Strongylophorine-26, a Rho-dependent inhibitor of tumor cell invasion that reduces actin stress fibers and induces nonpolarized lamellipodial extensions

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Abstract

Strongylophorine-26, a new meroditerpenoid, was recently identified as an inhibitor of cancer cell invasion. This study was undertaken to characterize its mechanism of action. We find that strongylophorine-26 inhibits the motility of MDA-MB-231 breast carcinoma cells on a plastic surface. Upon addition of strongylophorine-26, rapid cell contraction and depolarization occurred, followed by spreading and flattening of the entire cell. Treated cells exhibited increased membrane ruffling throughout and extended lamellipodia in all directions. Strongylophorine-26 induced a decrease in actin stress fibers, a dramatic increase in the size and number of focal adhesions, and the appearance of a dense meshwork of actin filaments around the cell periphery. Strongylophorine-26 caused a transient activation of the small GTPase Rho and treatment with the Rho inhibitor C3 exoenzyme abrogated the anti-invasive activity of strongylophorine-26. These effects are distinct from those of many motility and angiogenesis inhibitors that seem to act by a common mechanism involving the induction of actin stress fibers. This difference in mechanism of action sets strongylophorine-26 apart as an experimental anticancer agent and indicates that pharmacologic inhibition of cell migration may be achieved by mechanisms not involving the stabilization of actin stress fibers. [Mol Cancer Ther 2005;4(5):772–8]

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Introduction

Tissue invasion is a central process in tumor growth and metastasis. Tumor cells can invade surrounding tissues, including capillary walls, a key process in metastatic spread. Activated vascular endothelial cells also invade and generate new capillaries to irrigate tumors and provide conduits for metastatic spread. There are important similarities in the molecular mechanisms that enable tumor cells and endothelial cells to invade, and agents that inhibit invasion may inhibit both metastasis and neoangiogenesis (1).

Cell migration, a fundamental aspect of invasion and angiogenesis, is a dynamic process that involves regulated cyclical changes in cell shape, cell adhesion, and the cytoskeleton (2, 3). Directed movement is initiated by the protrusion of a broad leading membrane, termed the lamellipodium. Next, this membrane becomes anchored to the underlying substratum by small integrin-dependent adhesions called focal complexes. Coordinately, contraction within the cell body leads to detachment of trailing adhesions and retraction of the rear membrane, thus enabling translocation of the cell body in the forward direction. Cytoskeletal rearrangements are the underlying mechanism for these changes in morphology during the migration process. Actin polymerization occurs within a dense meshwork of actin filaments that make up the lamellipodium (4). Growing actin filaments push against the plasma membrane and provide the force for forward protrusions. Within the cell body, actin filament bundles, called stress fibers, terminate at focal adhesions. Actomyosin contractile forces generated from stress fibers pull against focal adhesions and induce retraction of the cell rear (5). Microtubules may also play a role in dynamic changes at the leading and trailing edges of motile cells by distributing regulatory proteins to the sites of actin reorganization (6, 7).

Rho-GTPases are important regulators of both actin dynamics and cell-substratum adhesions in migratory cells, and thus are critical proteins involved in tumor invasion and angiogenesis (8–10). The effects of Rho and Rac, two subgroups of the Rho-GTPases, on actin and adhesion dynamics have been studied extensively (11, 12). Rho induces the formation of stress fibers and mature focal adhesions. Rac induces lamellipodial extensions, also referred to as membrane ruffles, and the formation of smaller adhesions which are transient in nature, the focal complexes that line the lamellipodium at the leading edge of a migratory cell. Rac1 activity has been correlated with increased migratory and invasive ability (9). Both Rho and

Rac are overexpressed in some invasive cancers (13). However, there is increasing evidence that overactivation of Rho can lead to inhibition of cell migration through the formation of highly stable focal adhesions which impede adhesion turnover and thus the rate of cell movement (14–19).

