Emergence of potent inhibitors of metastasis in lung cancer via syntheses based on migrastatin

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Migrastatin is a biologically active natural product isolated from Streptomyces that has been shown to inhibit tumor cell migration. Upon completion of the first total synthesis of migrastatin, a number of structurally simplified analogs were prepared. Following extensive in vitro screening, a new generation of analogs was identified that demonstrates substantially higher levels of in vitro inhibitory activity, stability and synthetic accessibility when compared to the parent natural product. Herein, we describe two promising ether-derivative analogs, the migrastatin core ether (ME) and the carboxymethyl-ME (CME), which exhibit high efficacy in blocking tumor cell migration and metastasis in lung cancer. These compounds show an in vitro migration inhibition in the micromolar range (IC50: ME 1.5 to 8.2 μM, CME 0.5 to 5 μM). In a human small-cell lung carcinoma (SCLC) primary xenograft model, ME and CME compounds were found to be highly potent in inhibiting overall metastasis even at the lowest dosage used (degree of inhibition: 96.2% and 99.3%, respectively). Together these very encouraging findings suggest that these analogs have promise as potent antimetastatic agents in lung cancer.

Our laboratories have had a long-term interest in small-molecule natural products (SMNPs), from the standpoint of both synthesis and biological evaluation. At the level of chemical synthesis, these types of targets have provided a host of challenges, in terms of issues of gross structure and stereochemical presentation. Moreover, SMNPs often constitute unusually promising opportunities for discovery of novel biological profiles, which might, upon optimization, be exploitable for pharmaceutical ends. It was for such considerations that we took considerable interest in reports describing the isolation of a tumor cell migration inhibitor, termed migrastatin (1), from fermentation sources (1–4). From the perspective of a synthetic challenge, the structure of migrastatin is such that it fits in well with our ideas about modulating acyclic stereochemistry through systematic disconnection of pyranoid rings generated from the Lewis acid-catalyzed diene aldehyde cyclocondensation (LACDAC) reaction (5). Aside from the need to deal with the macrolactone matrix of migrastatin, a workable total synthesis must be responsive to the issue of appending the glutarimide containing side chain in a stereocontrolled manner.

In the context of providing a possible platform for drug discovery, migrastatin was of particular interest to us. The migration of metastatic tumor cells from the primary tumor to outlying sites and organs is a poor prognosis feature of many cancers and is associated with resistance to therapy. Cell migration is clearly relevant to a host of critical biological functions (cf. inter alia: embryonic development, organogenesis, and homeostasis). Unfortunately, the deregulation of this complex process is also involved in cancer progression (tumor angiogenesis and metastasis). Tumor angiogenesis (the angiogenic switch) is likely to set the stage for cancer cell invasion and metastasis. Though the activity of migrastatin in a wound-healing assay was only ca. 29 μM (IC50), it was hoped that our previously described modality of diverted total synthesis (DTS) might lead to enhanced potency in the context of a workable therapeutic index. The first stage in our SMNP-oriented discovery strategy is to focus on the total synthesis challenge. From there we go on to the exploration of “high pedigree” cognate molecular space, accessible through DTS. This discovery platform is in keeping with our notion that a molecular editing exercise with a biologically active natural product as the primary “first draft” is more likely to be fruitful than high-throughput exercises driven by stochastic modalities.

Critical to our synthetic planning goals was the design of a concise, scalable, and flexible route to migrastatin that would allow for verification of its reported structure and confirmation of its alleged biological functions while also providing maximum flexibility for exploring SAR profiles in surrounding high pedigree molecular space. From the planning phase there emerged a suitable strategy wherein migrastatin would be assembled in a convergent fashion from readily available building blocks. The reduction to practice of these deliberations is captured in Scheme 1. Thus, union of aldehyde 2, readily obtained from an inexpensive chiral pool source, and diene 3 selectively afforded the C4–C10 stereotriad (4) en route to the migrastatin macrocyclic core. Following our protocols of the 1980s, reduction of the ketone followed by a Ferrier rearrangement of the resultant glycal served to install the Z-trisubstituted olefin of migrastatin (see 5). This key intermediate was advanced to migrastatin (1) in a straightforward manner as shown. Key transformations included: stereocontrolled anti-aldol reaction of 7 and 6; introduction of the glutarimide functionality via Horner-Wadsworth-Emmons (HWE) reaction (9 + 10 → 11); and construction of the macro lactone through ring closing metathesis (RCM) (6). Indeed, the high level of congruence of the spectroscopic properties of the synthetic product and the fermentation-derived natural product left no cause to doubt its identity.

