Abstract. Background/Aim: Cytochalasin B is a mycogenic toxin that preferentially damages malignant cells through multiple mechanisms. The microfilament-disrupting agent inhibits cytokinesis, producing enlarged and multinucleated neoplastic cells without enlarging or producing multinucleated normal cells. In addition, cytochalasin B has been shown to induce apoptosis and to increase the mitochondrial activity of malignant cells. In spite of these pharmacological properties potentially exploitable in cancer chemotherapy, no cytochalasin congener or derivative and indeed no microfilament-directed agent has yet been examined in the clinic. Nevertheless, it will likely be necessary to combine microfilament-directed agents with other chemotherapeutic agents, and potentially with other anti-neoplastic modalities to amplify the mechanisms by which microfilament-directed agents inflict damage. These combinations could increase the likelihood of obtaining clinically useful activities with microfilament-directed agents and decrease the often inevitable emergence of drug resistance. Therefore, this study intends to determine appropriate chemotherapeutic agents to use concurrently with cytochalasin B and with other microfilament-directed agents. Materials and Methods: Since cytochalasin B has shown in vitro efficacy against anchorage-independent growth, as well as against attached malignancies, both U937 human monocytic leukemia and MCF7 human breast carcinoma cells were evaluated. These cell lines were assessed for their sensitivity to a comprehensive array of chemotherapeutic agents that could amplify the cytoskeletal effects of microfilament-directed agents or that could themselves be potentiated by the cellular effects of such agents. In addition, clinically-approved microtubule-directed agents, as well as clinically-active anti-neoplastic agents not specifically cytoskeletal-directed, were examined for their ability to potentiate cell enlargement, one of the hallmark features of microfilament-directed agents. Conditions for inducing optimal enlargement and multinucleation of neoplastic cells with cytochalasin B were also defined. Results: U937 and MCF7 cells have differing sensitivities to chemotherapeutic agents indicating that different regimens will likely be needed for various cell types in concomitant cytochalasin B-mediated chemotherapy. It was noted that microtubule-directed agents (paclitaxel and vincristine) would likely have a synergistic effect with cytochalasin B as they produced a substantial enlargement in viable cells at their 50% inhibitory (IC50) values. Interestingly, doxorubicin and mitomycin C also produced considerable cell enlargement, suggesting that nucleic acid-directed agents may be used to further enhance the cell-enlargement and multinucleation effects of microfilament-directed agents if appropriate sequences and concentrations can be found for the combination of agents. A subsequent publication in this series will examine the optimal combinations of chemotherapeutic agents with microfilament-directed agents in regards to drug concentrations and sequential timing. U937 cells exposed to cytochalasin B exhibited substantial cell enlargement and multinucleation that was still prevalent 8 days post-administration depending on the concentration used. Conclusion: Taken together, it appears that cytochalasin B has substantial synergistic potential with microtubule- and nucleic acid-directed agents.

This article is freely accessible online.

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Key Words: Chemotherapy, cytochalasin B, leukemia, breast carcinoma, cytoskeletal-directed agents, nucleic acid-directed agents, cell enlargement.

Although chemotherapeutic approaches have long exploited the aberrant cytoskeletal features of neoplastic cells as a method to obtain preferential damage, there has yet to be an effective microfilament-directed agent approved for clinical use. Cytochalasin B, a mycogenic toxin derived from a
variety of fungal sources has the potential to answer this need because it is lower in toxicity than certain other cytochalasin congeners and is more readily-available and well-studied. This microfilament-disrupting agent has the ability to bind microfilaments and block polymerization, subsequently preventing the elongation of actin (1). As a result of the inhibition of actin polymerization, cytochalasin B alters cellular morphology inhibiting cellular processes, such as cell division, producing greatly enlarged and multinucleated cells, and can even induce apoptosis (2-4).

