Inhibition of Tumor Cell Invasion and Angiogenesis by Motuporamines

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ABSTRACT

Tissue invasion is an important determinant of angiogenesis and metastasis and constitutes an attractive target for cancer therapy. We have developed an assay to identify agents that inhibit invasion by mechanisms other than inhibition of cell attachment or cytotoxicity. A screen of marine sponge extracts identified motuporamines as micromolar inhibitors of invasion of basement membrane gels by MDA-231 breast carcinoma, PC-3 prostate carcinoma, and U-87 and U-251 glioma cells. Motuporamine C inhibits cell migration in monolayer cultures and inhibits actin-mediated membrane ruffling at the leading edge of lamellae. Motuporamine C also reduces β1-integrin activation, raising the possibility that it interferes with “inside-out” signaling to integrins. In addition, motuporamine C inhibits angiogenesis in an in vitro sprouting assay with human endothelial cells and an in vivo chick chorioallantoic membrane assay. The motuporamines show little or no toxicity or inhibition of cell proliferation, and they are structurally simple and easy to synthesize, making them attractive drug candidates.

INTRODUCTION

The ability of cancer cells to invade adjacent tissues and to stimulate neovascularization is critical to tumor growth and metastasis (1). Tissue invasion allows primary tumors to disseminate and form metastases, the cause of 90% of cancer deaths (2). Tumors also stimulate the formation of new blood vessels without which they cannot grow beyond a size of 1–2 mm and cannot metastasize (3). Consequently, the elucidation of the mechanisms governing metastasis and angiogenesis and the development of therapies aimed at preventing these processes are the focus of intense research.

Invasion, angiogenesis, and metastasis all require cells to modify their adhesion to other cells and to the extracellular matrix, to break down the matrix, and to migrate through the breaches thus created (3, 4). Therefore, agents that inhibit the movement of tumor cells and endothelial cells through the extracellular matrix have the potential to be of considerable therapeutic value.

We have developed a quantitative assay suitable for testing crude natural extracts for inhibitors of this process. In an initial small-scale screen, we found that a family of marine sponge alkaloids, the motuporamines, inhibit the invasion of metastatic MDA-231 breast carcinoma cells. In subsequent testing, we demonstrate that motuporamine C interferes with the migration and leading edge ruffling of human breast cancer cells, prostate carcinoma cells, and glioma cells.

In addition, motuporamine C inhibits angiogenesis in both an in vitro sprouting assay and an in vivo chick chorioallantoic membrane assay. These properties, combined with its low cytotoxicity and ease of synthesis, make motuporamine C an attractive drug candidate.
SD of duplicate measurements of the effects of DMSO (negative control), 125 cells were measured using the MTT assay in which the absorbance at 570 nm provides a quantitative measure of the number of cells that failed to invade. Shown are averages and SD of duplicate measurements of the effects of DMSO (negative control), 125 mmol/L LY294002 (positive control), and 15 of the sponge extracts. A, B, structural formulae of motuporamins isolated from extract #1 from Xestospongia exigua.

**RESULTS**

**Three-step Screen for Invasion Inhibitors.** The “outgrowth” assay and the “Boyden chamber” assay are widely used to study invasion. In the former, cells are suspended in liquid Matrigel followed by maturation/evaporation of the combined CH2Cl2 extracts in vacuo. Water layer. Concentration of the n-butyl alcohol extracts in vacuo gave an active residue that was suspended in water adjusted to pH 7.2 by addition of NaOH. Extraction of the basic aqueous solution with CH2Cl2 followed by evaporation gave a residue active in the assay. The active residue was then further fractionated by reversed-phase high-performance liquid chromatography [elucent, 2% trifluoroacetic acid in methanol/water (11:9)] to give pure samples of the known compounds motuporamins A and C (6), a sample of monoacetylmotuporamine C (an artifact resulting from reaction of motuporamine C with ethyl acetate during purification), and mixtures of a number of minor related analogues. Diacetylated derivatives of the motuporamins were prepared by dissolving them in pyridine/acetic anhydride (3:1) and stirring at room temperature for 16 h. Removal of the reagents by evaporation in vacuo gave diacetylated motuporamins that were purified via reversed-phase high-performance liquid chromatography [elucent, acetonitrile/0.6% trifluoroacetic acid in H2O (2:3)].

