Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin

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Background: Angiogenesis, the formation of new blood vessels, is essential for tumor growth. The inhibition of angiogenesis is therefore emerging as a promising therapy for cancer. Two natural products, fumagillin and ovalicin, were discovered to be potent inhibitors of angiogenesis due to their inhibition of endothelial cell proliferation. An analog of fumagillin, AGM-1470, is currently undergoing clinical trials for the treatment of a variety of cancers. The underlying molecular mechanism of the inhibition of angiogenesis by these natural drugs has remained unknown.

Results: Both AGM-1470 and ovalicin bind to a common bifunctional protein, identified by mass spectrometry as the type 2 methionine aminopeptidase (MetAP2). This protein also acts as an inhibitor of eukaryotic initiation factor 2α (eIF-2α) phosphorylation. Both drugs potently inhibit the methionine aminopeptidase activity of MetAP2 without affecting its ability to block eIF-2α phosphorylation. There are two types of methionine aminopeptidase found in eukaryotes, but only the type 2 enzyme is inhibited by the drugs. A series of analogs of fumagillin and ovalicin were synthesized and their potency for inhibition of endothelial cell proliferation and inhibition of methionine aminopeptidase activity was determined. A significant correlation was found between the two activities.

Conclusions: The protein MetAP2 is a common molecular target for both AGM-1470 and ovalicin. This finding suggests that MetAP2 may play a critical role in the proliferation of endothelial cells and may serve as a promising target for the development of new anti-angiogenic drugs.

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Introduction

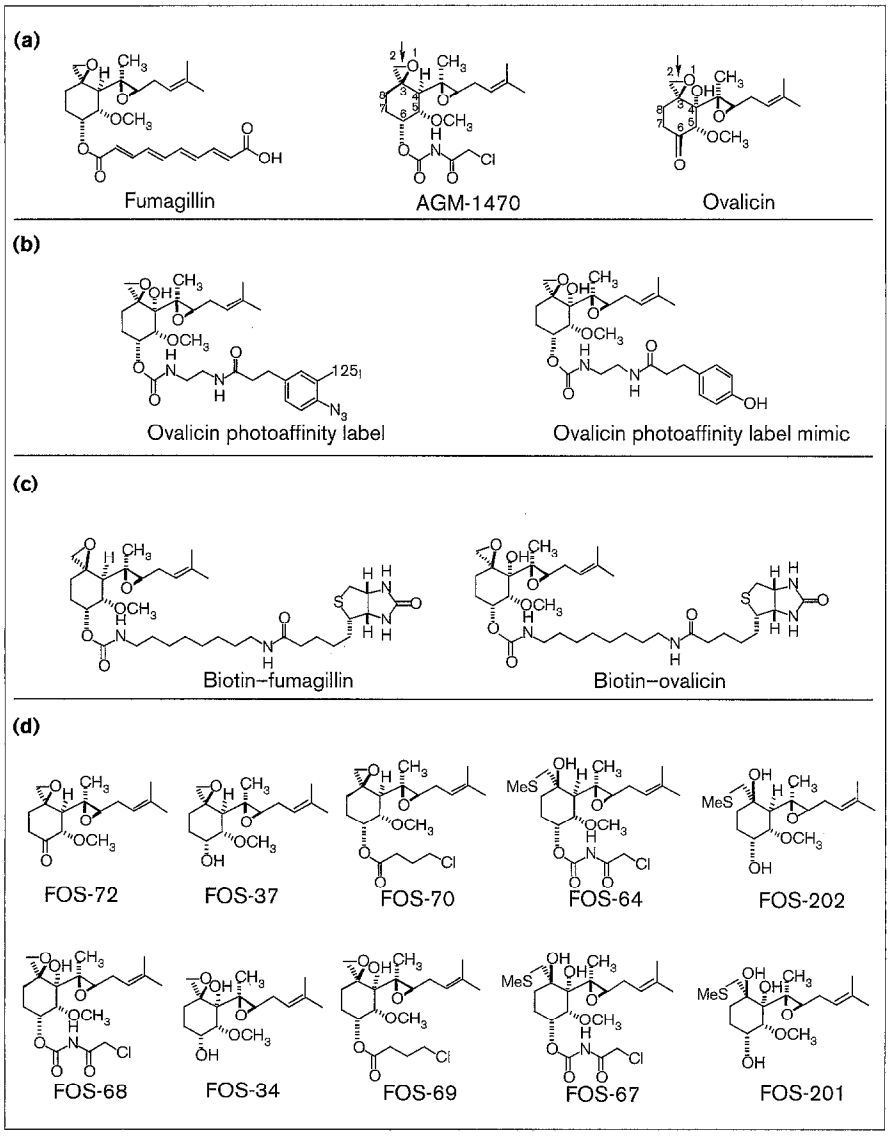
Angiogenesis, the process of new blood vessel formation, is known to play a pivotal role in both physiological states, such as wound healing, corpus luteum formation and embryonic development, and a variety of pathological states, including diabetic retinopathy, arthritis and inflammation [1–3]. In particular, angiogenesis has been demonstrated to be essential for the growth of solid tumors [4,5]. Without access to sufficient vasculature, tumor growth is restrained as a result of widespread cell death [6–8]. Inhibition of angiogenesis can therefore be used as an effective treatment for cancer.

Fumagillin (Figure 1a), a natural product of fungal origin [9,10], was discovered to act as a potent inhibitor of angiogenesis [11]. A synthetic analog of fumagillin, O-(chloroacetylcarbamoyl)fumagillol (AGM-1470, also known as TNP-470), was found to be less toxic and a 50-fold more potent inhibitor of angiogenesis than fumagillin [11]; this compound is currently undergoing clinical trials for a variety of cancers. AGM-1470 has been demonstrated to cytostatically inhibit capillary endothelial cell growth induced by both basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) [12], and preferentially inhibit endothelial cell growth in tumor vasculature *in vivo* [13]. The molecular mechanism of action of AGM-1470 remains unknown, however.

Ovalicin (Figure 1a), a sesquiterpene isolated from the fungus *Pseudorotium ovalis* [14,15], has been shown to exhibit antibiotic, antitumor and immunosuppressive activity [16,17]. Little is known about its molecular mechanism of action. The structural similarity of fumagillin and ovalicin led to the testing of ovalicin for anti-angiogenic activity. Ovalicin was found to inhibit angiogenesis with a potency that was comparable to that of AGM-1470 [18]. Similarly to AGM-1470, ovalicin cytostatically inhibits the proliferation of endothelial cells. Whether ovalicin and AGM-1470 share the same mechanism of action is not known.

As both AGM-1470 and ovalicin inhibit the proliferation of endothelial cells with high potency, they serve as excellent probes for elucidating the molecular mechanism of regulation of endothelial cell growth. Here, we report that

Figure 1



The chemical structures of fumagillin, ovalicin, ovalicin photoaffinity label, biotin conjugates and synthetic analogs. **(a)** Fumagillin, AGM-1470, and ovalicin. The epoxides at C-3 of AGM-1470 and ovalicin are highlighted by arrows and numbering of the six-membered ring system is shown. **(b)** Ovalicin photoaffinity label and its mimic. **(c)** Biotin–fumagillin and biotin–ovalicin conjugates. **(d)** Synthetic analogs of fumagillin and ovalicin.