Upon completing the first total synthesis of migrastatin, we confirmed the reported in vitro cell migration inhibitory activity with our synthetic material (6). We then set out to prepare and evaluate a range of analogs through reliance on the logic of DTS. Initial studies focused on discerning the biological roles of the glutarimide N-H functionality and the C2-C3 double bond, which has the potential, in principle, to act as a Michael acceptor. Interestingly, analogs incorporating either a methyl cap on the glutarimide moiety or saturation at C2-C3 showed approximately similar levels of migration inhibitory activity compared to the parent natural product, suggesting that these structural features are not central to the biological function of the natural product.
In light of these findings, we next designed a menu of structurally simplified migrastatin analogs lacking the glutarimide side chain. We note, in passing, that the success of our ongoing migrastatin program serves to illustrate the unique power and flexibility of the DTS approach to lead agent optimization.

With regard to SMNPs as a major resource for pharma development, it should be well appreciated that the reasons as to why organisms or plants biosynthesize such secondary metabolites are far from clear. It is likely that SMNPs emerge with proclivity for binding to biolevel targets, such as proteins, oligosaccharides, or nucleic acids—perhaps for regulatory purposes or as defensive resources against various challenges from potential predators. Certainly, there is no reason to believe that the SMNPs have been optimized for the purposes that the pharma scientist might have in mind. Moreover, the SMNP may not be optimized even for its intended mission. The structure of the SMNP that does arise may well reflect the limitations of the available biosynthetic pathway. These catabolic mechanisms are not readily diverted for purposes of med chem level analog synthesis.

Returning to migrastatin, we were particularly anxious to explore its adjoining molecular terrain. We sensed that approaches to analog synthesis, by manipulations starting with the intact natural product, would not readily permit access to the wide array of analogs that we sought. These are clear limitations in the available chemistry that would be needed to alter the sensitive migrastatin structure in the ways we envisioned. Modification of the biosynthetic machinery (via polyketide synthases) (8) to gain access to the substrates we wished to explore was not practical. Happily, we were able to synthesize what we needed from an advanced intermediate en route to migrastatin. As will be shown, several of our migrastatin-associated compounds, readily accessible through DTS, have shown great promise in preclinical settings. The overall program that emerged is summarized in Scheme 2.

The analogs shown in Scheme 3 were synthesized through DTS from a common advanced intermediate. We were pleased to find that several of the structurally simplified synthetic analogs exhibited higher levels of in vitro inhibitory activity than the parent natural product. Analysis of a series of macrolactone derivatives (13–16) revealed the migrastatin core (13) and 2,3-dihydro-migrastatin core (14) to be particularly active in an in vitro chamber cell migration assay. However, the hydrolytic instability of these analogs served to limit enthusiasm for their further development. Lactones 15 and 16, designed with a view toward enhancing hydrolytic stability, were found to exhibit poor in vitro activity (8).

In light of these findings, we sought to “edit” out the problematic lactone functionality. Thus, migrastatin core ketone (17), lactam (18), and ether (19) analogs (9) were prepared and evaluated in a chamber cell migration assay. As shown in Scheme 3, each of these compounds exhibited highly promising levels of in vitro migration inhibitory activity. A number of ketone-derived analogs also performed well in the migration assay (20–24).

On the basis of their demonstrated activity, stability, and synthetic accessibility, compounds 17, 18, and 19 were selected for further in vitro and in vivo evaluation.