**Integrin Activation State.** Integrin expression on the cell surface was analyzed by flow cytometry. PC3 cells were serum-starved overnight and then incubated for 1 h in medium without serum containing the indicated concentrations of motuporamine C. Cells were washed and harvested by scraping. Cells were suspended in 100 μl of PBS (pH 7.4), 20 mM glucose, and 1% BSA and incubated with a 4 μg/ml anti-activated β1 integrin (MAB2079Z; Chemicon) or total-β1 integrin (Upstate Biotechnology) monoclonal antibody. After a 45-min incubation at room temperature, cells were incubated with 1 μg of FITC-conjugated antimouse secondary antibody (Jackson Laboratories) for 30 min at room temperature. Cells were analyzed on a Coulter EXPO XL4 flow cytometer. Experiments were performed in duplicate and repeated three times.

**Endothelial Sprouting Assay.** Endothelial sprouting was assessed by a modification of the method used by Nellis and Drenckhahn (7). Briefly, microcarrier beads coated with denatured collagen (Cytodex 3; Sigma Chemical Co.) were seeded with HUVECs and embedded in fibrin gels in 96-well plates. For preparation of fibrin gels, bovine fibrinogen was dissolved in MCDB medium at a concentration of 2.5 mg/ml. Aprotinin was added at a concentration of 0.05 mg/ml, and the solution was passed through a 0.22-μm filter. The fibrinogen solution was supplemented with 15 ng/ml VEGF. As a control, fibrinogen solution without VEGF was used. Motuporamine C was also added at different concentrations, and the fibrinogen solutions were transferred to 96-well plates together with HUVEC-coated beads at a density of 50 beads/well. Clotting was induced by the addition of thrombin (1.2 units/ml). After clotting was complete, gels were equilibrated with MCDB medium containing 5% FBS at 37°C. After 60 min of incubation, the medium was replaced with medium with or without motuporamine C. After 3 days of incubation with daily changes of the medium, the number of capillary-like tubes formed/microcarrier bead (sprouts/bead) was counted by microscopy, monitoring at least 150 beads for each treatment. Only sprouts greater than 150 μm in length and composed of at least three endothelial cells were counted.

**Chick CAM Assay for Angiogenesis.** Fertilized White Leghorn chicken eggs were incubated at 37°C under conditions of constant humidity. On embryonic day 6, the developing CAM was separated from the shell by opening a small circular window at the broad end of the egg above the air sac. The opening was sealed with Parafilm, and the eggs were incubated for 2 more days. Motuporamine C was prepared in PBS supplemented with 30 ng/ml VEGF. On day 8, 20 μl was loaded onto 2-mm3 gelatin sponges (Gelfoam; Pharmacia Upjohn) that were placed on the surface of the developing CAM. Sponges containing vehicle alone (20 μl of PBS) were used as negative controls, whereas sponges containing 20 μl of 30 ng/ml VEGF in PBS were used as positive controls. Eggs were resealed and returned to the incubator. On day 10, images of CAM were captured digitally using an Olympus SZX9 stereomicroscope equipped with a Spot RT digital imaging system (Diagnostic Instruments).

