Depletion of Methionine Aminopeptidase 2 Does Not Alter Cell Response to Fumagillin or Bengamides

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Abstract
Inhibition of endothelial cell growth by fumagillin has been assumed to be mediated by inhibition of the molecular target methionine aminopeptidase 2 (MetAp2). New data show that depletion of MetAp2 by siRNA does not inhibit endothelial cell growth. Moreover, MetAp2-depleted endothelial cells remain responsive to inhibition by either fumagillin or a newly identified MetAp2 enzyme inhibitor. These data suggest that MetAp2 function is not required for endothelial cell proliferation.

Introduction
The methionine aminopeptidases (MetAp) are a class of proteases that selectively remove methionine from the NH2 terminus of newly synthesized proteins (1). Two isoforms of MetAps have been identified in humans (2–4). The human MetAp2 isoform has been of considerable interest as a target for cancer chemotherapy, after the discovery that the antiangiogenic natural compound fumagillin is a covalent inhibitor selective for MetAp2 (5–7). On the basis of the antiangiogenic activity observed in vitro, a fumagillin analog TNP-470 has progressed to clinical trials (8); however, the compound has demonstrated dose limiting neurotoxicity (9).

A second family of natural products, the bengamides, has recently been identified as inhibitors of both Met Ap isoforms (10). In contrast to fumagillin, bengamides and a synthetic analogue, LAF389, inhibit growth of tumor epithelial cells as well as endothelial cells. In an effort to test the hypothesis that inhibition of MetAp2 results in selective inhibition of endothelial cell growth, we prepared a series of bengamide analogues and tested their activity in MetAp enzyme and cell proliferation assays. The current data demonstrate that MetAp2 can be selectively inhibited by a bengamide analogue but that this compound does not selectively inhibit endothelial cell proliferation. Furthermore, endothelial cell proliferation is unaffected by selective depletion of MetAp2 through use of a targeting small interfering (si)RNA. Finally, endothelial cells depleted of MetAp2 by siRNA treatment remain sensitive to growth inhibition by either fumagillin or the MetAp2-selective bengamide. Taken together, these data show that MetAp2 is not the target for the antiangiogenic effects of fumagillin.

Materials and Methods

Cell and Enzyme Assays. A549 and H1299 non-small cell lung cancer cell lines were purchased from American Type Culture Collection. Fumagillin was purchased from Sigma. Human umbilical vein endothelial cells and human aortic endothelial cells were purchased from Clonetics and cultured as recommended by the vendor. LBM648 and LAF389 were synthesized as described previously (11, 12). LBM648, LAF389, and fumagillin were dissolved in DMSO at 1000-fold final concentrations and added to cells in culture. DMSO was included at 0.1% final concentration in all vehicle control samples. Cell culture, production of recombinant human MetAp1 and 2, and MetAp enzyme assays have been described previously (10). For Fig. 2, C and D, cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium assay. One-hundred percent growth refers to cell number of vehicle-treated cells after 72 h, corrected for initial plating density. Cell growth was normalized to initial cell number as described (10). For Figs. 2B and 3A–C, cell growth was measured by bromodeoxyuridine (BrdUrd) incorporation. BrdUrd was added to cells in the final 2 h of the indicated times of cell growth. BrdUrd incorporation into cells was measured using a cell proliferation ELISA kit (catalogue no. RPN250; Amersham Bioscience) according to manufacturer’s specifications. One-hundred percent growth refers to the incorporation of BrdUrd in cells treated with vehicle control. MetAp2 suppression was performed using an siRNA sequence designed as described previously (13). The targeting sequence was AAUGC-GUGACACAACAGUG (Dharmacon Research). The control mismatch sequence was AAUGCCGGCGCUACACACUGA. Duplex RNA was introduced into epithelial tumor cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Duplex RNA was introduced into endothelial cells by electroporation. All data are the average ± SE of triplicate determinations from a representative experiment repeated at least twice.

Human Endothelial Cell Response to Basic Fibroblast Growth Factor. Human umbilical vein endothelial cells and human aortic endothelial cells were transfected with siRNA as previously described (10), plated at 12,500 cells/well of a 24-well plate, and incubated overnight at 37°C, 5% CO2. Fresh media (EBM-2, Clonetics, + 2% fetal bovine serum) containing fumagillin were added for 30 min. After this pretreatment, cells received either vehicle control or 10 ng basic fibroblast growth factor and cultured for 72 h. Cells were trypsinized, and cell number was determined by Coulter counter.