The inhibitors of endothelial cell migration and angiogenesis fumagillin, TNP-470, thrombospondin-1, endostatin, EMAP II, and dihydromotuporamine C all increase focal adhesion and actin stress fiber densities (19, 20). This observation suggests that despite their structural dissimilarities and distinct direct targets, these agents may inhibit cell migration by a common mechanism. Using a phenotypic screen, we recently identified the new meroditerpenoid, strongylophorine-26, as an inhibitor of cancer cell invasion that shows no structural similarity to other inhibitors of invasion or angiogenesis (21). Here, we examine the mechanism of action of strongylophorine-26 and show that it inhibits cancer cell motility in a Rho-dependent manner, but that it causes a decrease in actin stress fiber density and the appearance of radial nonpolar lamellipodial protrusions, unlike the agents described above. This difference in mechanism of action sets strongylophorine-26 apart as an experimental anticancer agent and indicates that pharmacologic inhibition of cell migration may be achieved by mechanisms not involving the stabilization of actin stress fibers.

Experimental Procedures

Strongylophorine-26

Strongylophorine-26 was isolated from a methanol extract of the marine sponge *Petrosia* (*Strongylophora*) *corticata* as described recently (21).

Cell Culture, Migration, and Invasion Assays

MDA-MB-231 breast carcinoma cells were routinely maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen) and 5 μg/mL insulin (Sigma, St. Louis, MO). Cell migration and invasion assays were carried out exactly as described previously (22). Briefly, for invasion assays, cells were plated on reconstituted basement membrane gels (Matrigel, BD Biosciences, Mississauga, Canada) in the absence or presence of various compounds for 2 to 3 hours, and invasive morphology was assessed by phase contrast microscopy. The number of viable cells that did not invade the gel was determined quantitatively: noninvasive cells were removed from the top of the gel by light trypsinization, allowed to reattach to tissue culture plastic for 18 hours in separate wells, and viable cells were quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (22).

Immunofluorescence and F-actin Staining

Vinculin, tubulin, and actin staining was carried out as described previously (19).

Rho Activation Assay

Activated GTP-bound Rho proteins were isolated by coprecipitation with the Rho-binding domain of rhotekin and quantified by Western blotting for all Rho isoforms according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY) using reagents and protocols originally developed by Ren et al. (23). Thus, these experiments were designed to assess the aggregate activation state of all mammalian Rho isoforms. Blotting of unprecipitated whole cell lysates with the pan-Rho antibody was carried out to show that observed changes in activation state were not caused by changes in steady-state Rho protein levels.

C3 Exoenzyme Loading into Live Cells

The Rho inhibitor C3 exoenzyme from *Clostridium botulinum* (Calbiochem, San Diego, CA) was introduced into cells using the method of Renshaw et al. (24). Briefly, 5 μg/mL C3 exoenzyme was mixed with 5 μg/mL lipofectin (Invitrogen) 1:1 in medium lacking fetal bovine serum and incubated with MDA-MB-231 cells for 14 hours at 37°C before initiating the invasion assay. We have shown previously that this method of C3 loading results in significant Rho inhibition in MDA-MB-231 cells (19).

Results

Strongylophorine-26 Inhibits Tumor Cell Invasion and Cell Migration

In a recent report of the elucidation of the structure of strongylophorine-26 (Fig. 1A), we showed that 0.5 to 2.5 μg/mL strongylophorine-26 prevents MDA-MB-231 cells from invading Matrigel, a commercially available basement membrane gel (21). Without strongylophorine-26, cells attached and extended elongated invadopodial membrane protrusions into Matrigel as early as 2 hours after plating (Fig. 1B, *a*). When treated with 1 μg/mL strongylophorine-26 (2.2 μmol/L), the cells attached, but remained rounded on top of the gel, and did not form invadopodia (Fig. 1B, *b*). This morphologic effect was readily apparent 2 hours after strongylophorine-26 addition (Fig. 1B) and lasted for at least 12 hours (data not shown). At 1 μg/mL, strongylophorine-26 also caused easily quantifiable inhibition in the anti-invasion assay (Fig. 1C), and this concentration was selected for most experiments in this report. Strongylophorine-26 was slightly more potent than motuporamine C (active concentration range, 5–10 μmol/L), an invasion inhibitor that was identified previously using the same anti-invasion assay (22). Of note, strongylophorine-26 caused no detectable cytotoxicity at the concentrations used above during 24 hours of treatment but showed cytotoxicity at higher concentrations (IC_{50} = 5 μg/mL; data not shown).

