Selective inhibition of amino-terminal methionine processing by TNP-470 and ovalicin in endothelial cells

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Background: The angiogenesis inhibitors TNP-470 and ovalicin potently suppress endothelial cell growth. Both drugs also specifically inhibit methionine aminopeptidase 2 (MetAP2) *in vitro*. Inhibition of MetAP2 and changes in initiator methionine removal in drug-treated endothelial cells have not been demonstrated, however.

Results: Concentrations of TNP-470 sufficient to inactivate MetAP2 in intact endothelial cells were comparable to those that inhibited cell proliferation, suggesting that MetAP2 inhibition by TNP-470 underlies the ability of the drug to inhibit cell growth. Both drug-sensitive and drug-insensitive cell lines express MetAP1 and MetAP2, indicating that drug sensitivity in mammalian cells is not simply due to the absence of compensating MetAP activity. With a single exception, detectable protein N-myristoylation is unaffected in sensitive endothelial cells treated with TNP-470, so MetAP1 activity can generally compensate when MetAP2 is inactive. Analysis of total protein extracts from cells pulse-labeled with [^{35}S]-methionine following TNP-470 treatment revealed changes in the migration of several newly synthesized proteins. Two of these proteins were identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A. Purification and amino-terminal sequencing of GAPDH from TNP-470-treated cells revealed partial retention of its initiator methionine, indicating that methionine removal from some, but not all, proteins is affected by MetAP2 inactivation.

Conclusions: Amino-terminal processing defects occur in cells treated with TNP-470, indicating that inhibition of MetAP2 by the drug occurs in intact cells. This work renders plausible a mechanism for growth inhibition by TNP-470 as a consequence of initiator methionine retention, leading to the inactivation of as yet unidentified proteins essential for endothelial cell growth.

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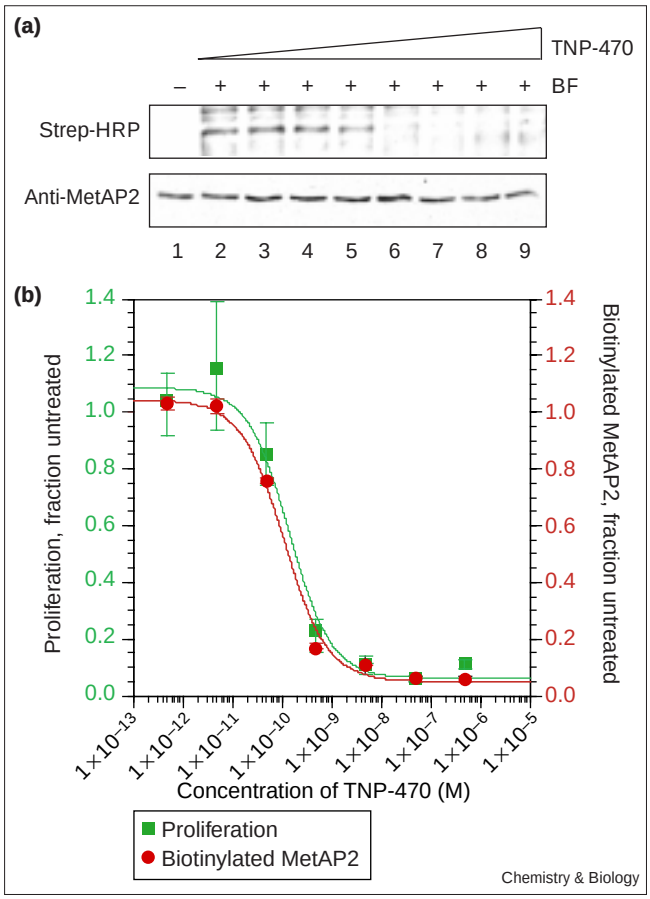
Introduction

TNP-470, a synthetic derivative of the fungal metabolite fumagillin, is a member of a novel class of anticancer drugs that act by inhibiting angiogenesis, the growth of new blood vessels, which is an event required for tumor progression [1–4]. The compound potently inhibits the growth of primary tumors and metastases in experimental animals and is currently in phase II clinical trials to treat a variety of cancers, including Kaposi's sarcoma, prostate cancer, cervical carcinoma, glioblastoma, pancreatic cancer and renal cancer [5]. TNP-470 inhibits the growth of endothelial cells and vascular smooth muscle cells in culture at picomolar concentrations. Much higher concentrations of the drug are required to substantially inhibit the growth of tumor cells in culture, however, suggesting that the effects of the drug *in vivo* are likely to be mediated by inhibition of angiogenesis [3,6,7].

TNP-470, fumagillin and the structurally related natural product ovalicin specifically and covalently inactivate methionine aminopeptidase 2 (MetAP2), one of two enzymes responsible for the cotranslational removal of the initiator methionine residue in eukaryotes [8,9]. The ability of fumagillin and ovalicin analogs to inhibit endothelial cell growth correlates strongly with their ability to inhibit MetAP2 activity, suggesting that inhibition of the enzyme underlies the growth-arresting effects of these drugs [8]. The mechanism by which inhibition of MetAP2 might lead to growth inhibition is not clear, however. Amino-terminal processing is essential for the activity of certain proteins that regulate cell growth. Removal of the initiator methionine residue is a prerequisite for post-translational modifications to the amino terminus, such as myristoylation and acetylation, which can be required for the proper localization and stability of proteins [10,11]. In addition, the identity of the amino-terminal residue of a protein can dramatically affect its rate of turnover by regulating its degradation by the N-end rule pathway or other mechanisms [12–14]. A defect in initiator methionine removal caused by MetAP2 inhibition

Figure 1



Inhibition of cell proliferation correlates with inactivation of MetAP2 in endothelial cells. **(a)** BAECs were pre-incubated in varying concentrations of TNP-470 (tenfold dilutions from 500 nM to 0.5 pM) for 24 h prior to lysis. Lysates were pre-cleared with immobilized streptavidin and incubated with excess (10 μM) biotin–fumagillin (BF) conjugate. Lysates were subjected to 8% SDS–PAGE and transferred to nitrocellulose. BF-bound MetAP2 was visualized using streptavidin-conjugated horseradish peroxidase (Strep-HRP) as previously reported [8,17]. Total MetAP2 present in the lysate was visualized by western blot. **(b)** The MetAP2 band from (a) was quantitated using NIH Image 1.60 software and plotted with cell proliferation as determined by incorporation of [^{3}H]-thymidine.

therefore might lead to aberrant levels of proteins important for cell proliferation.

