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Small molecule inhibitors of methionine aminopeptidase type 2 (MetAP-2) fail to inhibit endothelial cell proliferation or formation of microvessels from rat aortic rings *in vitro*

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Abstract

The protein processing enzyme, methionine aminopeptidase-2 (MetAP-2), has been identified as a molecular target of fumagillin and its derivative, TNP-470, compounds known to inhibit endothelial cell proliferation and angiogenesis. A high-throughput screening program was undertaken to identify selective, reversible inhibitors of MetAP-2 in an attempt to discover structurally novel anti-angiogenic agents for potential therapeutic use in oncology. Approximately 90 small-molecule, reversible, selective inhibitors of rhMetAP-2 were identified. The most potent of these compounds contained a singly-substituted triazole moiety which exhibited an IC_{50} of 8 nM (95% confidence limits 5 to 13 nM) and was highly selective for MetAP-2 over MetAP-1 (~ 60-fold dierence in IC_{50} values). Unlike fumagillin, these MetAP-2 inhibitors failed to significantly inhibit growth factor-stimulated endothelial cell (HUVEC) proliferation or to suppress angiogenesis in the *in vitro* aortic ring explant model of microvessel outgrowth. The MetAP-2-inhibitory activity of these compounds was dependent on the divalent cation used as the metalloenzyme activating cofactor for MetAP-2. These inhibitors were identified using cobalt(II)-activated recombinant human MetAP-2 for screening compound libraries. When manganese (Mn^{2+}) was substituted for cobalt following EDTA treatment and extensive dialysis of the MetAP-2 protein, these inhibitors were significantly less potent (40-fold increase in IC_{50}) as inhibitors of MetAP-2. These results support the recent hypothesis that cobalt may not be the relevant divalent metal ion cofactor for MetAP-2 in cells and may explain the observed absence of cell-based activity using potent triazole inhibitors of cobalt-activated MetAP-2.

Abbreviations: DMSO – dimethyl sulfoxide; DPP IV – dipeptidyl peptidase type IV; EDTA – ethylenediamine tetraacetic acid; HUVEC – human umbilical vein endothelial cells; LSGS – low serum growth supplement; MetAP – methionine aminopeptidase

Introduction

Methionine aminopeptidases (MetAPs) are protein-processing enzymes responsible for the removal of initiator methionine residues from polypeptide chains during protein synthesis [1]. Two primary MetAP isoforms, MetAP-1 and MetAP-2, are ubiquitously expressed in eukaryotic cells. While MetAP-1 appears to be a constitutive housekeeping enzyme, MetAP-2 expression is inducible by phorbol myristate acetate and expression levels correlate with cell growth [2, 3].

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Methionine aminopeptidase type-2 (MetAP-2) has been identified as a molecular target of the anti-angiogenic agent fumagillin and its clinically relevant derivative, TNP-470 [3–5]. Compounds of the fumagillin class inhibit MetAP-2 by irreversible binding in the active site of the MetAP-2 enzyme, but they do not bind to or inhibit MetAP-1 [3, 4]. Fumagillin and its analogs have been shown to be potent inhibitors of endothelial cell proliferation *in vitro*, arresting the transition from G1 to S phase of the cell cycle [6–8]. The anti-tumor and anti-angiogenic effects of TNP-470 have been attributed to cell-type specific, potent cytostatic inhibition of endothelial cell proliferation [9]. TNP-470 has undergone clinical trials as an anti-angiogenic cancer therapeutic with reported limited efficacy, due to its extremely short plasma half-life and reported toxicity [10].

The precise mechanism whereby inhibitors of MetAP-2 suppress the proliferation of endothelial cells is not well understood. Introduction of an antisense construct of MetAP-2 has been shown to block endothelial proliferation [11]. The effect of a reversible inhibitor of MetAP-2 on the *N*-terminal processing of the signaling adaptor protein 14-3-3γ has been described in a tissue culture system [12, 13]. Inhibition of the processing of 14-3-3γ presumably results in loss of function in cell proliferation-related signaling pathways involving 14-3-3γ. Other proteins involved in signaling pathways require N-terminal myristoylation, a process that would be blocked by retention of the N-terminal methionine [1].

