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# Selective Inhibition of Endothelial Cell Proliferation by Fumagillin Is Not Due to Differential Expression of Methionine Aminopeptidases

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**Abstract** The angiogenesis inhibitors fumagillin and TNP-470 selectively inhibit the proliferation of endothelial cells, as compared with most other cell types. The mechanism of this selective inhibition remains uncertain, although methionine aminopeptidase-2 (MetAP2) has recently been found to be a target for fumagillin or TNP-470, which inactivates MetAP2 enzyme activity through covalent modification. Primary cultures of human endothelial cells and six other non-endothelial cell types were treated with fumagillin to determine its effect on cell proliferation. Only the growth of endothelial cells was completely inhibited at low concentrations of fumagillin. MetAP1 and MetAP2 levels in these cells were investigated to determine whether differential enzyme expression plays a role in the selective action of fumagillin. Western blot analysis and RT-PCR data showed that MetAP1 and MetAP2 were both expressed in these different types of cells, thus, ruling out differential expression of MetAP1 and MetAP2 as an explanation for the cell specificity of fumagillin. Expression of MetAP2, but not of MetAP1, is regulated. Treatment of human microvascular endothelial cells (HMVEC) with fumagillin resulted in threefold increases of MetAP2 protein in the cells, while MetAP1 remained constant. Similar upregulation of MetAP2 by exposure to fumagillin was also observed in non-endothelial cells, eliminating this response as an explanation for cell specificity. Taken together, these results indicate that while MetAP2 plays a critical role in the effect of fumagillin on endothelial cell proliferation, differential endogenous expression or fumagillin-induced upregulation of methionine aminopeptidases is not responsible for this observed selective inhibition. J. Cell. Biochem. 77:465–473, 2000. 

**Key words:** angiogenesis; methionine aminopeptidase; p67; fumagillin; TNP-470; endothelial cells; MetAP2

Angiogenesis, the formation of new blood vessels from existing vasculature, is an essential component of a variety of pathological states, including tumor growth, diabetic retinopathy, macular degeneration, arthritis, and inflammation [Folkman, 1995; Yancopoulos et al., 1998]. Proliferation of normally quiescent endothelial cells is one of the critical steps required for angiogenesis. Fumagillin and its synthetic analogue TNP-470 (or AGM-1470) have been shown to block angiogenesis in both in vitro and in vivo models by their inhibition of endothelial cell proliferation [Ingber et al., 1990; Kusaka et al., 1994; Yamamoto et al., 1994]. TNP-470 has also been demonstrated to inhibit tumor growth and metastasis in a wide range of in vivo tumor models [Yamaoka et al., 1993; Bergers et al., 1999], and is under evaluation in clinical trials for cancer therapy [Castronovo et al., 1996; Kudelka et al., 1998].

Fumagillin or TNP-470 arrests endothelial cells in the G1 phase of the cell cycle [Abe et al., 1994]. A molecular target has recently been identified as methionine aminopeptidase-2 (MetAP2). Using a biotinylated fumagillin analogue [Sin et al., 1997] or an ovalicin photoaffinity label [Griffith et al., 1997], two groups have found that MetAP2 is specifically tar-

Abbreviations used: EC, endothelial cell; HMVEC, human neonatal dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; NHA, normal human astrocytes; NHDF, normal human dermal fibroblasts; NHEK, normal human epidermal keratinocytes; HMEpC, human mammary epithelial cells; PrEpC, human prostate epithelial cells; UASMC, human umbilical artery smooth muscle cells; MetAP2, methionine aminopeptidase-2; MetAP1, methionine aminopeptidase-1.

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geted by fumagillin and TNP-470. Furthermore, fumagillin and TNP-470 were shown to inhibit the enzymatic activity of MetAP2 by covalent complex formation. The crystal structures of human MetAP2 and its fumagillin adduct reveal that alkylation occurs at a single histidine residue (His231) at the enzyme active site [Liu et al., 1998]. Two forms of eukaryotic methionine aminopeptidases are known: MetAP1 and MetAP2. They share a similar enzymatic activity as both catalyze removal of the initiator methionine residue of newly synthesized proteins. Fumagillin and TNP-470 do not inhibit MetAP1 enzyme activity. Deletion of either the MetAP1 or MetAP2 gene results in viable, albeit slow-growing, yeast, while deletion of both genes gives rise to a lethal phenotype [Li and Chang, 1995a]. Fumagillin and TNP-470 selectively kill yeast lacking MetAP1, but they do not affect the growth of wild-type yeast or the MetAP2 deletion strain [Sin et al., 1997; Griffith et al., 1997]. A wide variety of fumagillin and related ovalicin analogues were tested for inhibition of endothelial cell proliferation and this potency was found to correlate strongly with the inhibitory activity of each compound against MetAP2 [Griffith et al., 1997]. These observations strongly demonstrate that MetAP2 is the anti-proliferate cellular target for fumagillin. However, the link between MetAP2 inhibition and endothelial cell cycle arrest by fumagillin and TNP-470 remains unclear.