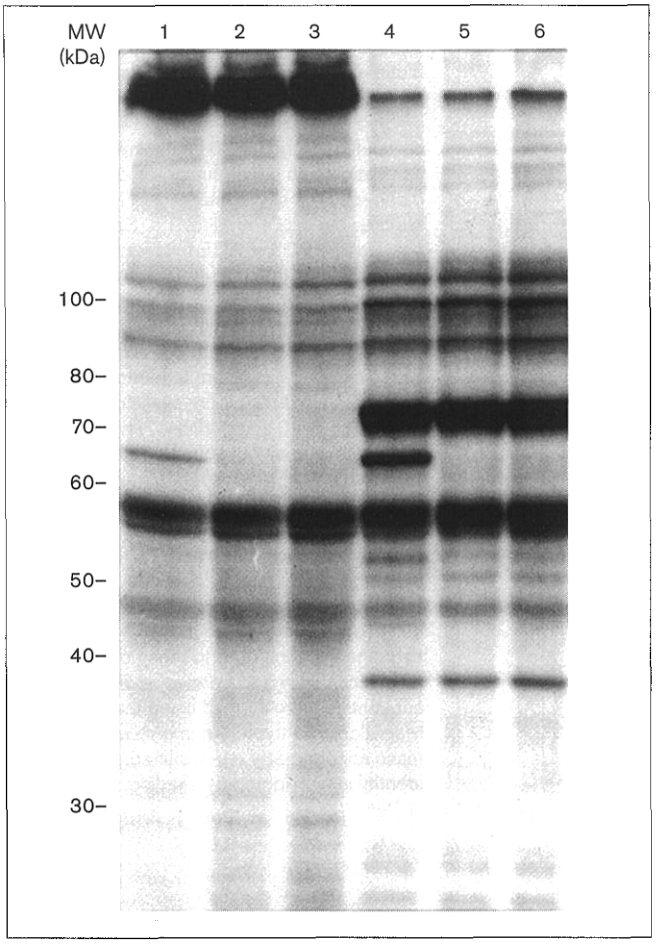
both AGM-1470 and ovalicin bind covalently to a bifunctional protein, the type 2 methionine aminopeptidase (MetAP2). The protein is also an inhibitor of eukaryotic initiation factor 2α (eIF-2α) phosphorylation. We show that both drugs potently inhibit the methionine aminopeptidase activity of the protein but neither affects its inhibition of eIF-2α phosphorylation. We demonstrate that AGM-1470 and ovalicin specifically inhibit type 2 but not type 1 methionine aminopeptidase. A significant correlation between the potency of inhibition of MetAP2 enzymatic activity and the inhibition of endothelial cell proliferation was observed for a number of fumagillin and ovalicin analogs. These results suggest that MetAP2 may play an important role in the proliferation of endothelial cells and serve as a target for identifying new inhibitors of angiogenesis.

Results

Detection of a 67 kDa protein that binds to both AGM-1470 and ovalicin

The structure/activity relationship of fumagillin, determined using a variety of fumagillin analogs [19], suggests that the sidechain at the C-6 position can tolerate a number of modifications without significant loss of activity (Figure 1a). We therefore attached a radioactive photoaffinity label to ovalicin at the corresponding position (Figure 1b). To ensure that this modification does not significantly abrogate the activity of ovalicin, we tested a mimic of the photoaffinity label in a bovine aortic endothelial cell (BAEC) proliferation assay [20] and found that the photoaffinity label mimic retained significant, albeit decreased, activity in comparison with ovalicin (data not shown). From extracts prepared from BAECs, a number of

Figure 2



Photoaffinity labeling of BAEC extracts reveals that p67 is a common binding protein for both AGM-1470 and ovalicin. Lanes 1–3, ovalicin photoaffinity labeling of BAEC extracts: 1, no competitor; 2, + 1 μM ovalicin; 3, + 1 μM AGM-1470. Lanes 4–6, ovalicin photoaffinity labeling of mouse embryo extract: 4, no competitor; 5, + 1 μM ovalicin, 6, + 1 μM AGM-1470.

proteins were labeled by the ovalicin photoaffinity probe (Figure 2, lane 1). Preincubation of cell extract with excess ovalicin led to the disappearance of a single labeled band of approximately 67 kDa (p67), suggesting that the labeling of p67 is mediated by the specific binding of ovalicin to the protein (Figure 2, lane 2). Significantly, treatment of cell extract with AGM-1470 also abrogated p67 labeling, suggesting that both AGM-1470 and ovalicin bind competitively to the same target protein (Figure 2, lane 3).

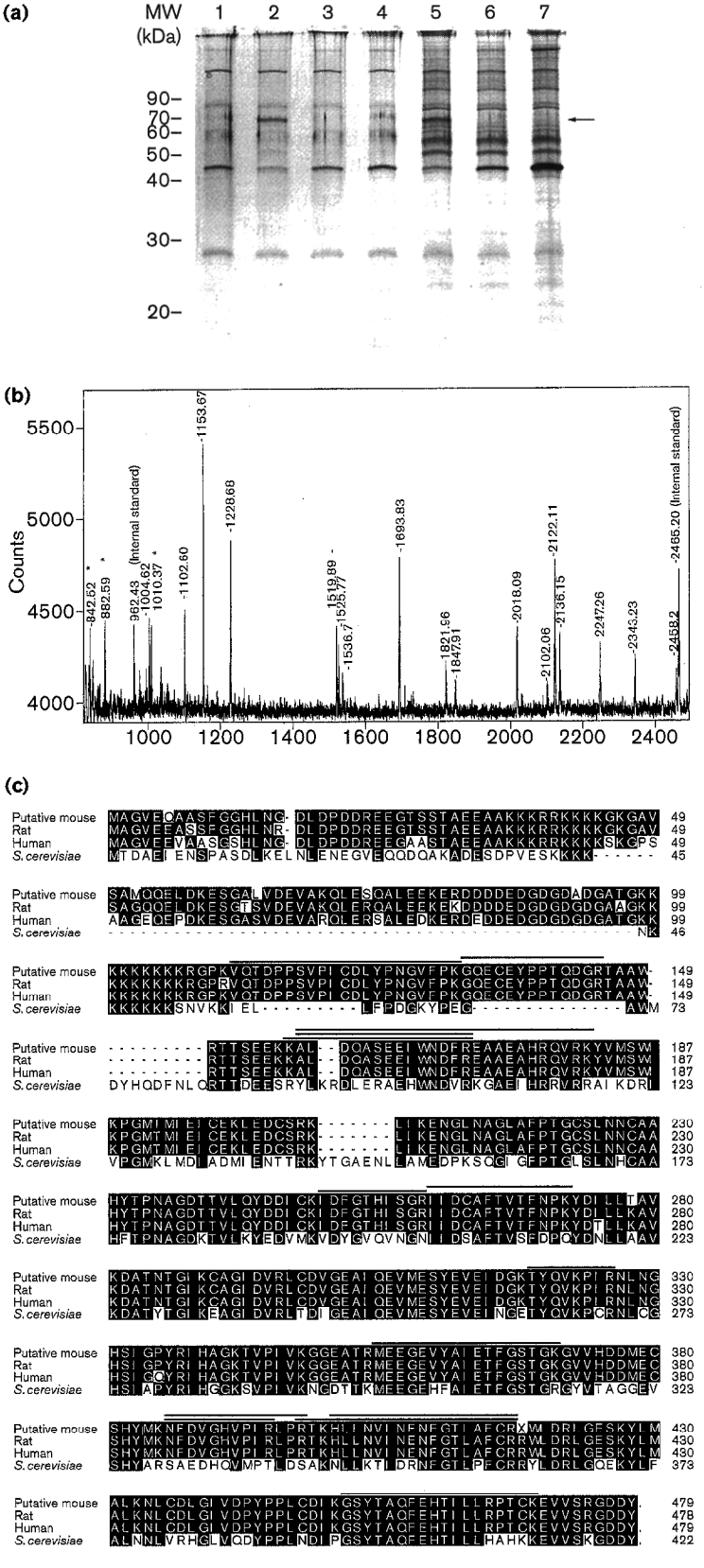
Isolation of p67 from mouse embryo extract