Further in vitro studies of migrastatin ketone (17), lactam (18) and core ether (ME, 19) revealed the ketone (17, IC50, = 0.023–0.35 μM) and ME (19, IC50, = 0.27–0.38 μM) to be generally more effective than the lactam (18, IC50, = 0.17–2.7 μM) in inhibiting transwell migration of a range of tumor cell lines. Importantly, we were pleased to observe that in all cases, an approximately 1,000-fold higher concentration of migrastatin analog was required to achieve comparable inhibition of normal human mammary epithelial MCF10A cells.

A range of preliminary in vivo studies was undertaken to further assess the therapeutic prospects of our migrastatin lead compounds. Due to its similarity to human breast cancer and its propensity to spontaneously metastasize to a variety of target organs, including the lung, bone, brain, and liver, the 4T1 mouse mammary tumor serves as a useful model. In a key study, BALB/c mice implanted with 4T1 cells were treated on day 10 following implantation with migrastatin core ketone (17) or lactam (18) at 10 mg/kg or 20 mg/kg (10). Following sacrifice 20 d later, the amount of lung metastasis in mice treated with either 17 or 18 was reduced by 91–99% relative to the control group. This effect is attributed, largely if not totally, to the inhibition of metastatic progression was observed following treatment with these drugs.

The migrastatin core ether (ME, 19), which we consider to be the most promising of the first-generation analogs on the basis of its in vitro activity, physical properties, ease of preparation, and apparent biological stability, has been the subject of a number of in vivo investigations. In one key study, NOD/SCID mice were injected in the abdominal mammary fat pad with MDA231 breast cancer cells. On day 1, groups of mice were treated with low-dose (40 mg/kg) or high-dose (200 mg/kg) ME (i.p. ×3 weekly). After four weeks, the primary tumors were resected and the mice were subjected to weekly bioimaging for metastatic tumor. One week following resection, the control mice (treated with PBS buffer) showed extensive metastases, while both groups of ME-treated mice presented significant reduction in metastasis (88–93%). Although the low-dose ME-treated mice showed liver and lung metastasis by nine weeks, no detectable metastatic growth was observed in the high-dose cohort. In accordance with our previous findings, treatment with ME did not significantly attenuate growth of the primary mammary tumor. Similarly positive results were observed with other breast cancer models, including the LM2 cell line, which demonstrate decreased metastasis in the lungs (11).

On the basis of these promising in vivo findings in breast cancer cell lines, we sought to expand the scope of our studies to encompass other types of metastatic tumors. We describe herein some encouraging findings that suggest that ME may serve as a viable tumor cell migration inhibitory agent against metastatic small-cell lung carcinoma (SCLC).

Moreover, in an effort to identify ME-based analogs with enhanced bioavailability and pharmacostability, we also synthesized...
Scheme 3. Migrastatin-based macrolides. Numbers in parentheses represent migratory inhibition activity (IC
demonstrate a very sensitive response in term of in vitro migra-
to the serum gradient. Comparatively, CME exhibited lower IC
tures to carry out to determine the IC
chemotaxis in a transwell migration assay. As shown in Fig. 1,
dies, in which
In Vitro Evaluation of ME (19) and CME (25).

Results and Discussion
In Vitro Evaluation of ME (19) and CME (25). In order to evaluate the antimitastatic properties of our recently synthesized ME analog, carboxymethyl-ME (CME, 25), we assessed its ability to inhibit in vitro cancer cell migration in a wound-healing assay and its chemotaxis in a transwell migration assay. As shown in Fig. 1, ME (19) and CME (25) at 100 μM almost completely blocked migration of human non-small-cell lung carcinoma (NSCLC) A549 cells in response to a scratch wound. At submicromolar concentrations, CME was still quite effective (32% inhibition of migration).

To further characterize these compounds, chemotaxis in response to a serum gradient in a modified Boyden chamber system was used to evaluate the panel of NSCLC lines. This assay provided reproducibly robust data, allowing dose-response studies to be carried out to determine the IC50. As shown in Fig. 2 and Table 1, ME and CME efficiently blocked the migration of human lung cancer cells through the 8 μm pore insert in response to the serum gradient. Comparatively, CME exhibited lower IC50 values (0.5 to 5 μM) than ME compound (1.5 to 8.2 μM) against A549, H1975, and H2999 cancer cells. Because we noticed a mild toxicity and effects on cell proliferation in the millimolar range, experiments with CME and ME at 1 mM or more were not included for the IC50 calculation. Collectively, these results demonstrate a very sensitive response in term of in vitro migration inhibition that is more than 2 orders of magnitude less than the concentration found to produce cytotoxicity.