MetAP2 is a bifunctional protein [Bradshaw et al., 1998]; it is identical to p67, a protein described to bind to eukaryotic initiation factor-2 (eIF2), and its C-terminal domain has enzymatic activity catalyzing hydrolysis of N-terminal methionine from proteins. eIF2 is required for protein synthesis, and p67(MetAP2) prevents its inactivation by blocking phosphorylation [Datta et al., 1988]. TNP-470 binds covalently to MetAP2 and inhibits its aminopeptidase activity, but the TNP-470-bound protein was shown to be able to inhibit eIF2 phosphorylation [Griffith et al., 1997], ruling out the possibility that modulation of eIF2 phosphorylation by MetAP2(p67) is directly responsible for the inhibition of endothelial cell proliferation by TNP-470 [Griffith et al., 1997]. Reduction of p67 protein levels by antisense transfection in rat hepatoma KRC-7 cells results in cell cycle arrest and apoptosis [Datta et al., 1999].

The in vitro cytostatic effect of fumagillin and TNP-470 at low concentration is highly specific for endothelial cells. In the present study, we provide further evidence that fumagillin selectively inhibits the growth of human endothelial cells, while it has minimal effect on all non-endothelial type normal human primary cultures. In an attempt to explore the role of MetAP2 in fumagillin action, we examined the expression of MetAP2 and the closely related enzyme, MetAP1, in these cells. We found that both MetAP2 and MetAP1 are ubiquitously expressed, but only MetAP2 protein is regulated during endothelial cell growth and by treatment with fumagillin.

## MATERIALS AND METHODS

Fumagillin was purchased from Sigma Chemical Company and was dissolved in dimethylsulfoxide (DMSO) at 1 mM; aliquots were stored frozen at −20°C. Other reagents were commercially obtained of the highest quality available.

### Cell Culture and Proliferation Assay

All normal human primary cells and their recommended culture media were purchased from Clonetics (San Diego, CA). The cells include human neonatal dermal microvascular endothelial cells (HMVEC), human umbilical vein endothelial cells (HUVEC), normal human astrocytes (NHA), normal human dermal fibroblasts (NHDF), normal human epidermal keratinocytes (NHEK), human mammary epithelial cells (HMEpC), human prostate epithelial cells (PrEpC), and human umbilical artery smooth muscle cells (UASMC). Cells were grown according to instructions from Clonetics, and cell proliferations were performed in 96-well plates using cells between passages 6 and 12. Cells were seeded at 3,000–5,000 cells/well and allowed to attach for 4 h. Fumagillin, diluted in culture medium, was added in quadruplicate, and cells were incubated for 3 days before MTS reagents (Promega, Madison, WI) were added to quantitate the live cells.

### RT-PCR

RNA from the cultured cells grown near confluence was isolated with Rneasy™ Total RNA kit purchased from Qiagen (Santa Clarita, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with Promega's

Access RT-PCR kit with 0.5 μg total RNA under the conditions recommended by the manufacturer. The primers used for MetAP1 are 5′–gcg gcc gtg gag acg cgg gtg t–3′ and 5′–tta aaa ttg aga cat gaa gtg agg ccg t–3′, and for MetAP2 are 5′–atg gcg ggc gtg gag gag gta gcg gcc t–3′ and 5′–tta ata gtc atc tcc tctg ctg aca act–3′. These primers were designed to generate full-length MetPA1 DNA product (~1160 bp) and full-length MetAP2 DNA product (~1440 bp). RT-PCR was carried out for 20, 25, 30, and 40 cycles, and 10 μl of the reaction was analyzed on 1.2% agarose gel.

### Antibodies

MetAP2 antibodies were generated in rabbits with purified recombinant human MetAP2 expressed in a baculovirus system. The IgG was purified with protein A-agarose. Antipeptide antibodies in rabbits against human MetAP1 peptide sequence ETWPDGWTAVTRDGKRS (amino acid 346–362) was produced and affinity-purified by Research Genetics (Huntsville, AL).

### Cell Lysates and Immunoblot

Cell lysates were made by lysing cells at 4°C for 30 min in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS), 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF, 1 mM NaF, and 1 mM sodium orthovanadate. The cell lysates were centrifuged at 10,000*g* for 10 min and stored frozen in aliquots at −70°C. The protein concentration of cell lysates was quantitated with BCA reagents (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard. 4 μg total protein was used in each lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Blots were blocked with casein in phosphate-buffered saline (PBS) (Pierce) and incubated with anti-MetAP1 IgG (3 μg/ml in blocking buffer) or anti-MetAP2 IgG (6 μg/ml in blocking buffer), followed by incubation with biotinylated goat anti-rabbit IgG (Amersham, Arlington Heights, IL) in blocking buffer. The blots were then incubated with streptavidin-HRP conjugate (Amersham), developed with Amersham's ECL plus reagents, and exposed to x-ray films. The images were scanned and the band intensity was quantitated with Bio-Rad model GS-710 Imaging Densitometer.