Having observed p67 as a putative target for both AGM-1470 and ovalicin in BAECs, we sought a more easily obtainable source of large amounts of proteins for isolation of p67. We examined extracts prepared from mouse embryos (14.5 days post conception), reasoning that the target of these compounds should be abundant during a period of embryogenesis known to involve extensive angiogenesis [21]. Using the ovalicin photoaffinity probe, an increased amount of p67 was indeed detected in mouse embryo extracts compared to BAEC extracts (Figure 2, lane 4). To facilitate the isolation of p67, we synthesized biotin conjugates of both ovalicin and fumagillin as affinity reagents (Figure 1c). When tested in the BAEC proliferation assay, both the biotin–fumagillin and biotin–ovalicin conjugates were found to retain significant activity (data not shown). The biotin conjugates were incubated with mouse embryonic extract and bound proteins were isolated by the addition of immobilized streptavidin. As shown in Figure 3a (lanes 2–4), a 67 kDa protein bound by biotin–fumagillin was visible upon silver staining of the sodium dodecylsulphate (SDS)–polyacrylamide gel, and its binding was competed by both AGM-1470 and ovalicin. Similarly, p67 was retained by the biotin–ovalicin conjugate bound to immobilized streptavidin in an AGM-1470- and ovalicin-sensitive manner (Figure 3a, lanes 5–7). Thus, the results obtained with the biotin conjugates are consistent with the observations made with ovalicin photoaffinity labeling, namely that p67 binds to both AGM-1470 and ovalicin.

Identification of p67 as MetAP2/inhibitor of eIF-2α phosphorylation

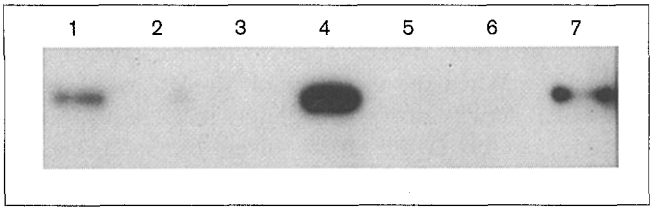
To obtain a sufficient amount of p67 for identification, we scaled up the biotin–fumagillin binding experiment and purified ~600 ng of p67 from mouse embryo extract. We subjected the partially purified p67 to SDS–polyacrylamide gel electrophoresis (PAGE) and excised the 67 kDa band after silver staining. This sample was subjected to in-gel digestion with trypsin and the resulting tryptic fragments were extracted from the gel [22]. The peptide mixture thus obtained was analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The resulting spectrum exhibited 22 distinct peaks corresponding to $[M+H]^+$ ions of peptides (Figure 3b). Those peaks at *m/z* 842.52, 882.59 and 1010.37 are common background peaks, and *m/z* 962.43 and 2465.2 represent the internal standards (adrenocorticotrophic hormone (ACTH) 4–10 and 18–39). The remaining 17 *m/z* values were used to search the EMBL protein database and this revealed that 16 of these values fit those predicted for both the rat and human MetAP2 (Swiss-Prot accession numbers P38062 and P50579, respectively). The rat and human enzymes have also been shown to inhibit the phosphorylation of eIF-2α, thus positively regulating protein synthesis [23–25]. These $[M+H]^+$ ions correspond to 15 different tryptic peptides derived from either human or rat MetAP2. The peaks at *m/z* 2136.15 and 2122.11 represent the same peptide (452–469) in which the carboxy-terminal Cys468 has reacted partly with monomeric acrylamide. The only peak unaccounted for, *m/z* 1228.68, may be a contaminant or a peptide from a region that bears a post-translational modification.

Figure 3



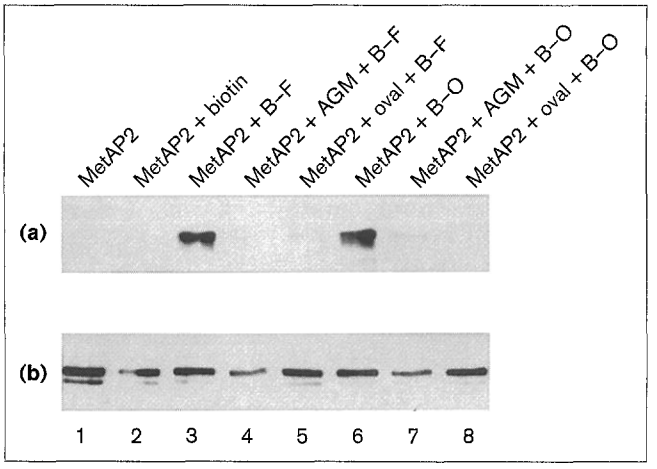
Isolation and identification of p67 from mouse embryo extracts using biotin–fumagillin and biotin–ovalicin conjugates. **(a)** Detection of p67 in mouse embryo extracts (arrow). Mouse embryo extracts were incubated with competing drugs for 30 min followed by incubation with biotin–fumagillin or biotin–ovalicin conjugate for 1 h. Immobilized streptavidin was added for 1 h. Samples were analyzed by SDS–PAGE followed by silver staining. Lane 1, immobilized streptavidin control; 2, biotin–fumagillin conjugate alone; 3, biotin–fumagillin conjugate with AGM-1470 competition; 4, biotin–fumagillin conjugate with ovalicin competition; 5, biotin–ovalicin conjugate alone; 6, biotin–ovalicin conjugate with AGM-1470 competition; 7, biotin–ovalicin conjugate with ovalicin competition.
(b) MALDI-TOF mass spectrum of a tryptic digest of p67. The *m/z* values are monoisotopic. Peaks marked (*) are also present in the spectrum from the blank experiment. See text for details.
(c) MetAP2 is highly conserved among eukaryotes. The putative mouse MetAP2 sequence is compared to those of rat, human and *Saccharomyces cerevisiae*. Protein sequences were aligned using the program MegAlign. The tryptic fragments identified by mass spectrometry are overlined. Sequence identity is highlighted by shading.