In addition to inhibiting invasion, strongylophorine-26 also prevented the migration of MDA-MB-231 cells in an *in vitro* wound closure assay. Confluent monolayers of cells grown on tissue culture plastic were scraped and then treated with or without strongylophorine-26. After 6 hours, untreated cells began to move into the wound, and after 12 hours, the wound was closed due to the influx of highly migratory cells (Fig. 2, *left*). In comparison, treatment with strongylophorine-26 almost

completely inhibited cell migration—after 12 hours in the presence of the compound, the size of the wound had not decreased appreciably (Fig. 2, *right*). Importantly, strongylophorine-26 did not exert its effects by preventing cell adhesion to the plastic substratum (Fig. 2).

Strongylophorine-26 Induces Cell Depolarization and Spreading

We next examined the effect of strongylophorine-26 on the morphology of MDA-MB-231 cells grown on tissue culture plastic. Cells were plated at subconfluent densities and allowed to adhere overnight prior to treatment. Under these conditions, untreated cells displayed a migratory, fibroblast-like morphology. Specifically, most cells were elongated and polarized, extending a broad lamellipodium at the leading edge and a narrow trailing membrane at the rear (Fig. 3A). When cells were treated with strongylophorine-26, they contracted and became depolarized rapidly, within 30 minutes (Fig. 3B). However, after 2 hours of treatment, the cells began to spread again and they formed a flattened apron that completely encircled the cells in a radial fashion (Fig. 3C). Phase contrast-dark membrane ruffles were prominent along the cell periphery, and the cells extended lamellipodia in all directions. After 6 hours of treatment, the cells were completely flat and not polarized, and the radial apron remained (Fig. 3D).

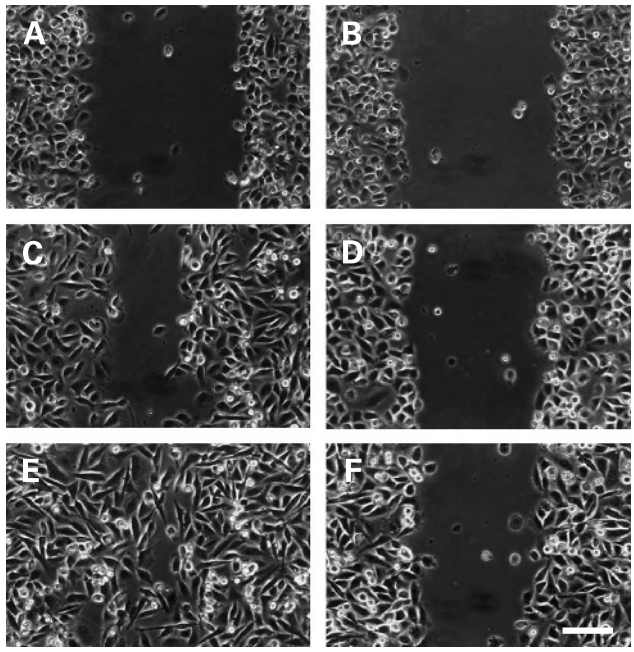

Figure 2. Strongylophorine-26 inhibits cell migration. A confluent monolayer was scraped with a sterile toothpick and migration into the wounded monolayer was assessed by phase contrast microscopy after 1 (**A** and **B**), 6 (**C** and **D**), and 12 h (**E** and **F**) in the presence of DMSO (**A, C,** and **E**) or 1 μg/mL strongylophorine-26 (**B, D,** and **F**; *bar*, 45 μm).