## RESULTS

### Selective Inhibition of Endothelial Cells Proliferation by Fumagillin

When treated with fumagillin, the growth of human microvascular endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) was effectively blocked (Fig. 1, HMVEC and HUVEC). The $IC_{50}$ value for the inhibition of endothelial cells proliferation is approximately 0.5 nM. Fumagillin showed partial or no inhibitory effect on the proliferation of non-endothelial type of cells (Fig.1, NHA, NHDF, NHEK, PrEpC, HMEpC, and UASMC), including normal human astrocytes (NHA), normal human dermal fibroblasts (NHDF), normal human epidermal keratinocytes (NHEK), human mammary epithelial cells (HMEpC), human prostate epithelial cells (PrEpC), and human umbilical artery smooth muscle cells (UASMC). The inhibition of HMVEC and HUVEC by fumagillin at low nM concentration was cytostatic but not cytotoxic because there was no reduction of cell density from the initial seeding (data not shown). Fumagillin may not have arrested all cells immediately because the cells were not cell cycle synchronized, and cells already in S-phase would continue to divide once and be arrested later at G1 phase [Abe et al., 1994]. Other types of endothelial cells, such as human umbilical artery endothelial cells, human lung microvascular endothelial cells, were inhibited by fumagillin in a manner similar to that for HMVEC and HUVEC (data not shown).

### MetAP1 and MetAP2 Protein Expression

All primary cells were used between passages 6 and10. Cell lysates were made from cells at 70–80% confluence. MetAP1 and MetAP2 protein levels were analyzed by Western blots using specific rabbit antibodies as described under Materials and Methods. Identical amount of total cell protein was applied on the gels. The data presented in Figure 2 show that both MetAP1 (apparent molecular weight of 48 kDa) and MetAP2 (apparent molecular weight of 67 kDa) were expressed in all cell types. MetAP2 was most abundant in epidermal keratinocytes (lane 4), mammary epithelial cells (lane 5), and human prostate epithelial cells (lane 6). Smooth muscle cells (lane 7) showed the lowest level of MetAP2, while endothelial cells (lane 1) had modest MetAP2 level. MetAP1 was present in all cells at