Figure 4



Western blot analysis confirms the identity of p67 as MetAP2. Samples were prepared as in Figure 3a, transferred to nitrocellulose and incubated with rabbit anti-human MetAP2 polyclonal antibodies followed by incubation with anti-rabbit IgG–HRP. Lane 1, biotin–fumagillin conjugate alone; 2, biotin–fumagillin conjugate with AGM-1470 competition; 3, biotin–fumagillin conjugate with ovalicin competition; 4, biotin–ovalicin conjugate alone; 5, biotin–ovalicin conjugate with AGM-1470 competition; 6, biotin–ovalicin conjugate with ovalicin competition; 7, recombinant MetAP2 (10 μg).

Figure 5



The biotin conjugates of fumagillin and ovalicin form stable adducts with MetAP2. Recombinant human MetAP2 was incubated with the biotin conjugates in the presence or absence of competitors. Samples were denatured, boiled in a buffer containing SDS and β-mercaptoethanol, and transferred to nitrocellulose. **(a)** The biotin conjugates bound to the protein were detected using streptavidin–HRP and **(b)** MetAP2 was detected using anti-human MetAP2 followed by anti-rabbit IgG–HRP. B–F, biotin–fumagillin; B–O, biotin–ovalicin; AGM, AGM-1470; oval, ovalicin.

The rat MetAP2, a 478 amino acid glycoprotein with a calculated molecular mass of 53 kDa, has been shown to migrate at 67 kDa by SDS–PAGE [23]. No mouse homolog of MetAP2 has yet been cloned, but by searching the expressed sequence tagged (EST) database, a putative open reading frame (ORF) was generated by aligning overlapping sequences. Comparison of this putative mouse ORF with the known sequences of human, rat, and yeast MetAP2 indicated that this protein is highly conserved among eukaryotes (Figure 3c). The three mammalian proteins show >93% sequence identity, and the yeast MetAP2 sequence has >55% identity with the human sequence. All 16 tryptic peptides identified by mass spectrometry matched the putative mouse ORF exactly. It therefore seemed likely that we had identified a mouse homolog of MetAP2.

To confirm the identity of the common binding protein for both AGM-1470 and ovalicin as MetAP2, we repeated the binding assay with both biotin–fumagillin and biotin–ovalicin conjugates and analyzed the protein retained on streptavidin beads by Western blot with anti-human MetAP2 polyclonal antibodies. We found that the mouse p67 which was bound by both biotin–fumagillin and biotin–ovalicin conjugates reacted with the anti-human MetAP2 antibodies (Figure 4, lanes 1,4). Competition with AGM-1470 and ovalicin led to the elimination of the cross-reacting band (Figure 4, lanes 2,3,5,6). The mouse p67 was also shown to migrate on SDS–PAGE at the same position as authentic human recombinant MetAP2. These experiments established that the common 67 kDa binding protein of both fumagillin and ovalicin is identical to MetAP2/inhibitor of eIF-2α phosphorylation.

AGM-1470 and ovalicin bind covalently to MetAP2

As both AGM-1470 and ovalicin possess potentially reactive epoxide groups (Figure 1a, arrows) that are capable of covalently modifying amino acid sidechains, we decided to determine whether these drugs indeed bind to MetAP2 covalently. Thus, the biotin conjugates were incubated with recombinant human MetAP2 alone, or in the presence of either AGM-1470 or ovalicin. The protein samples were boiled in a sample buffer containing SDS and β-mercaptoethanol, subjected to SDS–PAGE, and transferred to nitrocellulose. Probing directly with streptavidin–horseradish peroxidase (HRP) allowed the visualization of the protein samples that had been incubated with the biotin–fumagillin or biotin–ovalicin conjugates, but not those that had been incubated with free biotin or in the presence of competitors (Figure 5a). As a control, the presence of MetAP2 in each sample was confirmed using anti-MetAP2 antibodies (Figure 5b). As the drug–protein complex is maintained under denaturing conditions, these results strongly suggest that AGM-1470 and ovalicin bind to MetAP2 covalently.

Assessment of the effect of AGM-1470 and ovalicin on the two activities of MetAP2

As MetAP2 is a bifunctional protein, we wanted to assess the effect of AGM-1470 and ovalicin on its two activities. We first tested the effect of AGM-1470 and ovalicin on the methionine aminopeptidase activity of recombinant human MetAP2. Using a tetrapeptide substrate [26], we found that both drugs potently inhibit the methionine aminopeptidase activity of MetAP2. The IC_{50} values were estimated at 1 nM for AGM-1470 and 0.4 nM for ovalicin when 1 nM recombinant human MetAP2 was used in the assay (Table 1).

Table 1

Inhibition of BAEC proliferation and MetAP2 enzymatic activity by fumagillin and ovalicin analogs.

Compound	Proliferation IC_{50} (nM)	MetAP2 IC_{50} (nM)
AGM-1470	0.037±0.0024	1.0±0.3
Ovalicin	0.018±0.0059	0.4±0.2
FOS-72	0.013±0015	6±2
FOS-68	0.46±0.26	2.0±0.8
FOS-69	0.31±0.066	0.10±0.03
FOS-70	0.12±0.01	3.5±1.8
FOS-37	9.5±4.6	8±2
FOS-34	2.2±1.4	4±1
FOS-64	110±18	3,000±1,000
FOS-67	40±4	400±200
FOS-201	56±34	45±12
FOS-202	2,800±2,300	5,000±2,000

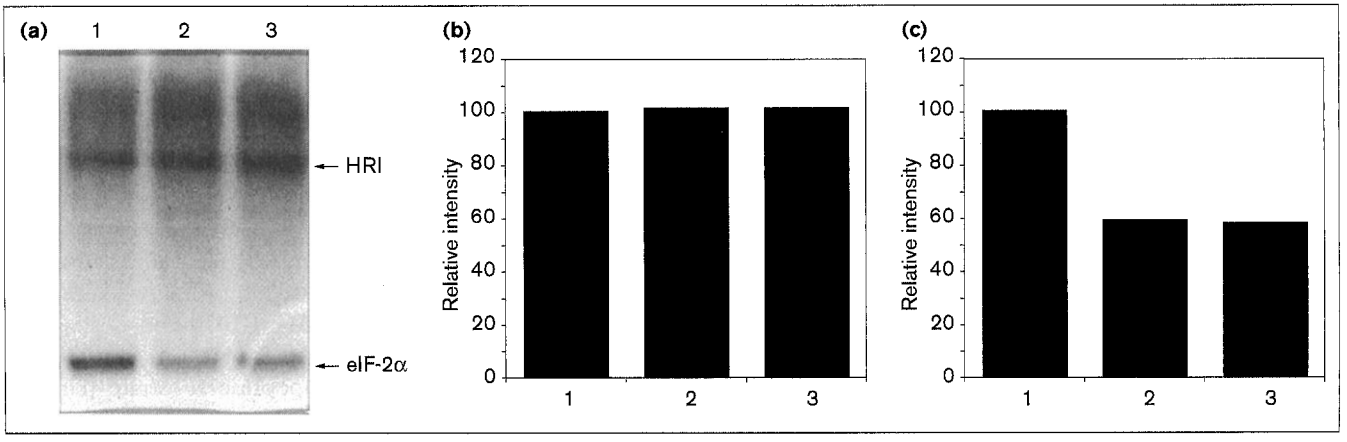
IC_{50} values were calculated as the average of at least three experiments fit using Deltagraph Pro 3.5 software.