We undertook a high-throughput screening (HTS) program to identify selective, reversible small molecule inhibitors of MetAP-2 as anti-angiogenic agents for potential therapeutic use in cancer. Using a dipeptidyl peptidase IV-coupled homogenous enzyme assay, potent small molecule inhibitors of MetAP-2 were identified with IC_{50} values as low as 8 nM. The most potent and type-selective inhibitors identified by HTS were further evaluated for cellular activity in *in vitro* endothelial cell proliferation and angiogenesis assays and were found to be inactive. Inhibitors discovered using a Co^{2+}-based enzymatic assay were less active when Mn^{2+} was substituted for Co^{2+} as the activating metal cofactor in the MetAP-2 assay. The observed lack of biological activity in cell-based assays may relate to the decreased potency of these inhibitors when Mn^{2+} was substituted for Co^{2+} as the divalent cation MetAP-2 cofactor.

Materials and methods

Methionine aminopeptidases

Recombinant human MetAP-1 and MetAP-2 were obtained from Dr Yie-Hwa Chang of Saint Louis University [14]. Briefly, full-length cDNA in pPAK8 vector was used to express HA-tagged hMetAP-1 or hMetAP-2 in insect cells.

Dipeptidyl peptidase

Dipeptidyl peptidase IV, a helper enzyme used to measure MetAP activity, was supplied by Dr Yie-Hwa Chang (Saint Louis University). The enzyme was purified from pig kidneys using standard biochemical purification methods and was substantially free of contaminating aminopeptidase activity.

Fluorophore-labeled substrate

An aminomethyl coumarin-labeled tripeptide substrate, Met-Gly-Pro-AMC, was synthesized using standard methods by Bachem Bioscience.

Methionine aminopeptidase enzyme assay

A simultaneous coupled enzyme assay developed in the laboratory of Dr Yie-Hwa Chang (Saint Louis University) as described in International Patent Publication number WO 01/27242 A2, was licensed for use with a fluorescent peptide substrate. Briefly, 2 μg of recombinant human enzyme (MetAP-1 or MetAP-2) were combined with 0.0025 activity units of porcine dipeptidyl peptidase in a final reaction volume of 50 μl. Inhibitors, when present, were added as 30% aqueous DMSO solutions in a volume of 2 μl. The final DMSO concentration was 1.2%. Reactions were initiated by addition of the fluorescent-labeled tripeptide substrate to give a final substrate concentration of 0.5 mM. The assay was carried out in flat-bottomed 96-well tissue-culture plates for 90 min at 37°C. Fluorescence was detected using a bench-top microtiter plate fluorometer. Excitation wavelength was set at 360 nm, and emission read at 460 nm.

Methionine aminopeptidase assay using 8mer peptide

An eight amino acid peptide derived from the N-terminal sequence of 14-3-3γ was synthesized by Sigma Bioscience using standard methods. Free methionine released from the substrate peptide was detected using l-amino-acid oxidase and horseradish peroxidase as previously described [15], except that the final reaction volume was reduced to 50 μl.

Caco-2 cell membrane permeability assay

Membrane permeability was assessed using Caco-2 monolayer cultures [16]. Briefly, Caco-2 confluent monolayers grown on microporous collagen-coated polycarbonate membranes in 12-well Costar Transwell plates were exposed to 10 μM compound in Hanks' Balanced Salt Solution. Unidirectional compound transport was assessed by LC/MS analysis of the receiver compartment. Controls to rule out cell damage and free diffusion through the culture containers were included.

