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Review

Exploiting the cytoskeletal filaments of neoplastic cells to potentiate a novel therapeutic approach

Matthew Trendowski *

Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

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ABSTRACT

Although cytoskeletal-directed agents have been a mainstay in chemotherapeutic protocols due to their ability to readily interfere with the rapid mitotic progression of neoplastic cells, they are all microtubule-based drugs, and there has yet to be any microfilament- or intermediate filament-directed agents approved for clinical use. There are many inherent differences between the cytoskeletal networks of malignant and normal cells, providing an ideal target to attain preferential damage. Further, numerous microfilament-directed agents, and an intermediate filament-directed agent of particular interest (withaferin A) have demonstrated *in vitro* and *in vivo* efficacy, suggesting that cytoskeletal filaments may be exploited to supplement chemotherapeutic approaches currently used in the clinical setting. Therefore, this review is intended to expose academics and clinicians to the tremendous variety of cytoskeletal filament-directed agents that are currently available for further chemotherapeutic evaluation. The mechanisms by which microfilament directed- and intermediate filament-directed agents damage malignant cells are discussed in detail in order to establish how the drugs can be used in combination with each other, or with currently approved chemotherapeutic agents to generate a substantial synergistic attack, potentially establishing a new paradigm of chemotherapeutic agents.

Contents

* Tel.: +1 3158078991.
E-mail address: mrtrendo@syr.edu.

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1. Introduction

Cytoskeletal-directed agents have been a mainstay in chemotherapy due to their ability to readily interfere with the rapid proliferation of neoplastic cells. Malignant cells have a perturbed cytoskeleton due to the effects of dysplasia and subsequent anaplasia [1,2]. With so many alterations present in malignant cells, the cytoskeleton provides an ideal opportunity to attain preferential damage. Ever since vincristine began demonstrating clinical efficacy in the 1960s [3], the idea of disrupting the cytoskeleton of malignant cells during the mitotic phase has become widely considered in chemotherapeutic protocols. Along with paclitaxel (taxol) and the closely related docetaxel (taxotere) that make up the taxane drug family [4], vinca alkaloids (vinblastine, vincristine, vindesine, vinflunine and vinorelbine) have been used extensively to treat a variety of cancers, particularly hematological malignancies [2,5]. In recent years, the discovery of epothilones has furthered the development of cytoskeletal-directed agents as they have very similar *in vivo* effects to taxanes, but with higher efficacy, and reduced toxicity [6,7].

However, despite this apparent diversity of cytoskeletal-directed agents available to oncologists, all currently approved cytoskeletal-directed agents used in the clinical setting are essentially microtubule-directed agents. Although it is true that these compounds act by distinct mechanisms (taxanes and epothilones stabilize microtubules, while vinca alkaloids disrupt polymerization), they all have the same cytoskeletal target. Since microtubules are pivotal for mitosis, cell trafficking, and in some circumstances cell movement, inhibiting the dynamic instability of these polymers can be absolutely devastating for rapidly proliferating cells, henceforth ideal for disrupting tumorigenic growths [8,9]. While microtubule-directed agents have also been shown to induce apoptosis [10,11], they are inherently limited to one component of the cytoskeleton. The other potential targets, intermediate filaments and microfilaments, remain as elusive clinical prospects.

Cytoskeletal filaments are indeed viable targets to exploit in chemotherapy. Actin is inherently required for cell motility, cytokinesis, and many other processes vital for malignant cell stability [12–15]. Intermediate filaments such as keratins are often overexpressed in carcinomas due to the aberrant effects of associated oncogenes [16,17], and vimentin has been shown to be vital for cell survival in numerous experiments [18–20]. A substantial variety of microfilament-directed agents and one intermediate filament-directed agent in particular (withaferin A) have shown profound anticancer activity in a variety of cancer cell lines. Despite these compelling data, there has yet to be a clinically approved intermediate filament-directed or microfilament-directed agent used in cancer therapy. Therefore, this review is intended to expose academics and clinicians to the tremendous variety of cytoskeletal filament-directed agents that are currently available for chemotherapeutic evaluation (Fig. 1). It is hoped that such an analysis will provide enough data to warrant further *in vivo*, preclinical and eventual clinical trials of these compounds, thereby potentiating a new paradigm of chemotherapeutic agents.

2. Microfilaments as chemotherapeutic targets

Actin is a globular multi-functional protein that can be present as either a free monomer known as globular actin (G-actin), or as part of a microfilament polymer called filamentous actin (F-actin). In addition to being an ATPase that helps dictate its structure, actin is able to carry out more interactions than any other protein, allowing it to perform a tremendous diversity of functions necessary for cellular life, including chemotaxis and cytokinesis [21–24]. Actin polymerization is stimulated by nucleating factors such as the Arp2/3 complex, which mimics a G-actin dimer in order to stimulate G-actin nucleation. The Arp2/3 complex binds forming microfilaments to form new actin branches off existing polymers [23,24]. As an ATPase, actin binds ATP to stabilize microfilament formation, and hydrolysis of this nucleotide stimulates depolymerization [21]. The growth of microfilaments is regulated by thymosin and profilin; thymosin binds G-actin to buffer the polymerizing process, while profilin binds G-actin to exchange ADP for ATP, promoting monomeric addition to the barbed, plus end of F-actin [25]. Unlike many biological polymers, microfilaments are formed through non-covalent bonding, which enables filament ends to readily release or incorporate monomers [21]. Therefore, microfilaments rapidly remodel and change structure in response to environmental stimulus, giving such structures an assembly dynamic very similar to microtubules.

Along with microtubules, microfilaments are vital for successful cell proliferation. Shortly after the initiation of chromatid separation during anaphase, a contractile ring of non-muscle myosin II and microfilaments is assembled at the cell cortex [12,26]. Myosin II uses ATP hydrolysis to move along F-actin, constricting the cell membrane to form the cleavage furrow. The ingression of the cleavage furrow ultimately potentiates the abscission (the process by which the cell bodies are cleaved) which is entirely dependent on septin filaments beneath the cleavage furrow, as they provide structural support to ensure the completion of cytokinesis [14,26].

Due to the absolute requirement of microfilaments during cytokinesis, disrupting actin polymerization can exert profound effects on cellular structure. Cytokinesis inhibitors such as cytochalasin B disrupt the actin cytoskeleton and interfere with the formation of the contractile ring, as well as the development of the cleavage furrow [27,28]. Consequently, the cell is unable to divide, permeating a weakened cytoskeletal network. However, the cell is still able to initiate another mitotic event, continuing to form nuclei, and eventually becoming grossly enlarged and multinucleated [29,30]. Substantial multinucleation increases the likelihood of apoptosis, as it only takes a single nucleus to undergo programmed cell death before a chain reaction is triggered, culminating in the cell's destruction [1]. Further, the multinucleated cells have an increased cell volume and weakened cytoskeleton, making them more susceptible to physical agitation [31]. Preferential damage to malignant cells is facilitated by the fact that normal cells exposed to cytochalasin B exit the cell cycle and typically enter the G_0 phase until sufficient actin levels are restored [28]. As indicated by cultured BALB/c mouse mammary gland epithelial cells, normal mammary gland cells remain predominantly mono- or binucleate when exposed to cytochalasin B, while highly tumorigenic cell lines derived from mammary tumors become extensively multinucleated when cultured under the same conditions [32]. Further, cell lines derived from bladder, kidney, and prostate carcinomas become multinucleated when grown in cytochalasin B-supplemented medium, whereas cells from corresponding normal tissue remain mono- or binucleate under comparable conditions [29]. Therefore, only malignant cells that have lost the ability to enter the rest phase become grossly enlarged and multinucleated. Such cells are ideal targets for concomitant chemotherapy, as they have reduced cytoskeletal integrity, multiple nuclei, and even increased mitochondrial activity [31].

Actin is also of substantial importance to cancer cell migration. Carcinomas are the most prevalent form of cancer, constituting ~85% of all cases annually worldwide [1]. It has been well documented that dedifferentiated epithelial cells will undergo an epithelial–mesenchymal transition (EMT) in order to readily detach and migrate toward nearby vasculature [33–35]. This transformation into a motile cell type is typically only reserved for embryonic development and wound healing [1], and is a marked sign of cancer progression. Since these transformed cells are dependent on the recruitment of matrix-degrading proteases to reach endothelial tissue, it has been postulated that potent protease inhibitors may be able to significantly delay or even reduce the rate at which metastasis is observed [33–36]. However, transformed epithelial cells are also capable of amoeboid migration that is typically seen in lymphocytes and neutrophils. In this type of migration, cell–substrate adhesions are weak, resulting in the cell presenting a rounded morphology. When rounded cells migrate through the extracellular matrix (ECM), they change shape and squeeze themselves into