Cytochalasin B appears to preferentially damage malignant cells through multiple mechanisms. Its ability to disrupt actin polymerization inhibits cytokinesis by interfering with formation of the contractile ring, as well as the development of the cleavage furrow. Consequently, the cell does not divide and an immature actin cytoskeleton remains. However, the cell continues to form nuclei and eventually becomes grossly enlarged and multinucleated (5). Such cells have more DNA targets, potentially increasing the likelihood of apoptosis when combined with DNA-directed agents. Preferential damage to malignant cells is facilitated by the fact that normal cells exposed to cytochalasin B exit the cell cycle and enter the G0 state until sufficient actin levels are restored (1). In contrast, malignant cells do not exit the cell cycle when the microfilament cytoskeleton is compromised.

Malignant cells that have become grossly enlarged and multinucleated might also be an ideal target for physicochemical therapeutic approaches, such as sonodynamic therapy (SDT). This is an ultrasound-mediated therapy in which specialized chemotherapeutic agents known as sonosensitizers are administered to increase the preferential damage elicited by ultrasound against neoplastic cells. SDT is an attractive prospect as the target cells are unlikely to acquire substantial resistance towards ultrasound. While neoplastic cells have a considerable propensity to acquire drug resistance to persistent chemotherapeutic exposure, it is very difficult for cytoskeletal integrity to be reinforced prior to or after exposure to a physical challenge, such as SDT (1). Further, the multinucleated cells have cell volumes much larger than normal cells that do not continue with nuclear synthesis in the absence of functional microfilaments. This makes the enlarged cancer cells more susceptible to direct physical destruction by challenge with ultrasound, thereby eliciting preferential damage (5).

We have previously shown that cytochalasin B treatments do indeed preferentially damage leukemia cells, leaving normal blood cells reasonably unaffected (5). U937 monocytic leukemia cells treated with 1 or 2 μM cytochalasin B for 2 days become grossly enlarged and multinucleated (Figure 1) providing an ideal target based on their size differential with normal blood cells. Further, we have shown that cytochalasin B substantially increases mitochondrial activity, opening up the opportunity to apply mitochondrial-directed agents that specifically target the organelle (5). This is particularly important since leukemia cells already demonstrate a dramatic increase in mitochondrial activity to sustain the metabolic rates needed for rapid cell proliferation (1). Therefore, mitochondria
present yet another target by which to preferentially damage malignant cells treated with cytochalasin B and other microfilament-directed agents. U937 cells also show a marked reduction in long-term clonogenicity after being exposed to cytochalasin B treatments (5).