**Cell Viability and Proliferation Assays.** HUVEC viability was determined as follows. Cells were plated in 96-well plates at 1.5 × 104 cells/well. When cells reached 95% confluency, motuporamine C was added at different concentrations and for different times, with daily change of medium and drug, and cell viability was measured by incubating cells with 100 μl of 0.005% neutral red in cell culture medium for 4 h. The medium was removed, and 100 μl of 1% acetic acid in 50% ethanol was added/well to solubilize the dye and absorbance was measured at 550 nm. HUVEC proliferation was determined as follows. Cells were plated in 96-well plates at 5 × 103 cells/well and incubated with different concentrations of motuporamine C, with daily change of medium and drug. Cell proliferation was measured at 0, 24, 48, and 72 h using neutral red as described above. MDA 231 proliferation was determined as follows. Cells were plated in 96-well plates at 1 × 105 cells/well and treated with different concentrations of motuporamine C for 24 h. Proliferation was measured at different times using the MTT assay (5).

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**Fig. 1.** Inhibition of invasion by two crude sponge extracts and chemical structures of motuporamins A and C. A, MDA-231 cells were incubated with Matrigel in the presence of sponge extracts for 2.5 h as described in “Materials and Methods.” Cells that failed to invade were recovered by trypsinization and replated. After overnight incubation, live cells were measured using the MTT assay in which the absorbance at 570 nm provides a quantitative measure of the number of cells that failed to invade. Shown are averages and SD of duplicate measurements of the effects of DMSO (negative control), 125 μM LY294002 (positive control), and 15 of the sponge extracts. **B,** structural formulae of motuporamins isolated from extract #1 from Xestospongia exigua.
gelling, and invasion is then assayed morphologically as the cells form outgrowths into the gel (8). In the latter, Matrigel is pre-gelled upon a porous filter support. Cells are then placed on the Matrigel, and invasion is quantified by measuring the number of cells that cross to the other side of the Matrigel/filter barrier, usually in response to a chemotactic agent (9). Both assays have proven very useful for studying mechanisms that regulate invasion, but they have drawbacks that make them less suited for screening for invasion inhibitors. The major drawback of outgrowth assays is the difficulty in quantifying changes in cell morphology. The Boyden chamber assays do generate quantitative data, but they are unable to discriminate between agents that affect invasion, adhesion, and cell viability.

Nature is a prime source of drug leads (10). However, an inherent problem with using crude natural extracts in cell-based screens is that 10–20% of the extracts are toxic to cells at the dilutions that generate optimal hit rates because of the high concentrations of salts and other materials they contain. To avoid unacceptably high numbers of false-positive results, a screen needs to distinguish invasion inhibition attributable to a specific inhibitor from that caused by cell death or toxicity. We have combined the principles of the Matrigel outgrowth and the Boyden chamber assays in a three-step screen for invasion inhibitors. The assay is quantitative and screens sequentially for compounds that prevent invasion of Matrigel, do not prevent cell attachment to Matrigel, and are not cytotoxic.

We first tested the suitability of the assay for drug screening using a small selection of crude methanol extracts from marine sponges. Two-hundred and thirty extracts were tested at 50–100 μg/ml. Two-hundred and twenty-eight extracts showed absorbance readings close to or below those of the DMSO carrier negative controls (0.025). Two extracts showed strong inhibition of invasion (Fig. 1A) higher than LY294002, a phosphatidylinositol 3-kinase inhibitor known to inhibit invasion (11).

Isolation and Identification of Motuporamines. The active compounds were purified from extract #1 as described in “Materials and Methods,” using the assay to guide fractionation. The sponge yielding extract #6 resembled the first sponge and contained the same active compounds. The active compounds were identified as motuporamine A and motuporamine C (Fig. 1B), two macrocyclic alkaloids with a spermidine-like “tail” (6). The compounds showed concentration-dependent activity with IC₅₀ of 3 μM for motuporamine A and 1 μM for motuporamine C. Only motuporamine C was obtained in sufficient quantities for further study. As expected from the design of the screening assay, motuporamine C did not inhibit the proliferation of MDA-231 cells at a concentration close to the IC₅₀ for invasion inhibition (Fig. 2). Higher concentrations of 4 and 8 μM showed only a mild inhibition of cell proliferation.