In addition to its methionine aminopeptidase activity, MetAP2 has been shown to inhibit the phosphorylation of eIF-2α by heme-regulated inhibitor kinase (HRI) *in vitro* [27,28]. Initial attempts to determine the effect of AGM-1470 and ovalicin on this activity of MetAP2 gave ambiguous results as these drugs appeared to stimulate both the autophosphorylation of HRI and its phosphorylation of eIF-2α. This result was probably due to the nonspecific modification of cysteine sidechains by both drugs because a number of thiol-modifying reagents, including N-ethylmaleimide, have been shown to stimulate the kinase activity of HRI [29]. To circumvent this problem, we took advantage of the irreversible nature of MetAP2 modification by AGM-1470 and ovalicin. We incubated recombinant human MetAP2 with AGM-1470 and dialyzed the modified protein extensively to remove residual free AGM-1470. When the drug-treated MetAP2 was assayed for MetAP activity, it was completely inactive, whereas a control treated with the carrier solvent (ethanol) retained full activity (data not shown). The dialyzed MetAP2 samples, either unmodified or modified with AGM-1470, were subsequently examined for their ability to inhibit eIF-2α phosphorylation by HRI. As shown in Figure 6, MetAP2 bound by AGM-1470 is as effective as unbound MetAP2 in inhibiting phosphorylation of eIF-2α by HRI, without affecting HRI autophosphorylation. This experiment ruled out the possibility that modulation of eIF-2α phosphorylation by MetAP2 is directly responsible for the inhibition of endothelial cell proliferation by AGM-1470.

Determination of the specificity of AGM-1470 and ovalicin for MetAP2

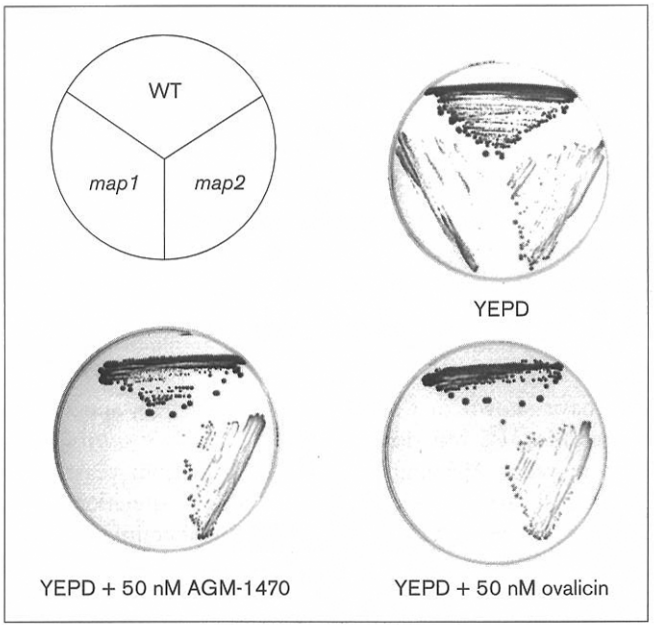
Two types of MetAPs have been found in eukaryotes, including the yeast *Saccharomyces cerevisiae* [24,25,30–32]. The fact that we detected binding of MetAP2, but not the type 1 enzyme MetAP1, to AGM-1470 and ovalicin using photoaffinity labeling and affinity purification suggests that these drugs are specific for MetAP2. To further test the specificity of these drugs, we plated both wild-type and mutant yeast strains lacking either MetAP1 (*map1*) or MetAP2 (*map2*) onto media containing the two drugs. Although wild-type and *map2* mutant yeast are resistant to either 50 nM AGM-1470 or 50 nM ovalicin, the growth of the *map1* mutant is completely inhibited by these drugs (Figure 7). These results indicate that yeast MetAP2, but not MetAP1, is a target for both AGM-1470 and ovalicin *in vivo*.

Figure 6



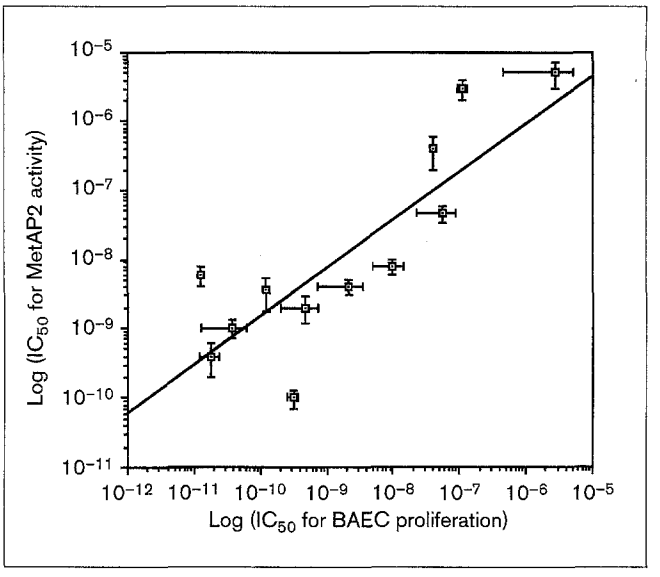
Drug binding does not alter the protective effect of MetAP2 on eIF-2α phosphorylation. **(a)** Autoradiogram of eIF-2α phosphorylation by HRI. Lane 1, eIF-2α + HRI; 2, eIF-2α + MetAP2 + HRI; 3, eIF-2α + MetAP2–AGM-1470 + HRI. **(b)** Quantification of HRI autophosphorylation. **(c)** Quantification of eIF-2α phosphorylation. Numbering for (b) and (c) is as for (a).

Figure 7



Specificity of AGM-1470 and ovalicin for MetAP2 in yeast. Wild-type (WT), *map1*, and *map2* yeast strains were plated on the yeast growth medium YEPD, or YEPD containing either 50 nM AGM-1470 or 50 nM ovalicin. The plates were incubated at 30°C for four days before being photographed.

Figure 8



Pharmacological correlation between the inhibition of the methionine aminopeptidase activity of MetAP2 and the inhibition of BAEC proliferation by fumagillin and ovalicin analogs. Values from Table 1 were plotted using Cricket Graph software.