Cytochalasin B has already exhibited substantial anticancer activity in both *in vitro* (5, 14, 15, 17, 18) and *in vivo* (13, 16) mammalian studies. Since clinical activity has not been achieved, it is likely necessary to combine cytochalasin B with other chemotherapeutic agents that amplify the mechanisms by which the microfilament-directed agent induces damage. Combination treatments should also significantly decrease the likelihood of inducing drug resistance. Therefore, this study intends to determine appropriate chemotherapeutic agents for use concurrently with cytochalasin B. Such agents were selected based on their likelihood to amplify the mechanisms by which the microfilament-directed agent damages neoplastic cells. We have established that cytochalasin B multinucleates MCF7 human breast carcinoma cells (Figure 2) indicating that the microfilament-directed agent may have chemotherapeutic applications for attached cell lines. As such, MCF7 cells were also examined for their response to the selected chemotherapeutic agents. In addition, the extent of cell enlargement and multinucleation potentiated by cytochalasin B was observed over the course of an 8-day period. This enabled a better characterization of the effects cytochalasin B has on U937 cells, which will be very important for determining agents likely to promote drug synergy. A subsequent study will then assess the efficacy of concomitant chemotherapy mediated by cytochalasin B-induced microfilament disruption.
<table>
<thead>
<tr>
<th>Chemotherapeutic agent</th>
<th>Drug class</th>
<th>Mechanism of action</th>
<th>Clinical applications</th>
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<tr>
<td>5-Fluorouracil (5-FU)</td>
<td>Nucleic acid-directed agent, antimetabolite</td>
<td>A pyrimidine analog used as a thymidylate synthase (TS) inhibitor. Interrupting TS blocks synthesis of the pyrimidine thymidine, a nucleoside required for DNA replication. TS also methylates deoxouridine monophosphate (dUMP) to form thymidine monophosphate (dTMP). Therefore, administration of 5-FU causes a scarcity in dTMP, perpetuating thymineless cell death. Calcium folinate provides an exogenous source of reduced folinates and hence stabilizes the 5-FU-TS complex, thereby enhancing the cytotoxicity of 5-FU.</td>
<td>Used systemically for anal, breast, colorectal, esophageal, stomach, pancreatic and skin cancers (particularly head and neck cancers). Can be administered intravenously or topically, depending on the cancer type.</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>Nucleic acid-directed agent</td>
<td>Acts through induction of DNA strand breaks and incorporation of thymidine into DNA strands. DNA cleavage by bleomycin depends on oxygen and metal ions. There are two proposed hypotheses in regards to the mechanism of bleomycin: (i) Bleomycin chelates metal ions, producing a pseudoenzyme that reacts with oxygen to produce superoxide and hydroxide free radicals that cleave DNA. (ii) Bleomycin binds at specific sites in the DNA strand and induces scission by abstracting hydrogen atoms from nucleotide bases resulting in strand cleavage. In addition, bleomycin mediates lipid peroxidation and oxidation of other cellular molecules.</td>
<td>Used in the treatment of Hodgkin’s lymphoma (as a component of the ABVD and BEACOPP regimen), squamous cell carcinomas and testicular cancer. Administered through intravenous infusion.</td>
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<tr>
<td>Carmustine, BCNU (bis-chloroethyl-nitroso-urea)</td>
<td>Nucleic acid-directed agent</td>
<td>As a bifunctional alkylating agent, BCNU alkylates DNA and RNA. Such reactions can cross-link DNA and inhibit several enzymes by carbamoylation. It is cell-cycle phase non-specific and is generally not cross-resistant with other alkylating agents (cross-resistance between carmustine and lomustine has occurred).</td>
<td>Used in the treatment of several types of brain cancer (including medulloblastoma and astrocytoma), multiple myeloma and lymphoma (Hodgkin’s and non-Hodgkin’s). BCNU is sometimes used in conjunction with alkyl guanine transferase (AGT) inhibitors, such as O6-benzylguanine as the AGT-inhibitors increase efficacy by inhibiting the Direct Reversal pathway of DNA repair. Administered through intravenous infusion.</td>
</tr>
<tr>
<td>Cisplatin (cis-diamminedichloroplatinum(II))</td>
<td>Nucleic acid-directed agent</td>
<td>Following administration, a chloride ligand is slowly displaced by water (aquation). The aqua ligand is itself readily displaced allowing the Pt atom to bind nitrogenous bases, particularly guanine. Subsequent formation of this guanine complex enables crosslinking to occur via displacement of the other chloride ligand, typically by another guanine. Cisplatin crosslinking activates DNA repair mechanisms in malignant cells, which in turn activate apoptosis when repair proves impossible. Note: although cisplatin is frequently designated as an alkylating agent, it has no alkyl group and so does not directly carry out alkylating reactions. It is correctly classified as alkylating-like.</td>
<td>Administered intravenously as short-term infusion in normal saline for treatment of solid malignancies. Used to treat various types of cancers, including sarcomas, some carcinomas (small cell lung carcinoma, ovarian carcinoma), lymphomas and germ cell tumors. Cisplatin is particularly effective against testicular cancers.</td>
</tr>
</tbody>
</table>
| Cytarabine (Cytosine Arabinoside)            | Nucleic acid-directed agent     | Combines a cytosine base with an arabinose sugar. Cytosine normally binds with deoxyribose to form deoxyctydine. Cytosine arabinoside is similar enough to human deoxyctydine to be incorporated into human DNA. Cytosine arabinoside triphosphate damages DNA during the S phase of the cell cycle. | Used mainly in the treatment of hematological malignancies, particularly acute myeloid leukemia (AML) and non-Hodgkin’s lymphoma. Cytarabine is rapidly
Table I. continued