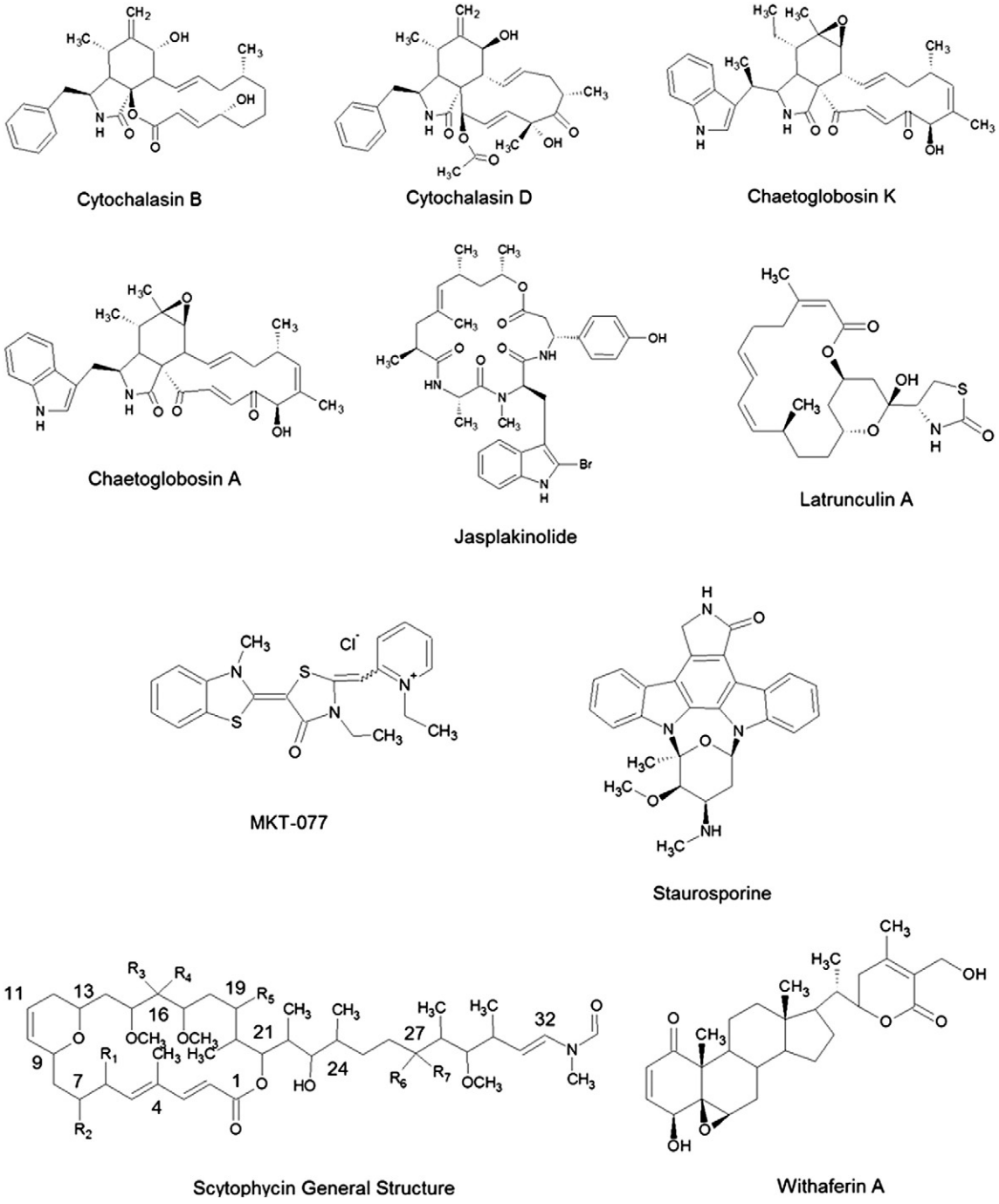

Fig. 1. A collection of cytoskeletal filament-directed agents. These compounds were reviewed to assess their chemotherapeutic potential. All compounds are microfilament-directed agents, except for withaferin A which is an intermediate filament-directed agent. The generalized structure of scytophycins is provided due to the novelty of the compounds. Bonds have been sequentially numbered to denote the naming system of the congeners. Scytophycins have seven R groups, indicating that the related compounds can still have substantial variability.

gaps in the ECM, thereby circumventing the need for proteases [33–36]. Cancer cell migration can convert between the mesenchymal and amoeboid types under certain conditions, particularly when exposed to protease inhibitors [37]. Therefore, it would be very difficult to repress cancer cell invasion by only targeting protease function.

However, the efficacy of protease inhibitors may be improved if amoeboid migration is sufficiently inhibited by targeting microfilaments needed for cell motility. As an example, amoeboid migration is driven by RhoA/ROCK-mediated bleb-like protrusions with active myosin/actin contractions and with cortical actin, but without the presence of stress fibers [38,39]. Inhibiting RhoA/ROCK signaling promotes the formation of multiple competing microfilament-derived lamellipodia that disrupt productive cell migration, thereby inhibiting amoeboid migration [38]. It is known that motile malignant cells unable to move through amoeboid migration will transition toward mesenchymal migration [33]. However, malignant cells exposed to protease inhibitors attempt to move through amoeboid migration. Using microfilament-disrupting RhoA/ROCK inhibitors in combination with protease inhibitors would simultaneously perturb both types of cancer cell migration. This would put metastasizing cells in a very difficult situation, as they

would have to traverse the ECM using deficient modes of motility. Such concomitant chemotherapy could substantially decrease metastatic efficiency, and is an area of research worth investigating.

Disrupting cancer cell migration may be further facilitated by adding tropomyosin isoforms such as Tm5NM1. Not only does the actin associated protein stabilize microfilaments and inhibit cell migration in two-dimensional culture systems [40–42], but it also stimulates down-regulation of Src kinase, substantially reducing pseudopodia formation [43]. Even more important to protease and RhoA/ROCK inhibitors is the fact that Tm5NM1 inhibits both the mesenchymal to amoeboid and amoeboid to mesenchymal transitions in HT-1080 human fibrosarcoma cells [43], suggesting that the addition of appropriate tropomyosin isoforms could produce considerable drug synergy.

The overexpression of microfilaments in metastatic cancers has been known for some time, as Schenk noted that carcinomas lacking abundant organized microfilaments did not promote invasion, while a prominent microfilament system was inherent in carcinomas producing metastases [44]. Mutations that affect cell migration are fundamentally pivotal in cancer progression, as more than 90% of cancer mortality is due to metastatic progression, rather than an invasive primary tumor [45,46]. Intravasation alone requires a series of biological events in which microfilaments are required, including tumor cell attachment to ECM components, the degradation of the matrix by tumor cell-associated metalloproteases, and tumor cell progression into the region where the matrix is modified by proteolysis [1,47]. In particular, invadopodia, ventral membrane protrusions seen in highly invasive carcinomas, are entirely dependent on actin and actin regulatory proteins [47]. Further, most transmembrane proteins, including growth factor receptors, adhesion proteins and ion channels, are either permanently or transiently associated with sub-membranous microfilaments [48]. Aberrant levels of growth factors, their associated receptors, and signaling intermediates are products of activated oncogenes, which stimulate microfilament expression, thereby perpetuating increased motility and cell proliferation. In other words, malignant cells often have abnormally high levels of microfilaments that promote phenotypes uncharacteristic of the cell type.

Due to the extreme importance of microfilaments in cell migration, it seems likely that preventing the formation of such structures would profoundly inhibit the motility of neoplastic cells. As shown in A549 human lung adenocarcinoma, MCF7 human breast carcinoma, and many other cancer cell lines, administering actin polymerization inhibitors substantially reduces cell motility, suggesting that chemotherapeutic intervention may potentially mitigate metastatic progression [49,50]. Further, inhibiting actin polymerization through the use of antigen-binding domains of Camelid heavy-chain antibodies substantially reduced the formation of invadopodia in MDA-MB-231 human breast carcinoma and PC3 human prostate carcinoma cells [51]. The nanobody delivery vehicles (or nanoparticles) used in the study specifically targeted fascin F-actin bundling, and also had a substantial influence on invadopodium array organization and turnover, matrix degradation, and cancer cell invasion, emphasizing the importance of stable actin bundles in the formation of these aberrant structures in malignant cells.

2.1. Cytochalasins

As indicated in both *in vitro* and *in vivo* experiments, microfilaments are of monumental importance to cancer progression, and are therefore an ideal target for chemotherapy. One of the most studied families of microfilament-directed agents has been the cytochalasins, mycogenic toxins known to disrupt the formation of actin polymers. Cytochalasins are characterized by a highly substituted perhydro-isoindolone structure that is typically attached to a macrocyclic ring. This macrocycle can vary tremendously between cytochalasins as carbocycles, lactones or even cyclic carbonates have been identified in these congeners [52]. In fact, more than 60 different cytochalasins from several species of fungi have been classified into various subgroups based on the size of the macrocyclic ring and the substituent of the perhydroisoindolyl-1-one residue at the C-3 position [53].

Although all cytochalasins demonstrate the propensity to bind microfilaments and block polymerization, the way in which each agent does so is unique [14]. Despite this diversity, only cytochalasins B and D have been extensively studied for their chemotherapeutic potential. While these congeners will be focused on, it is important to note that other cytochalasins have demonstrated anticancer activity. An *in vitro* study involving eight natural cytochalasins, and three hemisynthetic derivatives of cytochalasin B on six cancer cell lines indicated that most of the cytochalasins inhibit tumorigenic growths [53]. Particularly intriguing was the fact that the congeners were similarly effective against cancer cell lines displaying noticeable levels of resistance to pro-apoptotic stimuli when compared to cancer cell lines sensitive to these stimuli, tentatively suggesting that cytochalasins may be useful against malignancies known to circumvent apoptotic signaling.

In addition, some congeners that have yet to be extensively evaluated for anticancer activity demonstrate considerable activity against microtubules. Although cytochalasins are noted for their propensity to disrupt microfilaments, the unique α, β-unsaturated ketone moiety of cytochalasin A allows the congener to readily react with thiols [54,55]. As such, it has been shown that cytochalasin A reacts with critical thiol moieties on microtubules, with a binding site very similar to colchicine [56]. This interaction severely perturbs microtubules, thereby potentiating a novel mechanism by which the compound can damage malignant cells. Cytochalasin J is another potential microtubule drug synergizing agent, as it perturbs microtubules during mitosis, particularly the attachment of spindle microtubules with kinetochores [57–59]. This is supported by observations that the congener disrupts astral microtubules, and fragments spindle microtubules. Such interactions inhibit proper attachments to kinetochores, subsequently preventing proper chromosome congression [57–59]. This often results in chromosomes arranging near the periphery of the spindle or sometimes becoming completely detached from the spindle [58], and such a marked influence on mitosis could have profound toxicity for rapidly proliferating cancer cells. Further research will be needed to determine whether these and other congeners are also suitable candidates for chemotherapy.

Many studies that have examined the anticancer activity of cytochalasins concentrated their efforts on cytochalasin B as it appears to be a safer alternative to the more potent cytochalasin D; cytochalasin B is notably 20-fold less toxic than cytochalasin D in mice [60]. As such, there is sufficient experimental evidence to suggest that cytochalasin B is a potentially viable chemotherapeutic agent. Although cytochalasin B is most notably known for its propensity to preferentially induce cell enlargement and multinucleation in malignant cells [29,30,32], the microfilament-directed agent influences many other cellular processes as well. It has been long established that neoplastic cells often overexpress survivin and securin proteins, inhibiting apoptosis and promoting mitotic progression [61–63]. Survivin can also serve as a radio/chemoresistance factor during cancer therapy [64–66]. When tested on the human lung carcinoma cell lines A549 and H1299 known to highly express survivin proteins, cytochalasin B significantly decreased cell survival, inhibited cell growth, increased the levels of G2/M fractions, and induced multinucleation in both cell lines [67]. Further, cytochalasin B used concurrently with survivin small interfering RNA (siRNA) increased cytotoxicity and cell growth inhibition [67]. In addition, cytochalasin B has been shown to increase the sensitivity of U937 human monocytic leukemia cells to physicochemical therapeutic approaches such as ultrasound and X-radiation, as cells exposed to cytochalasin B have an enlarged and perturbed cytoskeleton, with aberrant actin bundles readily observed after treatment [31]. It has also been demonstrated that cytochalasin B substantially increases the mitochondrial activity of U937 cells [31]. This is a particularly pragmatic finding as leukemias and other cancers are well noted for dramatic increases in mitochondrial activity [1,2,28]. As such, using cytochalasin

B concomitantly with mitochondrial-directed agents may provide a dependable target to inflict preferential damage. U937 cells have even shown marked reductions in clonogenicity after being exposed to cytochalasin B treatments [31], indicating that the compound might effectively neutralize cancer's most prolific phenotypic characteristic: aberrant cell proliferation.