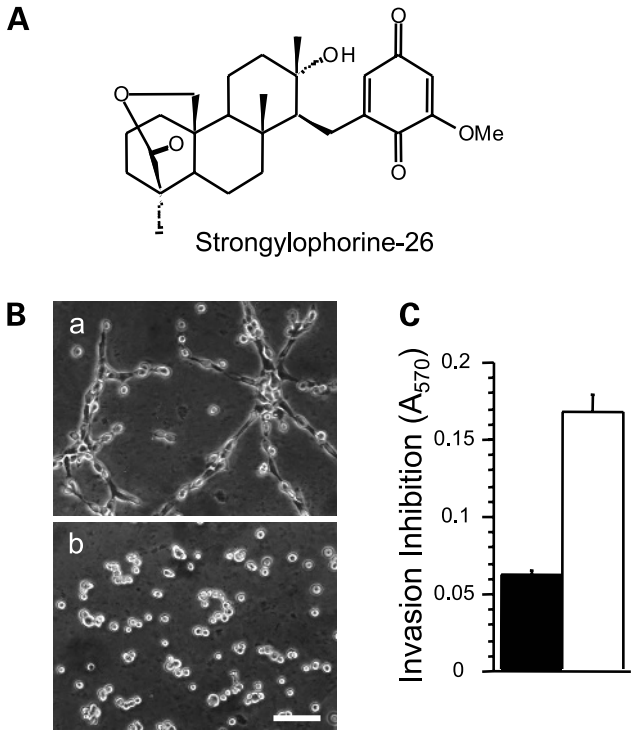

Figure 1. Strongylophorine-26 inhibits tumor cell invasion. **A,** structural formula of strongylophorine-26. **B,** MDA-MB-231 cells were plated on Matrigel in the presence of DMSO (*a*) or 1 μg/mL strongylophorine-26 (*b*) for 2 h, and photographed live by phase contrast microscopy (*bar*, 60 μm). **C,** cells were treated as in (**B**) without (*black column*) or with (*white column*) strongylophorine-26 and inhibition of tumor invasion was assessed quantitatively. The assay was done in triplicate and error bars represent SEM (P = 0.001).

Strongylophorine-26 Induces Reorganization of the Actin Cytoskeleton and Formation of Cell Adhesions

Regulation of cell shape and motility is governed in large part by the cytoskeleton, two major components of which are actin filaments and microtubules. Together, these cytoskeletal elements influence the formation of cell substrate adhesions (7, 25, 26), which are also fundamentally important for establishing cell morphology and a migratory phenotype.

In order to further characterize the effects of strongylophorine-26 on cell shape, the cytoskeleton and focal adhesions were examined by fluorescence microscopy. Strongylophorine-26 caused a significant decrease in F-actin-containing stress fibers, which was apparent within 30 minutes (Fig. 4, compare *a* with *c*). After longer incubation times, many cells displayed a dense meshwork of unpolarized actin filaments around the cell periphery (a 6-hour time point is shown in Fig. 4*e*). Of note, this effect was distinct from that of other previously described invasion and angiogenesis inhibitors, which instead increase actin stress fibers (19, 20). Strongylophorine-26 also induced a dramatic increase in the size and number of focal adhesions at the cell periphery, which became increasingly prominent during longer incubation (Fig. 4, compare *b*, *d* and *f*). By contrast, strongylophorine-26 had little effect on microtubules (data not shown).

Invasion Inhibition by Strongylophorine-26 Requires Rho Activity

Given the morphologically distinct phenotype of strongylophorine-26 treatment compared with other inhibitors and the involvement of Rho in dihydromotuporamine C activity (19), we next asked whether strongylophorine-26 influences Rho activity. Strongylophorine-26 induced a strong increase in Rho activity at 30 minutes, but this effect was transient, Rho activity diminished to basal levels within 6 hours (Fig. 5A).