<table>
<thead>
<tr>
<th>Chemotherapeutic agent</th>
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<th>Mechanism of action</th>
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<tbody>
<tr>
<td>Cytarabine also inhibits DNA and RNA polymerases, as well as nucleotide reductase enzymes needed for DNA synthesis (19).</td>
<td>Nucleic acid-directed agent</td>
<td>Alkylating agent that attaches an alkyl group ( (C_nH_{2n+1}) ) to DNA. The alkyl group is attached to the guanine base of DNA at the number 7 nitrogen atom of the purine ring, perturbing DNA synthesis (29).</td>
<td>Used in the treatment of various cancers, including malignant melanoma, Hodgkin’s lymphoma, sarcomas and islet cell carcinoma of the pancreas. Administered through intravenous infusion.</td>
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<tr>
<td>Doxorubicin (DOX)</td>
<td>Nucleic acid-directed agent, anthracycline</td>
<td>Intercalates DNA, preventing DNA replication and protein synthesis. DOX stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed, thereby inhibiting replication. Through intercalation, DOX can also induce histone eviction from chromatin (20). Has been shown to produce ROS (21) thereby inhibiting mitochondrial activity due to a cytotoxic increase of singlet oxygen.</td>
<td>Commonly used in the treatment of a variety of cancers, including hematological malignancies, carcinomas and soft tissue sarcomas. The drug is administered intravenously, as the hydrochloride salt.</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>Nucleic acid-directed agent, antimetabolite, antifolate</td>
<td>Causes competitive inhibition of dihydrofolate reductase, an enzyme that participates in tetrahydrofolate synthesis. This prevents production of thymidine, as well as all purine bases needed for DNA synthesis (24). Has been shown to affect the cytoskeleton of HeLa cells when used in SDT (25).</td>
<td>Effective in the treatment of a number of cancers including: breast, head and neck, leukemia, lymphoma, lung, osteosarcoma, bladder and trophoblastic neoplasms. Can be taken orally or administered by injection (intramuscular, intravenous, subcutaneous or intrathecal).</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Nucleic acid-directed agent</td>
<td>Crosslinks DNA through reductive activation followed by successive N-alkylations. Both alkylations are sequence-specific for a guanine nucleoside in the sequence 5'-CpG-3'. Potential bis-alkylating heterocyclic quinones have been synthesized to explore their antitumoral activities by bioreductive alkylation (22).</td>
<td>Administered intravenously to treat esophageal carcinoma, anal carcinoma and breast carcinoma. It is typically topically administered for bladder cancers and intraperitoneal tumors. A single instillation of mitomycin C within 6 hours of bladder tumor resection often prevents recurrence.</td>
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<tr>
<td>Paclitaxel (Taxol)</td>
<td>Cytoskeletal-directed agent, taxane</td>
<td>Cells treated with paclitaxel have defects in mitotic spindle assembly, chromosome segregation and cell division due to microtubule polymer stabilization. Inhibiting disassembly prevents chromosomes from achieving a proper metaphase spindle configuration. Prolonged activation of the subsequent mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell proliferation (35).</td>
<td>Used to treat patients with lung, ovarian, breast, head and neck carcinoma and advanced forms of Kaposi’s sarcoma. Administered through intravenous infusion.</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Cytoskeletal-directed agent, vinca alkaloid</td>
<td>Binds tubulin dimers, inhibiting assembly of microtubule structures and arresting mitosis in metaphase (31). In addition to being a mitotic poison, vincristine has also been shown to induce apoptosis in a variety of cancer cell types (31-33).</td>
<td>Used mainly in the treatment of hematological malignancies such as in non-Hodgkin’s lymphoma (CHOP), Hodgkin’s lymphoma (MOPP, COPP, BEACOPP) and the Stanford V chemotherapy regimen in acute lymphoblastic leukemia (ALL). Vincristine is also used for nephroblastomas. Administered through intravenous infusion.</td>
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Materials and Methods

**U937 cell and MCF7 cell preparation.** U937 human monocytic leukemia cells (ATCC® CRL-1593.2) were placed at 5.2×10^4 viable cells/ml in 20% fetal bovine serum (FBS) in Iscove’s medium without glutamine, with the following added: 200 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin sulfate, 40 μM glutamine (50 μl of 2 mM glutamine per 5 ml medium) and 240 μl amphotericin B (2.5 μg/ml concentration) per 5 ml of medium. MCF7 human breast carcinoma cells (ATCC® HTB-22) were kept and treated under the same conditions. Cells were incubated in 5% CO₂ in a humidified chamber at 37°C.

