasplakinolide were similar in both models. This is a very significant finding that will enable results from tumor-bearing zebrafish studies to be readily applied to later murine chemotherapeutic evaluations with an optimal use of these rare, highly expensive agents. Furthermore, establishing the tolerated doses of clinically-approved chemotherapeutic agents in zebrafish will enable cytochalasins to be evaluated for potential drug synergy with current chemotherapeutic protocols. This is an intriguing prospect, since cytochalasins are known cytokinesis inhibitors. Preventing rapidly proliferating neoplastic cells from successfully completing cytokinesis could be of substantial clinical importance, as such cells are sensitive to a variety of treatment modalities including physico-chemical approaches that preferentially exploit enlarged size and multinucleation leading to overproduction of intra-cellular nucleic acids. Malignant cells exposed to cytokinesis inhibitors have a highly perturbed cytoskeleton due to the disruption of actin polymerization, while concurrently developing multiple nuclei as a consequence of high proliferation rates (2-4, 38). This ultimately suggests that malignant cells exposed to cytokinesis inhibitors could have increased sensitivity to DNA-directed agents such as alkylators, antifolates, anthracyclines, and nucleoside analogs. Moreover, it has recently been demonstrated that actin polymerization plays a key role in cell-cell fusion (40) suggesting that agents that either disrupt or rigidify the microfilament cytoskeleton could affect cell fusion that contributes to the pathology of invasive cancer.

It also seems plausible that using microfilament-directed cytokinesis inhibitors in tandem with known microtubule-directed agents (epothilones, taxanes, and vinca alkaloids) could elicit important synergistic effects. In theory, this combination would present malignant cells with very limited opportunities to carry out mitosis successfully because the microtubule-directed agents would prevent proper formation of spindle fibers, while any cells that managed to evade this