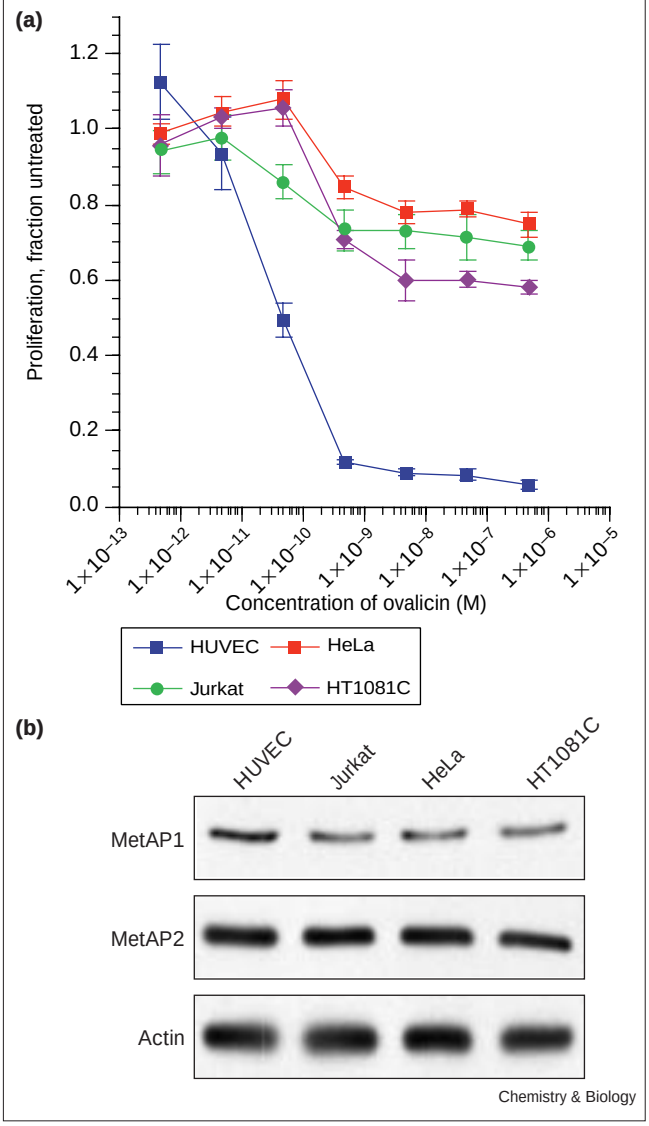
All eukaryotes appear to have two MetAPs: MetAP1 and MetAP2 [15,16]. Individual deletion of either MetAP gene in *Saccharomyces cerevisiae* results in viable yeast with a slow growth phenotype. Yeast in which both genes have been disrupted are inviable, however, indicating that MetAP activity is necessary for survival in eukaryotes [16]. As the growth of most mammalian cells and cell lines are not substantially inhibited by TNP-470, it would appear that, unlike yeast, most mammalian cells do not require MetAP2 for growth. The basis of the selective inhibition of endothelial cells by TNP-470 remains unclear. One simple explanation would be that these cell types do not express MetAP1, and therefore inactivation of MetAP2 results in complete loss of cellular MetAP activity. Alternatively, MetAP2-specific substrates essential for the growth of drug-sensitive cells may exist that require removal of the initiator methionine for proper activity or stability. Proteins that inhibit the growth of these cells may also be stabilized by retention of its initiator methionine, leading to protein accumulation and cell-cycle arrest. Here, we show that MetAP2 activity is inhibited in intact cells by TNP-470 at concentrations that inhibit cell growth. Furthermore, such inhibition is shown to result in altered amino-terminal processing of a subset of cellular proteins. These observations confirm the existence of specific MetAP2 substrates *in vivo* which cannot be efficiently processed in the absence of the enzyme, indicating that inhibition of MetAP2 by TNP-470 is a plausible mechanism for effecting cell-cycle arrest.

Results

For inhibition of MetAP2 to mediate the growth inhibitory effects of TNP-470 and ovalicin, the drugs must inactivate the enzyme intracellularly when applied to intact cells at concentrations that inhibit cell growth. To determine whether this is the case, we employed a previously described biotin–fumagillin conjugate which can be used to detect unbound MetAP2 in cell extracts [8,9,17]. Bovine aortic endothelial cells (BAECs) were treated for 72 hours with varying concentrations of TNP-470 and were either assayed for cell proliferation or lysed for determination of unbound MetAP2. Lysates were treated with biotin-fumagillin, labeling MetAP2 protein that remained unbound following TNP-470 treatment. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by blotting to nitrocellulose membrane. Bound biotin was detected by probing the membrane with streptavidin–horseradish peroxidase, and the signal was competed by cell treatment with increasing concentrations of TNP-470 (Figure 1). A concentration of 100 ± 13 pM TNP-470 results in modification of half of the MetAP2 in intact cells as determined using this assay. A similar drug concentration (161 ± 69 pM) was found to inhibit cell growth by 50%, indicating that inactivation of MetAP2 in endothelial cells occurs at concentrations that correlate with decreased proliferation. A similar correlation was also obtained in BAECs using FOS-37 [8], an ovalicin derivative with a 100-fold reduced potency (data not shown). Inactivation of MetAP2 in endothelial cells therefore appears to occur at drug concentrations that result in growth inhibition.

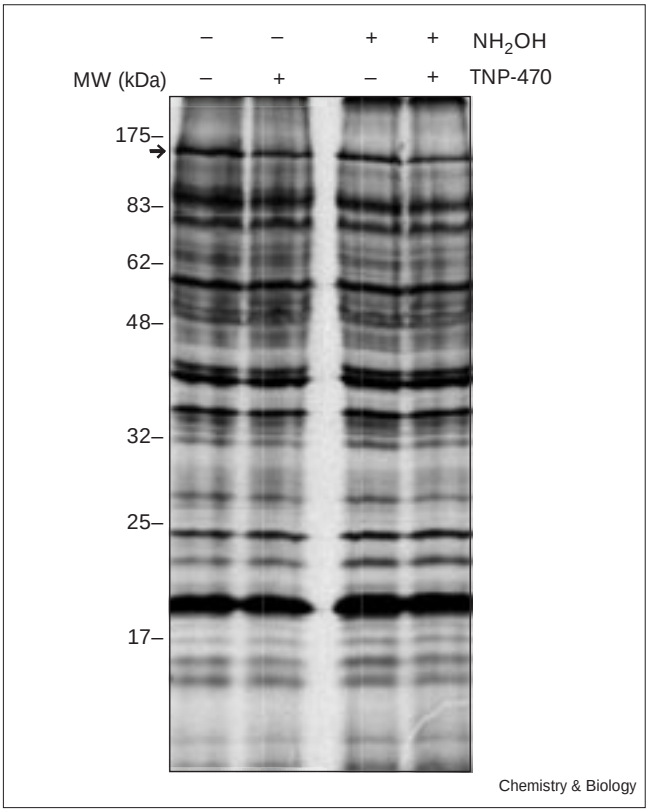
Whereas primary endothelial cells are sensitive to TNP-470 and ovalicin, many other cell types, including many transformed cells, show resistance to the compounds [3,18]. One possible explanation for this observation would

Figure 2



Expression of MetAP1 and MetAP2 does not account for the cell-type specificity of the compounds. (a) Sensitivity of human cell types to ovalicin. Cells were incubated with indicated concentrations of ovalicin for 32 h and pulsed with [^{3}H]-thymidine for the final 8 h. (b) Western blotting of cell extracts with MetAP1, MetAP2 and actin antibodies.

Figure 3

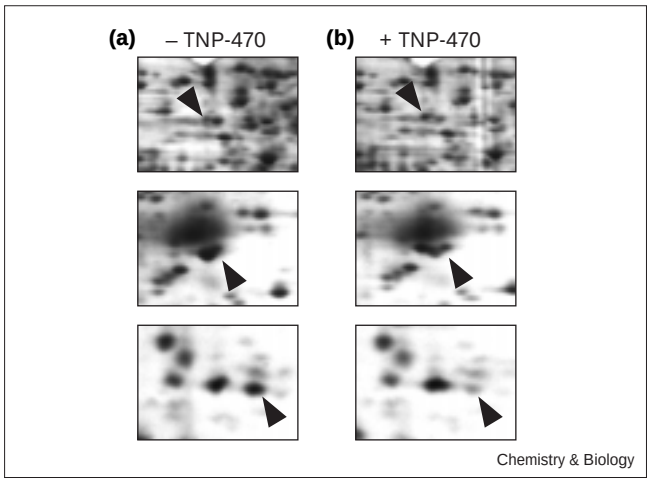


Analysis of protein myristoylation in BAECs in the presence or absence of TNP-470. Cells were pretreated with 50 nM TNP-470 or carrier solvent alone as indicated for 48 h prior to labeling for 4 h with [^{3}H]-myristic acid. Extracts were resolved using SDS–PAGE (10%), and lanes were treated with either 1 M Tris–HCl, pH 7.0, or 1 M hydroxylamine, pH 7.0, as indicated prior to fluorography. The band of ~150 kDa molecular weight, which decreases upon TNP-470 treatment, is indicated with an arrow.