**Determination of comparative inhibitory concentrations of potential synergistic agents.** In order to later test for synergistic interactions with cytochalasin B, chemotherapeutic agents currently being used in the clinical setting with mechanisms of action likely to synergize with cytochalasin B were selected to determine their inhibitory potential against MCF7 and U937 cells (Table I). Each selected drug preferentially damages microtubules or cellular nuclei of malignant cells; mechanisms that might potentially synergize with cytochalasin B. Cells were treated at various concentrations to determine the 50% inhibitory concentration (IC_{50}) for each chemotherapeutic agent. Such values become particularly important in combinatorial therapy where drug toxicities are often additive or even show enhanced toxicities when used in combination. These synergistic anti-neoplastic effects of agents in combination can be graphically evaluated using isobolograms where the IC_{50} or IC_{99} or other therapeutic indices for the separate and combined agents are plotted (36). Therefore, this study is interested in finding and defining the pharmacological properties of chemotherapeutic agents that not only could have a substantial synergistic effect with cytochalasin B, but that are themselves cytotoxic for neoplastic cells at low concentrations and have a high therapeutic index arising from manageable host toxicities.

**Cells shown in Table II were seeded at 1×10^3 cells/ml and evaluated in early and mid-log-phase growth.** Cells were Wright-Giemsa and DAPI (4',6-diamidino-2-phenylindole) stained to examine nuclear structure.

We have previously used MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays to assess mitochondrial activity of U937 cells (5). Such assays can also be used to determine cell viability as rapidly proliferating cancer cells exhibit high rates of MTT reduction leading to the generation of a purple (590 nm) color (37). MTT assays were used to assess the IC_{50} point of each chemotherapeutic agent.

**Potential synergy with the cell enlargement potential of cytochalasin B.** To determine whether cytoskeletal-directed agents other than cytochalasin B induce cell enlargement, two clinically approved microtubule-directed agents (paclitaxel and vincristine) were evaluated using U937 and MCF7 cells. U937 cells are substantially more sensitive to cell enlargement by cytoskeletal-directed agents than are MCF7 cells (unpublished data) indicating...
that cell enlargement after drug exposure should be more readily observed with U937 cells. Cells were grown under the same conditions as in the IC50 value experiments. Although methotrexate (MTX) is a known nucleic acid-directed agent, it has been shown to influence cytoskeletal features in HeLa human cervical carcinoma cells (25) suggesting that this, or other nucleic acid-directed agents, may also have the potential to produce enlarged U937 cells. Therefore, U937 cells were treated with MTX, doxorubicin (DOX) and mitomycin C to assess the cell enlargement potential of nucleic acid-directed agents. Cells were also exposed to 2 μM cytochalasin B to provide a benchmark for comparison. Enlarged cells were defined as ≥20 μm, as untreated U937 cell populations are typically between 13-18 μm in size (5). Cell populations were treated with the designated chemotherapeutic agent at their corresponding IC50 values for 48 h and then filtered through 20-μm nylon mesh. Fractions of the remaining cell populations were then used to determine the % viable large cells after trypan blue staining. In addition, cell populations were sized through the use of a Z2 and TC10 counters, along with a Bio-Rad® TC10 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Assessing the extent of cell enlargement and multinucleation potentiated by cytochalasin B. To characterize the long-term effects that cytochalasin B-induced cytokinesis inhibition has on rapidly proliferating cancer cells, log-phase U937 cells were treated with varying concentrations of the microfilament-directed agent, ranging from 0-4 μM, over the course of 8 days. Cell enlargement was characterized by the Z2 and TC10 counters, while nuclear content was visualized with DAPI and Wright-Giemsa staining.