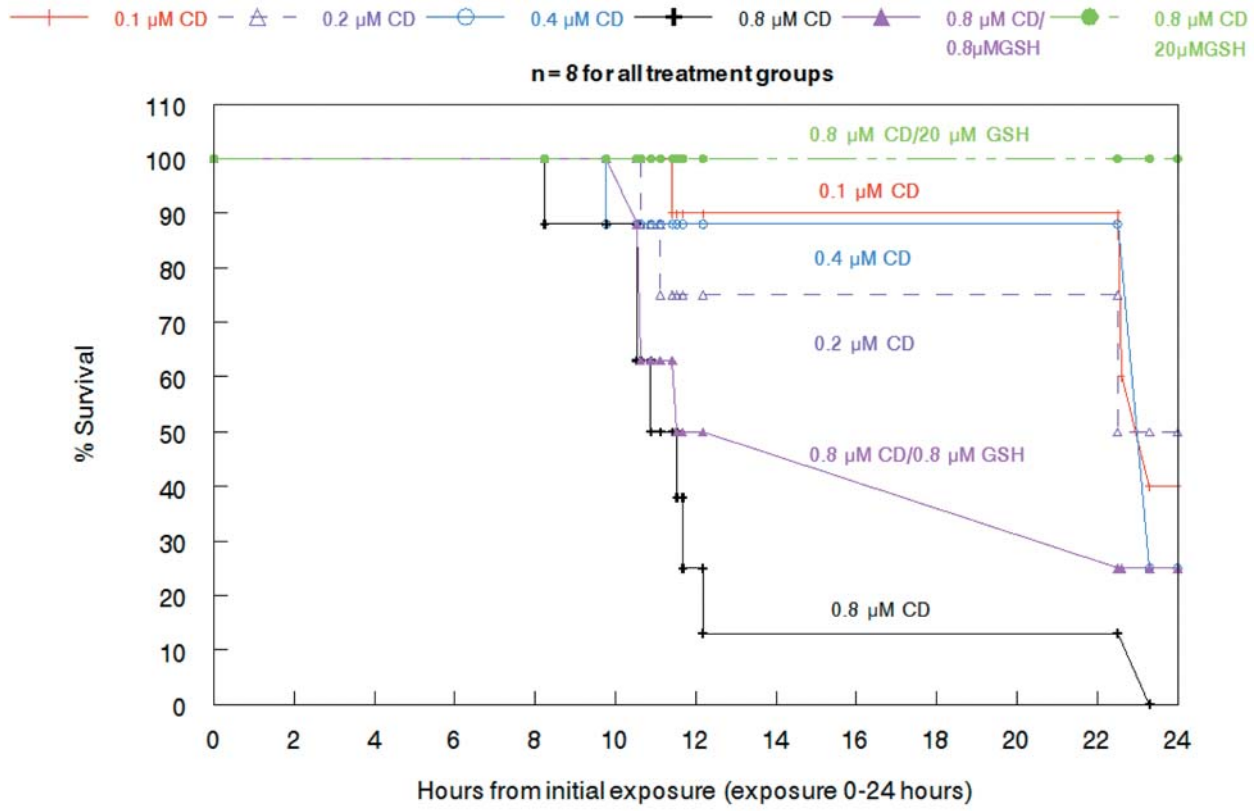

Figure 5. *Toxicity of cytochalsin D in zebrafish from 0.1 µM to 0.8 µM and the effect of glutathione. GSH=glutathione (γ-L-Glutamyl-L-cysteinylglycine).*

mechanism and replicate their nuclei would be unable to undergo cytokinesis. Such synergy has been demonstrated with cytochalasin B and vincristine (41), suggesting that this approach may be worth investigating in a pre-clinical model.

Another pivotal finding of this study is the significant reduction of cytochalasin B toxicity in the presence of GSH. It only took 5 µM GSH to substantially reduce the toxicity of 10 µM cytochalasin B in zebrafish. The proposed mechanism of this reduced toxicity may be explained by the hydroxyl group on C-20 of cytochalasin B. The C-20 hydroxyl group of cytochalasin B may oxidize to a ketone, thereby producing the highly toxic cytochalasin A (Figure 2). However, alkylating the hydroxyl group with a thiol through the use of GSH prevents cytochalasin A formation by sterically-hindering the formation of the ketone. Further, any cytochalasin A that does form would likely react with GSH, at the electrophilic α, β-unsaturated ketone group which reacts readily with thiols (35, 42, 43).

Such a mechanism is consistent with the experimental data. Modified GSH compounds that no longer contain a thiol (S-methyl and S-ethyl-glutathione) are unable to reduce the toxicity of cytochalasin B, thereby validating the importance of an active thiol nucleophile. The inability of a lipophilic thiol agent, TC, or of free cysteine to mitigate the toxicities of cytochalasin B suggests that glutathione has special properties in conferring protection. GSH is routinely used in physiological systems to neutralize reactive oxygen species (ROS) and other potentially cytotoxic electrophiles (42-45). More importantly, GSH preferentially reacts with soft electrophiles; precisely what is found at the C-20 of cytochalasin B. Soft electrophiles, such as hydroxyl groups found in the middle of cyclic carbon skeletons with no nearby electron withdrawing substituents, preferentially react with strong nucleophiles that have the propensity to polarize the electrophile for subsequent nucleophilic attack (46). As such, GSH or its conjugate base GS-, are ideal for reacting with the hydroxyl group at C-20 on cytochalasin B. Once alkylated, the functional group at C-20 would be substantially less likely to oxidize into a ketone, as is supported by the *in vivo* data. Whether this GSH protection will affect the anticancer efficacy of cytochalasin B *in vivo* is still undetermined, and will ultimately determine whether concomitant cytochalasin B/GSH treatments are applicable to tumor-bearing zebrafish and mouse models.

The *in vivo* tolerated dose of jasplakinolide in zebrafish is not yet clearly established. Although 0.5 µM jasplakinolide-