Human umbilical vein endothelial cell cultures

Primary endothelial cells were purchased from Cascade Biologics, Portland, Oregon. Cultures were maintained according to the supplier's protocols. For growth studies, nearly confluent cultures in 96-well culture plates were serum or growth-factor starved for 24 h prior to addition of inhibitors and either growth factor mixture (LSGS, Cascade Biologics) or purified basic fibroblast growth factor (bFGF). Cultures were incubated for 48 h in the presence of MetAP-2 inhibitors. Cell proliferation was assessed by incorporation of bromodeoxyuridine in cells during the final 24 h of incubation followed by enzyme-linked immunoassay for bromodeoxyuridine [17] using a reagent kit supplied by Roche Diagnostics.

Rat aortic ring explant culture

Angiogenesis was assessed *in vitro* using rat aortic ring explant cultures embedded in fibrin gels, as previously described [18]. Vessel outgrowth was quantified using an

automated image analysis system [19]. Briefly, thoracic aortas from young Sprague-Dawley rats were cleared of adhering fibroadipose tissue, extensively washed and cut into 1-mm sections. Each section was cultured in a small-volume fibrin gel matrix immersed in 5 ml serum-free MCDB 131 media for 8 days. Test compounds were added directly to the culture media and allowed to diffuse into the fibrin gel matrix. Media was replaced with fresh media every two to three days, and test compounds were again added after media replenishment. DMSO concentration was kept below 0.2% by volume.

Statistics

The Marquardt method of nonlinear regression was used to determine IC_{50} values from concentration response data. Dierences among treatment groups were evaluated using analysis of variance with Dunnet's multiple range test for group comparisons, with a *P*-value of 0.05 or less considered significant.

Results

A high-throughput screening program was undertaken to identify selective, reversible inhibitors of MetAP-2 in an attempt to discover structurally novel anti-angiogenic agents for potential therapeutic use in oncology. Using a dipeptidyl peptidase IV-coupled homogenous assay, 95 triazole-based compounds were identified that potently inhibit MetAP-2, with IC_{50} values less than 1 μM (Figure 1, representative structure). Compounds from this chemical series exhibited potency similar to that of the known irreversible inhibitor fumagillin (Figure 2a) and they were highly selective for MetAP-2, lacking inhibitory activity against the MetAP-1 isoenzyme at corresponding concentrations (Figure 2b). Compound JNJ-4929821 (Figure 1) was the most potent compound identified, yielding an IC_{50} of 8 nM (95% confidence limits, 5 to 13 nM), and was selected for further study. Estimated IC_{50} values for the direct comparison of JNJ-4929821 with fumagillin shown in Figure 2a were 14 nM for fumagillin (95% confidence limits, 9 to 21 mM) and 15 nM for JNJ-4929821 (95% confidence limits, 11 to 20 nM). Determinations of IC_{50} values were repeated a minimum of three times with comparable results. No inhibition of the assay reporter enzyme, DPP-IV, was observed in control assays using a fluorescent-labeled dipeptide substrate (data not shown).

The compounds identified as potent MetAP-2 inhibitors were discovered using a tri-peptide substrate containing an N-terminal methionine. To rule out potential artifacts produced by using an artificial tri-peptide substrate, inhibition of MetAP-2 by JNJ-4929821 was further characterized using a substrate consisting of 8 amino acids derived from the N-terminal sequence of 14-3-3γ (Figure 3). Free methionine released by the action of MetAP-2 was detected in a homogeneous assay by l amino-acid oxidase and horseradish peroxidase. Using this larger peptide substrate, inhibition

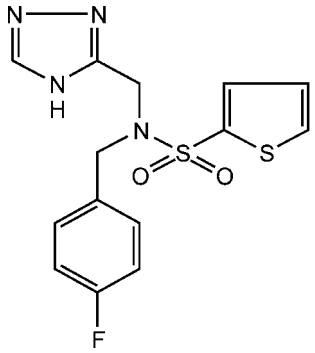

Figure 1. JNJ-4929821. Representative structure of triazole inhibitor of MetAP-2.

of MetAP-2 by JNJ-4929821 was readily detected. In this assay, the estimated IC_{50} of 25 nM (95% confidence limits, 19 to 31 nM) for JNJ-4929821 was comparable to the 15 nM IC_{50} value (Figure 2a) estimated using the tripeptide Met–Gly–Pro–AMC.