In addition to the yeast growth experiments *in vivo*, we also tested AGM-1470 and ovalicin with recombinant yeast MetAP1 and MetAP2 *in vitro*. We observed no inhibition of MetAP1 activity using up to 10 μM AGM-1470 and ovalicin. In comparison, the yeast MetAP2 enzyme was completely inhibited at concentrations as low as 5 nM, consistent with the *in vivo* results indicating that the drugs are specific for MetAP2.

Relationship between the inhibition of MetAP2 activity and the inhibition of endothelial cell proliferation by fumagillin and ovalicin analogs

Given that both AGM-1470 and ovalicin specifically inhibited the enzymatic activity of MetAP2 without affecting its protective effect on eIF-2α phosphorylation, we suspected that this effect mediated the anti-angiogenic activities of these drugs. As a first step to test this hypothesis, we synthesized a series of analogs of both fumagillin and ovalicin, including those substituted at the C-6 position and those in which the terminal epoxide has been opened (Figure 1d). These analogs were tested for the inhibition of MetAP2 activity *in vitro* and inhibition of BAEC proliferation in cell culture (Table 1). A significant correlation (Student's t test $P > 0.001$) was found between the potency of the inhibition of BAEC proliferation and the potency of the inhibition of methionine aminopeptidase activity (Figure 8). Importantly, no derivative was found which showed high potency in one assay but no activity in the other. This correlation provides further support to the hypothesis that the inhibition of MetAP2 enzymatic activity mediates the anti-angiogenic activity of AGM-1470 and ovalicin.

Discussion

We have detected a 67 kDa protein, p67, from BAECs and mouse embryonic extracts using a photoaffinity probe derived from ovalicin. The labeling of p67 by the ovalicin photoaffinity probe was sensitive to competition by both ovalicin and AGM-1470. Using biotin conjugates derived from the two inhibitors, we isolated p67 from mouse embryonic extracts and identified it as MetAP2 using MALDI-TOF mass spectrometry and a database search. The identity of p67 was confirmed using an anti-MetAP2 polyclonal antibody. MetAP2 is thus a common target for AGM-1470 and ovalicin.

MetAP2 is a bifunctional protein. In addition to its methionine aminopeptidase activity, MetAP2 has been shown to regulate protein synthesis by affecting the phosphorylation state of eIF-2α. We showed that the binding of AGM-1470 does not affect this regulatory activity of MetAP2. This result is in agreement with earlier studies demonstrating that AGM-1470 did not block proliferation by affecting gross protein synthesis [33]. Thus, the anti-angiogenic activity of AGM-1470, and probably that of ovalicin, are not directly mediated by inhibition of general protein synthesis.

Both AGM-1470 and ovalicin were shown to potently inhibit the methionine aminopeptidase activity of MetAP2 using a peptide substrate. A series of analogs were synthesized from fumagillin and ovalicin with different potencies for the inhibition of endothelial cell proliferation. When these analogs were tested for the inhibition of MetAP2 aminopeptidase activity *in vitro*, a strong correlation was observed between these two activities. We note that the IC_{50} values for MetAP2 inhibition *in vitro* are an order of magnitude higher than the corresponding IC_{50} values for inhibition of endothelial cell proliferation. This discrepancy is due in large part to the higher concentrations of protein required for the enzymatic assay *in vitro*. The small deviation of some compounds from this correlation may be attributable to differences in cell permeability and metabolism within the cell. Although the small number of analogs tested precludes the establishment of an absolute correlation, no compound that is active in one assay and not in the other has yet been found, supporting the notion that inhibition of MetAP enzymatic activity is responsible for the anti-proliferative effects of AGM-1470 and ovalicin.

Earlier work has focused on the clarification of the anti-proliferative effects of AGM-1470 at the cellular level. AGM-1470 has been demonstrated to potently inhibit endothelial cell proliferation induced by both bFGF and VEGF [12]. The inhibition of endothelial cell proliferation by these inhibitors exhibits a biphasic character. The inhibition was cytostatic in the first phase with an IC_{50} of 45 pM. At concentrations above 90 μM, AGM-1470 became cytotoxic [33]. The cytostatic inhibition has been shown to block the entry of cells into the G1 phase of the cell cycle [20]. At the molecular level, it has been demonstrated that the treatment of vascular endothelial cells with AGM-1470 inhibits the activation of the cyclin-dependent kinases cdc2 and cdk2 and the phosphorylation of the retinoblastoma gene product [34]. Early G1 events such as protein tyrosyl phosphorylation and the expression of immediate early genes such as *c-fos* and *c-myc* were unaffected, however. AGM-1470 does not affect cdk activity *in vitro*, arguing that AGM-1470 exerts its effects prior to cdk activation. Inhibition of MetAP2 may therefore lead to the disfunction of key molecules involved in the cell cycle progression of endothelial cells and other sensitive cell types prior to cdk activation, leading to cell cycle arrest.

Methionine aminopeptidase is an essential enzyme in both prokaryotes and eukaryotes. A single gene for MetAP is known in *Escherichia coli* and its knockout leads to inviability [35]. The removal of the amino-terminal methionine is known to be important for post-translational modification of many proteins in eukaryotes. Amino-terminal myristoylation requires the processing of the amino-terminal methionine and has been shown to affect the function of many proteins, including protein kinases such as Src [36], protein phosphatases such as calcineurin B [37], and proteins involved in the regulation of protein secretion such as ADP-ribosylation factors [38]. Another important function of processing of the amino-terminal methionine is the regulation of protein stability and turnover according to the N-end rule [39]. Inhibition of methionine aminopeptidase activity by these drugs therefore may disrupt endothelial cell cycle progression due to the disregulation of post-translational processes required for the function of MetAP2 substrates. It remains to be established whether the inhibition of endothelial cell proliferation is mediated through the inhibition of either post-translational modification, disregulation of protein stability, or both.

We have shown that AGM-1470 and ovalicin specifically target MetAP2 but do not affect MetAP1 *in vitro* and *in vivo* in yeast. Although the two MetAPs from yeast show limited substrate specificity *in vitro* [32], specificity may exist *in vivo* as a result of their different subcellular localization. Both MetAP1 and MetAP2 possess domains at their amino termini that are thought to be involved in mediating protein–protein interactions. MetAP1 possesses two zinc finger motifs at the amino terminus which have been shown to be required for full function *in vivo* [40], whereas MetAP2 amino-terminal sequences contain polylysine blocks and an aspartic acid block that are also present in human eIF-2β and the yeast protein Sui 3 [23]. These residues may mediate the specific association of MetAP2 with the eIF-2 complex or other cellular proteins. In yeast, the knockout of MetAP1 or MetAP2 leads to a slow growth phenotype, and disruption of both MetAPs is lethal [32]. Although MetAP1 can substitute for MetAP2 in yeast, the situation is likely to be different in drug-sensitive mammalian cell types. There could be MetAP2-specific substrates that have a unique role in drug-sensitive cells. It is also possible that the compounds are metabolized differently in different cell types; the metabolism of these compounds could be affected by several factors including the expression level of epoxide hydrolases that may serve to inactivate these epoxide-containing drugs [41].