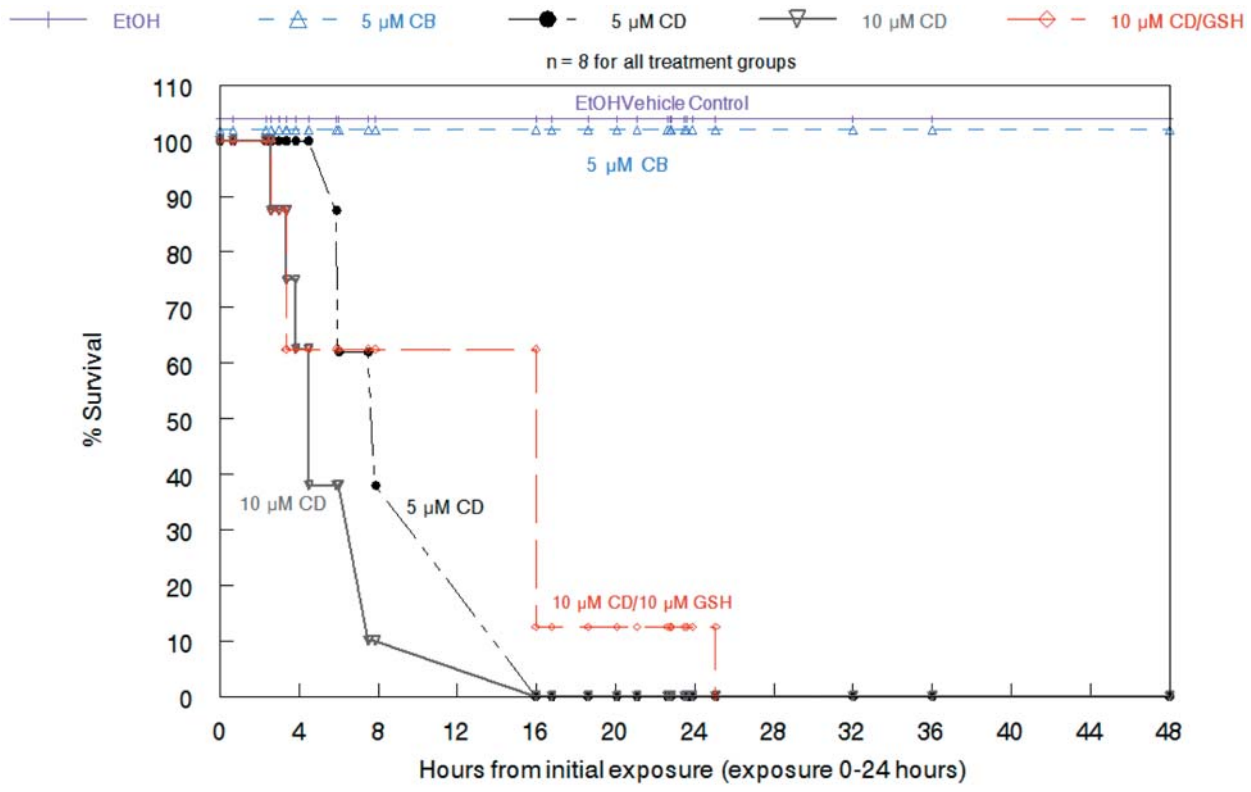

Figure 6. *Toxicity of cytochalsin D in zebrafish at 5 and 10 µM and the effect of glutathione. GSH=glutathione (γ-L-Glutamyl-L-cysteinylglycine).*

Table I. *Tolerated doses for clinically-active chemotherapeutic agents in zebrafish. Comparison with tolerated doses in mice.*

Chemotherapeutic Agent	Concentration (µM)	Fish Survival	Murine Tolerated Dose *i.p.* (mg/kg)
Cytochalasin B	5	8/8	50 (36)
Cytochalasin C	1	8/8	20-25 (20)
Cytochalasin D	0.2	4/8	1.9-2.6 (20, 37)
21,22-Dihydrocytochalasin B	10	8/8	-
Jasplakinolide	0.5	1/2*	20-21 (38)
Cisplatin	10	2/2	6.6 (Teva MSDS)
	50	2/2	
Dacarbazine (DTIC)	10	2/2	567 (Bedford Labs MSDS)
	28	2/2	
Vincristine	1	2/2	3 (Pfizer MSDS)
	3	2/2	
Vinblastine	1	2/2	2.7 (Bedford Labs MSDS)
Methotrexate (MTX)	45	2/2	50 (Bedford Labs MSDS)
5-Fluorouracil (5-FU)	80	2/2	100 (GeneraMedix MSDS)
Doxorubicin (DOX)	12	2/2	11.16 (Bedford Labs MSDS)
Paclitaxel (Taxol)	0.25	3/4	128 (Bedford Labs MSDS)
	0.5	1/4	
	1	1/8	
	2	0/4	
Kolliphor EL (Paclitaxel Vehicle)	-	2/2	-

Zebrafish were exposed to each chemotherapeutic agent individually for 24 h, with a 96 h follow-up to observe viability after exposure. References for *in vivo* murine tolerated doses are given in parentheses following the concentration. Doses given for murine intraperitoneal (*i.p.*) injection. *While one zebrafish treated with 0.5 µM jasplakinolide survived the 96 h follow-up assessment, it died shortly after.