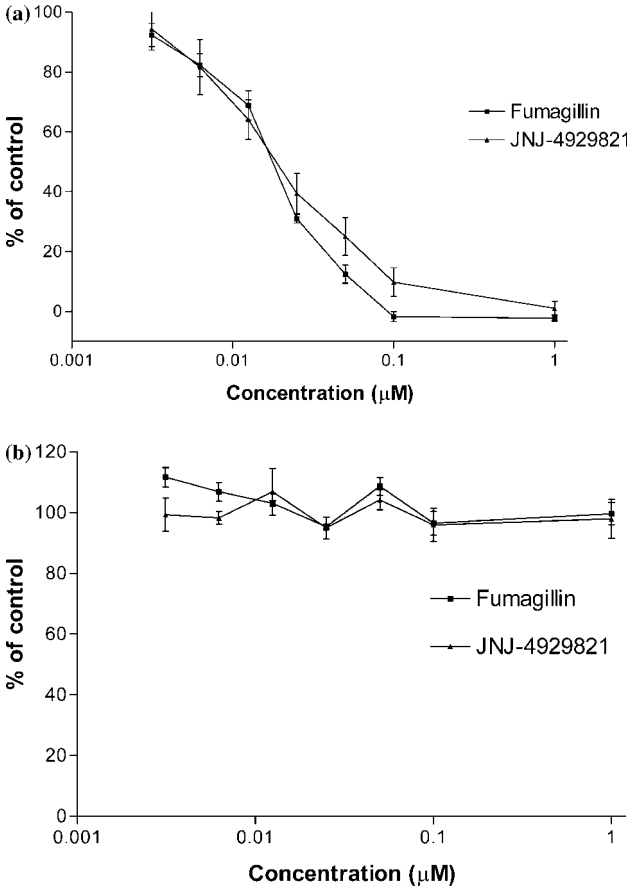

Figure 2. Differential inhibition of MetAP-1 and MetAP-2 by JNJ-4929821 and fumagillin. (a) Accumulation of free fluorescent product after 90 min incubation of substrate with MetAP-2, DPP IV helper enzyme and inhibitor compounds at the indicated concentrations. (b) Accumulation of fluorescent product after 90 min incubation of substrate with MetAP-1, DPP IV helper enzyme and inhibitor compounds at the indicated concentrations. Error bars represent standard deviation for three replicates per data point. Similar results were obtained in at least three independent experiments.

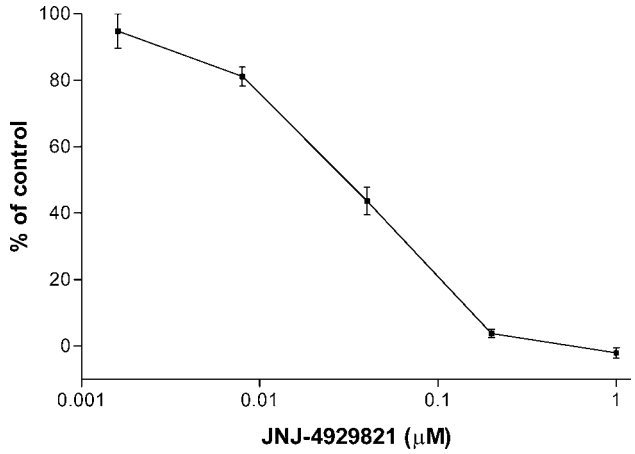

Figure 3. Inhibition of MetAP-2 processing of N-terminal 8 amino-acid peptide fragment derived from the sequence of 14-3-3γ. Activity was measured using l-amino oxidase/peroxidase detection of free methionine. Error bars represent standard deviation for four replicates at each concentration.