be that endothelial cells lack MetAP1, which would mean that treatment with TNP-470 or ovalicin would result in complete loss of MetAP activity in these cells. Cells would thereby be unable to proliferate, by analogy with yeast lacking MetAP1 [8]. To address this possibility, we examined expression levels of both MetAP1 and MetAP2 in a series of human cell lines. Whereas human umbilical vein endothelial cell (HUVEC) proliferation was potently inhibited following ovalicin treatment, several other human cells lines including HeLa, Jurkat T lymphocytes, and HT1081C, a human fibrosarcoma cell line, showed a proliferation decrease of only approximately 30% (Figure 2). Western blotting of the cell extracts revealed no significant differences in expression of MetAP1 or MetAP2. Furthermore, similar concentrations of TNP-470 were shown to inactivate MetAP2 in drug-sensitive and drug-insensitive cell types (data not shown). It is therefore unlikely that TNP-470 treatment blocks all MetAP activity in sensitive cells or that the expression level of either protein confers resistance in insensitive cell types.

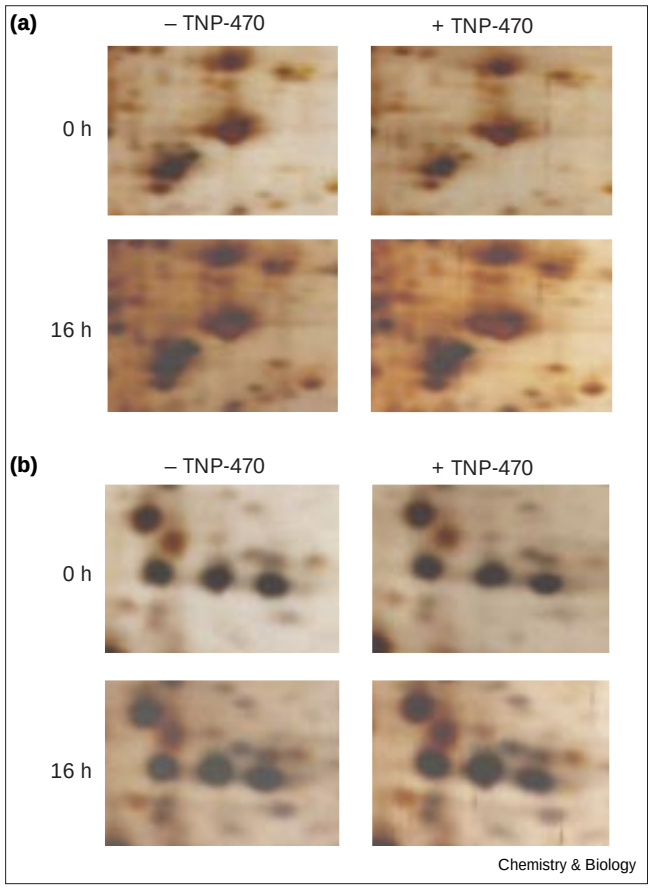
Given that differences in expression of the two MetAP enzymes did not appear to account for the cell-type specificity of the compound, we postulated that drug-sensitive cells may have specific MetAP2 substrates that cannot be processed by the type 1 enzyme. Several approaches were therefore taken to determine whether cells treated with the drugs have specific defects in methionine processing. First, protein myristoylation was examined, as N-myristoylation requires the prior removal of the initiator

Figure 4



TNP-470 affects the migration of several protein spots by two-dimensional electrophoresis. Cells were pretreated with either (a) carrier solvent alone or (b) 50 nM TNP-470 for 4 h prior to pulse labeling with [^{35}S]-methionine. Extracts were prepared immediately following the pulse and analyzed using two-dimensional electrophoresis (NEPHGE/SDS–PAGE) followed by autoradiography. Altered migration of protein spots of 55 kDa (top panels), 37 kDa (middle panels), and 16 kDa (bottom panels) are shown. The 16 kDa spot shifts to co-migrate with the spot immediately to its left upon treatment with TNP-470.

Figure 5



TNP-470 only affects the migration of the 37 kDa and 16 kDa proteins synthesized subsequent to drug treatment. Cells were treated for 4 h with either TNP-470 or carrier solvent alone as indicated prior to pulse labeling with [^{35}S]-methionine. Extracts were prepared either immediately or 16 h following the pulse as indicated and analyzed by two-dimensional electrophoresis followed by silver staining. (a) Altered migration of the 37 kDa protein is visible by silver stain only at the 16 h time point. (b) Migration of the 16 kDa protein is not visibly affected over the course of 20 h of drug treatment.

methionine to expose an amino-terminal glycine residue. BAECs were pretreated with TNP-470 and then labeled with [^{3}H]-myristic acid, which is metabolically converted to myristoyl CoA and incorporated into proteins as N-myristoyl groups [19]. Labeled cell extracts were subjected to SDS–PAGE followed by fluorography. Samples were run in duplicate and treated with either Tris buffer or 1 M hydroxylamine, which cleaves thioester bonds, to ensure that the incorporated radioactivity was due to myristoylation and not due to S-palmitoylation as a consequence of metabolic conversion of the labeled myristate to palmitate. A series of myristoylated protein bands could be detected using this procedure (Figure 3). For most of the detected proteins, no differences in the intensity of labeling were observed between untreated cells and cells treated with TNP-470. The only exception was a single protein band of ~150 kDa molecular weight, which decreased ~twofold in intensity upon treatment with TNP-470. This band may correspond to endothelial nitric oxide synthase (eNOS), which has recently been reported to be affected by TNP-470 treatment [20]. To determine whether the myristoylation of a subset of proteins that could not be visualized by one-dimensional electrophoresis was affected by TNP-470 treatment, extracts were also analyzed by two-dimensional electrophoresis, and no differentially myristoylated proteins were detected (data not shown). Though it is likely that there are scarce myristoylated proteins that could not be detected by this method, it would appear that for the great majority of proteins destined for myristoylation MetAP1 can compensate in the absence of MetAP2 activity.

Given the precedent for effects on turnover rate as a consequence of changes in the amino terminus of a protein, we also examined whether TNP-470 treatment affected the half-life of cellular proteins. Cells were pre-treated with TNP-470 or carrier solvent alone and then pulse labeled with [^{35}S]-methionine. Following the pulse, cells were exchanged into fresh media containing excess cold methionine and extracts were prepared at various times thereafter. Extracts were analyzed using two-dimensional electrophoresis followed by autoradiography to allow the observation of as many proteins as possible. Despite detection of several rapidly degraded proteins (with half-lives of

Table 1

Molecular weights of tryptic peptides from the 37 kDa protein identify it as bovine GAPDH.

Measured mass (Da)	Calculated mass (Da)	GAPDH peptide	Location in sequence
783.344	–	–	–
795.426	795.425	LTGMAFR	225–231
806.397	–	–	–
977.55	977.542	KAITIFQER	70–77
1033.61	–	–	–
1213.58	–	–	–
1358.71	1358.681	VVDLMVHMASKE	321–332
1369.78	1369.744	GAAQNIIPASTGAAK	198–212
1461.87	1461.832	LEKPAKYDEIKK	246–257
1556.83	1556.811	VPTPNVSVVDLTCR	232–245
1570.88	1570.826	VPTPNVSVVDLTCR	232–245*
1615.93	1615.881	AITIFQERDPANIK	70-83
1763.83	–	–	–
1795.83	–	–	–
1848.01	1848.936	IVSNASCTTNCLAPLAK	143–159*
2213.08	2213.11	VIISAPSADAPMFVMGVNHEK	116–136

*Peptides predicted to be modified on cysteine residues by monomeric acrylamide.