Cytochalasin B has also shown substantial *in vivo* activity. When cytochalasin B was injected subcutaneously (s.c.) at 10 or 100 mg/kg single doses 24 h after s.c. challenge of $B6D2F_1$ mice with trocar implants of B16F10 murine melanoma cells, the appearance of measurable tumor nodules was delayed by 93 and 157% respectively and extended host survival by 26 and 65% [67]. Tumorigenic growth was also delayed when cytochalasin B treatment was given 24 h after the appearance of notable tumor nodules in the mice. To supplement these findings, the same study introduced Madison 109 murine lung carcinoma cells into $CD2F_1$ mice s.c. before cytochalasin B was injected s.c. at 100 or 150 mg/kg 24 h after initial tumor challenge. As a result, cytochalasin B-treated mice showed a 66% delay in the median day of tumor nodule appearance. When administered under these conditions or at the time of nodule appearance, cytochalasin B markedly inhibited the rate of tumor growth, prevented tumor invasion at day 23, extended life span by 23%, and significantly inhibited spontaneous lung metastases measured 28 days after tumor challenge [68]. Although cytochalasin B can cause immunosuppression in mice, it is readily reversed through the introduction of human recombinant interleukin-2 (rhIL-2) [69]. In addition, cytochalasin B can be encapsulated in liposomes at dosages substantially higher (three times the maximum tolerated dose in mice) than those which normally suppress immune responses without inducing any appreciable immunosuppression [69].

Cytochalasin B also demonstrates a marked propensity to inhibit glycolysis *via* inhibition of GLUT transporters [70–73]. Considerable attention has been paid toward the chemotherapeutic potential of glycolysis transporters due to the observation that most cancer cells predominantly rely on elevated rates of glycolysis to sustain metabolic activity, rather than waiting on pyruvate oxidation in mitochondria for eventual oxidative phosphorylation [74–76]. Exploiting such an inherent difference between the malignant and normal states of many cell types is a sensible prospect to attain preferential damage in the clinical setting. It is possible that cytochalasin B may exert anticancer activity through glycolysis inhibition. Although this has yet to be critically examined, work from my laboratory has shown that cytochalasin B synergizes with doxorubicin (DOX) against DOX-resistant P388/ADR murine leukemia in a mechanism that is likely independent of glucose transport inhibition (unpublished data). This is due to the observation that 21,22-dihydrocytochalasin B (DiHCB), a reduced congener that does not inhibit glucose transport [77,78], appeared to be more synergistic with DOX in reducing the clonogenicity of P388/ADR cells. Nevertheless, this is a preliminary finding for a very specific case of drug interaction, and the potential anticancer effects of cytochalasin B potentiated through glucose inhibition warrant further investigation.

Although there is some concern over the potency of cytochalasin D, studies examining its potential for use as a chemotherapeutic agent have found promising results. In an *in vivo* study involving BALB/c mice challenged with murine colorectal carcinoma CT26 cells, mice were injected intravenously (i.v.) with various doses of cytochalasin D (12.5, 25, 50 and 100 mg/kg in 200 μL DMSO) [79]. The agent readily inhibited CT26 tumor cell proliferation in a time and dose dependent manner and induced significant CT26 cell apoptosis. The level of apoptosis was so high that it almost reached the level induced by the positive control TACS-nuclease. Further, cytochalasin D effectively inhibited tumor angiogenesis, indicating that the agent can attack tumorigenic growths through multiple mechanisms. Evidence of apoptotic events induced by cytochalasin D was also found in a study that observed the effects of cytochalasin D, LY294002 (morpholine-containing PI3K inhibitor), and olomoucine (purine derivative that inhibits cyclin-dependent kinases, and induces G1/S and G2/M arrest) on 5 separate melanoma cell lines [80]. While the drugs had only moderate effects when administered alone, concomitant chemotherapy was very effective in inducing caspase-3 activity as well as reducing cell viability. In particular, the triple combination of cytochalasin D/LY294002/olomoucine produced substantial caspase-3 activity and apoptosis, suggesting that a synergistic approach of cytochalasin D and other chemotherapeutic agents could be a potentially effective clinical strategy for melanoma patients.

As with its congener, cytochalasin D can also be encapsulated in polyethylene glycol (pegylated) liposomes to prevent immunological recognition until it reaches an intended tumor. In a comparative study between pegylated cytochalasin D and its natural form, pegylated cytochalasin D was more readily dissolved in water for intravenous injection, accumulated in tumor tissues more efficiently than natural cytochalasin D, and even had a significantly longer plasma $t_{1/2}$ (4 h *vs.* 10 min) [81]. All of these benefits were observed in liposomal cytochalasin D, while still retaining the antitumor activity of its parental compound. Significant inhibition of tumor angiogenesis and apoptosis was observed in mice treated with liposomal cytochalasin D (B16 melanoma model in C57BL/6N mice, and colorectal carcinoma CT26/hepatoma H22 models in BALB/c mice) [81]. It is important to note that no significant side effects were observed in mice treated with liposomal cytochalasin D, suggesting that this enhanced form of cytochalasin D may be a viable chemotherapeutic agent.

2.2. Chaetoglobosins

Chaetoglobosins are very close derivatives of cytochalasins, the main difference being an indol-3-yl group replacing the phenyl group at C-3 of the core carbon skeleton (perhydroisoindolone and macrocyclic ring). Due to structural similarities, chaetoglobosins exhibit many of the *in vitro* and *in vivo* effects on cells as do cytochalasins such as inhibiting actin polymerization and the capping of lymphocytes [82]. Further, chaetoglobosins have been shown to multinucleate malignant cells, indicating that these congeners are also cytokinesis inhibitors [83,84].

While chaetoglobosins have not been studied as extensively as cytochalasins in regard to chemotherapeutic potential, enough studies have been compiled to warrant further investigation. Chaetoglobosin K has shown particular anticancer activity in Ras-dependent cancer cell lines. The compound caps the plus-ends of F-actin, which contributes to its anti-Ras oncogenic activity through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-mediated pathway by preventing Ras from activating Rac [85]. This is important for influencing neoplastic cell transformation as Rac causes uncapping of F-actin at the plus-ends, through phosphatidylinositol 4,5 bisphosphate (PIP2), and eventually induces formation of a motile cell surface that contains a meshwork of newly polymerized actin filaments (membrane ruffling) [86,87]. In fact, it has been shown that the Ras-induced malignant phenotype of anchorage-independent growth is suppressed by overexpression of tensin which is normally used in cells to cap the plus-end of F-actin [85]. Chaetoglobosin K exhibited particular efficacy in WB-Ras1 rat liver epithelial cells, as even a non-cytotoxic dose inhibited both anchorage-dependent and anchorage-independent growth, as well as induced substantial multinucleation [88]. The study also confirmed that chaetoglobosin K decreases the level of phosphorylation of Akt kinase, a key signal transducer of the PI3K pathway. Since Ras mutations appear in at least 30% of human cancers [89,90], finding a chemotherapeutic agent that inhibits oncogenic overproduction of microfilaments, while inducing multinucleation for concomitant chemotherapy with nucleic acid agents would be of substantial clinical utility.

Although it has been less studied, the congener chaetoglobosin A also appears to exhibit substantial anticancer activity. An *in vitro* testing of chaetoglobosin A against 89 individual cell cultures taken directly from patients suffering from chronic lymphoid leukemia (CLL) revealed effective targeting of CLL cells by the compound independent of bad prognosis characteristics, such as 17p deletions or TP53 mutations

[91]. This is particularly important as current chemoimmunotherapeutic approaches reciprocate minimal clinical benefit against such prognostic factors, and patients presenting with these genotypes have little chance of survival [92,93]. As expected, chaetoglobosin A targeted microfilaments in CLL cells, thereby inducing cell-cycle arrest, as well as inhibiting cell migration. Further, it appeared that the compound prevented CLL cell activation and sensitized the cells to treatment with PI3K and Bruton's tyrosine kinase (BTK) inhibitors. Using current chemotherapeutic protocols, CLL is a treatable but incurable disease that often shows relapse due to the development of drug resistant cells. Therefore, chaetoglobosin A could be of particular benefit to CLL chemotherapy, hopefully helping to establish a protocol that can effectively cure patients.

2.3. Jasplakinolide

Contrary to cytochalasins and the closely related chaetoglobosins, jasplakinolide does not inhibit actin polymerization. Rather, it induces polymerization, and then rigidifies the formed microfilaments to prevent the inherent tendency of actin depolymerization [94,95]. The differences between cytochalasin congeners and jasplakinolide are akin to the vinca alkaloids and taxanes' method of attacking microtubules, as vinca alkaloids inhibit polymerization, while taxanes stabilize the polymers [2]. As with microtubules, stabilizing formed polymers can have just as much of a deleterious effect on a cell as inhibiting polymerization, suggesting that jasplakinolide is also a likely candidate for chemotherapeutic evaluation.

Unlike cytochalasins which are fungal in origin, jasplakinolide is derived from marine sponges [96]. The compound is a cyclo-depsipeptide containing a tripeptide moiety linked to a polypeptide chain. When cells are treated with jasplakinolide at nontoxic dosages, observations after drug removal indicate that a misshapen cytoskeleton forms, and protrusions on the cell surface become readily apparent [97–99]. When applied during mitosis, the compound can induce the formation of binucleate cells, although multinucleated cells are a rare occurrence [98]. Interestingly enough, jasplakinolide can induce bundling of F-actin in organisms that hardly ever exhibit this process [99], demonstrating that the compound substantially stimulates microfilament formation.