Importance of the Spermidine-like Tail for Activity. A conspicuous feature of motuporamines is their spermidine-like tail, which carries positive charges at physiological pH. To examine the role of the tail in invasion inhibition, motuporamine C was acetylated at one or both amines of the tail, and the compounds were tested for activity.

Fig. 3. Importance of the tail amines for invasion inhibition. Motuporamine C and derivatives were prepared and tested at different concentrations in the invasion inhibition assay.

Fig. 4. Motuporamine C inhibits invasion of basement membrane gels and subtly affects cell morphology in monolayer culture. A, MDA-231 cells were plated on Matrigel in the presence of the vehicle control (DMSO; 0.1%) or the indicated concentrations of motuporamine C for 4 h (bar, 20 μm). B, MDA-231 cells were pre-spread in monolayer culture after attachment to tissue culture plastic and then treated with the vehicle control (DMSO) or 5 μM motuporamine C or 1 μg/ml cytochalasin D (CD) for 4 h. Cell morphology was then assessed by phase contrast microscopy of live cultures (parts a–c), and actin was visualized by rhodamine-phalloidin staining (parts d–f, arrowheads, small discontinuous aggregations of actin at cell edges; bar, 15 μm).
Acetylation of the terminal amino group had no effect on activity, whereas acetylation of both the terminal and central amines caused a complete loss of activity (Fig. 3). HCl treatment of diacetylated motuporamine C removed both acetates and restored activity (Fig. 3). Therefore, the terminal amino group is dispensable for activity, but the central amine is absolutely required.

Morphological Analysis of Invasion Inhibition by Motuporamine C. MDA-231 cells are highly invasive, both in vivo and in vitro (12). These cells become spindle-shaped and move into basement membrane gels within 4 h. Incubation of the cells with motuporamine C at the time of plating prevented these morphological responses (Fig. 4A). It is noteworthy that the motuporamine C concentration that strongly inhibited basement membrane invasion (5 μM) did not cause the cells to detach from the matrix. Instead, the cells remained rounded, attached, and, for the most part, single (Fig. 4A, part d).

Motuporamine C (5 μM) had similar morphological effects on prostate carcinoma (PC-3) and glioma (U-251 and U-87) cells (Table 1).

Therefore, the activity observed in the initial three-step screen using MDA-231 cells correctly identified motuporamine C as a cancer cell invasion inhibitor, and the follow-up morphological experiments with other cell lines indicates widespread in vitro efficacy.

The actin cytoskeleton regulates changes in cell shape (13). The

Table 1 Comparison of the ability of motuporamine C (5 μM) to inhibit basement membrane invasion, inhibit migration in wounded monolayers, and disrupt leading edge actin-based ruffles in four different tumor lines

<table>
<thead>
<tr>
<th>Activity</th>
<th>MDA-231 breast carcinoma</th>
<th>PC-3 prostate carcinoma</th>
<th>U-87 glioma</th>
<th>U-251 glioma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of basement membrane invasion</td>
<td>++*</td>
<td>+*</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Inhibition of migration</td>
<td>++</td>
<td>+</td>
<td>ND*</td>
<td>++</td>
</tr>
<tr>
<td>Disruption of leading edge ruffles</td>
<td>++</td>
<td>++</td>
<td>ND*</td>
<td>++</td>
</tr>
</tbody>
</table>

* For each category, MDA-231 was designated the standard with a value of ++.

† At 4 h after treatment, the inhibitory effect on PC-3 was less than for MDA-231; however, the inhibition was greater for PC-3 at later time points (i.e., 24 h).

ND, not done; this cell line did not form confluent monolayers that could be wounded.

ND, not done; this cell line did not form clearly discernible leading edge ruffles under control conditions.