Table 2

Molecular weights of tryptic peptides from the 16 kDa protein indicate it to be bovine cyclophilin A.

Measured mass (Da)	Calculated mass (Da)	Cyclophilin A peptide	Location in sequence
686.371	686.399	HVVFGK	125–130
737.349	737.358	TAENFR	31–36
777.341	777.347	GSCFHR	49–54*
1055.53	1055.541	VSFELFADK	19–27
1132.61	1132.604	KITIADCGQI	154–163*
1140.57	1140.558	FDDENFILKH	82–90
1379.79	1379.758	VSFELFADKVPK	19–30
1505.78	1505.746	VKEGMNIVEAMER	131–143
1564.76	–	–	–
1612.76	1612.761	IIPGFMCQGGDFTR	55–68*
1817.95	1817.896	SIYGEKFDDENFILK	76–90
1946.01	1946.002	VNPTVFFDIAVDGEPLGR	1–18

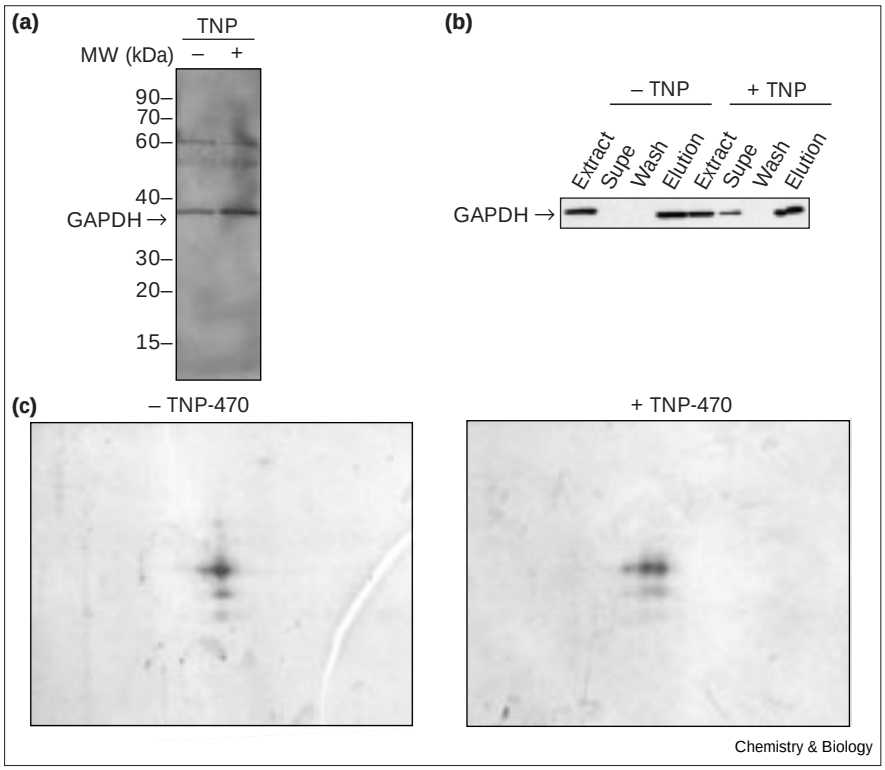
*Peptides predicted to be modified on cysteine residues by monomeric acrylamide.

less than 16 hours), no clear differences in the turnover rate of any protein were observed upon TNP-470 treatment (data not shown). However, several protein spots, which migrated with molecular weights of roughly 16 kDa, 37 kDa and 56 kDa, displayed altered mobility upon drug treatment when visualized by autoradiography (Figure 4). Interestingly, these changes in mobility were not evident upon silver staining of extracts prepared immediately following the pulse. The mobility of the 37 kDa and 56 kDa proteins when visualized by silver staining began to change at later time points (20 hours after initiation of drug treatment), whereas no change in migration was evident for the 16 kDa protein during the same time period (Figure 5 and data not shown). These observations suggested that the altered mobility observed was due to modifications that only occurred to protein molecules synthesized subsequent to drug treatment. The ability to detect a partial change in mobility of the 37 kDa and 56 kDa proteins by silver stain at later times probably indicates that turnover of a detectable fraction of the protein has occurred by that time.

To study these modifications further, the 37 kDa and 16 kDa proteins, which were relatively abundant as judged by the intensity of silver staining, were identified using matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry analysis of in-gel tryptic digests [21]. Mass spectra of tryptic peptides from the 37 kDa protein revealed 16 peptides, which were used to search the protein databank. Of the 16 masses, nine matched predicted peptides from the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with high accuracy (Table 1). Two additional peptides appear to result from modification of GAPDH cysteine residues with monomeric acrylamide. The nature of the remaining five peptides is not known but may reflect allelic differences between the isolated protein and the sequence in the protein databank or possibly unknown post-translational modifications. The mass spectrum from the tryptic digest of the 16 kDa protein contained 12 peaks distinct from background. Of these, 11 matched peptides derived from the peptidyl prolyl *cis–trans* isomerase cyclophilin A, including three that were modified by acrylamide as above (Table 2). In addition, both protein assignments were consistent with the observed molecular weights of 37 and 16 kDa, respectively.

Both bovine GAPDH and cyclophilin A have their initiator methionine residues removed co-translationally to yield mature proteins with unmodified valine residues at their amino termini, raising the possibility that the shifts in mobility following drug treatment are due to retention of the initiator methionine. Furthermore, varying the concentration of TNP-470 treatment confirmed that the appearance of the altered form of GAPDH occurred at drug concentrations comparable to those which were found to inactivate MetAP2 in endothelial cells (data not shown). To investigate this possibility, we chose to purify GAPDH from cells treated with TNP-470. GAPDH was chosen over cyclophilin A as its rate of turnover appeared to be more rapid (Figure 5). BAECs were treated for 72 hours with either 50 nM TNP-470 or carrier solvent alone, and extracts were prepared by hypotonic lysis.

Figure 6



Purification of GAPDH from endothelial cells treated with or without TNP-470. Cells were treated for 72 h with 50 nM TNP-470 or carrier solvent alone. GAPDH was isolated from extracts by AMP-agarose affinity chromatography. **(a)** Silver staining of purified material. **(b)** Western blotting with GAPDH antibodies. Equal portions of the extracts, AMP-agarose bead supernatants, column wash and 1 mM NAD^+ elution fractions were loaded in each lane. **(c)** Two-dimensional electrophoresis of purified GAPDH from cells treated with carrier solvent alone (left panel) or TNP-470 (right panel).

GAPDH was purified using affinity chromatography on AMP agarose [22] (Figure 6a). As judged by immunoblotting, TNP-470 treatment had little effect on levels of the enzyme, and recovery from extracts of both drug-treated and untreated cells was nearly quantitative (Figure 6b). Two-dimensional gel analysis of the purified protein from TNP-470-treated cells revealed an ~1:1 mixture of the two closely migrating forms, whereas the material from untreated cells was almost exclusively a single species (Figure 6c).