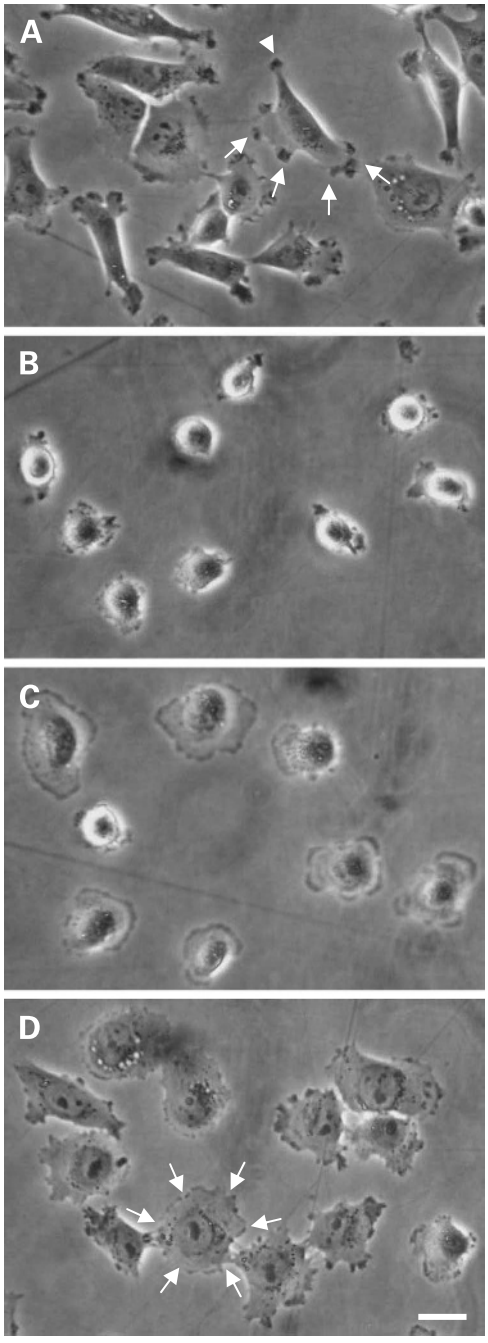

Figure 3. Strongylophorine-26 induces cell depolarization and spreading. Cells were treated with DMSO (**A**) or strongylophorine-26 for 30 min (**B**), 2 h (**C**), or 6 h (**D**) and phase contrast photographs were taken of live cells. Notice the polarized lamella at the leading membrane (*arrows*) and the narrow retracting membrane (*arrowhead*) of an untreated cell in (**A**). In contrast, strongylophorine-26-treated cells seem flattened and spread, and exhibit nonpolarized lamellar extensions around the entire cell periphery in (**C** and **D**), shown with arrows in (**D**; *bar*, 15 μm).

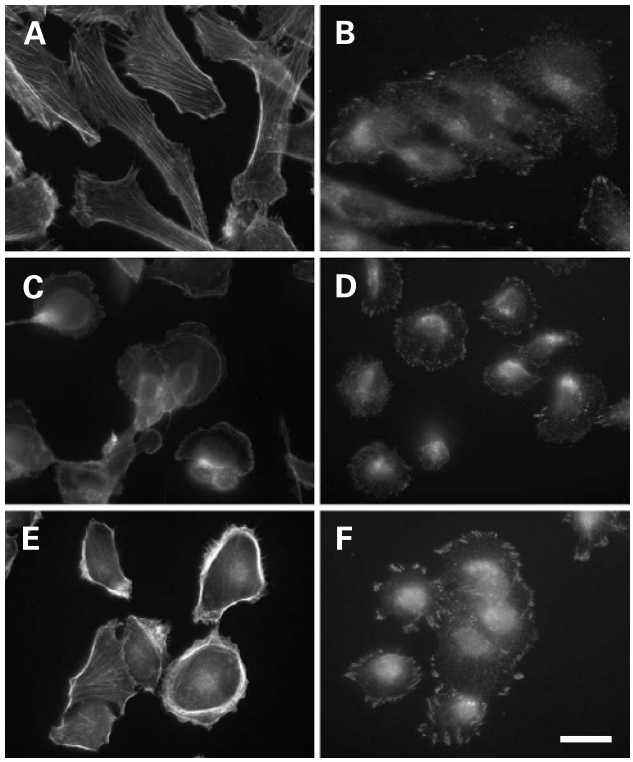

Figure 4. Strongylophorine-26 induces peripheral actin bundling and adhesion complex formation. MDA-MB-231 cells were treated with DMSO (**A** and **B**) or 2 μg/mL strongylophorine-26 for 30 min (**C** and **D**) or 6 h (**E** and and **F**). They were fixed and stained for F-actin (**A, C,** and **E**) and the focal adhesion protein vinculin (**B, D,** and **F**; *bar*, 10 μm).