Although atypical of most microfilament-directed agents, jasplakinolide has also demonstrated substantial anticancer activity. In an *in vitro* study involving three human prostate carcinoma cell lines (LNCaP, PC3, and TSU-Pr1), only 41 nM jasplakinolide was needed to potently inhibit cell growth, with the mechanism of growth inhibition being directly associated to its influence on the actin cytoskeleton [100]. Jasplakinolide also appears to be a potent radiation sensitizer as observed *in vitro* and *in vivo* with DU-145, LNCaP, and PC3 human prostate carcinoma, as well as murine Lewis lung carcinoma cell lines [101]. Concomitant use of jasplakinolide and X-radiation produced a marked diminution in the shoulder of the survival curve of normally oxygenated PC3 cells. Further, the compound appeared to be a potent radiation sensitizer of hypoxic DU-145 cells and hypoxic PC3 cells. *In vivo*, jasplakinolide displayed substantial antitumor activity against Lewis lung carcinoma and DU-145 prostate carcinoma xenografts. In particular, the compound markedly increased survival of the mice, and reduced lung metastases due to Lewis lung carcinoma. Metastases were further decreased when jasplakinolide was administered along with X-radiation in s.c. primary tumors. In addition, concomitant jasplakinolide/X-radiation markedly delayed tumor growth. However, in the DU-145 tumor, the effects of jasplakinolide and fractionated radiation for 1–2 weeks appeared to be primarily additive, and not synergistic. Nevertheless, jasplakinolide might still be a beneficial addition to current radiation sensitizers, as the microfilament-directed agent damages malignant cells by a mechanism novel to radiation oncology.

2.4. Latrunculins

As with jasplakinolide, latrunculins are microfilament-directed agents derived from marine sea sponges. However, their mechanisms of action are much more similar to cytochalasins as the compounds bind actin monomers near the nucleotide binding cleft with 1:1 stoichiometry, thereby inhibiting polymerization [102]. It should also be noted that latrunculins typically bind G-actin as opposed to cytochalasins which have a high affinity for F-actin [103–105]. Consequently, while cytochalasins affect the kinetics of microfilament polymerization at both the barbed and pointed ends, latrunculins preferentially associate with actin monomers, thereby preventing subunits from repolymerizing into filaments.

Latrunculins are unique in structure as the 14 or 16 membered macrolide base is attached to the rare 2-thiazolidinone moiety which can be oxidized to form oxalatrunculin derivatives [106]. This structural component appears to be pivotal for latrunculin binding as the crystal structure of G-actin/latrunculin A complexes shows that the macrolide binds above the ATP-binding site between the two major domains of G-actin, with its unique 2-thiazolidinone moiety buried deep in the cleft [107]. Further, structural variations in the 2-thiazolidinone moiety among various latrunculin congeners confer substantial differences in binding affinity to G-actin, validating its importance in the inhibition of actin polymerization [108]. Once bound to G-actin monomers, latrunculin prevents actin polymerization by hindering the rotation of the two major domains associated with the G- to F-actin transition, inhibiting microfilament formation.

In regard to chemotherapeutic potential, latrunculin A has shown particular *in vitro* and *in vivo* efficacy against gastric cancer. The microfilament-directed agent was shown to induce substantial apoptosis *in vitro* in MKN45 and NUGC-4 gastric adenocarcinoma cells through activation of the caspase-3/7 pathway [109]. The importance of microfilaments in neoplastic cell homeostasis was readily apparent as cells treated with 5 μM latrunculin A for 1 h were swollen, and contained abnormal accumulation of vesicles within the cytoplasm. After 12 h, intracellular organelles became substantially compressed and erupted within the cells, inflicting considerable damage on the plasma membrane. Further, treated cells initially developed irregular filopodia, lamellipodia, and microvilli protruding from the cell surface before the fine actin network and stress fibers virtually disappeared after 24 h of treatment. *In vivo*, MKN45 and NUGC-4 cell-challenged mice exhibited significantly improved survival rates without any major side-effects after i.p. injection of latrunculin A [109]. Although the incidence and mortality of gastric carcinomas have decreased markedly over several decades due to improvements in sanitation conditions [1], it still remains prevalent in Asia as there is no effective therapy once peritoneal dissemination is observed [110–112]. Therefore, latrunculin A could potentially be used to supplement current chemotherapeutic protocols for gastric cancer if further *in vivo* evaluation warrants clinical trials.

Synthesized derivatives of latrunculin A also appear to potentiate a substantial anticancer effect, as exhibited by latrunculin A-17-O-carbamates in PC3 human prostate and T47D human breast carcinoma cells. Prepared by reacting latrunculin A with the corresponding isocyanate, the congeners exhibited potent anti-invasive activity against PC3 cells in a Matrigel™ assay [113]. While 1.5 μM latrunculin A decreased the disaggregation and cell migration of PC3-CT+ spheroids by three-fold, two different carbamate derivatives at the same concentration were two and half and five-fold more active than the progenitor compound. Further, latrunculin A and its 17-O-N-(benzyl)carbamate suppressed hypoxia-inducible factor-1 (HIF-1) activation in T47D cells, a clinically relevant finding as no chemotherapeutic agents have been clinically approved to specifically target tumor cells that have become hypoxic [113–115]. Similar results have been found in other analogs of latrunculin A (acetylated, esterified, and N-alkylated) that were specifically designed to modulate the binding affinity toward G-actin [116]. As with the carbamate series, these synthesized analogs demonstrated

anti-proliferative and anti-invasive properties against MCF7 and MDA-MB-231 cells. It should be noted that derivatives of latrunculin A were developed due to the observation that the hydrogen bond donation of the thiazolidinone NH is obligatory to latrunculin/G-actin binding [116]. In effect, the compounds were developed to optimize the activity of latrunculins' lactol hydroxyl and thiazolidinone NH groups, thereby conferring a higher binding affinity in malignant cells. Potentiating latrunculin/G-actin binding affinity could make the congeners more potent chemotherapeutic agents, and is an area of research worth further evaluation.

2.5. MKT-077

MKT-077 (1-ethyl-2-[[3-ethyl-5-(3-methyl-2(3H)-benzothiazolylidene)-4-oxo-2-thiazolidinylidene]methyl]-pyridinium chloride) is a highly water-soluble (>200 mg/mL) rhodacyanine dye that has significant antitumor activity in a variety of cancer cell types [117–119]. It has gained substantial notoriety due to its ability to inhibit the mitochondrial hsp70 (heat shock protein, 70 kDa) chaperones, including mortalin (mot-2), inducing the selective death of cancer cells [120], and was the first delocalized lipophilic cation with a favorable pharmacological and toxicological profile in preclinical studies [117]. Although it is more commonly known for its ability to induce apoptosis through mitochondrial pathways, MKT-077 is also a potent F-actin inhibitor, exerting a substantial influence on actin polymerization dynamics within malignant cells [121].

MKT-077 was first examined for its potential to induce mitochondrial-linked apoptosis due to the unique properties of lipophilic cations. The large membrane potential (150–180 mV) across the mitochondrial inner membrane can be exploited as a method by which to deliver molecules to mitochondria [122,123]. As such, MKT-077 readily traverses the lipid bilayers of mitochondria, as its charge is dispersed over a large surface area, allowing the potential gradient to drive its accumulation into the negatively-charged mitochondrial matrix. In fact, the uptake of lipophilic cations into mitochondria increases 10 fold for every 61.5 mV of membrane potential at 37 °C, leading to a 100–500 fold accumulation [122], and enabling such molecules to profoundly influence mitochondrial physiology. It has since been determined that MKT-077 is an allosteric inhibitor of Hsp70 chaperones, preferentially inducing apoptosis in neoplastic cells as Hsp70 chaperones are significantly up-regulated in tumors [124]. Along with neutralizing conformational changes in aberrant proteins, Hsp70 chaperones specifically inhibit cell death pathways, providing a viable chemotherapeutic target.

However, this is only half of the story. In addition to allosterically inhibiting Hsp70 chaperones, MKT-077 is known to substantially crosslink F-actin, producing aberrant microfilaments within malignant cells. It has been observed that MKT-077 binds a 45-kD protein (p45) and a 75-kD protein (p75) in Ras-transformed neoplastic cells, but not in parental normal cells [121,125]. It was subsequently demonstrated that p45 and p75 co-migrate with actin in SDS-PAGE, and that MKT-077 binds directly to purified G- and F-actin. Further, as with F-actin bundling proteins, the compound suppresses Ras transformation by blocking membrane ruffling [125]. As expected of F-actin crosslinkers/bundlers, sufficient concentrations of MKT-077 (300 μM) superprecipitates most microfilaments within treated cells. In accordance with these findings, the compound substantially lowers the specific viscosity of F-actin, and electron microscopy has confirmed that MKT-077 induces actin bundle formation, indicative of microfilament crosslinking [125].

Unlike other microfilament-directed agents, MKT-077 has been clinically evaluated in preliminary Phase I studies, and substantial data on patient toxicity and pharmacokinetics have been acquired. In one study that assessed the tolerability and pharmacokinetic behavior of MKT-077, 13 patients with advanced solid malignancies were administered MKT-077 as a 30 min i.v. infusion weekly for 4 weeks every 6 weeks at doses ranging from 42 to 126 mg/m^2/week [126]. The principal toxicity was renal magnesium wasting, which was subsequently improved with i.v. magnesium supplementation. However, dose escalation above 126 mg/m^2 was not considered feasible, and the recommended dose conjectured for this schedule of MKT-077 is 126 mg/m^2/week. Fortunately, this dosing schedule of MKT-077 produced a toxicity profile consistent with preferential accumulation of the chemotherapeutic agent within tumor cell mitochondria, and biologically relevant plasma concentrations were achieved. Pharmacokinetic data acquired for all patients revealed a low Cl_s (plasma clearance; 39 ± 13 L/h/m^2), large V_{ss} (apparent volume of distribution at steady state; 685 ± 430 L/m^2), and C_{max} (maximum serum concentration; 1.2 ± 0.31 to 6.3 ± 5.3 mg/mL) values that exceeded IC_{50} concentrations required for human CX-1 colorectal, MCF7 breast, CRL-1420 pancreas, EJ bladder, and LOX melanoma tumor cell lines *in vitro* (0.15 to 0.5 mg/mL).

Despite these promising results, another Phase I study noted that severe renal toxicity might potentially limit the utility of MKT-077 in the clinical setting [127]. In the study, 10 patients with advanced solid malignancies were treated at three dose levels: 30, 40 and 50 mg/m^2/day for a total of 18 cycles. In patients that experienced perturbed renal function, nephrotoxicity appeared by day 5 of the first cycle of treatment and recurred with each cycle of treatment. However, there was no evidence for cumulative renal toxicity, even in the patient who developed nephrotoxicity in each of the 11 treatment cycles. In regard to pharmacokinetics, the distribution of MKT-077 after termination of treatment was rapid, and the terminal $t_{1/2}$ was between 19 and 25 h. The elimination characteristics for the chemotherapeutic agent were similar to what were observed after single and weekly 30 min i.v. infusions. In addition, pharmacodynamic monitoring using ^{31}Phosphorus magnetic resonance spectroscopy (MRS) on skeletal muscle mitochondrial function revealed that MKT-077 inhibited mitochondrial function in patients, indicating that the chemotherapeutic agent did reach its intended target. Although substantial nephrotoxicity was observed, the study indicated that it is feasible to target mitochondria with MKT-077, suggesting that it or other rhodacyanine analogs can be used in the clinical setting if higher therapeutic indices can be developed. However, two preliminary Phase I clinical trials with contrasting results are insufficient to fully evaluate the clinical efficacy of MKT-077, and further study is required. Further, syntheses of MKT-077 derivatives that have increased activity against MDA-MB-231 and MCF7 cells, as well as a much higher theoretical plasma $t_{1/2}$ due to protection against oxidation at the benzothiazole and pyridinium rings, are promising potential chemotherapeutic agents [128], and may be able to reach the therapeutic indices necessary for clinical utility.