Results

Comparative chemotherapeutic efficacies. As expected, there were marked differences in the IC50 values obtained for the MCF7 and U937 cell lines (Table II). MCF7 cells appeared to be much more responsive to nucleic acid-directed agents exhibiting lower IC50 values for most drugs under this category (with exception to cytarabine, DOX and MTX) than did U937 cells. By contrast, MCF7 and U937 cells were similarly sensitive to cytoskeletal-directed agents. Many of the non-cytoskeletal-directed chemotherapeutic agents had IC50 values >50 μM indicating relatively low cytotoxicities. It is worth noting that five out of nine of the nucleic acid-directed agents were ineffective against U937 cells. Four nucleic acid-directed agents showed potential as effective inhibitors of multinucleated U937 cells exposed to cytochalasin B. Cytarabine had an IC50 value of 8 nM, while MTX (38 nM), DOX (100 nM) and mitomycin C (200 nM) also had reasonable efficacy with U937 cell populations.

Cell enlargement potential of cytoskeletal-directed and nucleic acid-directed agents. Both 6 nM paclitaxel and 4 nM vincristine produced a substantial proportion of U937 cells ≥20 μm (28% and 33%, respectively) (Figure 3). However, neither agent produced as high of a proportion of cell enlargement as was observed with 2 μM cytochalasin B where 92% of treated cells were ≥20 μm. Although MTX induced only a moderate increase in U937 cell size (Figure 3), two nucleic acid-directed agents, DOX and mitomycin C induced substantial cell enlargement in U937 cells 4 days post-administration (Figure 4). In particular, 38% of the mitomycin C-treated cells were ≥20 μm, while 72% of the DOX-treated cells were ≥20 μm after 4 days of exposure.

Table II. Differences in IC50 values between MCF7 and U937 cells.

<table>
<thead>
<tr>
<th>Chemotherapeutic agent</th>
<th>MCF7 48-h exposure</th>
<th>U937 48-h exposure</th>
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</thead>
<tbody>
<tr>
<td>5-Fluorouracil (5-FU)</td>
<td>12 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>~10 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Carmustine (BCNU)</td>
<td>&gt;50 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.5 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>500 nM</td>
<td>8 nM</td>
</tr>
<tr>
<td>Daclaborazine (DTIC)</td>
<td>&gt;50 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Doxorubicin (DOX)</td>
<td>~150 nM</td>
<td>100 nM</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>&gt;50 μM</td>
<td>38 μM</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>60 nM</td>
<td>~200 nM</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>28 nM</td>
<td>6 nM</td>
</tr>
<tr>
<td>Vincristine</td>
<td>5 nM</td>
<td>4 nM</td>
</tr>
</tbody>
</table>

Results were assessed for 5 independent populations of each cell type. Cells were seeded at 1x10^3 cells/ml for accurate MTT readings.
Figure 5. Comparison of varying concentrations of cytochalasin B to potentiate U937 cell enlargement. Cells were treated at concentrations ranging from 0-4 μM over the course of 8 days. A) The percentage of cells ≥20 μm. B) The percentage of cells ≥25 μm. The percentage of cells is based on a sample of 50 cells per treatment group.
Further, 6% of the mitomycin C-treated cells were ≥31 μm, substantially larger than the typical size range of U937 cells (13-18 μm). It should be noted that DOX was administered at twice the concentration of its IC50 value in U937 cells and cells were allowed to enlarge for 4 days, while mitomycin C was administered at its approximate IC50 value.