In contrast to the action of fumagillin, JNJ-4929821 was not active in suppressing mixed (LSGS) growth factor-stimulated proliferation of cultured human umbilical vein endothelial cells at 1.0 and 10 μM final concentration (Figure 4). Out of 95 triazole compounds inhibiting MetAP-2 at concentrations below 1 μM, 34 compounds were similarly assessed in endothelial cultures under varying conditions; none inhibited cell proliferation. Endothelial cell proliferation stimulated by purified recombinant basic fibroblast growth factor was also not inhibited by triazole MetAP-2 inhibitors at concentrations up to 10 μM (data not shown).

In addition to endothelial cell proliferation, compounds were assessed for anti-angiogenic activity using the *in vitro* rat aortic ring explant assay of microvessel outgrowth [18]. The triazole JNJ-4929821 as well as other small molecule MetAP-2 inhibitors of varying chemical structure failed to suppress microvessel outgrowth when tested at a final concentration of 1.0 μM, approximately 100 times the IC_{50} for inhibition of MetAP-2 by JNJ-4929821 (Figure 5a). Other compounds included in Figure 5a had IC_{50} values ranging from 37 to 670 nM in the MetAP-2 assay. In contrast, fumagillin produced statistically significant, concentration-dependent inhibition of microvessel outgrowth at concentrations as low as 0.1 nM (Figure 5b). Assessment of compounds in the aortic ring model was completed in two independent experiments without detecting eects on microvessel outgrowth.

In an attempt to better understand this lack of activity in cell-based assays, the cellular penetration of JNJ-4929821 was confirmed using the Caco-2 cell membrane permeability assay (data not shown). JNJ-4929821 exhibited good membrane permeability in this assay, suggesting that poor cellular penetration was not the direct cause for the lack of cellular activity.

Recently, the identity of the physiologically relevant cation in the bimetallic active site of MetAP-2 has been questioned [20]. Since these inhibitors were identified using a cobalt (II)-activated MetAP-2 enzyme assay, we assessed the influence of the divalent metal ion cofactor on the activity of triazole inhibitors of MetAP-2. For this study recombinant human MetAP-2 was 'stripped' of metal cation with 1 mM EDTA followed by extensive dialysis against a buffer lacking divalent metal ions. Enzymatic activity was then recovered by supplementing the assay buffer with either 0.5 mM manganese chloride or 0.5 mM cobalt chloride [14]. Comparable control MetAP-2 enzymatic activity was detected in the presence of Mn^{2+} or Co^{2+}, and enzyme activity was not reduced by the stripping and

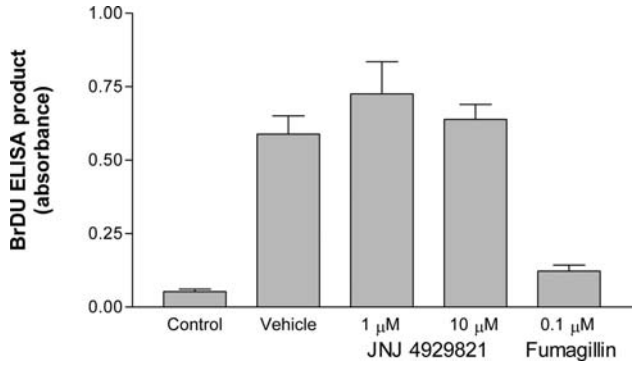

Figure 4. Human endothelial cell DNA synthesis stimulated by low serum growth supplement (LSGS). Bromodeoxyuridine labeling was assessed by ELISA after 48 h of incubation in the presence of LSGS and JNJ-4929821 or DMSO vehicle. No LSGS was added to control cultures. This experiment is representative of repeated attempts under varying conditions to demonstrate effects on endothelial cultures by JNJ-4929821. Error bars represent standard deviation for four wells per treatment.