2.6. Staurosporine

Staurosporine is an antibiotic product of the bacterium *Streptomyces staurosporeus* [129]. The compound is a member of the indolocarbazoles, an alkaloid sub-class of bisindoles. The indolocarbazoles are frequently found as indolo(2,3-a)pyrrole(3,4-c)carbazoles which can be divided into two major classes; halogenated with a fully oxidized C-7 carbon and only one indole nitrogen containing a β-glycosidic bond, and non-halogenated, consisting of both indole nitrogen glycosylated compounds, and those with a fully reduced C-7 carbon [130,131]. Staurosporine is non-halogenated and contains a fully reduced C-7, and is therefore associated with the second non-halogenated class.

As with MKT-077, staurosporine is a microfilament-directed agent more commonly known for other influences on cellular physiology. The compound is a potent protein kinase inhibitor that exerts its effects by preventing ATP binding [132–134]. As a competitive inhibitor, staurosporine has a much stronger affinity to the ATP-binding site on the kinase than ATP. However, structural analysis of kinase pockets has indicated that main chain atoms which are conserved in their relative positions to staurosporine contribute to its affinity [135]. As such, concern has arisen that the compound will indiscriminately inhibit

kinases in malignant, as well as normal cells, although it has been demonstrated that leukemic lymphocytes are more sensitive to staurosporine than normal lymphocytes [134].

It has been established that staurosporine can induce apoptosis by activating caspase-3 proteases, which then cleave Forkhead box O3a (FOXO3a) [136–140]. This is important as FOXO3a is known to trigger apoptosis through up-regulating genes necessary for cell death, such as the Bcl-2-like protein 11 (Bim) [141,142] and the p53 upregulated modulator of apoptosis (PUMA) [139,143,144], as well as down-regulating anti-apoptotic proteins such as the FLICE-like inhibitory protein (FLIP) [145,146]. In addition, staurosporine has been shown to activate the caspase-dependent apoptotic pathway in human melanoma cells (Me4405, Me1007, IgR3, Mel-FH, Mel-RMu, Mel-RM, Mel-CV, and MM200) by release of cytochrome c and Smac/DIABLO from mitochondria, as well as cleavage of poly(ADP-ribose) polymerase (PARP) [147]. The study also indicated that a second, caspase independent apoptotic pathway may be involved in late apoptotic events induced by staurosporine, as overexpression of Bcl-2 inhibited the early onset of apoptosis, but not the later, caspase-independent pathway.

Although staurosporine is clearly a protein kinase inhibitor, it has also been demonstrated that the compound has a profound effect on the actin cytoskeleton of a variety of cell types. The protein kinase inhibitor was found to dramatically alter microfilaments in PTK2 epithelial cells, Swiss 3T3 fibroblasts, and human foreskin fibroblasts [148]. In particular, cells exposed to 20 nM staurosporine exhibited a progressive thinning and loss of cytoplasmic actin bundles within 60 min, while microtubule and intermediate filament systems remained intact. Similar results of microfilament perturbation have been observed in rat osteoblasts [149]. Not only did actin depolymerizers enhance the effects of staurosporine, but fluorescence labeling showed that staurosporine caused a substantial dissolution of microfilaments, again leaving microtubules and intermediate filaments intact in perturbed cells. While further studies are needed to confirm the extent of microfilament disruption in malignant cells, it is no doubt a mechanism by which staurosporine inflicts damage.

The potential indiscriminate inhibition of protein kinases elicited by staurosporine may be alleviated by liposomal encapsulation, akin to reducing the toxicity of cytochalasins through the same process. In one study, a novel reverse pH gradient liposomal loading method for staurosporine was developed [150], which produced 70% loading efficiency with good retention, and substantial *in vivo* antitumor activity in U87 human glioblastoma cells challenged in the flanks of nude mice. Biodistribution analyses revealed that the compound preferentially accumulated within tumors (although high accumulations were found in the spleen), and body weight data were unaffected by liposomal staurosporine, as opposed to the free compound which did reduce body weight. *In vitro*, liposomal staurosporine was shown to block Akt phosphorylation, induce PARP cleavage, and increase levels of apoptosis in U87 cells. Such results are intriguing, but further evaluation of this novel staurosporine derivation is needed to determine whether such results can be repeated. Nevertheless, brain cancers, particularly glioblastoma multiforme, are very problematic in the clinic, and finding novel compounds to supplement current chemotherapeutic and radiation protocols (particularly temozolomide and X-radiation) are of significant interest.

2.7. Scytophycins

Tumors often exhibit innate or acquired resistance to chemotherapeutic agents due to the overexpression of ATP-binding cassette (ABC) transporters, which efflux a substantial variety of compounds across cellular compartments [1,2]. In particular, the plasma membrane-spanning proteins permeability glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) confer resistance to vinca alkaloids, taxanes, and other bulky chemotherapeutic agents due to drug efflux [151–153]. However, scytophycins, a novel class of natural cytotoxins isolated from cyanobacteria of the family Scytonemataceae [154], are microfilament-directed agents that do not appear to be modulated by ABC transporters, a surprising find given the structural bulk of the compounds.

Scytophycins consist largely of polyketides and numerous 1,3-diol units that vary in structure and stereochemistry [154]. Due to the novelty of these compounds, a generalized carbon skeleton structure is presented in Fig. 1. It has been determined that scytophycins are potent, cytokinesis inhibitors with mechanisms very similar to cytochalasins. Treatment of KB HeLa contaminant carcinoma cells with the scytophycin tolytoxin (2–16 nM) results in profound morphological changes, including marked zeiosis (localized decoupling of the cytoskeleton from the plasma membrane, inducing membrane blebbing), and substantial nuclear protrusion [155]. Further examination revealed that tolytoxin inhibited actin polymerization, as well as depolymerized and fragmented F-actin. In L1210 murine lymphoid leukemia cells, cytokinesis was shown to be inhibited by 2 nM tolytoxin, inducing multinucleation. In addition, tolytoxin specifically disrupted microfilament organization in A10 rat myoblasts, while having no apparent effect on microtubules or intermediate filaments. Particularly intriguing was the observation that tolytoxin exerts very similar effects on microfilaments as cytochalasin B, but was effective at concentrations 1/1000 that of the known cytokinesis inhibitor [155].

Although limited data are available on the anticancer activity of scytophycins, a study demonstrating cytoxicity against drug sensitive (SKOV3) and drug-resistant (SKVLB1) human ovarian carcinoma cells is of particular interest [156]. In a comparison with cytochalasin B, microfilaments in SKOV3 and SKVLB1 cells were depolymerized by similar concentrations of tolytoxin, while cytochalasin B exhibited less potency. While SKVLB1 cells demonstrated >150 and 10,000-fold decreases in sensitivity to DOX and vinblastine, respectively, both cell lines were equally sensitive to the anti-proliferative effects of tolytoxin and a few other scytophycins. In regard to circumventing ABC transporter drug resistance, both tolytoxin and cytochalasin B synergized considerably with vinblastine toward SKVLB1 cells. While this does not directly indicate reversal of drug resistance, it appears that tolytoxin and other scytophycins are not subject to Pgp-mediated efflux from SKVLB1 cells exhibiting multidrug resistance due to overexpression of this transport protein, suggesting that the compounds may have clinical applications for drug-resistant tumors. Extensive *in vivo* assessment is needed to determine whether scytophycins exhibit the same antitumor activity as the mechanistically related cytochalasins.

3. Intermediate filaments as chemotherapeutic targets

Along with microfilaments, intermediate filaments are the other component of the cytoskeleton that has yet to be exploited in the clinical setting. All intermediate filaments have a central alpha-helical rod domain that is composed of four alpha-helical segments (named as 1A, 1B, 2A and 2B) separated by three linker regions [157]. The N and C-termini of intermediate filaments are non-alpha-helical regions that are considerably diverse in terms of length and polypeptide sequence [157]. As opposed to microtubule and microfilament formation, intermediate filaments are constructed from a parallel and in-register dimer, which forms through the interaction of the rod domain to form a coiled coil [158,159].

Intermediate filaments typically assemble into non-polar unit-length filaments (ULFs) that can then interact to form staggered, antiparallel, soluble tetramers [160]. These structures associate head-to-tail into protofilaments that pair up laterally into protofibrils; four of these protofibrils then wind together into an intermediate filament. Interestingly, the N-terminal head domain binds DNA, and vimentin (type III intermediate filament) heads are routinely used in normal cell physiology to alter nuclear architecture and chromatin distribution [161,162]. Intermediate filaments are also distinct from other cytoskeletal components in that its anti-parallel orientation of tetramers does

not enable polarity, as is found in microfilaments and microtubules. Therefore, intermediate filaments are not routinely involved in cell motility and intracellular transport [157]. Also, as opposed to actin or tubulin, intermediate filaments do not contain a binding site for a nucleoside triphosphate [157,158]. However while intermediate filaments do not undergo treadmilling (one end of the structure grows in length while the other end shrinks) like microfilaments and microtubules, formed structures are dynamic, and are used in intracellular crosstalk with other components of the cytoskeleton [163,164].

Due to their tremendous diversity in structure, intermediate filaments exhibit tissue-specific expression. Whereas microfilaments and microtubules are polymers of actin and tubulin, respectively, intermediate filaments are composed of a variety of proteins that are expressed in different types of cells. In fact, more than 50 different intermediate filament proteins have been identified and classified into 6 types based on similarities in amino acid sequence [157]. In regard to potential chemotherapeutic targets, the intermediate filaments that have shown the most promise are keratins, nestin and vimentin. The three will be described in detail in order to elucidate how valuable these potential targets are to malignant cell viability.