**Time-course effects of cytochalasin B on U937 cell enlargement and multinucleation.** The effects of cytochalasin B on potentiating cell enlargement and multinucleation in U937 cells were dose dependent. While 1 μM cytochalasin B-only produced modest cell enlargement that did not last throughout the 8-day observational period, 4 μM cytochalasin B induced substantial cell enlargement (Figure 5). For comparison, the maximum percentage of cells reaching ≥20 and ≥25 μm after treatment with 1 μM cytochalasin B was 40 and 22%, respectively. Further, no 1 μM cytochalasin B-treated cells were ≥20 μm after 8 days. By contrast, 4 μM cytochalasin B-treated cells reached a maximum of 90% ≥20 μm and 75% ≥25 μm. In regards to sustainability, 57% of the 4 μM cytochalasin B-treated cells were ≥20 μm after 8 days and 35% were ≥25 μm after the same length of time.

The dose-dependent effects of cell enlargement potential elicited by cytochalasin B were paralleled by its ability to induce multinucleation (Figure 6). Two days post-1 μM cytochalasin B administration, 84% of the cells were mononucleated and, by day 8, 95% were mononucleated. By contrast, only 58% of the 4 μM cytochalasin B-treated cells were mononucleated 2 days post-administration and only 30% were mononucleated after 8 days. This indicates that most U937 cells remained multinucleated 8 days after treatment with 4 μM cytochalasin B. Further, 5% of cells had >6 nuclei 2 days after treatment with 4 μM cytochalasin B and 15% of the cells had >6 nuclei after 8 days of treatment.

**Discussion**

It is apparent that chemotherapeutic agents for cytochalasin B-mediated concomitant chemotherapy against breast carcinoma will likely not be the same as the ones used in leukemia protocols. Experimental data acquired from this study revealed that while nucleic acid-directed agents are particularly effective against MCF7 cells, only three of the nine proposed agents attained lower IC50 values in U937 cell populations (Table II). Cytoskeletal-directed agents inhibited MCF7 and U937 cell growth in a similar fashion suggesting that concomitant use of cytoskeletal-directed agents with cytochalasin B would be effective against both cell lines.

Both microtubule-directed and nucleic acid-directed agents would benefit cytochalasin B-mediated concomitant chemotherapy because the microfilament-disrupting agent acts through multiple mechanisms. Cytochalasin B perpetuates the formation of multinucleated cells that are much more likely to undergo apoptosis as only a single nucleus has to be severely damaged to activate a chain reaction within the cell (1, 5, 10). Further, cytochalasin B is well-known for its propensity to inhibit actin polymerization, thereby halting cytokinesis within malignant cells. Since cytochalasin B is a microfilament-directed cytokinesis inhibitor, it seems likely that using the agent in combination with a known microtubule-directed mitotic inhibitor could elicit a substantial profound synergistic effect. In theory, this provides malignant cells very few opportunities to carry out a successful mitosis as the microtubule-directed agents...
would prevent proper formation of a spindle fiber, while any cells that managed to evade this mechanism and replicate their nuclei would be unable to undergo cytokinesis. Therefore, any malignant cells that manage to circumvent the microtubule-directed agents would likely be disrupted by the microfilament-directed agents. Both microtubule-directed agents (paclitaxel and vincristine) have considerable cell enlargement potential (Figure 3) suggesting that substantial drug synergy with cytochalasin B may be achieved as the two drug classes have distinct cytoskeletal targets. It should also be noted that the nucleic acid-directed agent MTX does appear to have at least some influence on cell enlargement (Figure 3), while DOX and mitomycin C exhibited a substantial cell enlargement effect (Figures 3 and 4). This is particularly intriguing and suggests that certain nucleic acid-directed agents could synergize with cytochalasin B through perturbation of the cytoskeleton, as well as increasing the likelihood of DNA/cell-cycle arrest-mediated apoptosis.