To confirm that this mobility shift resulted from alterations at the amino-terminus of GAPDH, purified material from both preparations was separated using SDS–PAGE and electrophoretically transferred to PVDF membrane. The amino-terminal sequences were subsequently determined by Edman degradation. The amino-terminal sequence of the protein purified from untreated cells was identical to the predicted sequence for bovine GAPDH, with complete removal of the initiator methionine [23]. The protein from TNP-470-treated cells, however, appeared to be a mixture of GAPDH with the methionine retained and with the methionine removed in roughly equal amounts (Table 3). This result establishes that complete processing of the amino-terminal methionine from GAPDH requires MetAP2 activity, and that this activity is inhibited in intact cells by TNP-470. Taken together with the observation that protein myristoylation is not generally effected by the drug, this result indicates that the amino-terminal processing of some, but not all, proteins is diminished in TNP-470 treated cells. It is formally possible, however, that the defect in GAPDH processing was simply due to an overall decrease in MetAP activity in cells, which may have led to subtle decreases in myristoylation not detected in our experiments.

In order to observe directly whether MetAP2 was required for complete amino-terminal methionine removal from all proteins, we chose to examine the amino terminus of another cytosolic protein. Bovine glutathione S-transferase-π (GST-π) is normally processed by removal of its initiator methionine to produce a mature protein with an unblocked amino-terminal proline residue [24]. We isolated GST-π from cells treated with or without 50 nM TNP-470 using affinity chromatography on glutathione sepharose. To ensure that the protein analyzed was synthesized subsequent to drug treatment, cells were plated at low density and allowed to grow to confluence under both conditions. As TNP-470 decreases the growth rate of BAECs dramatically, drug-treated cells did not reach confluence until 7 days following replating, while carrier treated cells reached confluence after only 2 days. Western blotting confirmed that ~75% of the protein present in extracts was synthesized during the last 6 days

Table 3

Amino-terminal sequence analysis of GAPDH purified from TNP-470-treated and untreated BAECs.

Cycle	Literature sequence	Control cells	TNP-470-treated cells
1	V	V (44.1), M (0)	V (13.6), M (17.0)
2	K	K (47.7), V (0)	K (19.1), V (10.6)
3	V	V (42.2), K (3.0)	V (15.7), K (17.0)
4	G	G (34.3), V (2.0)	G (17.2), V (12.2)
5	V	V (36.7), G (2.4)	V (16.4), G (14.8)
6	N	N (23.1), V (4.8)	N (9.9), V (13.5)

Purified GAPDH from TNP-470-treated cells was run on SDS–PAGE, blotted to PVDF membrane, and sequenced by Edman degradation. The picomolar amount of each amino acid is given for each cycle in parentheses.

Table 4

Amino-terminal sequence of purified GST-π from cells grown to confluence in the presence or absence of TNP-470.

Cycle	Literature sequence	Control cells	TNP-470-treated cells
1	P	P (11.7), M (1.6)	P (19.2), M (2.8)
2	P	P (12.7)	P (19.5)
3	Y	Y (13.4), P (2.1)	Y (20.5), P (3.8)
4	T	T (9.7), Y (3.3)	T (14.1), Y (5.5)
5	I	I (13.6), T (1.9)	I (22.8), T (3.3)
6	V	V (10.8), I (2.8)	V (17.6), I (5.9)

GST-π isolated from BAECs treated with or without TNP-470 was electrophoresed, blotted onto PVDF membrane and sequenced by Edman degradation. The molar amount of each amino acid present in each cycle is indicated.

of drug treatment, as there was fourfold more GST-π present in equal proportions of extracts prepared 7 days after initiating drug treatment compared with extract from an identical plate prepared one day following drug treatment (data not shown). Amino-terminal sequencing of the purified protein revealed that approximately 80% of the protein had its initiator methionine removed, whereas ~20% retained the methionine regardless of whether it was from drug-treated or untreated cells (Table 4). This result indicates that, in contrast with GAPDH, MetAP2 activity is dispensable for normal processing of GST-π, illustrating that specific MetAP2 substrates do exist within endothelial cells.

To determine whether the substrate specificity observed *in vivo* could be recapitulated *in vitro*, the activity of the recombinant enzymes towards a series of model peptides was examined. Recombinant human MetAP1 and MetAP2 were produced and purified from baculovirus-driven insect cells. As bovine cyclophilin A and GAPDH are processed to reveal an amino-terminal valine and processing of GST-π reveals an amino-terminal proline residue, it was possible that the identity of the second translated residue was the basis for the observed specificity. To determine whether the specificity between the two enzymes was based on the identity of the second substrate residue, the activity of each enzyme was measured using a series of peptides based on the amino terminus of hemoglobin (MVHTLPEE; using single-letter amino-acid code) differing at the second residue. In addition, the activity of the enzymes towards peptides corresponding to the amino termini of bovine eNOS, GAPDH, and GST-π were also examined. The activity of each enzyme was normalized to that for the reference tetrapeptide substrate Met–Gly–Met–Met to simplify interpretation, although MetAP2 showed a 4.5-fold higher activity for this peptide than did MetAP1. Both MetAP1 and MetAP2 showed activity towards peptides that have a small, uncharged second residue, as has been observed previously using peptide substrates in other *in vitro* studies [16,25–27] (Table 5). Although some differences were apparent between the two enzymes, specificity appeared to be dictated by more than simply the identity of second residue. For example, the trend in relative activity between MetAP1 and MetAP2 was not the same for the two peptides that have a glycine residue in the second position. Likewise, the presence of valine in the second position could not be used to predict the relative ability of a peptide substrate to be cleaved by MetAP1 versus

Table 5

Comparison of substrate specificity of human MetAP1 and MetAP2.

Peptide	Human MetAP1 relative activity (%)	Human MetAP2 relative activity (%)
MGMM	100 ± 6	100 ± 4
MGHTLPEE	100 ± 7	14 ± 5
MAHTLPEE	530 ± 15	79 ± 5
MPHTLPEE	260 ± 10	76 ± 6
MVHTLPEE (hemoglobin)	185 ± 8	63 ± 6
MSHTLPEE	93 ± 8	39 ± 5
MTHTLPEE	93 ± 8	38 ± 5
MLHTLPEE	0*	0*
MFHTLPEE	0*	0*
MDHTLPEE	0*	0*
MRHTLPEE	0*	0*
MGNLKSVG (eNOS)	54 ± 6	152 ± 8
MPPYTIVY (GST-π)	62 ± 8	63 ± 6
MVKVGVNG (GAPDH)	0*	25 ± 5

Enzyme activity was determined under standard assay conditions using peptide substrates at a final concentration of 2 mM. The activity of each enzyme was normalized to that for the reference substrate Met–Gly–Met–Met. Data reported as mean activity ± S.D. *No detectable cleavage.

MetAP2. Interestingly, the specificity observed for the bovine proteins *in vivo* could be reconstituted *in vitro* using the corresponding amino-terminal peptides. When the activities of MetAP1 and MetAP2 towards these peptide substrates were directly compared without the normalization factor, peptides corresponding to both eNOS and GAPDH showed strong preferences for cleavage by MetAP2 (> tenfold), whereas the peptide based on GST-π showed only a modest preference for MetAP2 cleavage. The observed *in vivo* specificity of MetAP2 therefore can be recapitulated with recombinant protein in an *in vitro* system, paving the way for the future investigation of the rules governing substrate specificity for this class of enzymes.

Discussion

Recent reports have identified MetAP2 as a cellular target for a class of small-molecule angiogenesis inhibitors structurally related to the natural product fumagillin [8,9]. Inhibition of MetAP2 aminopeptidase activity could result in growth inhibition by blocking the activity of key polypeptide substrates required for cell proliferation. Given that MetAP2 appears to be a ubiquitously expressed housekeeping enzyme, however, this model must account for the known selectivity of this class of compounds for endothelial cells [3,18]. One simple explanation would be that sensitive cell types lack the related type-1 enzyme, whereas in drug-insensitive cell types MetAP1 compensates for the loss of MetAP2 activity. This situation has been observed in yeast strains lacking MetAP1 [8,9]. Surprisingly, expression levels of the two enzymes showed no significant differences in sensitive and resistant cell types, arguing against this model. In drug-sensitive mammalian cell types, unlike in yeast, MetAP1 activity therefore cannot compensate for the loss of MetAP2 aminopeptidase activity. In addition, the observation that similar concentrations of TNP-470 were required to inactivate MetAP2 in intact cells regardless of cell sensitivity to the compounds argues against differential drug permeability or metabolism in drug-insensitive cells. Factors that affect cell sensitivity to TNP-470 therefore appear to act subsequent to the inactivation of MetAP2.