Evaluation of the Antimetastatic Activity of ME (19) and CME (25) in a Human SCLC Xenograft Model. We next examined the capacity of our lead compounds to inhibit tumor metastasis in a human SCLC primary xenograft model. These cells, in primary and subsequent in vivo passages in NOD/SCID mice, tend to form liver metastases. The tumor cells were stably transduced with a triple-fusion protein reporter construct (AC3-TGL) and then transplanted by subcutaneous injection with matrigel into NOD/SCID IL2R gamma null (NSG) mice.

Starting 1 d prior to the inoculation of tumor cells, groups of mice were treated with ME at doses of 10 mg/kg, 40 mg/kg, or 200 mg/kg, or with CME at 12 mg/kg or 49 mg/kg (note: dosage levels were adjusted to account for differences in molecular weight: 1 mg ME = 1.2 mg CME). Control mice were treated with DMSO vehicle. The compounds were administered by i.p. injection every 3 d, from day 1 to day 55 following cell injection (n ≥ 5 mice per group). During this time, tumor burden and metastatic spread were monitored by serial noninvasive bioluminescent imaging (BLI) at days 14, 23, 30, 40, and 50. At day 55, the mice were sacrificed and the metastatic spread of tumor cells

Fig. 1. Inhibition of A549 lung cancer cell lines migration in an “in vitro wound-healing” assay by migrastatin ether (ME) and carboxymethyl-migrastatin ether (CME). A549 cancer cells were grown as a nearly confluent monolayer culture and then starved overnight in medium containing 0.5% FCS. Cell monolayers were then scratched using pipette tip, photographs, and incubated with a 6 log-scaled concentration range of ME and CME from 10−3 to 10−6 M, with or without 2% FCS. After 12 h, areas were fixed, stained, and photographed using computer-assisted microscopy. Micrographs (4X magnification) are presented, showing the A549 cancer cell migration across the scratches in absence of serum (no migration), presence of serum (migration), and in the presence of serum plus CME or ME at 100 μM or 100 nM.
potent than the parent compound, ME, in inhibiting in vivo metastases. The luciferase activity quantified by ex-vivo BLI. Compared to the control group, the mice treated with CME, which we view as a particularly promising candidate in the treatment of tumor metastasis. Though the compounds do not appear to attack the primary tumors, one could readily imagine considerable clinical benefits from an agent hypothesized that appendage of a carboxymethyl functionality onto the ME scaffold provides enhanced migratory inhibition activity. We are currently pursuing further preclinical testing of CME, which we view as a particularly promising candidate in the treatment of tumor metastasis.

In summary then, collaborative chemistry and cell biology studies have led to the identification of several compounds worthy of serious consideration as inhibitors of metastasis. Though the compounds do not appear to attack the primary tumors, one could readily imagine considerable clinical benefits from an agent to the lungs, liver, heart, kidneys, and spleen was assessed by ex-vivo BLI on the removed organs.

As expected, treatment with ME and CME did not significantly affect tumor growth kinetics at the primary site of injection (Fig. S1). In the ME-treated cohorts, some toxicity was observed, resulting in the deaths of three mice in the 200 mg/kg group and two mice each in the 10 mg/kg and 40 mg/kg groups prior to the study endpoint. In contrast, no toxicity was observed in either cohort of CME-treated mice.

At the study endpoint, the potential metastatic sites (liver, lungs, spleen, heart, and kidneys) were surgically resected and the luciferase activity quantified by ex-vivo BLI. Compared to the control group, the mice treated with 10 mg/kg or 40 mg/kg ME exhibited significantly decreased levels of overall metastasis, with calculated inhibition factors of 96.2% (p-value = 0.0095) and 99.1% (p-value = 0.0043), respectively (Fig. 3 and Table S1). An even greater degree of metastasis inhibition (99.8%) was seen in the two surviving mice treated with high-dose ME (200 mg/kg). Bioimaging of mice after removal of the various organs showed no other metastatic sites.