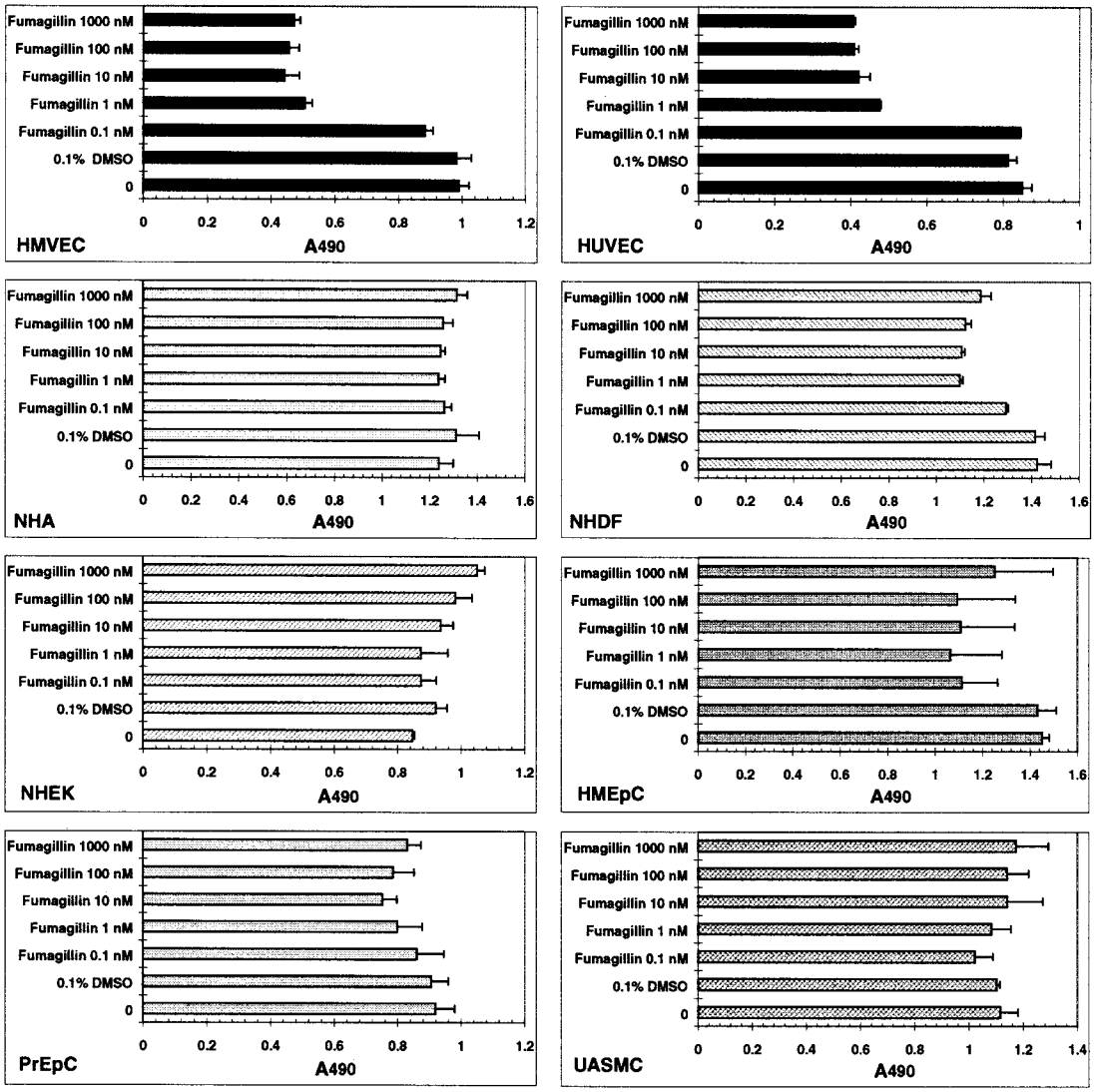



**Fig. 1.** Effect of fumagillin on proliferation of normal human primary cells. Primary cells were grown in the growth media that contain growth factors and 4% fetal bovine serum (FBS) as recommended by Clonetics. Cells between passages 6 and 12 were used. For proliferation assays, cells were seeded in 96-well plate at 3,000–5,000 cells/well. After incubation at 37°C, 5% $CO_2$ for 4 h, fumagillin diluted in culture media for final concentration of 0, 0.1, 1, 10, 100 and 1000 nM was introduced to wells in quadruplicates. Controls with 0.1% DMSO (corresponding to DMSO concentration in 1000 nM fumagillin wells) were also performed. The plates were returned to the incubator for 3 days. Live cells in each well were quantitated with MTS reagents. Absorbance at 490 (A490) was then recorded, which correlates with the number of live cells in the corresponding wells. Data shown are from one of the three separate experiments for each cell, and they represent the average of the quadruplicates. HMVEC, human neonatal dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; NHA, normal human astrocytes normal human dermal fibroblasts (NHDF); NHEK, normal human epidermal keratinocytes; HMEpC, human mammary epithelial cells; PrEpC, human prostate epithelial cells; UASMC, human umbilical artery smooth muscle cells.

a roughly similar level. There is no correlation between MetAP2 or MetAP1 level and cytostatic sensitivity to fumagillin.

### RT-PCR Analysis of MetAP1 and MetAP2 Expression

The mRNA for MetAP1 and MetAP2 was also analyzed in these cells. Primers were designed for RT-PCR to produce full-length cDNA products of MetAP1 (1160 bp) and MetAP2 (1440 bp). Various PCR cycles were used to make sure the reactions were not saturated when the products were analyzed on the gels (Fig. 3). As shown Figure 3A, MetAP1 mRNA was present in all cells at similar level. Likewise, MetAP2 (Fig. 3B) was also expressed in

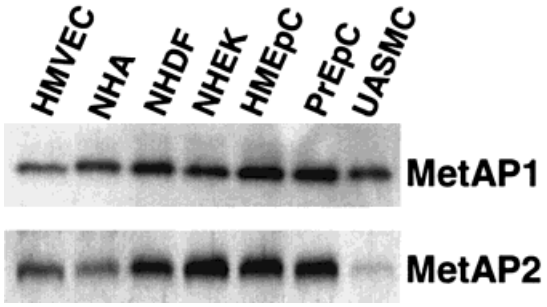



**Fig. 2.** MetAP1 and MetAP2 protein expression in normal human primary cell cultures. Primary cells were grown in the growth media as recommended by Clonetics. Cells between passages 7 and 10 were used. Cells in T75 flasks at 70–80% confluence were washed with cold PBS 3 times and lysed with 1 ml cold lysis buffer as described under Materials and Methods; 4 μg total proteins was used for each lane in the blots. HMVEC, human neonatal dermal microvascular endothelial cells; NHA, normal human astrocytes normal human dermal fibroblasts (NHDF); NHEK, normal human epidermal keratinocytes; HMEpC, human mammary epithelial cells; PrEpC, human prostate epithelial cells; UASMC, human umbilical artery smooth muscle cells.

all cells analyzed. MetAP2 mRNA was relatively more abundant than MetAP1 mRNA, as indicated by the number of cycles needed to generate similar amount of DNA products (30 cycles for MetAP1 vs. 25 cycles for MetAP2).

### Regulation of MetAP2 Protein in Endothelial Cells

The above data showed MetAP1 and MetAP2 were not differentially expressed in the primary culture of any the various types of human cells; we therefore proceeded to examine the regulation of both MetAP1 and MetAP2 in endothelial cells. HMVEC cells were normally grown in growth medium (EGM2) containing growth factors and 4% serum. When incubated with basic medium (EBM2) for 24 h, the cells became quiescent and the MetAP2 protein level decreased (Fig. 4A, 0). When the EGM2 growth medium was added back to the starved cells, MetAP2 was upregulated as shown in Figure 4A. MetAP2 protein doubled at 10 h and increased by threefold after incubation with EGM2 for 24 h as compared with that of starved cells. By contrast, MetAP1 protein remained unchanged. Fumagillin inhibits HMVEC proliferation with an $IC_{50}$ value of 0.5 nM, but it does not show cellular toxicity. Fumagillin inhibits MetAP2 enzyme activity by covalent modification. Cells treated with fumagillin in the presence of serum and growth factors for