Anti-invasion assays were next used to determine whether the Rho inhibitor C3 exoenzyme could abrogate the anti-invasive activity of strongylophorine-26. Cells were loaded with or without C3 exoenzyme and then plated on Matrigel in the presence or absence of strongylophorine-26. Quantitative assessment of noninvasive cells revealed that treatment with C3 exoenzyme blocked strongylophorine-26 activity (Fig. 5B). Cells that had been simultaneously treated with strongylophorine-26 and C3 exoenzyme extended protrusions into the Matrigel, whereas cells treated with strongylophorine-26 alone remained rounded (Fig. 5C, compare *c* with *d*). These results indicate that Rho activation is required for the anti-invasion activity of strongylophorine-26.

Interestingly, we observed a morphologic difference in the protrusions of cells treated with C3 exoenzyme alone compared with those of cells treated with strongylophorine-26 and C3 exoenzyme together. In the latter condition, the membrane extensions were broader and less extensive, whereas in the former, cells had elongated spindle-like

protrusions that are characteristic of low levels of active Rho (ref. 27; Fig. 5C, compare *b* with *d*). Although C3 exoenzyme was able to restore invasive characteristics to cells in the presence of strongylophorine-26, the cells showed a different morphology than control cells, indicating that complete abrogation of strongylophorine-26 effects did not occur.