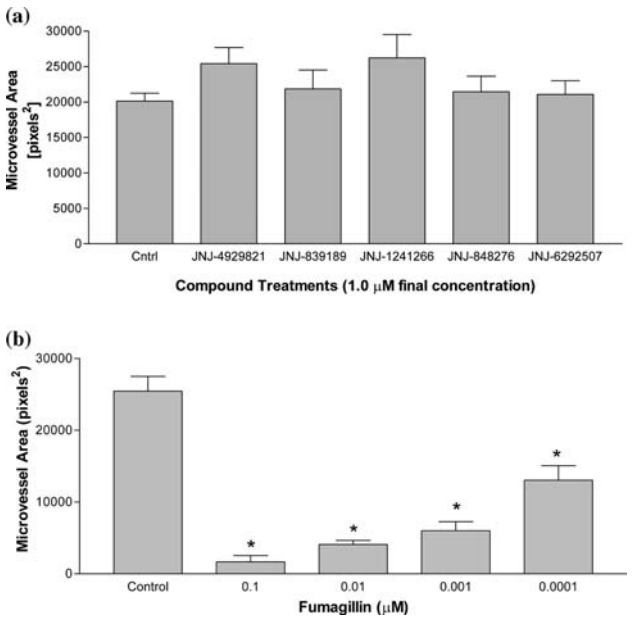

Figure 5. Effect of small molecule MetAP-2 inhibitors on microvessel outgrowth from rat aortic rings. Microvessels were quantified by automatic image analysis and expressed as the mean area +/− SEM for groups of 9 or 10 rings per condition. (a) Selected small molecule inhibitors of Co^{2+}-activated MetAP-2. (b) Fumagillin. *P< 0.05 by analysis of variance, Dunnet's multiple range test.

reconstitution procedure (Figure 6a). Fumagillin inhibition was not dependent on the metal ion cofactor (Figure 6a), whereas, the IC_{50} of JNJ-4929821 was reduced approximately 40-fold in the assay supplemented with manganese, compared to its activity in the cobalt-activated assay (Figure 6b). Inhibition concentration-response curves were generated in at least three independent experiments involving two independently prepared batches of MetAP-2 converted to manganese activation with comparable results (data not shown).