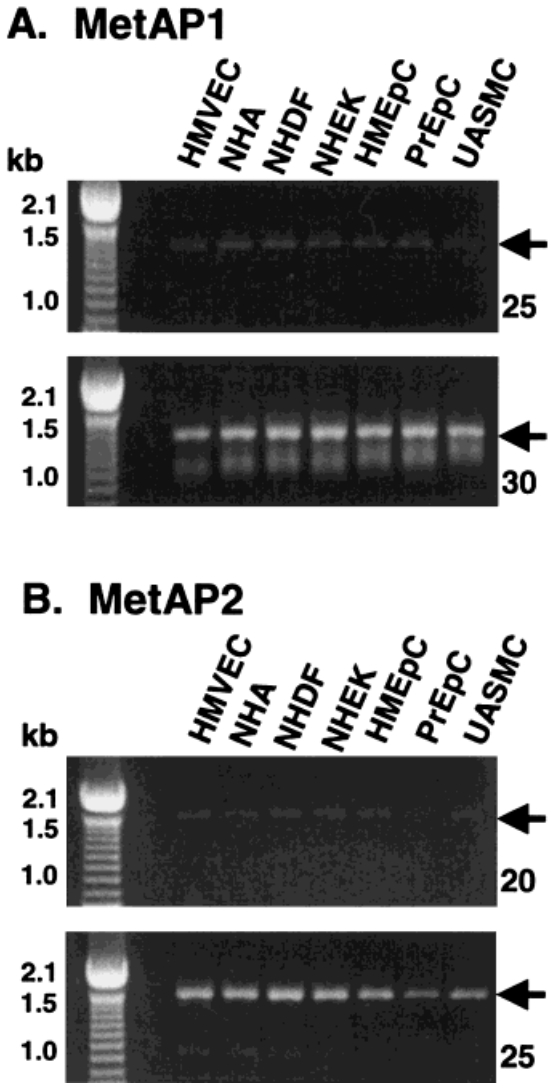
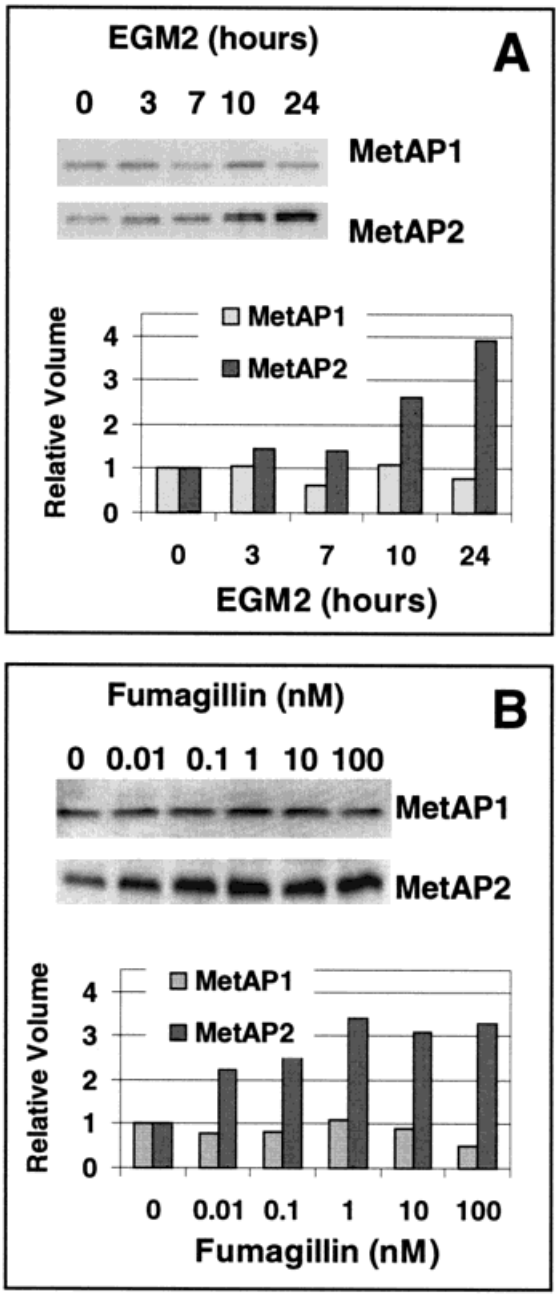



**Fig. 3.** RT-PCR analysis of MetAP1 and MetAP2 expression. Primary cells were grown in the growth media that contain growth factors and 4% FBS as recommended by Clonetics. Cells between passages 7 and 10 in T75 flasks at 70–80% confluence were used for RNA isolation with Qiagen's RNeasy kit. 0.5 μg total RNA was used in each RT-PCR reaction as described in Materials and Methods. The specific DNA products for MetAP2 or MetAP1 are indicated by the arrows; the numbers on the right show that 25 and 30 cycles of PCR products for MetAP1 (**A**), and 20 and 25 cycles of PCR products for MetAP2 (**B**) were used. Both MetAP1 and MetAP2 messages were expressed in all cells.