Another model to account for cell-type selectivity of the compounds postulates the existence of MetAP2-specific substrates in drug-sensitive cells that are important for proliferation. However, no MetAP2-specific substrates had yet been identified prior to this study, and work with yeast deficient in either enzyme revealed that these two enzymes are largely redundant. Examination of protein myristoylation provided data consistent with a largely overlapping specificity for these enzymes. With a single exception, detectable protein N-myristoylation remains unaffected in TNP-470-treated cells, indicating that MetAP1 activity can largely compensate for MetAP2. It was recently reported that myristoylation of eNOS is decreased twofold in BAECs treated with TNP-470 [20]. However, high concentrations (5 μM) of the drug, which correlated with cytotoxicity rather than growth arrest, were used in this study, raising the possibility that inhibition of eNOS myristoylation (and cell death) may have resulted from nonspecific effects such as inhibition of MetAP1 as well as MetAP2. The molecular weight of eNOS is consistent with the protein observed in our experiments and a peptide based on the eNOS amino terminus shows a strong preference for MetAP2, indicating the possibility that eNOS myristoylation in cells is hampered by specific inhibition of MetAP2. Experiments to examine this possibility are currently underway.

Other proteins affected by TNP-470 treatment were identified using two-dimensional gel electrophoresis of cell extracts pulse-labeled with [^{35}S]-methionine following drug treatment. Although no changes were observed in the half-lives of detectable proteins, drug treatment resulted in changes in the migration of several newly synthesized proteins. Two of these proteins were identified, using mass spectrometry, as GAPDH and cyclophilin A. Purification and sequencing of GAPDH from cells treated with TNP-470 revealed partial retention of the initiator methionine compared with untreated cells. Although only half of the GAPDH isolated from drug-treated cells had failed to undergo amino-terminal processing, the fraction of the enzyme with altered mobility by two-dimensional electrophoresis appears closer to 70% when examined by autoradiography, a ratio that reflects the constitution of protein synthesized only during the pulse period when MetAP2 activity was absent (see Figure 4). A portion of the protein isolated was therefore likely to be present prior to drug treatment, indicating that an even smaller fraction the protein (~30%) synthesized in the absence of MetAP2 activity undergoes methionine removal. In contrast, TNP-470 treatment had no effect on initiator methionine cleavage of GST-π, confirming that methionine retention is not simply due to an overall decrease in cellular MetAP activity. GAPDH therefore represents, to our knowledge, the first demonstration of a MetAP2-specific substrate *in vivo* and the first direct demonstration that TNP-470 treatment of cells results in amino-terminal processing defects.

Using recombinant human MetAP1 and MetAP2 with methionine-containing peptides as substrates, we gained significant, albeit incomplete, insight into the substrate specificity for this class of enzymes. The two types of amino-terminal methionine-processing enzymes appear to have distinct substrate specificity (Table 5). Whereas MetAP1 prefers alanine, proline and valine, MetAP2 favors glycine, alanine and proline as the second residue. It will be critical in the future to elucidate the rules governing substrate specificity for this class of enzymes. Importantly, we have shown in this study that the specificity

observed *in vivo* can apparently be reconstituted in an *in vitro* system using recombinant enzymes. In addition, specificity in the *in vitro* system appeared to be dictated by sequence determinants as well as by the first two amino-terminal residues as shown by the dramatic decrease in activity of MetAP2 towards MGHTLPEE in comparison with MGMM (Table 5). This situation is probably the case *in vivo* as well; whereas all N-myristoylated proteins are processed to reveal an amino-terminal glycine residue, only a single detectable myristoylated protein was affected by TNP-470 treatment of endothelial cells. On the basis of the crystal structures of MetAPs from *Escherichia coli* to human, the putative substrate-binding pocket in MetAPs are relatively shallow, fitting about two amino acid residues [28,29]. The additional interaction between substrates and MetAPs would have to result from interactions with residues on MetAP surfaces around the binding pocket. A more comprehensive study of the sequence determinants of specificity for this class of enzymes will be required to reveal the basis of this specificity between MetAP1 and MetAP2.

Given the nature of our approach to identify MetAP2-specific substrates, we have identified only relatively abundant cellular proteins. Drug-induced defects in GAPDH or cyclophilin A processing are not likely to play any direct role in the inhibition of cell proliferation by TNP-470. The time required to accumulate substantial amounts of the altered forms of these proteins is inconsistent with the rapid growth arrest observed in drug-treated cells [30]. Additionally, TNP-470 treatment does not affect cellular GAPDH activity (data not shown). Other more relevant MetAP2-specific substrates probably remain below the limits of detection for the methods used in this study, and it is not known at this time what fraction of cellular proteins are specifically processed by MetAP2. Crucial components to the understanding of the mechanism of action of TNP-470 and ovalicin will be the future identification of these specific MetAP2 substrates and the rules that govern MetAP1 and MetAP2 specificity *in vivo*.

Significance

TNP-470, a synthetic derivative of the natural product fumagillin, acts as a potent inhibitor of angiogenesis and endothelial cell proliferation. The compound is currently being evaluated in clinical trials for treatment of a variety of cancers. Recently, this class of compounds has been found to inhibit the type 2 methionine aminopeptidase (MetAP2) *in vitro,* and structure–activity relationships suggest that MetAP2 inhibition mediates the anti-angiogenic effects of this class of compounds. Here, we have found that concentrations of TNP-470 sufficient to inactivate MetAP2 in intact endothelial cells are comparable to drug concentrations that inhibit cell proliferation, providing further evidence that inhibition of MetAP2 accounts for the ability of TNP-470 to block cell growth. Moreover, we have shown that amino-terminal processing defects occur in cells treated with TNP-470, demonstrating inhibition of MetAP activity in intact cells. This effect was specific for a subset of cellular proteins, providing the first demonstration of *in vivo* specificity between MetAP1 and MetAP2. One cellular MetAP2 substrate affected by TNP-470 treatment was identified as glyceraldehyde-3-phosphate dehydrogenase, resulting in a detectable mobility shift by two-dimensional gel electrophoresis. This mobility shift now provides a novel *in vivo* assay for MetAP2 inhibition, which should aid in the development of specific MetAP2 inhibitors as angiogenesis inhibitors. As amino-terminal processing is known to be required for the proper function of some cellular proteins, this work renders plausible a mechanism for growth inhibition by TNP-470 as a consequence of initiator methionine retention, leading to the inactivation of as yet unidentified proteins that are essential for endothelial cell growth.

Materials and methods

[Methyl-^{3}H]-Thymidine, EXPRESS [^{35}S]-methionine/cysteine mixture, [γ-^{32}P]-ATP, and ENHANCE fluorographic enhancer were purchased from DuPont/NEN Life Science. [9,10(n)-^{3}H]-myristic acid was obtained as an ethanolic solution from Amersham and was concentrated to dryness and resuspended to 10 mCi/ml in EtOH prior to use. Antibodies to GAPDH and GST-π were obtained from Biodesign International and Medical and Biological Laboratories (Nagoya), respectively. Antibodies to actin were obtained from Boehringer Mannheim. Polyclonal antibodies against MetAP2 have been reported previously [8] and those against MetAP1 were generated in a similar fashion (Y-H.C., unpublished observations).