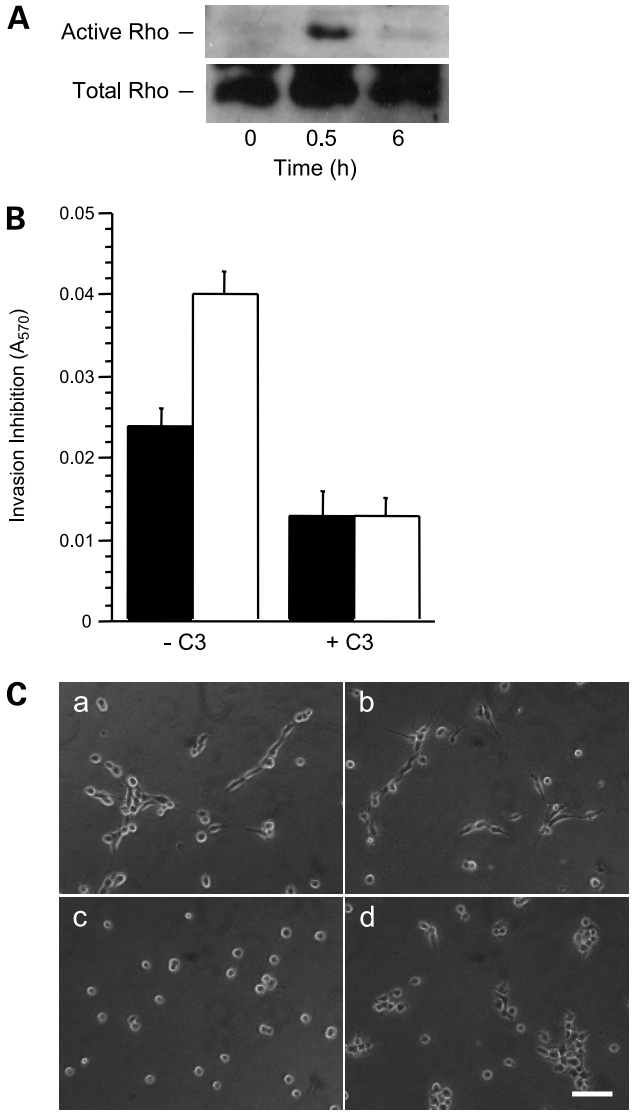

Figure 5. Strongylophorine-26 activates Rho and inhibits invasion in a Rho-dependent manner. **A,** MDA-MB-231 cells were treated with 2 μg/mL strongylophorine-26 for the indicated times and Rho activation was assessed. **B,** MDA-MB-231 cells were preloaded with or without the Rho inhibitor C3 exoenzyme, and cells were plated on Matrigel in the presence of DMSO (*black column*) or 1 μg/mL strongylophorine-26 (*white column*). Invasion inhibition was quantified as described in Materials and Methods. The assay was done in triplicate; *bars*, SEM (strongylophorine-26 versus strongylophorine-26 + C3 = $P < 0.001$). **C,** cells were treated as in (**B**) for 4 h and phase contrast photographs of live cells were taken. Untreated cells without (*a*) or with (*b*) C3 exoenzyme invaded the gel. Invasion was inhibited by strongylophorine-26 (*c*), but this effect was abrogated by pretreatment with C3 exoenzyme (*d*; *bar*, 60 μm).

Discussion

Metastatic progression of tumor cells and angiogenesis both require the acquisition of a motile and invasive phenotype. Thus, inhibiting tissue invasion by interfering with cell motility is a promising approach to antimetastasis therapy. In this study, we characterized the anti-invasion effect of strongylophorine-26 and determined that it inhibits cell motility and leads to a depolarized cell morphology. Strongylophorine-26 causes a dramatic rearrangement of the actin cytoskeleton and induces the formation of large, peripherally localized focal adhesions. We also determined that strongylophorine-26 activates Rho, and that Rho activation is required for its anti-invasion activity.

At early time points, treatment with strongylophorine-26 induces Rho activation, cell contraction, and rounding. It is possible that these effects are caused by Rho-induced stimulation of actomyosin contractility. However, after longer treatments, Rho is not activated and strongylophorine-26 induces radial nonpolar lamellipodial protrusions in cells on tissue culture plastic, a morphology that may indicate elevated levels of the Rho-GTPase family member Rac. Rho activation is classically associated with the formation of thick actin stress fibers, as reported for the Rho activator and invasion inhibitor dihydromotuporamine C (19). However, in this study, induction of stress fibers by strongylophorine-26 was not observed. One explanation might be that signaling cascades induced by strongylophorine-26 and dihydromotuporamine C feed through distinct Rho effectors. ROCK (Rho-kinase) and mDia are two Rho effectors that cooperate to organize the formation and alignment of actin stress fibers (28). Activation of ROCK leads to actin stabilization and the formation of thick centrally located stress fibers (29, 30), whereas mDia reduces central stress fibers and induces actin polymerization (31, 32), a process required for lamellipodial extension. In the case of strongylophorine-26, it is possible that signaling cascades through mDia and feeds back to activate Rac, as seen in a study by Tsuji et al. (33), where ROCK signaling in lysophosphatidic acid–treated Swiss 3T3 cells was abrogated using the inhibitor Y-27632. Cells under these conditions exhibited membrane ruffles and focal complexes, and stress fiber formation was suppressed. The phenotype was shown to be dependent on Rac1. A similar feedback mechanism through mDia to Rac could explain the delayed cell flattening and lamellipodial extensions that were observed in this study. In the case of dihydromotuporamine C, inhibition of ROCK blocks stress fiber formation and tumor invasion,[4] which suggests that dihydromotuporamine C exerts its effect primarily by signaling through the ROCK pathway. Thus, divergent signaling downstream of Rho is an attractive explanation for the distinct cellular effects of strongylophorine-26 and other agents such as dihydromotuporamine C. Cdc42 is a third GTPase of the Rho family that has been well

[4] L.M. McHardy, unpublished observations.