3.1. Keratins

Keratins (formerly known as cytokeratins) are proteins of keratin-containing intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue [165]. These intermediate filaments are either found as the acidic type I keratins, or the basic/neutral type II keratins. Complete keratins are typically found in pairs comprising a type I keratin and a type II keratin, and expression of these structures is frequently organ or tissue specific. This is derived from the fact that subsets of keratins expressed in epithelial cells depend on the type of epithelium, the time of terminal differentiation, and the stage of development [166]. Such specificity produces a unique keratin fingerprint for different types of epithelia, allowing classification to be based on this protein expression profile. This specificity even holds true in most carcinomas, as the keratin profile tends to remain consistent when epithelial cells develop neoplastic characteristics [166]. Therefore, the keratin profile can be exploited by immunohistochemistry techniques as a tool for tumor diagnosis and characterization in surgical pathology [167,168].

However, keratins may have much more utility to cancer treatment than diagnostic measures. It has been shown that introducing a keratin network into mouse fibroblasts confers a MDR phenotype [169]. Further, insertion of keratins has perpetuated resistance to mitoxantrone, DOX, melphalan, bleomycin, and mitomycin C in different keratin-positive cell lines [170]. In addition, keratin-positive cell lines were protected from apoptosis against mitoxantrone exposure, while cell lines without keratins exhibited marked levels of apoptosis after being exposed to the chemotherapeutic agent [169]. Multiple studies have supported these findings [169,171,172], and it has been further demonstrated that keratins play a prominent protective role in TNF and Fas mediated apoptosis after exposure to chemotherapeutic agents [173]. These studies indicate that acquisition of drug resistance may be attributed to a keratin-conferred protection against apoptosis.

Keratins of particular interest to cancer therapy are keratin 8 (K8) and keratin 18 (K18), the most common and characteristic members of intermediate filaments expressed in single layer epithelial tissues [17,174]. Oncogenes which activate Ras signaling stimulate expression of K18 through transcription factors, and elevated expression of K8 and K18 has been associated with an escape from the suppressive epigenetic mechanisms of DNA methylation and chromatin condensation [175,176]. In other words, overexpression of K8 and K18 is stimulated by multiple oncogenes that have been shown to be activated through only a limited number of transcription factors. As well as being associated with drug resistance, aberrant K8 and K18 expression has been noted in particularly invasive carcinomas [173,177], prompting investigation to determine how these intermediate filaments are involved in tumorigenesis, and apoptotic expression. Although it has been known for years that nuclear lamins are cleaved by caspases, it has been shown that K18 is also a substrate of the cysteine-aspartic proteases during epithelial apoptosis [173]. While the importance of keratin in apoptosis needs to be further characterized, it is apparent that aberrant keratin expression found in many cancers presents a novel chemotherapeutic target that warrants further investigation.

3.2. Nestin

Nestin is a type VI intermediate filament that is expressed in many cell types during development, but is typically not expressed in adults. One exception is the expression of nestin in stem cells, particularly neuronal precursor cells which has been associated with the radial growth of axons [178,179]. Nevertheless, embryonic levels of nestin often return in a variety of cancer types, and is now being considered as a potential diagnostic factor. Nestin expression is currently used as a stem cell marker [179], indicating that dedifferentiation of adult cells can be tracked using appropriate intermediate filaments. Indeed, nestin is highly expressed in several cancers, including gliomas, osteosarcomas, and colorectal and prostate carcinomas [180–184]. It has been postulated that nestin promotes the migration of prostate carcinoma cells and metastasis [179]. This is in accordance with data that suggest that nestin knockdown decreases the motility of cells derived from metastasized prostate carcinomas and the ability of these metastatic cells to migrate following xenograft transplantation [181]. Although the underlying mechanism has not yet been elucidated, it has been speculated that nestin interacts with Cyclin-dependent kinase 5 (Cdk5) in which Cdk5 regulates nestin organization, and nestin, in turn, regulates Cdk5 localization and activity. This is supported by the observation that nestin acts as a survival factor to inhibit Cdk5-mediated apoptosis [185,186]; critical for successful development, but also as potentially vital for cancer progression.

3.3. Vimentin

Vimentin is a type III intermediate filament that is found in a variety of cell types, including fibroblasts, smooth muscle cells, and leukocytes [157]. Further, vimentin is the major cytoskeletal component of mesenchymal cells and is used for maintaining cell shape, as well as stabilizing cytoskeletal interactions such as supporting and anchoring the position of organelles [187–189]. Due to its prevalence in mesenchymal cells, it is often used as a marker for cells undergoing EMT during both normal physiological processes and metastatic progression [190,191].

As with K8, K18, and nestin, vimentin has been shown to be overexpressed in a variety of carcinomas, including those of the breast, central nervous system, gastrointestinal tract, and lung, as well as melanoma [192]. This is particularly intriguing for cancers derived from epithelial tissue, as many of these cell types have a high expression of vimentin during fetal development that substantially decreases with age [192]. Overexpression of vimentin in these cell types can therefore be seen as a characteristic sign of dedifferentiation, validating the use of intermediate filaments as diagnostic and prognostic factors in a variety of carcinomas. Further, overexpression of vimentin is associated with increased tumor growth, invasion, and a poor prognosis [193–195].

Due to the prevalence of vimentin overexpression in carcinomas, it should come as no surprise that the intermediate filament is vital for EMT. It has been recently shown that overexpression of oncogenic H-Ras-V12G and Slug induces vimentin expression and cell migration in pre-malignant breast epithelial cells [196]. Even more intriguing is that vimentin expression is necessary for Slug- or H-Ras-V12G-induced EMT-associated migration. Silencing vimentin expression in breast epithelial cells reduces invasiveness-related gene expression, and gene expression profiling analyses reveal that vimentin expression correlates positively/negatively with these genes in multiple breast carcinoma cell lines and breast cancer patient samples. Induction of

vimentin by EMT is also associated with upregulation of Axl expression, thereby enhancing migratory activity in pre-malignant breast epithelial cells [196]. Similar results have been found in lung carcinomas [197], with vimentin expression being perpetuated by HIF-1, facilitating EMT, and subsequent metastasis. In addition, vimentin forms a complex with 14-3-3 proteins (regulatory molecules that bind a multitude of functionally diverse signaling proteins) and beclin 1 in lung and prostate carcinomas to inhibit autophagy *via* an AKT-dependent mechanism, thereby regulating various intracellular signaling and cell cycle control pathways by depleting the availability of free 14-3-3 [197,198].

3.4. Withaferin A

Although no potential chemotherapeutic agent has been identified to specifically target aberrant keratin or nestin levels in malignant cells, withaferin A has shown promise as a potent vimentin inhibitor. Withaferin A is a steroidal lactone that was initially isolated from winter cherry (*Withania somnifera*), and was the first member of the withanolides to be discovered [199]. While other withanolides have shown anticancer activity, withaferin A has demonstrated the most efficacy, likely due to its unique structure: an α, β-unsaturated ketone in the first ring, a 5-β, 6-β-epoxy group in the second ring, and a nine carbon side chain with an α, β-unsaturated δ-lactone group. As expected, the 28 carbon steroidal lactone contains the characteristic steroid ring system that is connected to a lactone *via* a methanetriyl carbon. This lactone is a cyclic ester, characterized by a closed ring consisting of five carbon atoms and a single oxygen atom. In all withanolides, the lactone moiety is built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring.

While withaferin A is known to influence a variety of proteins, the most extensively studied target protein has been vimentin. The compound binds vimentin at Cys328 located in the conserved α-helix and lying in close proximity with the C-3 and C-6 sites of the first two rings in withaferin A [200]. This covalent modification of vimentin in the conserved α-helical coiled coil domain inhibits its assembly, thereby preventing formation of the complete intermediate filament. Affinity purification has confirmed this interaction with vimentin, as well as with desmin, and glial fibrillary acidic protein (GFAP) [201–203]. In addition, withaferin A down-regulates the expression of these filament proteins, as well as peripherin [204], inducing substantial cytoskeletal perturbation [201–204] in affected cells, and suggesting that withaferin A is a potent inhibitor of all type III intermediate filaments.

However, withaferin A is much more than a potent intermediate filament inhibitor. Withaferin A has been shown repeatedly to inhibit angiogenesis [201,205–207]. It has been demonstrated that withaferin A inhibits human umbilical vein endothelial cell (HUVEC) sprouting by inhibiting NF-κB, as well as cyclin D1 expression [206]. It has also been shown that withaferin A inhibits NF-κB through interference with the ubiquitin-mediated proteasome pathway, exerting potent anti-angiogenic activity at doses as low as 7 μg/kg/day i.p. in C57BL/6J mice [206]. These *in vivo* observations can be tied directly back to the propensity of withaferin A to inhibit vimentin, as site-specific modification of vimentin by withaferin A induces endothelial cell apoptosis through interference with the conserved rod 2B domain. In addition, withaferin A substantially down-regulates vascular endothelial growth factor (VEGF) expression [208,209], further inhibiting angiogenesis.

Withaferin A has also demonstrated potent anticancer activity by directly inhibiting tumorigenic growth. The compound inhibited growth of murine, as well as patient-derived mesothelioma cells in part by decreasing the chymotryptic activity of proteasomes, consequently perpetuating increased levels of ubiquitinated proteins and pro-apoptotic proteasome target proteins (p21, Bax, IkBa) [210]. Withaferin A suppression of mesothelioma growth was also attributed to elevated levels of apoptosis induced by activation of pro-apoptotic p38 stress activated protein kinase (SAPK) and caspase-3, and cleavage of PARP. In addition, gene-array based analyses further revealed that withaferin suppressed oncogene activity in tumors, including c-myc [211]. Withaferin A has also shown substantial anticancer activity against MDA-MB-231 and MCF7 cells by suppressing XIAP, cIAP-2, and survivin protein levels [212]. *In vivo* activity was also exhibited in MDA-MB-231 xenografts, but only suppression of survivin was observed.

Perhaps most intriguing is that withaferin A has demonstrated significant synergistic effects with the multikinase-targeted inhibitor sorafenib in thyroid carcinoma models (human papillary (BCPAP) and anaplastic (SW1736) cells) [212]. Concomitant use of sorafenib and withaferin A at IC_{50} levels decreased cell viability to 19%, as opposed to 50% for each agent administered separately. Further, apoptosis levels in SW1736 cells increased significantly from 0–2% (sorafenib- or withaferin A-alone treatments) to 89% when administered together, as assessed by annexin V/propidium iodide flow cytometry. Synergy was also observed in the ability to down-regulate the BRAF, Raf-1, and extracellular signal-regulated kinase (ERK) signaling pathways, while each agent administered individually exerted only minimal influence on regulatory control. This ultimately suggests that concomitant chemotherapy of sorafenib and withaferin A may exert potent anticancer activity, with lower overall doses of the moderately toxic sorafenib required to significantly inhibit neoplastic growths.