Results
A number of analogues of the bengamide LAF389 were prepared and screened for MetAp2 specificity. A methyl-substituted analogue of LAF389 (Fig. 1), despite a relatively modest structural modification, was found to be specific for the desired isoform. LBM648 inhibited MetAp2 with a 0.38 μM IC50, equivalent to LAF389 but had no activity on MetAp1, even at 10 μM (Table 1). The effect of LBM648 on cell proliferation was assayed on several epithelial cell lines and on human umbilical vein endothelial cells. Compared with the nonspecific MetAp inhibitor LAF389, LBM648 was 3–10-fold less potent on epithelial tumor lines (Table 2). The increase in MetAp2 enzyme selectivity of LBM648 did not result in increased selectivity for endothelial growth inhibition, compared with LAF389. Several additional MetAp2-selective bengamide analogues were also as potent on epithelial cells as on endothelial cells (data not shown), thus challenging the hypothesis that small molecule inhibition of MetAp2 necessarily results in selective inhibition of endothelial cell growth.

In an alternative approach to address the role of MetAp2 in cell proliferation, we prepared cells depleted in MetAp2 using siRNA. As has previously been reported (10), treatment of epithelial cells with MetAp2 siRNA reduced MetAp2 protein within 24 h to a level at or
below the detection level of Western blots (Fig. 2). In H1299 epithelial cells, reduction of MetAp2 by the targeting sequence was specific; MetAp2 protein levels were not reduced in cells treated with a sequence containing 3 mismatch bases, and actin levels were identical in cells treated with the MetAp2 targeting sequence and the mismatch sequence. Reduction of MetAp2 protein by siRNA caused appearance of an unprocessed form of 14-3-3, showing that MetAp2 enzyme function was also substantially reduced in these cells. No difference in growth was observed in H1299 cells depleted of MetAp2, as measured by incorporation of BrdUrd (Fig. 2B), suggesting that in these cells MetAp2 activity is not limiting for cell growth. Surprisingly, H1299 cells depleted of MetAp2 by siRNA remained completely responsive to growth inhibition by LBM648; growth inhibition curves were superimposable in H1299 cells treated with the mismatch siRNA sequence versus cells treated with the MetAp2 siRNA sequence (Fig. 2C). A549 cells treated with MetAp2 siRNA also showed no change in response to LBM648 compared with cells treated with the mismatch sequence (Fig. 2D).

A more stringent test of MetAp2’s role in cell growth was performed using primary endothelial cells. Endothelial cells have been suggested to have an absolute requirement for MetAp2 function for growth based on the antiproliferative effects seen with fumagillin and analogues (6, 7, 14). This would suggest that reduction of MetAp2 protein should inhibit endothelial cell growth. To test this hypothesis, MetAp2 protein levels were specifically reduced by treatment with siRNA in human umbilical vein and human aortic endothelial cells. In both types of endothelial cells, proliferation in complete growth medium was unaffected by reduction of MetAp2 protein to <5% of control levels (Fig. 3A). This experiment could not rule out the possibility that the remaining levels of MetAp2 protein in siRNA-

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<tr>
<th>Table 1</th>
<th>IC50 for LAF389 and LBM648 on human methionine aminopeptidase (MetAp1 and MetAp2)</th>
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<td>MetAp1</td>
<td>MetAp2</td>
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<tr>
<td>LAF389</td>
<td>0.70 ± 0.01</td>
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<td>LBM648</td>
<td>&gt;10</td>
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Table 2 | Antiproliferative IC50 for LAF389 and LBM648 |
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<tr>
<td></td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IC50, μM (average ± SE)</td>
<td>A549</td>
</tr>
<tr>
<td>LAF389</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>LBM648</td>
<td>0.74 ± 0.20</td>
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Fig. 2. Methionine aminopeptidase 2 (MetAp2) small interfering (si)RNA effects on epithelial tumor cells. A, H1299 cells were treated with MetAp2 siRNA (2) or control siRNA (2C) and analyzed by Western blotting as described in “Materials and Methods.” Uncleaved 14-3-3γ was recognized using a monoclonal antibody specific for the unprocessed, methionine containing form (10). Total 14-3-3γ was recognized using a gamma isoform-specific antibody (Santa Cruz Biotechnology). B, effects of siRNA treatment for 48, 72, and 96 h on cell proliferation were measured by incorporation of bromodeoxyuridine (BrdUrd). Western blots were performed in parallel on cell lysates to confirm that MetAp2 down-regulation persisted for 96 h (data not shown). C and D, growth of siRNA-treated H1299 (C) and A549 (D) cells was measured after 72-h exposure to LBM648 using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium. Insets show MetAp2 and actin levels measured in cells after 72 h.
treated cells were sufficient for endothelial cell growth. Human aortic endothelial cells treated with MetAp2-specific siRNA were therefore challenged with the MetAp2 enzyme inhibitors LBM648 (Fig. 3B) and fumagillin (Fig. 3C). The ability of both compounds to inhibit proliferation of human aortic endothelial cells was unaltered in cells depleted of MetAp2 protein compared with either control, untreated human aortic endothelial, or to human aortic endothelial cells treated with the mismatch siRNA sequence. Human umbilical vein endothelial cells depleted of MetAp2 also retained the ability to respond to fumagillin, whether growth was measured under serum-depleted conditions, or in response to a basic fibroblast growth factor stimulus (Fig. 3D).