As hoped, the CME analog was found to be significantly more potent than the parent compound, ME, in inhibiting in vivo tumor metastasis. Thus, as shown in Fig. 3, treatment with low-dose CME (12 mg/kg) led to suppression of metastasis by 99.3%, rendering CME approximately four times more potent than ME at the lowest dosage level. Moreover, at 49 mg/kg, tumor metastasis was inhibited by 99.8%. These findings, which are consistent with our in vitro results, lend support to our hypothesis that appendage of a carboxymethyl functionality onto the ME scaffold provides enhanced migratory inhibition activity.

| Table 1. Inhibition of transwell lung cancer cell lines migration by migrastatin ether (ME) and carboxymethyl-migrastatin ether (CME) (IC50 in μM) |
|-----------------|-----------------|-----------------|
| Cell line       | ME              | CME             |
| A549            | 1.93 ± 0.41     | 0.66 ± 0.20     |
| H1975           | 1.51 ± 0.69     | 0.51 ± 0.42     |
| H299            | 8.20 ± 1.75     | 5.02 ± 1.13     |

Lung cancer cell line chemotaxis was performed in cell culture inserts after serum starvation and an overnight preincubation with an incremental logarithmic scale of drug concentration (ME and CME from 10^-3 to 10^-10 M). Cancer cell migration in response to a serum gradient was measured after a 12-hour-long incubation in presence of different concentrations of ME or CME in both upper and lower chamber (three wells at each dose). Cells were then fixed and stained with crystal violet and photographed. Data show the half maximal inhibitory concentration (IC50, in μM) for ME and CME for A549, H1975, and H299 lung cancer cells. Data are expressed as the mean ± SEM of three independent experiments. Each experiment was performed in triplicate.

Fig. 3. CME comparison with ME for inhibition of metastasis in a human primary SCLC xenograft model. AC3-TGL cells were transplanted by subcutaneous ventral injection into NSG mice. Xenografted mice were treated with indicated dosages of CME or ME every three days started one day prior to the inoculation of tumor cells: ME 10 mg/kg (ME10 n = 5), 40 mg/kg (ME40, n = 5), 200 mg/kg (ME200, n = 5), CME 12 mg/kg (CME12 n = 5), 49 mg/kg (CME49 n = 5), and DMSO vehicle (control, n = 6). At day 55, the mice were sacrificed. Three mice in the ME200 group died before the end of treatment. Tumor metastasis at endpoint: At day 55, mice were analyzed for metastasis by ex vivo BLI quantifying luciferase activity in the excised lungs (L), liver (L), heart (H), kidneys (K), and the spleen (S). Measurements for each mouse are presented in A (black-filled circle), with the average per group (red line) and expressed as photon flux (flux in photon/sec) in log scale. P values were obtained using two-tailed Mann-Whitney U test. Pictures of bioluminescence signal measured on the organs from one mouse of each group are presented in B. Each picture is presented with the same settings (4 min exposure; photon signal; color scale from 3.10^6 (min) to 5.10^9 (max)).
that blocks metastasis. Applications of an antimetastatic agent might well be particularly helpful following resection of the primary tumor via surgery, chemotherapy, or radiation. Of course, the effort could gain much from an insight as to the mode of action, at least of our lead compounds. A recent claim by Huang and coworkers that has attracted much attention identified the intracellular protein, FASCIN, as the primary target (12). In vivo wound-healing assay. Migration of cancer cells was measured using an "in vitro wound-healing assay" (or scratch assay) performed in a 12-well plate (Becton Dickinson). Briefly, cancer cells were seeded at a density of 5-10 × 10^4 cells per well, grown to near confluent monolayers in 10% serum-supplemented M5 medium, and then starved overnight in a low serum medium (0.5% FCS). Perpendicular wounds were scratched through the cell monolayer using a sterile 200 μL pipette tip. The cells were then washed twice very gently using PBS, and the scratched areas were photographed at 4X and 20X magnification using computer-assisted microscopy. PBS was removed and replaced with 2 mL of media with or without 2% FCS, and containing drugs or DMSO (vehicle control) at 0.2% (v/v). After 12-24 h in a humidified incubator at 37 °C with 5% CO2, cells were fixed with 3.7% formaldehyde, permeabilized with ice cold methanol, and stained with a 0.2% crystal violet solution. Each well was photographed at 4X and 20X magnification and the pictures analyzed with CellProfiler2.0 cell image analysis software as previously described (15). The migration in response to the test condition was calculated relative to the DMSO vehicle control.