The actin cytoskeleton regulates changes in cell shape (13). The

Fig. 5. Motuporamine C inhibits cell migration and perturbs actin ruffling in leading lamellae. A, confluent MDA-231 cell monolayers were wounded with a sterile toothpick (vertical orientation in the micrographs), and cells were allowed to migrate into the wound over a 24-h period in the presence of the vehicle control (DMSO) or 5 μM motuporamine C (MP; bar, 30 μm). B, MDA-231 monolayers were wounded and maintained for 8 h in the presence of DMSO or 5 μM motuporamine C and then photographed live by phase contrast microscopy or stained for filamentous actin with rhodamine-phalloidin. The wound is located in the top portion of each photomicrograph. The black arrow on the right indicates the direction of cell migration (white arrows, continuous membrane ruffles in control cultures; white arrowheads, discontinuous ruffles in motuporamine treated cultures; bar, 20 μm for parts a and b and 10 μm for parts c and d).
motuporamine C also inhibits angiogenesis. This was tested using an in vitro endothelial sprouting assay and an in vivo CAM assay. In the endothelial sprouting
assay, HUVECs are seeded onto collagen-coated beads. Exposure to VEGF stimulates the formation of capillary-like tubes, the number and length of which may be measured over time by microscopy (Fig. 7). In two separate experiments, endothelial sprouting quantitated at 72 h was inhibited by 88% and 93% with 5 μM motuporamine C and was inhibited completely with 10 μM motuporamine C (see Fig. 7 for examples). In the CAM assay, angiogenesis in response to VEGF (30 ng/ml) was reduced by treatment with 2.5 μM and 5 μM motuporamine C and was completely inhibited at 10 μM motuporamine C (Fig. 8). Motuporamine C was not toxic in the CAM assay when tested at concentrations of up to 25 μM (data not shown).

Finally, we examined the effects of motuporamine C on the survival and proliferation of HUVECs. Incubation of rapidly proliferating HUVECs with different concentrations of motuporamine C for up to 3 days did not inhibit cell proliferation (Fig. 9A). Exposure of confluent, nonproliferating HUVEC monolayers to different motuporamine concentrations for up to 3 days also had no effects on cell survival (Fig. 9B). Therefore, as was the case for invasion, motuporamine C does not inhibit angiogenesis through toxic or antiproliferative effects.

**DISCUSSION**

Invasion and angiogenesis are important determinants of tumor progression and, as such, constitute attractive targets for cancer therapy. Extracts from marine invertebrates, particularly sponges, are a rich source of secondary metabolites with potential as lead compounds for the development of therapeutic drugs (16). To search for inhibitors of invasion and angiogenesis in natural extracts, we sought to establish a quantitative assay that would be simple yet able to eliminate cell death and inhibition of attachment as causes of invasion inhibition. The assay described here achieves this by screening sequentially for compounds that inhibit invasion into Matrigel, do not prevent cell attachment to Matrigel, and do not kill the cells. A small-scale screen showed activity in an extract of the Papua, New Guinea sponge *Xestospongia exigua* (Kirkpatrick), and assay-guided fractionation led to the isolation of motuporamines as potent and efficacious inhibitors of invasion and angiogenesis.

The motuporamines are a family of relatively simple macrocyclic alkaloids containing a spermidine-like substructure (6). Comparison of the activities of the natural compounds and simple chemical modification of the tail of motuporamine C provided initial structure-activity information. The positively charged amine in the middle of

![Fig. 8. Motuporamine C inhibits angiogenesis in vivo. Photographs of developing CAMs incubated for 2 days with VEGF (A) or VEGF and motuporamine C at 2.5 μM (B), 5 μM (C), or 10 μM (D). The arrows indicate the corners of the gelatin sponges containing VEGF and the compounds.](image)

![Fig. 9. Motuporamine does not inhibit HUVEC proliferation or survival. Different concentrations of motuporamine C were added at day 0 to rapidly dividing HUVECs (A) or near-confluent HUVECs (B), and medium and compound were changed daily. Proliferation and survival were measured at the indicated times as described in "Materials and Methods."](image)
the tail is a critical determinant of activity because its acetylation completely abrogated anti-invasion activity. However, acetylation of the terminal amino group had no detectable effect. The size of the macrocyclic ring had an influence on activity because motuporamines with smaller rings were slightly less active. Simple and inexpensive motuporamine synthetic schemes have been published recently (17, 18) that will make possible further structure-activity study and eliminate dependence on natural sources.