24 h showed increased levels of MetAP2 (Fig. 4B). Fumagillin at 1 nM induced MetAP2 by threefold, while MetAP1 protein was not affected. MetAP2 protein in the fumagillin-treated cells remained at similar elevated levels at 48 and 72 h posttreatment (data not shown).

Upregulation of MetAP2 protein by fumagillin was not unique to endothelial cell lines but

was also seen in non-endothelial cell types (Fig. 5). After exposure to 1 nM fumagillin for 24 h, all cell types increased their MetAP2 protein level by two- to sixfold. Smooth muscle cells showed the greatest increase; however, they normally had the lowest background level of MetAP2 (Figs. 1, 5). In spite of this low background level of MetAP2 UASMC was not sensitive to fumagillin (Fig. 1). MetAP1 protein remained unchanged during the fumagillin treatment in all cells. Upregulation of MetAP2 seemed to be a common phenomenon in cells treated with fumagillin. Such a response does not provide an explanation for fumagillin specificity of cytostatic inhibition of endothelial cells, although it indicates that all cells detect the loss of MetAP2 catalytic activity and respond by attempting to restore this activity. This adds further support for the central role of MetAP2 in fumagillin action.




## DISCUSSION

The cell type selectivity of fumagillin became evident when a panel of normal human primary cells was used in this study. Fumagillin completely inhibited HMVEC and HUVEC proliferation at low nanomolar concentration, while it showed partial or no effect on non-endothelial cells at concentrations up to 1 micromolar. The specificity of endothelial cell inhibition by fumagillin and TNP-470 has been reported previously, mostly by comparing endothelial cells to immortalized cells and tumor cells [Ingber et al., 1990; Kusaka et al., 1994]. Our data strongly support that fumagillin and TNP-470 may inhibit angiogenesis by specifically blocking endothelial cell proliferation.

Fumagillin was first identified in 1990 as an inhibitor of endothelial cell proliferation, tumor-induced angiogenesis, and tumor growth in mice [Ingber et al., 1990], although it was known for nearly 40 years as a naturally secreted antibiotic of *Aspergillus fumigatus* fresenius used to treat human amoebiasis [Killough et al., 1952]. A synthetic analogue of fumagillin, TNP-470 or AGM-1470, is now in clinical trials for the treatment of a variety of

**Fig. 4.** Regulation of MetAP2 protein in HMVEC. HMVEC cells were grown in the growth medium EGM2, which contains growth factors and 4% FBS as recommended by Clonetics. **A:** Cells at 70–80% confluence were washed 3 times with basic medium EBM2 and incubated with EBM2 for 24 h at 37°C, 5% $CO_2$. Cells were then feed with EGM2 for 0, 3, 7, 10, and 24 h. Cell lysates were made at each time point and analyzed by Western blotting with MetAP1 and MetAP2 antibodies as detailed under Materials and Methods. **B:** Cells at 70 - 80% confluence grown in the growth medium EGM2 containing serum and growth factors were treated with 0, 0.01, 0.1, 1, 10, and 100 nM fumagillin in the same EGM2 medium for 24 h. The MetAP1 and MetAP2 protein levels were analyzed by Western blotting and densitometry, which measures the intensity of the bands in terms of volume. Data shown are from one of three independent experiments, which displayed a similar pattern of MetAP2 regulation.

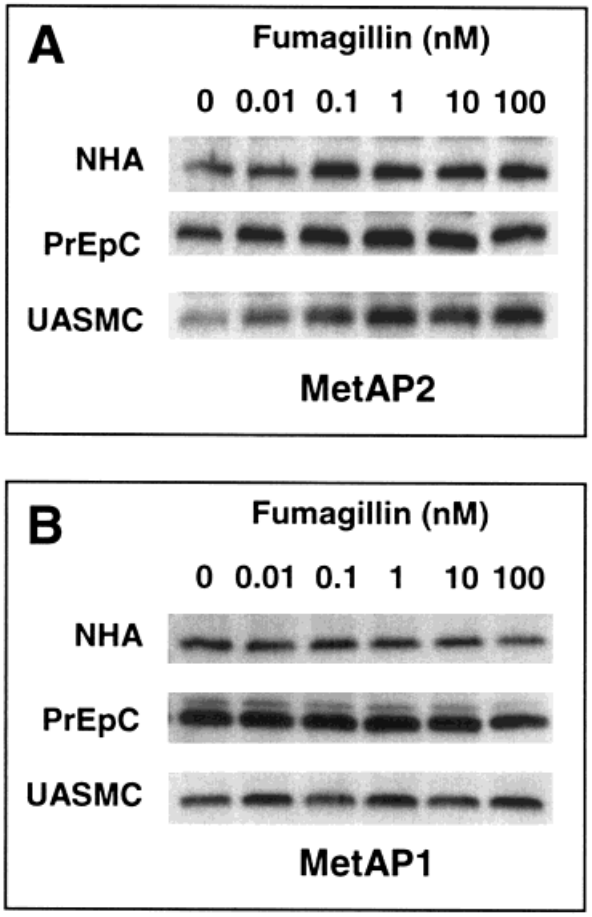



**Fig. 5.** Upregulation of MetAP2 protein by fumagillin in non-endothelial cells. Cells were grown in the growth media as recommended by Clonetics. When at 70–80% confluence, cells were exposed to 0, 0.01, 0.1, 1, 10, and 100 nM fumagillin for 24 h. Cell lysates were made and the MetAP2 (**A**) and MetAP1 (**B**) protein levels were analyzed by Western blot analysis.

cancers [Castronovo et al., 1996; Kudelka et al., 1998; Gervaz and Fontolliet, 1998]. The molecular mechanism for the action of fumagillin or TNP-470 was unknown until the discovery that these epoxide-containing molecules bind to, and covalently inactivate, MetAP2 [Sin et al., 1997; Griffith et al., 1997]. However, it is not clear why the cytostatic inhibition of endothelial cell proliferation by fumagillin and TNP-470 is endothelial cell specific.