treated zebrafish did not show any noticeable acute deleterious effects over a three-day period following a 24-h exposure to the agent, delayed toxicity was observed with one fish dying 4 days after the cessation of drug exposure, and the second one after 6 days. This suggests that jasplakinolide does not have significant acute toxicity, but could have a deleterious effect on long-term survival. Since lipophilic agents readily concentrate inside fish models after being administered through the water, it is conceivable that prolonged absorption for the 24 hour exposure time could allow for a high level of partitioning of the lipophilic jasplakinolide from the aqueous medium into the fish and could exert substantial damage to zebrafish physiological functioning. However, the sample size (n=2) for jasplakinolide is too small to propose definitive conclusions. The remarkably delayed toxicity of jasplakinolide is itself noteworthy and may reflect *in vivo* effects of microfilament rigidification that could be important in establishing the roles of microfilaments *in vivo*. Further testing of jasplakinolide using higher sample sizes will be needed to confirm these observations.

Based on the results of the present study, it is now feasible to examine cytochalasins and other rare cytoskeletal-directed natural products such as jasplakinolide for pre-clinical anticancer activity in a zebrafish model. In particular, cytochalasins B, C and DiHCB present favorable prospects for zebrafish-mediated chemotherapeutic trials, especially once the *in vivo* toxicity of cytochalasin B is modulated with GSH. The *in vivo* toxicity of cytochalasin D can also be modulated by GSH, but the ratio of GSH (20 μM) to cytochalasin D (0.8 μM) that is required to obtain protection may not be feasible *in vivo*. Nevertheless, cytochalasin D has demonstrated substantial efficacy *in vitro* and it is worth further examination. If microfilament-directed agents do prove to have clinical relevance, they could be concomitantly used with currently approved chemotherapeutic approaches to increase the efficacy of such protocols based on the multiple mechanisms by which these compounds damage malignant cells. These agents can also be used in conjunction with externally applied low frequency ultrasound to exploit the enlarged cell size and weakened cytoskeletal structure that is produced in neoplastic cells by the use of microfilament-directed agents (3).

Acknowledgements

Approval for zebrafish experiments was provided by the IACUC Protocol (#97-021). The Authors would like to thank Poniard Pharmaceuticals for providing additional cytochalasin B, as well as Dr. Katharine Lewis for providing zebrafish for the study.

Conflicts of Interest

The Authors declare no conflict of interest.

References

1 Weinberg RA: The biology of cancer, 2nd Edition. Garland Science 2013.

2 Van Goietsenoven G, Mathieu V, Andolfi A, Cimmino A, Lefranc F, Kiss R and Evidente A: *In vitro* growth inhibitory effects of cytochalasins and derivatives in cancer cells. Planta Med *77(7)*: 711-717, 2011.

3 Trendowski M, Yu G, Wong V, Acquafondata C, Christen T and Fondy TP: The real deal: using cytochalasin B in sonodynamic therapy to preferentially damage leukemia cells. Anticancer Res *34*: 2195-2202, 2014.

4 Kolber MA and Hill P: Vincristine potentiates cytochalasin B-induced DNA fragmentation *in vitro*. Cancer Chemother Pharmacol *30(4)*: 286-290, 1992.

5 Bogyo D, Fondy SR, Finster L, Fondy C, Patil S and Fondy TP: Cytochalasin-B-induced immunosuppression of murine allogeneic anti-tumor response and the effect of recombinant human interleukin-2. Cancer Immunol Immunother *32(6)*: 400-405, 1991.

6 Medina D, Oborn CJ and Asch BB: Distinction between preneopastic and neoplastic mammary cell populations *in vitro* by cytochalasin B-induced multinucleation. Cancer Res *40(2)*: 329-333, 1980.

7 Matesic DF, Villio KN, Folse SL, Garcia EL, Cutler SJ and Cutler HG: Inhibition of cytokinesis and akt phosphorylation by chaetoglobosin K in ras-transformed epithelial cells. Cancer Chemother Pharmacol *57(6)*: 741-754, 2006.

8 Tikoo A, Cutler H, Lo SH, Chen LB and Maruta H: Treatment of Ras-induced cancers by the F-actin cappers tensin and chaetoglobosin K, in combination with the caspase-1 inhibitor N1445. Cancer J Sci Am *5(5)*: 293-300, 1999.

9 Knudsen PB, Hanna B, Ohl S, Sellner L, Zenz T, Döhner H, Stilgenbauer S, Larsen TO, Lichter P and Seiffert M: Chaetoglobosin A preferentially induces apoptosis in chronic lymphocytic leukemia cells by targeting the cytoskeleton. Leukemia *28(6)*: 1289-1298, 2014.