4. Potential pitfalls

As with currently approved chemotherapeutic agents, cytoskeletal filament-directed agents that eventually reach the clinical setting will invariably be limited by drug toxicity. Although mitigating cell motility is an important chemotherapeutic target for microfilament-directed agents, many physiological functions are inherently dependent on such capabilities. Leukocytes also have the need to extravasate in great quantity over a short period in order to combat infection [213]. In the course of this process known as diapedesis, leukocytes adhere *via* selectins to the endothelium and perform a form of rolling adhesion to interact with endothelial cells, which in turn enables the passage of appropriate immune cells [213,214]. In a manner similar to malignant cells, leukocytes are also capable of amoeba migration, meandering their way through the ECM to reach a site of infection or inflammation [215,216]. Although 1.5 μM cytochalasin B does not appear to have a substantial effect on human leukocytes *in vitro* [31], 50 mg/kg cytochalasin B *in vivo* does produce substantial immunosuppression in C57B1/6 mice [68,69]. In addition, the same dosage is marked by splenomegaly, supported by tissue distribution analyses which confirm high accumulation rates in the spleen, as well as blood cells [217]. Nevertheless, elevated concentrations do not typically last longer than 24 h [217], and cytochalasin B immunosuppression can be readily ameliorated with rhIL-2, with less marked splenomegaly [69].

There are also concerns about the acute organ toxicity microfilament-directed agents may have. Cytochalasins [218–221] and jasplakinolide [222,223] have been noted for cardiac toxicity, MKT-077 for marked renal impairment [127], and staurosporine for neurotoxicity [224,225]. Therefore it may be necessary to find effective drug vehicles that can ensure successful delivery, while reducing unintended toxicity. Liposomes have been extensively examined for their abilities to improve drug delivery and have been used for a substantial variety of medicines, including cytotoxic agents, antibiotics and antifungal agents [226]. In regard to cancer therapy, liposomal incorporation has been shown to reduce side effects of a variety of chemotherapeutic agents, as well as promote targeted tumor damage due in part to their ability to substantially aggregate at tumor sites by leaking through pores and defects in tumor capillary endothelium [226]. Liposomal incorporation has been shown to improve the delivery, while reducing aberrant toxicity in cytochalasins B [69] and D [81], and staurosporine [150], suggesting that it may be an appropriate method by which to administer many microfilament-directed agents in the clinic. In addition, curcumin, a plant extract used

in traditional medicine has shown to mitigate the toxicity of staurosporine [227]. However, the protein kinase inhibitor has shown potential against malignant brain cancers [150,228,229], and whether the plant extract would also mitigate anticancer activity has yet to be assessed.

Although withaferin A has shown considerable anticancer activity *in vitro* and *in vivo*, the compound is relatively novel to chemotherapeutic investigation, and a comprehensive toxicity profile has yet to be assembled. From the limited toxicity data available, it appears that the compound is not deleterious to pancreatic islet cells [230,231], but can have immunosuppressive effects on human B and T lymphocytes, as well as on mice thymocytes [232]. A comprehensive preclinical pharmacokinetic and pharmacodynamic study of withaferin A has yet to be performed, and such data would be pivotal for assessing its potential clinical utility.

A practical limitation for cytoskeletal filament-directed agents, particularly those that affect microfilaments is the expense. Many of these compounds are natural toxins derived from a diversity of organisms, such as bacteria (scytophycins, staurosporine), flowers (withaferin A), molds (cytochalasins, cheatoglobosins), and marine sponges (latrunculins, jasplakinolide). As such, compounds relevant for chemotherapeutic assessment have to be extracted and purified, resulting in a considerable expense for interested laboratories. This is epitomized by jasplakinolide, as the microfilament-stabilizing agent varies from $289.00 to 498.50 for 100 μg when purchased from commercial suppliers. Scytophycins have the additional problem of being relatively novel compounds, and they are not readily available commercially. Nevertheless, successful application of these compounds will inevitably lower the cost of production due to demand, as demonstrated by the vinca alkaloids and taxanes.

The costs for comprehensive mammalian studies can be significantly reduced if the laboratory is willing to isolate the compound directly from the organism, as has been done in my laboratory. To compare, a matte of *Drechslera dematioidea* that can produce the gram quantities of cytochalasin B needed for extensive murine study costs ~$354.00. By contrast, 50 mg cytochalasin B from a commercial supplier can cost around $1245.00. Further, interested laboratories can seek donations from the Natural Products Branch of the National Cancer Institute as was done in [101], or other providers of natural products. In addition, partial or total syntheses of many cytoskeletal-filament directed agents have been described [233–240], and are another alternative to commercial purchases. The promise of these potential chemotherapeutic agents will hopefully enable much easier access in the coming future.

5. Concomitant use of cytoskeletal filament-directed agents and other chemotherapeutic agents

This review has comprehensively examined the mechanisms by which microfilament directed- and intermediate filament-directed agents damage malignant cells. A summary of these mechanisms is provided in Table 1 for reference. In addition, the binding sites of the proposed agents are depicted in Figs. 2 and 3 (microfilament-directed agents and intermediate filament-directed agents, respectively). This summarization was done in order to elucidate the potential of using these drugs in combination with each other, or with currently approved chemotherapeutic agents to generate a substantial synergistic attack. For example, while it is true that cytochalasins and latrunculins disrupt microfilament formation, thereby causing the formation of multinucleated cells, they do so by different mechanisms. Cytochalasins (as well as chaetoglobosins and scytophycins) bind F-actin, and depolymerize actin polymers; latrunculins bind G-actin monomers, inhibiting polymerization before it initiates. It would be very difficult for malignant cells sensitive to microfilament-directed agents to circumvent both destabilizers in order to form viable microfilaments. Preventing rapidly proliferating cells from successfully completing cytokinesis could be of monumental clinical importance, as such cells are sensitive to a variety of treatment modalities. 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 [27,29,98,102]. This ultimately suggests that malignant cells exposed to cytokinesis inhibitors could be substantially sensitive to DNA-directed agents such as alkylators, antifolates, anthracyclines, and nucleoside analogs. Further, cells exposed to cytochalasin B have markedly increased rates of mitochondrial activity [31] . Therefore, combining MKT-077 or other known mitochondrial-directed agents with microfilament-directed agents, as well as DNA-directed agents could induce potent drug synergy, especially since MKT-077 is known to perturb microfilament formation.

Microfilament-directed agents also could be used to enhance the efficacy of physicochemical therapeutic approaches such as X-radiation or the experimental sonodynamic therapy (SDT) [28], as malignant cells exposed to such agents develop a perturbed cytoskeleton. As shown with U937 human monocytic leukemia cells exposed to 1.5 μM cytochalasin B, malignant cells that become grossly enlarged and multinucleated are sensitized to physical agitation [31]. It has even been shown that SDT increases reactive oxygen species (ROS) content within malignant cells, making them more sensitive to mitochondrial-directed agents, as many cancer types have substantially lower concentrations of thiol buffers than normal cells [256–258]. Therefore, combining physicochemical approaches with cytochalasin B, mitochondrial-directed agents and DNA-directed agents could exert substantial preferential damage on tumorigenic growths.

It is also extremely pragmatic to emphasize the fact that the only cytoskeletal-directed agents currently used in the clinical setting perturb microtubule function. Microtubule-directed agents such as taxanes and vinca alkaloids primarily act as mitotic poisons, subsequently inducing cell cycle arrest, and eventual apoptosis. Since many microfilament-directed agents are potent cytokinesis inhibitors, it seems likely that using these agents in tandem with a known microtubule-directed mitotic inhibitor could elicit a 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 evade this mechanism by mitotic escape and replicate their nuclei would be unable to undergo cytokinesis, and potentially even metastasis due to the effects microfilament-directed agents have on actin polymerization. Such synergy has been demonstrated with cytochalsin B and vincristine *in vitro* [243], suggesting that this approach may be viable in the clinical setting. It has also been shown that some microfilament-directed agents (particularly tolytoxin) are resistant to drug efflux mediated through ABC transporters. Combining microtubule-directed agents also known to be resistant to drug efflux, such as epothilones, with these microfilament-directed agents might be a particularly useful method to combat drug resistant tumors, and warrants further investigation.

As microfilaments and microtubules do not constitute the entire cytoskeleton, intermediate filament-directed agents such as withaferin A are very promising drug leads. Withaferin A is a potent angiogenesis inhibitor that induces apoptosis in endothelial cells, as well as down-regulates essential angiogenic proteins. Using withaferin A to supplement statins and other known anti-angiogenic agents could be more effective at preventing tumor vascularization. An ample blood supply is absolutely critical for sustaining the elevated metabolic rates of rapidly proliferating cancer cells, and finding novel approaches to mitigate angiogenesis has significant clinical utility. Further, as shown with sorafenib, withaferin A may be used to increase the efficacy of protein kinase inhibitors (including staurosporine) or other chemotherapeutic agents. Investigating this potential synergy could result in novel chemotherapeutic protocols for cancers that respond to protein kinase inhibitors, many of which have particularly grim prognoses (hepatocellular,

Table 1
Prospective cytoskeletal filament-directed agents for use in chemotherapy.