The effects of AGM-1470 and ovalicin are not entirely confined to endothelial cells. AGM-1470 has been shown, for example, to inhibit the proliferation of human embryonic fibroblasts [11]. Both AGM-1470 and ovalicin have also been shown to modulate the immune response. Ovalicin was initially shown to be active in several assays for immunosuppression. It reduces the number of antibody-producing cells in the spleens of mice, it is active in experimental encephalomyelitis in rats, and it prolongs the survival of skin allografts [42–44]. At the cellular level, it was shown to inhibit the mixed lymphocyte reaction (MLR) at concentrations as low as 3.0 pM [45]. Fumagillol (FOS-37; Figure 1d), the alkaline hydrolysis product of fumagillin, has also been shown to suppress a mouse MLR with an IC_{50} of 250 nM [46]. Inversion of the stereo-center

at C-6 and demethylation at C-5 greatly increase this effect, giving an IC_{50} of 34 nM for the corresponding natural product FR65814 [46]. On the other hand, AGM-1470 has been shown to stimulate B-cell proliferation upon stimulation with phytohemagglutinin (PHA) in the presence of T-cells [47,48]. It remains to be established whether ovalicin also stimulates B-cell proliferation and whether AGM-1470 inhibits MLR. Whether inhibition of MetAP2 activity by these compounds underlies their immunomodulatory effect also awaits further investigation.

We have shown that both AGM-1470 and ovalicin form covalent complexes with MetAP2. The two biotin conjugates were shown to remain associated with the protein under denaturing conditions. In addition, incubation of MetAP2 with AGM-1470 rendered the protein enzymatically inactive even after extensive dialysis. This covalent interaction is likely to be mediated by the reactive epoxides present in these compounds [49]. Indeed, opening of the terminal epoxide reduces the anti-angiogenic activity and the inhibition of methionine aminopeptidase activity of the compounds by several orders of magnitude (Figure 1d; Table 1), suggesting that the terminal epoxide may be involved in the covalent modification of MetAP2. This terminal epoxide may react with a cysteine residue present in or near the active site of MetAP2. Interestingly, the recombinant human MetAP2 has been shown to be sensitive to sulfhydryl-modifying reagents such as $HgCl_2$ and *p*-hydroxymercuribenzoic acid (Y.-H.C., unpublished observations). One major difference between the two types of eukaryotic MetAPs is an insertion of 64 amino acid residues towards the carboxyl terminus of the catalytic domain of the type 1 enzyme. It is possible that this insertion is responsible for the selectivity of AGM-1470 and ovalicin for the type 2 enzyme. Interestingly, there is a cysteine within this insertion, conserved between yeast, rat, human and the putative mouse MetAP2, that may be a candidate for covalent modification by these inhibitors [24,25]. The exact nature of the interaction between AGM-1470 or ovalicin and MetAP2 awaits further characterization.

Two types of MetAPs are presently known in eukaryotes. A database search of the entire genome of yeast *S. cerevisiae* did not reveal any new members of this family, indicating that yeast possesses only two types of MetAPs. In higher eukaryotes, however, the MetAP family is likely to be larger. In fact, a mouse proliferation-associated protein was recently cloned and found to have significant homology to human MetAP2 [50]. It is not known whether the proliferation-associated protein possesses MetAP activity and whether it is required for cell cycle progression. Although we did not observe binding of a 38 kDa protein to either the photoaffinity probe of ovalicin or the biotin conjugates of both AMG-1470 and ovalicin, it remains to be verified whether this MetAP2 homolog binds to AGM-1470 and ovalicin, and if it does, whether the binding mediates the inhibition of endothelial cell proliferation and immunomodulation by these drugs. Once MetAP2 or its homolog is proven to be the physiological target for AGM-1470 and ovalicin, elucidation of the physiological function of the target protein in cell cycle progression, including its relevant substrates in endothelial cells, will offer insight into the regulation of endothelial cell proliferation and facilitate the identification of new anti-angiogenic agents for the treatment of cancer.

Significance

Angiogenesis plays a pivotal role in tumor growth and metastases. The inhibition of angiogenesis is emerging as a promising new strategy for the treatment of cancer. Because endothelial cell proliferation is necessary for angiogenesis, an understanding of the molecular mechanism of regulation of endothelial cell proliferation will offer new targets for inhibiting angiogenesis. Two structurally related inhibitors, AGM-1470 and ovalicin, potently inhibit endothelial cell proliferation, thus serving as useful molecular probes for elucidating the mechanism of endothelial cell proliferation. We found that both AGM-1470 and ovalicin bind to a common protein target, the type 2 methionine aminopeptidase (MetAP2), which also possesses a second function, inhibition of eukaryotic initiation factor 2α (eIF-2α) phosphorylation. Both drugs were found to bind covalently to MetAP2 and inhibit its methionine aminopeptidase activity without affecting its inhibition of eIF-2α phosphorylation. The type 1 enzyme, MetAP1, was not inhibited by the drugs, suggesting that the interaction between the drugs and MetAP2 is highly specific. These results revealed that MetAP2 may play an important role in endothelial cell proliferation and it may serve as a target for identifying novel inhibitors of angiogenesis for the development of anticancer drugs.

Materials and methods

Synthesis of fumagillin and ovalicin analogs

All analogs of fumagillin, including AGM-1470, were synthesized according to literature procedures [19]. All analogs of ovalicin and the biotin–fumagillin and biotin–ovalicin conjugates were synthesized using modified procedures. All compounds were characterized by ^1H nuclear magnetic resonance (NMR), IR and mass spectrometry. The photoaffinity label of ovalicin was synthesized from its corresponding free amine by analogy to previous reports [51]. The details of the synthesis and characterization of the analogs will be reported elsewhere.

Photoaffinity labeling

Cell or tissue extract (10 µl; 10 mg/ml of total protein concentration) was added to 5 µl labeling buffer (20 mM Tris·HCl, pH 7.5, 100 mM NaCl), 5 µl 5 × cold competitor drug or carrier control (1% EtOH in labeling buffer), and 5 µl ovalicin photolabel (0.2 µCi/µl in 20% MeOH/ddH_2O; 2010Ci/mmol) in the absence of direct light. Reaction mixtures were incubated on ice in the dark for 1 h and then irradiated at 254 nm (0.2 J/cm^2). Reactions were quenched by adding 1.5 µl β-mercaptoethanol followed by 7.5 µl 5 × SDS sample buffer and heated in a boiling water bath for 3 min. Samples were analyzed by 10% SDS–PAGE, followed by autoradiography.