Taken together, these data demonstrate that MetAp2 is not required for endothelial cell proliferation and that two different classes of MetAp2 enzyme inhibitors remain effective inhibitors of cell growth on cells depleted of their putative target. One question left unanswered by these surprising results is the nature of the target(s) for fumagillin and MetAp2-depleted endothelial cells. The activity of the compounds in MetAp2-depleted endothelial cells is particularly puzzling because both classes of inhibitors have been cocrystallized with human MetAp2 (10, 15). One possibility is that another member of the MetAp family exists in humans; the current data could be resolved by showing the presence of a third MetAp enzyme expressed and active in endothelial cells but able to be inhibited by both fumagillin and bengamide analogues. Indeed, a recent publication has proposed such an enzyme based on sequence homology, although no enzymatic activity of the putative new MetAp isoform, was shown (16). Regardless of the existence of a third MetAp isoform or some other unrelated antiproliferative target, the current data are sufficient to challenge the hypothesis that inhibition of MetAp2 by fumagillin or bengamide analogues is the mechanism by which these compounds exert their antiangiogenic effects. Given that clinical trials are in progress with fumagillin analogues (17), additional target-finding experiments are clearly called for to elucidate the molecular targets of these compounds.

References
Corrections

p53 and BCNU Resistance in Astrocytes

In the article on p53 and BCNU Resistance in Astrocytes in the June 15, 1996 issue of Cancer Research (1), the title was incorrect. The title should have read “Wild-Type p53 Renders Mouse Astrocytes Resistant to 1,3-Bis(2-chloroethyl)-1-nitrosourea Despite the Absence of a p53-dependent Cell Cycle Arrest.”


AChE in Apoptosis

In the article on AChE in Apoptosis in the April 15, 2004, issue of Cancer Research (1), there is an error on page 2652, in the section under “Materials and Methods” on “siRNA Transfection”. The AChE target sequence should have read 5’-AAGAGUGUCUGCUAC-CAAUAU-3’.


Depletion of Methionine Aminopeptidase 2

In the article on Depletion of Methionine Aminopeptidase 2 in the May 1, 2004, issue of Cancer Research (1), there is an error on page 2984, in the section under “Materials and Methods” on “Cell and Enzyme Assays”. The text near the end of the section should have read the following: “The targeting sequence was AAUGC CGUGA-CACAACAGUA (Dharmacon Research). The control mismatch sequence was AAUGCCGGCGCUACACAGUA.”


NIS Gene Therapy of Hepatocarcinoma

In the article on NIS Gene Therapy of Hepatocarcinoma in the November 1, 2004, issue of Cancer Research (1), a note should have been included indicating that J. Faivre and J. Clerc contributed equally to the study.


Novel Functions of BRAK

In the article on Novel Functions of BRAK in the November 15, 2004, issue of Cancer Research (1), the following grant support information should have appeared:

This work was supported in part by the University of Texas M.D. Anderson Cancer Center SPORE in Head and Neck Cancer NIH-NCI P50 CA097007 (G. Clayman and M. Frederick), NIH R01 DE013954 (G. Clayman), Cancer Center Support Grant NIH P30 CA016672, Alano J. Ballantyne Distinguished Chair in Head and Neck Surgery Award (G. Clayman), Michael A. O’Bannon Endowment for Cancer Research (G. Clayman), Betty Berry Cancer Research Fund (G. Clayman), and NIH INRS Award T32 CA060374 (G. Clayman).

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