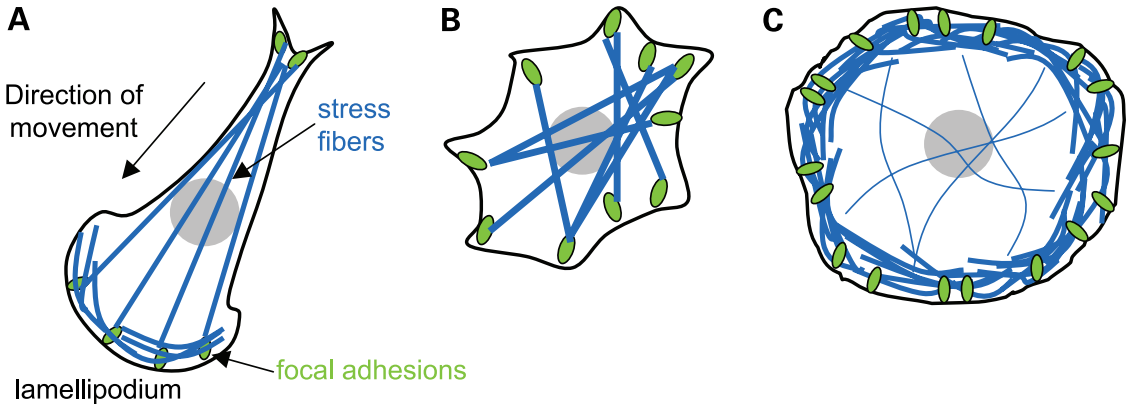

Figure 6. Diagram comparing the morphologic effects of strongylophorine-26 with those of other inhibitors of cell motility and angiogenesis. **A,** migrating cells with a lamellipodium extending in the direction of cell migration, focal adhesions, a meshwork of lamellipodial actin filaments and actin stress fibers. **B,** dihydromotuporamine C and several inhibitors of cell motility and angiogenesis disrupt cell polarity and prevent cell migration by stabilizing focal adhesions and actin stress fibers (14, 15). **C,** strongylophorine-26 disrupts cell polarity and prevents cell migration by stabilizing focal adhesions and inducing the formation of radial protrusions resembling lamellipodia that contain a dense meshwork of peripheral actin filaments and few stress fibers.

characterized for its central role in establishing cell polarity in all eukaryotic cells (34). Cdc42 is required to induce polarization, although levels of activity must be restricted both spatially and temporally in order for complete polarization to occur. In the case of strongylophorine-26, it is possible that the compound is affecting Cdc42 activity or localization, which could result in the loss of polarity that was observed in this study.

Classically, the literature reports that lamellipodial protrusions increase in rapidly migrating cells. So, why then, does strongylophorine-26 inhibit cell migration? We observed that strongylophorine-26 induced lamellipodia in a nonpolarized manner. The entire cell periphery was extended and flattened with increased ruffling throughout. Cells also exhibited numerous, large focal adhesions at the cell periphery. Effectively, the cells are spread and ''stuck'' (Fig. 6). In this case, the lack of polarized phenotype is most likely responsible for the inhibition of motility.

The necessity for balanced levels of Rho GTPase signaling is an emerging theme in the field of cell migration. This is best exemplified in the literature concerning Rho; both inhibiting and stimulating Rho activity can inhibit cell migration (16, 19, 35–37). Although the activities of Rac and Cdc42 have primarily been shown to promote cell migration, this requirement for balanced signaling may be applicable to all of the Rho-GTPase family members. In the case of strongylophorine-26, further analysis of Rac and Cdc42, as well as the downstream Rho effectors ROCK and mDia is required to better understand whether this balanced signaling is a universal principle which governs cell migration and tumor invasion.

Tumor angiogenesis is another fundamental aspect of the metastatic process requiring tissue invasion, but by normal vascular endothelial cells rather than tumor cells. Recently, Keezer et al. (20) analyzed a panel of structurally dissimilar angiogenesis inhibitors and found that they all act by a common mechanism that involves induction of stress fibers in migratory endothelial cells. Fittingly, dihydromotuporamine C also inhibits angiogenesis (22). The effect of strongylophorine-26 on angiogenesis has not yet been determined. However, because the compound does not induce stress fiber formation, its effect on angiogenesis may be distinct from those of angiogenesis inhibitors described to date. Thus, it will be important to determine whether strongylophorine-26 shows antimetastatic or antiangiogenic activity *in vivo* and whether its activity might be different from that of other angiogenesis inhibitors.

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