In Vivo Xenograft Model of Lung Cancer Metastasis. A human primary SCLC xenograft model was developed in 10- to 14-week-old male NSG mice. Primary tumor samples were obtained after patient informed consent under an MSKCC IRB approved protocol. AC3-TGL tumor cells growing as clumps were dissociated into a single-cell suspension by Trypsin/Collagenase IV (Invitrogen) sequential treatment. Xenografts were performed by subcutaneous ventral injection of 500 AC3-TGL cells in serum-free medium mixed with Matrigel (Becton Dickinson) at the ratio of 1:1.

One day prior to the tumor cell injection, mice were pretreated with either: (i) migrastatin ether (ME) at a dose of 10 mg/kg, 40 mg/kg or 200 mg/kg per mouse; (ii) carboxymethyl-ME (CME) at a dose of 12 mg/kg or 49 mg/kg; or (iii) DMSO vehicle control (n = 5 mice per group). Drug treatment was delivered by intraperitoneal injection every 3 days starting from day 1 after cell injection to day 55. During this time, the tumor burden and metastatic spread were monitored by bioluminescence imaging (BLI) at day 14, 23, 30, 40, and 50. At day 55 (endpoint), the mice were killed and the metastatic spread of tumor cells to the lungs, liver, heart, kidneys, and spleen was assessed by ex vivo BLI on the surgically resected organs.

All animal experiments were done in accordance with a protocol approved by the MSKCC Institutional Animal Care and Use Committee. All animals were maintained in a pathogen-free animal housing facility at MSKCC.

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Supporting Information

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In Vivo Xenograft Model of Lung Cancer Metastasis. The human primary SCLC xenograft model was developed in 10- to 14-week-old male NSG mice. Briefly, xenografts were performed by subcutaneous ventral injection of 500 AC3-TGL cells in serum-free medium mixed with Matrigel (Becton Dickinson), into mice pre-treated with miglurast analogs at a dose of either 10 mg, 40 mg, and 200 mg/kg for ME, or 12 mg and 49 mg/kg for CME per mouse or with DMSO vehicle control (n ≥ 5 mice per group). Drug treatment was delivered by intraperitoneal injection every three days started from day −1 after cell injection to day 55. During this time, the tumor burden and metastatic spread were monitored by bioluminescence imaging (BLI) at day 14, 23, 30, 40, and 50. At day 55 (endpoint), the mice were killed and the metastatic spread of tumor cells to the lungs, liver, heart, kidneys, and spleen was assessed by ex-vivo BLI on the surgically resected organs.

All animal experiments were done in accordance with a protocol approved by the MSKCC Institutional Animal Care and Use Committee. All animals were maintained in a pathogen-free animal housing facility at MSKCC.

![Tumor growth diagram](image)

Fig. S1. Tumor growth. Every five days, the tumor burden was monitored by in vivo Bioluminescence Imaging (BLI) and data is expressed as photon flux (flux in photon/sec) ± S.E.M. in log scale.

| Table S1. Inhibition factors of overall metastasis by ME/CME treatment |
|--------------------------|-----------------|
| Group        | IF     |
| ME10         | 96.2   |
| ME40         | 99.1   |
| ME200        | 99.8   |
| CME12        | 99.3   |
| CME49        | 99.8   |

Inhibition factors (IF) represent the percentage of reduction of overall metastasis at the endpoint, calculated after the quantification of the luciferase activity in the resected organs (liver, lung, spleen, heart, and kidney) by ex vivo bioluminescence imaging, reported to the control.