Chemotherapeutic agent	Drug class	Mechanism of action	Cancer types exhibiting sensitivity
Cytochalasin B	MFD	Depolymerizes F-actin and inhibits nucleation by binding to the barbed end of F-actin filaments [14,15], as well as interacting with capping proteins (CAPZA1 and others in the F-actin capping protein α subunit family) [241,242].	Breast carcinoma [27,29,32] Colorectal carcinoma [67] Leukemia [31] Lymphoma [243] Lung carcinoma [27,67,68] Prostate carcinoma [27] Mastocytoma [243] Melanoma [27,68]
Cytochalasin D	MFD	Depolymerizes F-actin and inhibits nucleation by binding to the barbed end of F-actin filaments [244–246], potentially activating P53 in cancers with an active form of the tumor suppressing gene [247]. Induces actin dimer formation in the presence of $Mg2^{+}$, thereby eliminating the polymerization lag phase due to accelerated nucleation by the dimers [245,248]. ATP hydrolysis is also stimulated, preventing actin polymerization [249,250].	Colorectal carcinoma [79,81] Melanoma [80,81] Hepatocellular carcinoma [81]
Chaetoglobosin K	MFD	Depolymerizes F-actin and inhibits nucleation by binding to the barbed end of F-actin filaments [84,85,88]. Shows particular anticancer activity in Ras-dependent cancer cell lines, as the compound caps the plus-ends of F-actin, which confers anti-Ras oncogenic activity through the PI3K-mediated pathway by preventing Ras from activating Rac [88].	Ras-dependent cancers [84,85,88]
Chaetoglobosin A	MFD	Depolymerizes F-actin and inhibits nucleation by binding to the barbed end of F-actin filaments [91]. Sensitizes malignant cells to PI3K and BTK inhibitors [91].	Leukemia [91]
Jasplakinolide	MFS	Induces actin polymerization by binding F-actin at three G-actin sites, and then rigidifies the formed microfilaments to prevent the inherent tendency of depolymerization [94,97,98].	Lung carcinoma [101] Prostate carcinoma [100,101]
Latrunculin A	MFD	Binds actin monomers near the nucleotide binding cleft, thereby inhibiting polymerization [105]. Preferentially associates with G-actin, thereby preventing subunits from repolymerizing into filaments [103–105,107,108]. Activates the caspase-3/7 pathway [109].	Breast carcinoma [113,116] Gastric adenocarcinoma [109] Prostate carcinoma [113]
MKT-077	MFC, Hsp70 chaperone inhibitor	Crosslinks F-actin, producing aberrant microfilaments within malignant cells [121,125]. Suppresses Ras transformation by blocking membrane ruffling [125]. Allosterically inhibits Hsp70 chaperones, inducing apoptosis [124].	Breast carcinoma [117,119,120,128] Bladder carcinoma [117] Colorectal carcinoma [117,119] Osteosarcoma [119] Pancreatic carcinoma [117] Leukemia [118] Melanoma [117] Ras-dependent cancers [120,125]
Staurosporine	MFD, protein kinase inhibitor	Binds actin polymers, inducing depolymerization, thereby perpetuating thinning and loss of cytoplasmic actin bundles [148,149]. Competitively inhibits ATP from binding protein kinases, as it has a much stronger affinity to the ATP-binding site [135–137].	Cervical carcinoma [132] Fibrosarcoma [132] Leukemia [132,134] Melanoma [147]
Tolytoxin	MFD	Depolymerizes F-actin and inhibits filament nucleation [154,155]. Produces marked morphological changes, including zeiosis, and nuclear protrusion [155].	Cervical Carcinoma [116] Leukemia [155] Ovarian Carcinoma [156]
Withaferin A	IFD	Binds vimentin at Cys328 located in the conserved α-helix [200]. The resulting covalent modification of vimentin in the conserved α-helical coiled coil domain inhibits its assembly, thereby preventing formation of the complete intermediate filament [200,201]. Known to be a potent inhibitor of angiogenesis [206,208].	Breast carcinoma [211,251–253] Mesothelioma [210] Ovarian carcinoma [254] Thyroid carcinoma [212]

MFC: microfilament-crosslinking agent; MFD: microfilament-disrupting agent; MFS: microfilament-stabilizing agent; IFD: intermediate filament-disrupting agent. Agents are listed in the order that they appear in the review.

renal and anaplastic thyroid carcinomas). It may even be possible to combine withaferin A with microfilament- and microtubule-directed agents in cancers that overexpress vimentin to inflict irreversible damage to cytoskeletal structure and function. This appears feasible, as microfilaments, microtubules, and intermediate filaments often act in concert, rather than isolation [163,259,260]. Since the cytoskeleton is fundamental for many vital cell signaling processes, interfering with this intricate system of interaction with mechanistically distinct cytoskeletal agents could result in highly effective chemotherapeutic protocols.

6. Conclusion

The mechanisms by which cytoskeletal filament-directed agents inflict preferential damage on malignant cells are as diverse as the molecular structures of these proposed compounds. They could be used to fill gaps within the arsenal of clinicians, providing more comprehensive, and therefore more effective therapeutic protocols. However, many of these compounds lack the necessary *in vivo* data for clinical evaluation. Microfilament- and intermediate filament-directed disruption is an intriguing area of pharmacological research, and could potentially revolutionize chemotherapy or other treatment modalities currently used against cancer. While it is true that most promising drug leads fail to exhibit clinical efficacy, cytoskeletal-filament directed agents have yet to be critically evaluated at this stage, and have demonstrated potential in numerous *in vitro* and *in vivo* preclinical studies. Critical assessment of these novel chemotherapeutic agents is indeed warranted, and hopefully will establish a new avenue of cancer therapy.

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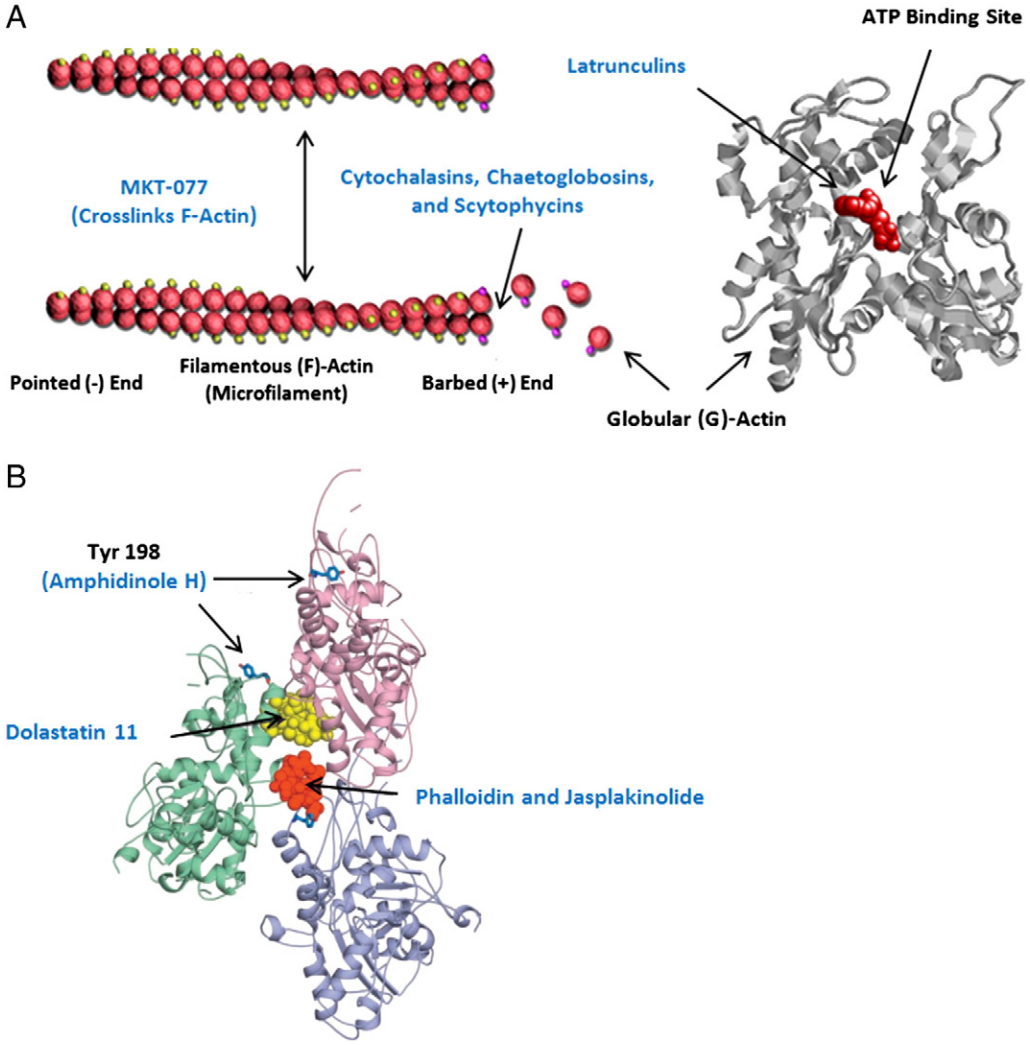

Fig. 2. Binding sites of microfilament-directed agents. A) The binding sites of microfilament-disrupting and microfilament-crosslinking agents are shown. While cytochalasins, chaetoglobosins, and scytophycins bind the barbed (+) end of F-actin, latrunculins bind G-actin at the nucleotide binding cleft. MKT-077 crosslinks adjacent microfilaments in the cytoskeleton. B) The binding sites of microfilament-stabilizing agents are shown. Note that amphidinole H, dolostatin 11, and phalloidin were not discussed in this review, but demonstrate the importance of the positioning of three G-actin monomers in F-actin for stabilizing microfilaments. Staurosporine was not shown as its binding site on microfilaments has yet to be determined.
Panel B was adapted from [255].

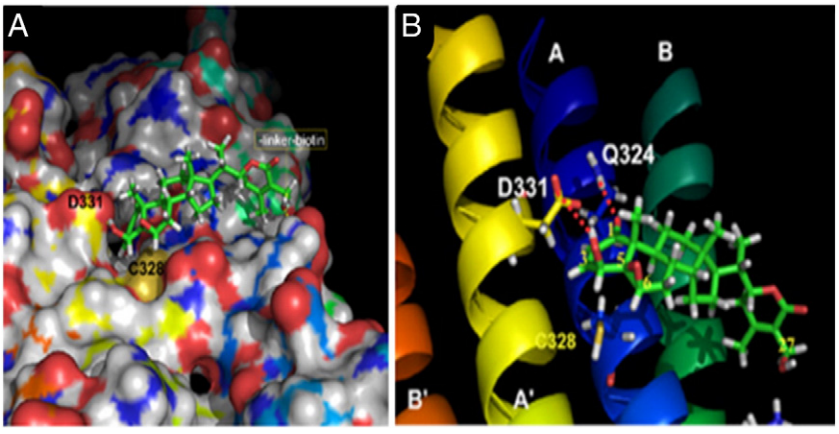

Fig. 3. Binding site models of withaferin A on tetrameric vimentin. A) The close-up image shows the A-ring twist-boat and B-ring half-chair conformation of withaferin A is accommodated deep within the binding cleft of the vimentin tetramer, allowing for proper orientation with Cys328 (yellow) to form a covalent bond with the C3 or C6 electrophilic carbon centers of withaferin A. The exocyclic C27 hydroxyl group of withaferin A was conjugated to the linker-biotin for affinity labeling. The biotin affinity tag was linked to withaferin A *via* a long hydrocarbon chain residue. It is orientated toward the solvent-side of the binding cleft, enabling the withaferin A B-ring to bind tetrameric vimentin. B) The model shows hydrogen bonding between Gln324 of the vimentin A-helix and the C1 position oxygen atom (2.3 Å), and Asp331 of the vimentin A′ helix and the C4 hydroxyl group (1.7 Å).
Images were adapted from [201].

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