Cell culture

Cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2. Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were obtained from Clonetics and cultured in EGM-2 and EGM-MV (Clonetics), respectively. HT1081C cells were cultured in DME containing 10% fetal bovine serum (FBS) and 50 units/ml penicillin plus 50 µg/ml streptomycin (P/S).

Cell proliferation assay

Cells prepared as described above were seeded into 96-well plates and grown in the presence of drug or carrier alone (0.5% EtOH) for 32 h. Cells were pulsed with [^{3}H]-thymidine (6.7 Ci/mmol, 1 µCi per well) for the final 8 h of culture and harvested with a semi-automated cell harvester onto glass fiber papers for scintillation counting.

Electrophoresis

SDS–PAGE was performed as described by Laemmli [31], using 2% SDS, 50 mM Tris HCl, pH 6.8, 5% β-mercaptoethanol, with 0.1% bromophenol blue as loading buffer. Isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE) were performed as described previously [32,33], except that ampholytes used were purchased from Bio-Rad Laboratories. For IEF, samples were made up to 1% SDS with 20 mM dithiothreitol (DTT), heated in a boiling water bath for 3 min, and cooled to ambient temperature after which 1/10th volume of two-dimensional gel loading buffer (9 M urea, 4% CHAPS, 1% DTT, 2% ampholytes, pH 3–10) was added.

In vivo *binding assay*

BAECs were incubated in varying concentrations of TNP-470 (tenfold dilutions from 500 nM to 0.5 pM) or EtOH carrier (0.1% final) for 24 h. Cells were harvested or pulsed for 8 h with [^{3}H]-thymidine to

measure cell proliferation. BAECs (10 cm plate) were washed twice with cold PBS and lysed in 500 μl lysis buffer (0.2% Triton X-100, 20 mM Tris–HCl, pH 7.2, 100 mM KCl, 1 mM PMSF, 200 μg/ml aprotinin, 25 μM leupeptin, and 10 μg/ml pepstatin A). Lysates were incubated at 4°C for 10 min and centrifuged at 16,000 × g for 10 min to remove cell debris. Samples were pre-cleared by adding 25 μl of pre-equilibrated immobilized streptavidin beads (Boehringer Mannheim) and mixing at 4°C for 1 h. Samples were normalized for protein concentration, diluted in lysis buffer to 1 mg/ml, and incubated with biotin–fumagillin (10 μM) or EtOH carrier alone (0.5% final) for 2 h at 4°C. Samples were subjected to SDS–PAGE, transferred to nitrocellulose, and blotted with streptavidin-horseradish peroxidase or anti-MetAP2 antibodies as previously described [8].

Analysis of protein myristoylation

BAECs were trypsinized from confluent monolayers, seeded into fresh plates (10^5 cells/cm^2), and allowed to recover 4 h at 37°C. After drug or EtOH carrier treatment for the indicated time, the media was removed and replaced with labeling media (DME with low glucose, 5% dialyzed FBS, and 5 mM sodium pyruvate) containing drug or carrier. [^{3}H] myristic acid (54 Ci/mmol, 10 mCi/ml in EtOH) was added to a final concentration of 50 μCi/ml, and cells were incubated for the indicated times. After washing once with PBS, cells were harvested by direct lysis into either SDS–PAGE loading buffer or two-dimensional gel loading buffer. Extracts were incubated at room temperature for 2 h, frozen on dry ice/EtOH and stored at –20°C until analysis by either one- or two-dimensional gel electrophoresis as described above. Gels were stained with Coomassie blue, impregnated with an autoradiography enhancer (NEN Enhance), dried, and exposed to X-ray film to visualize spots.

Analysis of total protein synthesis and turnover

BAECs were plated into 6-well dishes and grown to 50% confluence. Cells were treated for 4 h with TNP-470 (50 nM) or EtOH carrier alone (0.5 %), at which time the media was removed and replaced with labeling media (methionine-free DME containing 5% dialyzed FBS, 1 ml). After incubating 10 min at 37°C, the media was replaced with fresh labeling media (0.5 ml) containing 0.5 mCi/ml [^{35}S] methionine (NEN EXPRESS label, > 1000 Ci/mmol) including TNP-470 or carrier. Cells were incubated 30 min at 37°C, washed once with complete media, and then cultured in fresh complete media for various times. Cells were washed with PBS and harvested directly into 150 μl two-dimensional gel loading buffer either immediately or at 1 h, 4 h or 16 h following the pulse. Extracts were kept at room temperature 1 h, frozen on dry ice EtOH, and stored at –20°C until analysis by two-dimensional gel electrophoresis (NEPHGE/SDS–PAGE). Gels were silver stained, dried and exposed to BioMax film.

Mass spectrometry

Samples were prepared from silver-stained polyacrylamide gels and analyzed using MALDI–TOF mass spectrometry as previously described [8,21].

Purification of GAPDH for amino-terminal sequence analysis

BAECs were plated at 1/3 confluence and treated with either 50 nM TNP-470 or 0.1% EtOH carrier alone in 15 cm plates (six of each type) for 72 h. Cells were detached by treating with 1 mM EDTA in PBS, pelleted, washed once with PBS, and lysed by homogenization in three packed cell volumes of hypotonic buffer (10 mM Tris HCl, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 200 μg/ml aprotinin, 25 μM leupeptin and 10 μg/ml pepstatin A). Lysates were centrifuged at 16,000 × g for 10 min at 4°C. Supernatants were dialyzed for 18 h with one change into dialysis buffer (30 mM sodium phosphate, pH 6.0, 1 mM DTT and 1 mM EDTA). Samples were assayed for protein by Bradford assay (Bio Rad), and 1 mg of protein was brought to a volume of 2 ml with dialysis buffer and added to 0.35 ml of AMP-agarose beads. Samples were rotated at 4°C for 20 h. The bead suspensions were transferred to columns, drained, washed with five column volumes of dialysis buffer, and then eluted with four 350 μl portions of elution buffer (30 mM sodium phosphate, pH 7.5, 1 mM DTT, 1 mM EDTA and 1 mM NAD^+). Eluates were concentrated to 125 μl under reduced pressure, and then subjected to SDS–PAGE. Samples were transferred to PVDF membrane in 10 mM CAPS, pH 11, 10% MeOH at 20 V for 2 h at 4°C. The membrane was stained with Ponceau S (0.2% in 1% HOAc) and destained with ddH_2O. Bands were excised and submitted to the MIT Biopolymers laboratory for sequence analysis.

Purification of GST-π for amino-terminal sequence analysis

BAECs were plated at low density (20% confluence) and allowed to grow to confluence in the presence or absence of 50 nM TNP-470. Cells treated with TNP-470 were exchanged into fresh media containing drug after 3 days. Cells were washed once with cold PBS and then extracted into lysis buffer (0.5% Triton X-100, 50 mM HEPES, pH 7.3, 250 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 μg/ml aprotinin, 25 μM leupeptin, 1 mM PMSF, 1 ml per 15 cm plate) by scraping into a microcentrifuge tube and rotating at 4°C for 30 min. Extracts were centrifuged 10 min at 16,000 × g at 4°C to remove debris, frozen on dry ice/EtOH, and stored at –80°C until use. To purify GST, 1.2 ml of each lysate was mixed with 100 μl of glutathione Sepharose 4B (Pharmacia) for 2 h at 4°C. Beads were pelleted and washed three times for 5 min at 4°C with lysis buffer, suspended in SDS–PAGE loading buffer, and heated in a boiling water bath for 10 min. Samples were prepared for N-terminal analysis as described above for GAPDH, except that blots were stained with Coomassie Brilliant Blue (0.1% in 1% HOAc, 40% MeOH) to visualize protein bands.