Two classes of methionine aminopeptidases, MetAP1 and MetAP2, are known to be present in eukaryotes. MetAP2 was initially described in mammalian cells as p67, a cellular protein of 67 kDa apparent molecular weight that associates with eukaryotic initiation factor-2 (eIF2) involved in protein synthesis [Datta et al., 1988]. p67 binds eIF2 and protects it from phosphorylation by heme-regulated inhibitors; thus, it is a positive regulator for protein synthesis. It was not known that p67 and MetAP2 were identical until the molecular cloning and sequencing of rat p67 [Wu et al., 1993] and human MetAP2 [Li and Chang, 1995b; Arfin et al., 1995]. Thus, MetAP2 is a multifunctional protein involved in protein translation and co-translational or posttranslational modification. MetAP1 is another methionine aminopeptidase that shares a similar enzyme activity to that of MetAP2. However, fumagillin and TNP-470 do not inhibit MetAP1 enzyme activity, although fumagillin was shown to modify *Escherichia coli* MetAP1 by using high concentrations and long incubation time [Lowther et al., 1998]. MetAP2 expression in endothelial cells is highly regulated while MetAP1 is not. MetAP2 protein levels decrease in starved HMVEC cells and increase upon growth stimulation. A similar observation was also reported for p67 in rat hepatoma cells [Ray et al., 1992]. In distinct contrast, MetAP1 levels do not change with the growth status of cells, and Thus, MetAP1 and MetAP2 may have distinct functions in spite of catalyzing the same reaction. While upregulation of MetAP2 in cells exposed to fumagillin may be just a homeostatic compensation due to loss of MetAP2 catalytic function, this response suggests a role for MetAP2 in fumagillin-mediated HMVEC proliferation inhibition.

The data reported in this study support the conclusion that endothelial cells are exceptionally sensitive to the inhibition of MetAP2 activity. One of the hypotheses to account for the cell-selective action of fumagillin's MetAP2 mediated cytostatic activity is that there may be differential expression of MetAP1 and MetAP2 in various types of cells [Sin et al., 1997]. However, Western blot analysis shows that both types of human methionine aminopeptidases are present in all cells studied. RT-PCR data also indicate the ubiquitous expression of both MetAP1 and MetAP2 in all cells. Thus, endothelial cells are not susceptible because they are unusually deficient in either MetAP1 or MetAP2. We observed upregulation of MetAP2 upon presumed loss of its catalytic activity via fumagillin exposure. It could be hypothesized that the cells quickly detect the loss of this enzyme activity. It could also be speculated that the upregulation response might itself

contribute to cytostasis, possibly via excessive increase in the ribosomal regulatory (p67) function of increased MetAP2 protein in free form or bound to fumagillin. The TNP-470-bound MetAP2 retains the ability to protect eIF2 required for protein synthesis [Griffith et al., 1997]. However, this seems unlikely in view of the observation that many non-susceptible cell types also upregulate their MetAP2 levels when exposed to fumagillin.

Other possible mechanisms by which MetAP2 can play an essential role in endothelial cell proliferation include protein co-translational or posttranslational processing and myristoylation, as well as regulation of protein stability. Present in cytoplasm and also tightly associated with the ribosome, where it removes N-terminal methionine co-translationally, MetAP2 displays strong catalytic preference for proteins where the penultimate residue is one of the smallest amino acids [Bradshaw et al., 1998]. These sequences represent a small subset of proteins and are especially notable in being typical of key signal transducing proteins, including the MARCKS protein kinase, many of the src family tyrosine kinases and others whose function requires co-translational modification for trafficking to sites of action at the cell membrane. Myristoyl transferase cotranslationally catalyzes irreversible N-terminal lipid modification of these nascent kinases on the ribosome, in tandem with methionine removal, and commits them to further conjugation and movement to the outer membrane. It is likely that disruption of post-translational modification and trafficking of these kinases via failure to myristoylate on the ribosome contributes to EC cytostasis. Because of the metabolic positioning of MetAP-2 in this cascade, a decrease in its activity is expected to inhibit signal transduction and selectively interfere with those cells requiring active signaling, such as those with specialized functions or poised near a commitment to apoptosis. Such downstream target(s) may be uniquely present in or essential for endothelial cells and some tumor cells that are sensitive to fumagillin or TNP-470. The observation that MetAP2 up-regulation by fumagillin occurs in all cell types supports the idea of EC-specific downstream effects of MetAP2 action. However, further work is needed to provide direct evidence that MetAP2 is the molecule solely responsible for all the effect by TNP-470 or fumagillin. It will be of great interest to determine whether MetAP2 overexpression would protect cells from inhibition by fumagillin and TNP-470, and whether MetAP2 inhibition leads to disruption of signal transduction specifically mediated by N-terminally modified proteins present in endothelial cells.