10 Konishi H, Kikuchi S, Ochiai T, Ikoma H, Kubota T, Ichikawa D, Fujiwara H, Okamoto K, Sakakura C, Sonoyama T, Kokuba Y, Sasaki H, Matsui T and Otsuji E: Latrunculin a has a strong anticancer effect in a peritoneal dissemination model of human gastric cancer in mice. Anticancer Res *29(6)*: 2091-2097, 2009.

11 Sayed KA, Khanfar MA, Shallal HM, Muralidharan A, Awate B, Youssef DT, Liu Y, Zhou YD, Nagle DG and Shah G: Latrunculin A and its C-17-O-carbamates inhibit prostate tumor cell invasion and HIF-1 activation in breast tumor cells. J Nat Prod *71(3)*: 396-402, 2008.

12 Stingl J, Andersen RJ and Emerman JT: *In vitro* screening of crude extracts and pure metabolites obtained from marine invertebrates for the treatment of breast cancer Cancer Chemother Pharmacol 30(5): 401-406, 1992.

13 Senderowicz AM, Kaur G, Sainz E, Laing C, Inman WD, Rodríguez J, Crews P, Malspeis L, Grever MR and Sausville EA: Jasplakinolide's inhibition of the growth of prostate carcinoma cells *in vitro* with disruption of the actin cytoskeleton. J Natl Cancer Inst *87(1)*: 46-51, 1995.

14 Bousquet PF, Paulsen LA, Fondy C, Lipski KM, Loucy KJ and Fondy TP: Effects of cytochalasin B in culture and *in vivo* on murine Madison 109 lung carcinoma and on B16 melanoma. Cancer Res *50(5)*: 1431-1439, 1990.