The motuporamines show some resemblance to squalamine, an angiogenesis inhibitor (19) currently in Phase II clinical trials for the treatment of advanced non-small cell lung cancer (20). Squalamine was isolated from dogfish shark liver and is a much more complex molecule composed of spermidine attached to C-3 of a steroid core with a sulfated side chain. The observation that both classes of compounds decrease cell migration, inhibit angiogenesis, and are composed of a macrocyclic ring attached to a polyamine raises the possibility that they act in a similar fashion. However, although the mechanism of action of squalamine is still unclear, it appears to inhibit both cell proliferation and migration (19). Motuporamine C inhibits migration with little effect on proliferation, suggesting that the two compounds may have distinct cellular targets.

In this study, we carried out preliminary cell biological studies in an effort to broadly define the mechanism of action of motuporamine C. We found that doses of motuporamine C that inhibit both tumor cell invasion and endothelial cell angiogenesis are not cytotoxic to cancer cell lines and HUVECs. This characteristic, which was built into the original screen, is an important consideration with respect to the possible therapeutic usefulness of the drug. Invasive tumor cells in malleable basement membrane gels and endothelial cells in malleable fibrin gels remained rounded in the presence of motuporamine C. This could have been explained by global disruption of the actin cytoskeleton. However, experiments in monolayer culture do not support this possibility because cells were able to spread and form cytoplasmic actin stress fibers.

The ability of motuporamines to decrease tumor cell movement through basement membrane gels and endothelial cell movement through fibrin gels suggests that the drug acts to inhibit cell-mediated degradation of extracellular matrix and/or inhibit cell motility. It has not yet been determined whether motuporamine C alters matrix degradation. However, we have demonstrated that the compound decreased tumor cell motility and subtly altered the organization of filamentous actin at the cell margin. In particular, we observed an impairment of actin-mediated membrane ruffling in the leading lamellae of cells induced to migrate by wounding. This suggests at least two possible classes of molecular targets. The first class is the Rho-family GTPases, most specifically Rac, the activity of which is required for the formation of leading lamellae and ruffles (21, 22). The second class consists of molecules that regulate actin polymerization within ruffles. These include the Arp2/3 complex and regulators such as WASP (23). Cellular adhesion to the extracellular matrix itself leads to the activation of Rho family GTPases and subcellular actin rearrangements, including those associated with leading edge ruffling (24). Importantly, these events also provide feedback via an as yet poorly defined “inside-out” signaling mechanism to regulate the affinity of cell surface integrin receptors for their extracellular matrix ligands (25). It has been proposed that such changes in integrin affinity help regulate the maturation of transient focal complexes at the leading edge of migrating cells into more mature, stable focal complexes in the cell body (26). Motuporamine C may act to dampen this inside-out signaling because it subtly decreased the affinity state of β1 integrins. Despite this modest decrease, we did not observe any disruption of tumor cell attachment to either basement membrane gels or tissue culture plastic. To our knowledge, compounds with analogous structure have not been documented to have effects on adhesion. We are currently address-

ing this by examining focal adhesion complex formation and signaling on a number of defined extracellular matrices.

Compounds that have the potential for inhibiting tumor cell invasion and angiogenesis are attractive candidates for cancer therapy. In combination with conventional cytotoxic chemotherapy agents, they may prove to be efficacious in controlling cancer progression. We are now carrying out experiments to evaluate the antitumor, antiangiogenesis, and antimetastasis activity of motuporamine C in animals and structure-activity studies to identify additional compounds for in vivo testing. Motuporamines showing in vivo activity will become attractive novel drug candidates.

ACKNOWLEDGMENTS

We thank Edmund Au and Lindsay Chung for technical assistance and Hilary Anderson for critical reading of the manuscript.

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Cancer Res 2001;61:6788-6794.

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