BAEC proliferation assay
BAECs were detached from tissue culture flasks by trypsin and plated into 96 well plates at a density of 2000 cells per well. After the cells adhered to the plate, compounds dissolved in ethanol (final concentration of 0.5%) were added to the cultures. Three days later, 25 µl of 2.5 mg/ml (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution was added to the cultures. After an additional 4 h incubation, 100 µl of 10% SDS/0.01 N HCl solution was added to the culture. The absorbance at 600 nm was determined 12 h later using a Titertek Multiscan Plus plate reader.

Affinity binding assay
Mouse embryo extracts were prepared from 14.5 days post conception. mouse embryos. Embryos were dissected and dounce homogenized (30 strokes) in 4 ml/g lysis buffer (20 mM Tris·HCl, pH 7.1, 100 mM KCl, 0.2% Triton X-100, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µg/ml soybean trypsin inhibitor). Lysates were centrifuged at 10,000 × g for 20 min. The resulting supernatant was centrifuged at 50,000 × g for 30 min. The supernatant was either used immediately or frozen at −80°C for storage. An aliquot of extract (200 µl) was incubated for 30 min with 50 µM competitor or ethanol control at 4°C. Following competition, the extract was incubated with the conjugate ligands (0.2–1 µM final concentration) for 1 h at 4°C. Immobilized streptavidin (40 µl; 1:1 in lysis buffer) was added and the mixture was incubated at 4°C for 1 h. The beads were pelleted at 10,000 rpm in a microcentrifuge for 5 min and washed twice with 600 µl lysis buffer for 5 min. 40 µl of 1 × SDS sample buffer was added and the samples were boiled for 10 min. 25 µl of the mixture was loaded on a 12% SDS–PAGE gel and silver stained.

Identification of p67 by mass spectrometry
The affinity binding experiment was scaled up by using 3 ml of mouse embryo extract (16 mg/ml) and increasing the amount of biotin–fumagillin, immobilized streptavidin and other reagents and solutions proportionally. The partially purified p67 was released from immobilized streptavidin by boiling in sample buffer for 10 min before loading onto a 10% SDS–polyacrylamide gel. After electrophoresis, the gel was silver stained to visualize p67. The 67 kDa band was excised, reduced and alkylated with iodoacetamide, followed by digestion with trypsin and extraction as previously described [52].

The extract of the tryptic peptide mixture was dried in a Speedvac and the residue was dissolved in 3.5 µl of 7% aqueous formic acid. About 0.5 µl of this solution and 0.5 µl of the standard (ACTH 4–10 and 18–39, 50–100 fmol each) were placed onto a thin film of α-cyano-4-hydroxycinnamic acid deposited on the sample plate of a PerSeptive Biosystems Voyager-Elite MALDI-TOF mass spectrometer and evaporated to dryness. Figure 3b represents the spectrum obtained by summation of 256 N_2 laser pulses. The instrument was operated in the reflectron mode with delayed extraction [53]. Under these conditions, the resolution is >4000, sufficient to resolve the isotopic multiplets with the mass accuracy being over 50 ppm.

The human MetAP2 sequence (Swiss-Prot accession number P50579) was used to search the EST database. A total of 13 overlapping mouse clones were found (Accession numbers: AA175951, AA172540, AA023796, AA185067, AA175099, AA138570, L26708, AA204267, AA175131, AA212018, AA242695, AA408613 and D21545). They were assembled into single cDNA encoding the full length MetAP2.

Western immunoblotting
Recombinant human MetAP2 was expressed and purified as previously reported [26]. Samples were transferred to nitrocellulose at 50 V for 1 h at 4°C. The nitrocellulose was treated overnight with blocking solution (5% BSA, 2% nonfat milk, 0.02% NaN_3 in PBS). The membrane was incubated with rabbit anti-human MetAP2 polyclonal antibodies (1:500) for 1 h at room temperature, followed by incubation with sheep anti-rabbit IgG–HRP. MetAP2 was visualized with the chemilluminescent ECL kit (Amersham) as described in the manufacturer's instructions.

Detection of biotin–fumagillin and biotin–ovalicin covalently associated with MetAP2
Recombinant human MetAP2 (100 ng) was incubated in 40 µl binding buffer (20 mM Tris•HCl, pH 7.1, 100 mM KCl, 0.2% Triton X-100) in the presence or absence of competitors for 1 h followed by incubation with the biotin conjugates (1 µM) at 4°C for 2 h. An aliquot of 2 × SDS sample buffer (40 µl) was added, and the samples were boiled for 10 min. Following SDS–PAGE, the samples were transferred to nitrocellulose at 50 V for 1 h at 4°C and blocked overnight in blocking solution (5% BSA, 2% nonfat milk, 0.02% NaN_3 in PBS). The membrane was incubated with rabbit anti-human MetAP2 antibodies (1:500) for 1 h at room temperature, followed by incubation with sheep anti-rabbit IgG–HRP or incubated with streptavidin–HRP (1:1000) for 1 h and visualized with the chemilluminescent ECL kit (Amersham), according to the manufacturer's instructions.

MetAP enzymatic assay
Recombinant human MetAP2 was expressed and purified from insect cells as previously described [26]. To determine the effect of ovalicin and AGM-1470 as well as their derivatives on MetAP activity, various amounts of these inhibitors was added to buffer H (10 mM Hepes, pH 7.35, 100 mM KCl, 10% glycerol, and 0.1 M Co^{2+}) containing 1 nM of purified human MetAP2, and incubated at 37°C for 30 min. To start the enzymatic reaction, Met–Gly–Met–Met was added to a concentration of 1 mM to the reaction mixture. Released methionine was quantified at different time points (0, 2, 3 and 5 min) using the method of Zuo *et al.* [40].

Phosphorylation assay for eIF-2α
Recombinant human MetAP2 was incubated with AGM-1470 or ethanol carrier alone and dialyzed into 20 mM Tris•HCl, pH 7.8, 100 mM KCl. Modified or control MetAP2 (0.6 µg) was incubated with purified eIF-2 (0.3 µg) in 20 mM Tris•HCl, pH 7.8, 40 mM KCl, and 2 mM $MgAc_2$ on ice for 1 h. Recombinant HRI (0.25 ng) and [γ-^{32}P]ATP were then added to a final total volume of 20 µl and the reaction mixture was further incubated at 37°C for 10 min. The labeled eIF-2α was analyzed by 10% SDS–PAGE followed by autoradiography. The phosphorylated bands were quantified by NIH Image 1.60 software.

Effect of AGM-1470 and ovalicin on yeast growth
The wild-type [YPH500 (MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1), the *map1* null [XLP101 (map1:: HIS3)], and the *map2* null [XLP201 (map2::URA3)] yeast cells were grown in YEPD at 30°C to A_{600} of 1, and plated out on a YEPD plate, and YEPD plates containing 50 nM ovalicin or AGM-1470. The plates were incubated at 30°C for four days.

Note added in proof
A paper reporting the identification of MetAP2 as the binding protein for fumagillin has appeared (Sin N., *et al.*, & Crews, C.M. (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP2. *Proc. Natl Acad. Sci USA* **94**, 6099-6103). We thank Craig Crews for communicating information to us prior to publication.

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