Peptide synthesis

Peptides were synthesized at the MIT Combinatorial Peptide Synthesis Core Facility on an Applied Biosystems automated peptide synthesizer using standard Fmoc chemistry. Peptides were purified by preparative reversed phase HPLC and characterized by electrospray mass spectrometry.

MetAP assay

Recombinant human MetAP2 was expressed and purified as previously described [17]. Human MetAP1 was PCR-amplified from a cDNA clone (KIAA0094, accession #: D42084) using a 5′ primer (5′-GATC-G*AATT*CATGGCGGCCGTGGAGACG) tagged with an *Eco*RI site (italicized) and a 3′ primer (5′-GATC*CTCGAG*TTAAAATTGAGACA-TGAAGTGAGGCCG) tagged with an *Xho*I site (italicized). The product was digested and ligated into pFastBac HTa (Gibco/BRL). Recombinant protein was generated by analogous procedures as for MetAP2. MetAP assays were performed as in Griffith *et al.* [17] using peptide substrates at a final concentration of 2 mM. Released methionine was quantitated according to the method of Zuo *et al.* [34].

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References

1. Folkman, J. (1995). Tumor angiogenesis. In *The Molecular Basis of Cancer.* (Mendelsohn, J., Howley, P.M., Israel, M.A. & Liotta, L.A., eds) pp. 206-232, W.B. Saunders, Philadelphia.
2. Fan, T.D. (1994). Angiosuppressive therapy for cancer. *Trends Pharmacol. Sci.* **15**, 33-36.
3. Ingber, D., *et al.*, & Folkman, J. (1990). Synthetic analogs of fumagillin that inhibit angiogenesis and suppress tumor growth. *Nature* **348**, 555-557.

4. Nelson, N.J. (1998). Inhibitors of angiogenesis enter phase III testing. *J. Natl Cancer Inst.* **90**, 960-963.
5. Castronovo, V. & Belotti, D. (1996). TNP-470 (AGM-1470): Mechanisms of action and early clinical development. *Eur. J. Cancer* **32A**, 2520-2527.
6. Kusaka, M., *et al.*, & Folkman, J. (1991). Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem. Biophys. Res. Commun.* **174**, 1070-1076.
7. Koyama, H., *et al.*, & Morii, H. (1996). The fumagillin analogue TNP-470 inhibits DNA synthesis of vascular smooth muscle cells stimulated by platelet-derived growth factor and insulin-like growth factor-I: possible involvement of cyclin-dependent kinase 2. *Circ. Res.* **79**, 757-764.
8. Griffith, E.C., *et al.*, & Liu, J.O. (1997). Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors TNP-470 and ovalicin. *Chem. Biol.* **4**, 461-471.
9. Sin, N., Meng, L., Wang, M.Q.W., Wen, J., Bornmann, W.G. & 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.
10. Kendall, R.L., Yamada, R. & Bradshaw, R.A. (1990). Cotranslational amino-terminal processing. *Methods Enzymol.* **185**, 398-407.
11. Bradshaw, R.A., Brickey, W.W. & Walker, K.W. (1998). N-Terminal processing: the methionine aminopeptidase and N^{α}-acetyl transferase families. *Trends. Biochem. Sci.* **23**, 263-267.
12. Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. *Proc. Natl Acad. Sci. USA* **93**, 12142-12149.
13. Nishizawa, M., Okazaki, K., Furuno, N., Watanabe, N. & Sagata, N. (1992). The 'second-codon' rule and autophosphorylation govern the stability and activity of Mos during the meiotic cell cycle in *Xenopus* oocytes. *EMBO J.* **11**, 2433-2446.
14. Hämmerle, M., *et al.*, & Wolf, D.H. (1998). Proteins of newly isolated mutants and the amino-terminal proline are essential for ubiquitin-proteasome-catalyzed catabolite degradation of fructose-1,6-bisphosphatase of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 25000-25005.
15. Arfin, S., *et al.*, & Bradshaw, R.A. (1995). Eukaryotic methionyl aminopeptidases: two classes of cobalt-dependent enzymes. *Proc. Natl. Acad. Sci. USA* **92**, 7714-7718.
16. Li, X. & Chang, Y.-H. (1995). 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.
17. Griffith, E.C., Su, Z., Niwayama, S., Ramsay, C.A., Chang, Y.-H., & Liu, J.O. (1998). Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc. Natl. Acad. Sci. USA* **95**, 15183-15188.
18. Antoine, N., *et al.*, & Castronovo, V. (1994). AGM-1470, a potent angiogenesis inhibitor, prevents entry of normal but not transformed endothelial cells into the G_1 phase of the cell cycle. *Cancer Res.* **54**, 2073-2076.
19. Magee, A.I., Wootton, J. & de Bony, J. (1995). Detecting radiolabeled lipid-modified proteins in polyacrylamide gels. *Methods Enzymol.* **250**, 330-337.
20. Yoshida, T., Kaneko, Y., Tsukamoto, A., Han, K., Ichinose, M. & Kimura, S. (1998). Suppression of hepatoma growth and angiogenesis by a fumagillin derivative TNP-470: possible involvement of nitric oxide synthase. *Cancer Res.* **58**, 3751-3756.
21. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide. *Anal. Chem.* **68**, 850-858.
22. Alexander, M., Curtis, G., Avruch, J. & Goodman, H.M. (1985). Insulin regulation of protein biosynthesis in differentiated 3T3 adipocytes: regulation of glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **260**, 11978-11985.
23. Kulbe, K.D., Jackson, K.W. & Tang, J. (1975). Evidence for a liver-specific glyceraldehyde-3-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **67**, 35-42.
24. Ahmad, H., Sing, S.V., Medh, R.D., Ansari, G.A.S., Kurosky, A. & Awasthi, Y.C. (1988). Differential expression of α, μ and π classes of isozymes of glutathione S-transferase in bovine lens, cornea and retina. *Arch. Biochem. Biophys.* **256**, 416-426.
25. Chang, Y.-H., Teichert, U. & Smith, J.A. (1990). Purification and characterization of a methionine aminopeptidase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 19892-19897.
26. Kendall, R.L. & Bradshaw, R.A. (1992). Isolation and characterization of the methionine aminopeptidase from porcine liver responsible for the co-translational processing of proteins. *J. Biol. Chem.* **267**, 20667-20673.
27. Li, X. & Chang, Y.-H. (1996). Evidence that the human homologue of a rat initiation factor-2 associated protein (p67) is a methionine aminopeptidase. *Biochem. Biophys. Res. Commun.* **227**, 152-159.
28. Roderick, S. L. & Matthews, B. W. (1993). Structure of the cobalt-dependent methionine aminopeptidase from *Escherichia coli*: a new type of proteolytic enzyme. *Biochemistry* **32**, 3907-3912.
29. Liu, S., Widom, J., Kemp, C.W., Crews, C.M. & Clardy, J. (1998) Structure of human methionine aminopeptidase-2 complexed with fumagillin. *Science* **282**, 1324-1327.
30. Abe, J., *et al.*, & 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.
31. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
32. O'Farrell, P.Z. & Goodman, H.M. (1976). Resolution of simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: heterogeneity of the major capsid protein. *Cell* **9**, 289-298.
33. O'Farrell, P.Z., Goodman, H.M. & O'Farrell, P.H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142.
34. Zuo, S., Guo, Q. & Chang, Y.-H. (1994). A protease assay via precolumn derivatization and high-performance liquid chromatography. *Anal. Biochem.* **222**, 514-516.

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