15 Scherlach K, Boettger D, Remme N and Hertweck C: The chemistry and biology of cytochalasans. Nat Prod Rep *27(6)*: 869-886, 2010.
16 Huang FY, Mei WL, Li YN, Tan GH, Dai HF, Guo JL, Wang H, Huang YH, Zhao HG, Zhou SL, Li L and Lin YY: The antitumour activities induced by pegylated liposomal cytochalasin D in murine models. Eur J Cancer *48(14)*: 2260-2269, 2012.
17 Huang FY, Li YN, Mei WL, Dai HF, Zhou P and Tan GH: Cytochalasin D, a tropical fungal metabolite, inhibits CT26 tumor growth and angiogenesis. Asian Pac J Trop Med *5(3)*: 169-174, 2012.
18 Stracke ML, Soroush M, Liotta LA and Schiffmann E: Cytoskeletal agents inhibit motility and adherence of human tumor cells. Kidney Int *43(1)*: 151-157, 1993.
19 Małecki JM, Bentke A, Ostrowska B and Laidler P: Cytochalasin D, LY294002 and olomoucine synergize in promoting death of melanoma cells through activation of caspase-3 and apoptosis. Melanoma Res *20(1)*: 52-58, 2010.
20 Walling EA, Krafft GA and Ware BR: Actin assembly activity of cytochalasins and cytochalasin analogs assayed using fluorescence photobleaching recovery. Arch Biochem Biophys *264(1)*: 321-332, 1988.
21 Singh J and Hood RD: Effects of protein deficiency on the teratogenicity of cytochalasins in mice. Teratology *35(1)*: 87-93, 1987.
22 Terada Y, Simerly C and Schatten G: Microfilament stabilization by jasplakinolide arrests oocyte maturation, cortical granule exocytosis, sperm incorporation cone resorption, and cell-cycle progression, but not DNA replication, during fertilization in mice. Mol Reprod Dev *56(1)*: 89-98, 2000.
23 Henquin JC, Mourad NI and Nenquin M: Disruption and stabilization of β-cell actin microfilaments differently influence insulin secretion triggered by intracellular Ca^{2+} mobilization or store-operated Ca^{2+} entry. FEBS Lett *586(1)*: 89-95, 2012.
24 Chabner BA and Longo DL: Cancer chemotherapy and biotherapy: Principles and practice, 5th Edition. Lipincott Williams & Wilkins 2011.
25 Scott VR, Boehme R and Matthews TR: New class of antifungal agents: jasplakinolide, a cyclodepsipeptide from the marine sponge, Jaspis species. Antimicrob Agents Chemother *32(8)*: 1154-1157, 1988.
26 Makioka A, Kumagai M, Ohtomo H, Kobayashi S and Takeuchi T: Effect of jasplakinolide on the growth, encystation, and actin cytoskeleton of Entamoeba histolytica and Entamoeba invadens. J Parasitol *87(2)*: 399-405, 2001.
27 Holzinger A: Jasplakinolide. An actin-specific reagent that promotes actin polymerization. Methods Mol Biol *161*: 109-120, 2001.
28 Holzinger A and Meindl U: Jasplakinolide, a novel actin targeting peptide, inhibits cell growth and induces actin filament polymerization in the green alga Micrasterias. Cell Motil Cytoskeleton *38(4)*: 365-372, 1997.
29 Wing DA, Powers B and Hickok D: U.S. Food and Drug Administration drug approval: slow advances in obstetric care in the United States. Obstet Gynecol *115(4)*: 825-833, 2010.
30 Johnson JR, Ning YM, Farrell A, Justice R, Keegan P and Pazdur R: Accelerated approval of oncology products: the food and drug administration experience. J Natl Cancer Inst *103(8)*: 636-644, 2011.
31 Feitsma H and Cuppen E: Zebrafish as a cancer model: Mol Cancer Res *6(5)*: 685-694, 2008.
32 Epperly MW, Bahary N, Quader M, Dewald V and Greenberger JS: The zebrafish – Danio rerio – is a useful model for measuring the effects of small-molecule mitigators of late effects of ionizing irradiation. In Vivo *201226(6)*: 889-897, 2012.
33 Liu S and Leach SD: Zebrafish models for cancer: Annu Rev Pathol *6*: 71-93, 2011.
34 White R, Rose K and Zon L: Zebrafish cancer: the state of the art and the path forward. Nat Rev Cancer *13(9)*: 624-636, 2013.
35 Lagunoff D: The reaction of cytochalasin A with sulfhydryl groups. Biochem Biophys Res Commun *73(3)*: 727-732, 1976.
36 Lipski KM, McQuiggan JD, Loucy KJ and Fondy TP: Cytochalasin B: preparation, analysis in tissue extracts, and pharmacokinetics after intraperitoneal bolus administration in mice. Anal Biochem *161(2)*: 332-340, 1987.
37 Austin WL, Wind M and Brown KS: Differences in the toxicity and teratogenicity of cytochalasins D and E in various mouse strains. Teratology *25(1)*: 11-18, 1982.
38 Takeuchi H, Ara G, Sausville EA and Teicher B: Jasplakinolide: interaction with radiation and hyperthermia in human prostate carcinoma and Lewis lung carcinoma. Cancer Chemother Pharmacol *42(6)*: 491-496, 1998.
39 Martineau SN, Andreassen PR and Margolis RL: Delay of HeLa cell cleavage into interphase using dihydrocytochalasin B: retention of a postmitotic spindle and telophase disc correlates with synchronous cleavage recovery. J Cell Biol *131(1)*: 191-205, 1995.
40 Shilagardi K, Li S, Luo F, Marikar F, Duan R, Jin P, Kim JH, Murnen K and Chen EH: Actin-propelled invasive membrane protrusions promote fusogenic protein engagement during cell-cell fusion. Science *340(6130)*: 359-363, 2013.
41 Kolber MA and Hill P: Vincristine potentiates cytochalasin B-induced DNA fragmentation *in vitro*. Cancer Chemother Pharmacol *30(4)*: 286-290, 1992.
42 Armstrong JS, Steinauer KK, Hornung B, Irish JM, Lecane P, Birrell GW, Peehl DM and Knox SJ: Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line. Cell Death Differ *9(3)*: 252-263, 2002.
43 Davis Jr. W, Ronai Z, and Tew K: Cellular Thiols and Reactive Oxygen Species in Drug-Induced Apoptosis. J Pharmacol Exp Ther *296(1)*: 1-6, 2001.
44 Moran LK, Gutteridge JM and Quinlan GJ: Thiols in cellular redox signalling and control. Curr Med Chem *8(7)*: 763-772, 2001.
45 Deneke SM: Thiol-based antioxidants: Curr Top Cell Regul *36*: 151-180, 2000.
46 Ketterer B, Coles B and Meyer DJ: The role of glutathione in detoxication. Environ Health Perspect *49*: 59-69, 1983.

Received June 23, 2014
Revised August 18, 2014
Accepted August 22, 2014