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## REFERENCES

Abe J, Zhou W, Takuwa N, Taguchi J, Kurokawa K, Kumada M, Takuwa Y. 1994. A fumagillin derivative angiogenesis inhibitor, AGM-1470, inhibits activation of cyclin-dependent kinases and phosphorylation of retinoblastoma gene product but not protein tyrosyl phosphorylation or protooncogene expression in vascular endothelial cells. Cancer Res 54:3407–3412.

Arfin SM, Kendall RL, Hall L, Weaver LH, Stewart AE, Matthews BW, Bradshaw RA. 1995. Eukaryotic methionyl aminopeptidases: two classes of cobalt-dependent enzymes. Proc Natl Acad Sci USA 92:7714–7718.

Bergers G, Javaherian K, Lo KM, Folkman J, Hanahan D. 1999. Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science 284:808–812.

Bradshaw RA, Brickey WW, Walker K.W. 1998. N-terminal processing: the methionine aminopeptidase and Nα-acetyl transferase families. Trends Biochem Sci 23:263–267.

Castronovo V, Belotti D. 1996. TNP-470 (AGM-1470): mechanisms of action and early clinical development [review]. Eur J Cancer 32A:2520–2527.

Datta B, Datta R. 1999. Induction of apoptosis due to lowering the level of eukaryotic initiation factor 2-associated protein, p67, from mammalian cells by antisense approach. Exp Cell Res 246:376–383.

Datta B, Chakrabarti D, Roy AL, Gupta NK. 1988. Roles of a 67-kDa polypeptide in reversal of protein synthesis inhibition in heme-deficient reticulocyte lysate. Proc Natl Acad Sci USA 85:3324–3328.

Folkman J. 1995. Clinical applications of research on angiogenesis. N Engl J Med 333:1757–1763.

Gervaz P, Fontolliet C. 1998. Therapeutic potential of the anti-angiogenesis drug TNP-470. Int J Exp Pathol 79: 359–362.

Griffith EC, Su Z, Turk BE, Chen S, Chang YH, Wu Z, Biemann K Liu JO. 1997. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. Chem Biol 4:461–471.

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. 1990. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348:555–557.

Killough JH, Magill GB, Amith RC. 1952. The treatment of amebiasis with fumagillin. Science 115:71–72.

Kudelka AP, Verschraegen CF, Loyer E. 1998. Complete remission of metastatic cervical cancer with the angiogenesis inhibitor TNP-470 [letter]. N Engl J Med 338: 991–992.

Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, Ingber D, Folkman J. 1994. Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). Br J Cancer 69:212–216.

Li X, Chang, Y.-H. 1995a. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc Natl Acad Sci USA 92:12357–12361.

Li X Chang Y-H. 1995b. Molecular cloning of a human complementary DNA encoding an initiation factor 2-associated protein (p67). Biochim Biophys Acta 1260: 333–336.

Liu S, Widom J, Kemp CW, Crews CM, Clardy J. 1998. Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science 282:1324–1327.

Lowther WT, McMillen DA, Orville AM, Matthews BW. 1998. The anti-angiogenic agent fumagillin covalently modifies a conserved active-site histidine in the *Escherichia coli* methionine aminopeptidase. Proc Natl Acad Sci USA 95:12153–12157.

Ray MK, Datta B, Chakraborty A, Chattopadhyay A, Meza-Keuthen S, Gupta NK. 1992. The eukaryotic initiation factor 2-associated 67-kDa polypeptide (p67) plays a critical role in regulation of protein synthesis initiation in animal cells. Proc Natl Acad Sci USA 89:539–543.

Sin N, Meng L, Wang MQ, Wen JJ, Bornmann WG, Crews CM. 1997. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proc Natl Acad Sci USA 94:6099–6103.

Wu S, Gupta S, Chatterjee N, Hileman RE, Kinzy TG, Denslow ND, Merrick WC, Chakrabarti D, Osterman JC, Gupta NK. 1993. Cloning and characterization of complementary DNA encoding the eukaryotic initiation factor 2-associated 67-kDa protein (p67). J Biol Chem 268: 10796–10781.

Yamamoto T, Sudo K Fujita T. 1994. Significant inhibition of endothelial cell growth in tumor vasculature by an angiogenesis inhibitor, TNP-470 (AGM-1470). Anticancer Res 14:1–3.

Yamaoka M, Yamamoto T, Masaki T, Ikeyama S, Sudo K, Fujita T. 1993. Inhibition of tumor growth and metastasis of rodent tumors by the angiogenesis inhibitor O-(chloroacetyl-carbamoyl)fumagillol (TNP-470; AGM-1470). Cancer Res 53:4262–4267.

Yancopoulos GD, Klagsbrun M, Folkman J. 1998. Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border. Cell 93:661–664.