Despite the limited efficacy of most nucleic acid-directed agents against U937 cells, there were four that exhibited particular promise. In particular, cytarabine appeared to be a potent U937 cell inhibitor as it only took 8 nM to reach an IC\textsubscript{50} value (Table II). These results are expected as cytarabine is used predominantly to treat hematological malignancies (19). In addition, cytochalasin B has been shown to further potentiate the cytotoxicity of cytarabine in BHK/IV3 hamster renal tumor cells (38). Therefore, cytarabine can elicit a potentially useful synergistic effect with cytokinesis inhibitors, such as cytochalasin B, in the treatment of neoplastic cells. DOX and MTX also showed promise as U937 cell inhibitors at relatively small concentrations (~100 nM for DOX, 38 nM for MTX) were needed to reach an IC\textsubscript{50} value. While mitomycin C had a higher IC\textsubscript{50} value in U937 cells than with MCF7 cells (~200 nM for U937 cells, 60 nM for MCF7 cells), the relatively low concentration still makes the agent viable for cytochalasin B-mediated concomitant chemotherapy. It should also be noted that DOX has a considerable effect on malignant cell mitochondrial activity as it is a known reactive oxygen species (ROS) agent that produces singlet oxygen when combined with SDT (21). Since cytochalasin B increases the already high metabolic activity of U937 cells (5), it is likely that DOX would also be valuable in concomitant chemotherapy.

In regards to finding effective doses for concomitant chemotherapeutic evaluation \textit{in vitro}, cytochalasin B appears to exert its effects on neoplastic cells in a dose-dependent manner. Although 1 \textmu M cytochalasin B does have a noticeable short-term influence on cell enlargement (Figure 5) and nuclear content (Figure 6) in U937 cells, these effects are moderate and readily reversed within a few days of treatment. By contrast, the relatively small increase to 4 \textmu M substantially perturbs cytokinesis in most U937 cells. Not only does the higher concentration result in a much higher percentage of cell enlargement (Figure 5) and multinucleation (Figure 6), but the effects are still observed in a high proportion of treated cells 8 days after administration. We have established that normal blood cells are not influenced by 1.5 \textmu M cytochalasin B, indicating that the microfilament-disrupting agent does act preferentially towards malignant cells (5). However, it is unclear whether a dose elevation to 4 \textmu M would have a noticeable effect on normal cell viability and this will need to be confirmed before using the higher concentration in multidrug protocols. It should be noted that cytochalasin B has relatively low toxicity \textit{in vivo} (50 mg/kg intraperitoneally in mice (13-16) and 5 \mu M in zebrafish (39) are well-tolerated), suggesting that higher concentrations will be well-tolerated. Further, the maximum tolerated dose (MTD) of cytochalasin B in murine models can be increased by at least three-fold through the use of liposome encapsulation or co-administration with human recombinant interleukin-2 (rHIL-2) (16).

As demonstrated by this study, there appears to be clinically-approved chemotherapeutic agents that could exhibit substantial drug synergy with cytochalasin B. Although the regimens would vary based on the tumor type (as indicated by the difference between MCF7 and U937 cells), it appears that the multi-mechanism attack of cytochalasin B could be used in combination with a substantial diversity of chemotherapeutic agents. Since cytochalasin B-treated cells are considerably more sensitive to physical disturbances, such as ultrasound exposure (5), combining chemotherapeutic agents with physically-based modalities that exploit cell size and cytoskeletal integrity could provide new opportunities for combination chemotherapy. Indeed, SDT has shown to have both \textit{in vitro} and \textit{in vivo} efficacy with a variety of cell lines (40-43) and might eventually be used to supplement current protocols.

Regardless of the potential use of ultrasound in therapeutic measures, cytochalasin B appears to be a viable chemotherapeutic agent. Finding clinically-tested agents to supplement the mechanisms by which the microfilament-directed agent preferentially damages malignant cells could be important for breast carcinoma or leukemia therapy. Before that, cytochalasin B needs to be tested with other chemotherapeutic agents to see whether a substantial synergistic effect can be attained. As indicated by O’Neill - (38) and Kolber and Hill (18) cytochalasin B demonstrates considerable drug synergy with currently approved chemotherapeutic agents (cytarabine and vincristine, respectively). If cytochalasins and other microfilament-directed agents do prove to have clinical relevance, they could be used in combination with currently approved chemotherapeutic agents to increase the efficacy of such protocols.

**Conflicts of Interest**

The Authors declare no conflicts of interest.
References


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