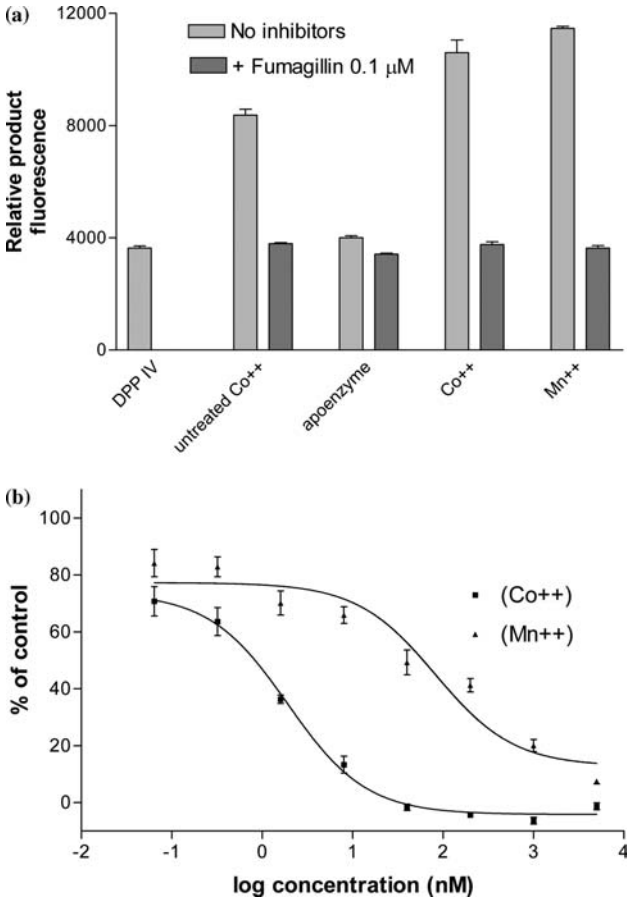

Figure 6. Effect of conversion of active site divalent cations to manganese. (a) Recombinant MetAP-2 was treated with EDTA and extensively dialyzed to remove cation cofactors (apoenzyme). Cobalt and Manganese were added back as chloride salts at 0.5 μM. For comparison, the untreated cobalt-containing enzyme was included at equivalent enzyme concentration. (b) Inhibition of cobalt-reconstituted or manganese-reconstituted MetAP-2 activity by JNJ-4929821. Curves were fit to data by nonlinear regression. Error bars represent standard deviations for four replicates at each data point shown.

Discussion

In this communication, we report that potent, selective small molecule inhibitors of MetAP-2 activity were identified through high-throughput screening. Further, we show that these triazole compounds are as potent as the irreversible inhibitor fumagillin and are highly selective for MetAP-2 vs the MetAP-1 isozyme. However, these small molecule inhibitors of MetAP-2 failed to inhibit the growth factor-stimulated proliferation of human umbilical vein endothelial cells at concentrations three orders of magnitude higher than the IC_{50} for inhibition of rhMetAP-2. Furthermore, these compounds failed to suppress the formation of microvessels in an *in vitro* aortic ring explant culture model of angiogenesis at concentrations up to 1.0 μM. These cellular results contrast greatly with the activity of the macrolide antibiotic fumagillin, that was demonstrated to significantly inhibit HUVEC proliferation and angiogenesis in the rat aortic ring assay. The most potent triazole MetAP-2 inhibitor discovered in this study, JNJ-4929821, was shown to cross cell membranes in the Caco-2 cell permeability assay, suggesting that poor cellular penetration was not the explanation for the lack of cellular activity. We were unable to demonstrate inhibition of endothelial cell proliferation in spite of confirmed cell penetration by the most active representative of the small molecule inhibitors reported here. At least 34 structurally related triazole-containing inhibitors of Co^{2+}-activated MetAP-2 were shown to lack the ability to inhibit endothelial cell proliferation under varying conditions (data not presented).

In order to rule out the possibility that the potency of inhibitors we found in screening was due to the use of a small artificial tripeptide substrate, we confirmed inhibition using a longer 8 amino acid peptide substrate derived from the N-terminal sequence of 14-3-3γ. The signaling molecule 14-3-3γ has been shown to retain N-terminal methionine in epithelial cells and endothelial cells treated with a reversible MetAP inhibitor [12, 13]. Triazole compounds tested using this peptide as substrate inhibited MetAP-2 with potency comparable to the screening assay using the tripeptide substrate.

In a recent report, Wang et al. [20] demonstrated that activity of structurally related small molecule inhibitors of MetAP-2 was dependent on the divalent cation used to activate the human recombinant MetAP-2 preparation. When Mn^{2+} was substituted for Co^{2+}, representative compounds bearing a triazole moiety were no longer potent inhibitors of MetAP-2. We have confirmed this observation with the inhibitors reported herein. Although cobalt is very eective as a metal cofactor for activation of recombinant MetAP-2, and this metal ion forms the bimetallic active site in the three-dimensional structures reported for both mammalian and bacterial methionine aminopeptidases [21, 22], the identity of the physiologically relevant metal ion cofactor for MetAP-2 remains controversial [20]. Rigorous purification of MetAP-2 from a natural source in a manner that avoids extraneous metal contamination has not yet been reported. The lack of significant activity in cell-based models with potent inhibitors of the cobalt-containing enzyme suggests that cobalt may not be the divalent metal cofactor required for MetAP-2 activity in cells.

The absence of cellular activity for compounds found to potently inhibit cobalt-containing MetAP-2 does not invalidate this enzyme as a potentially useful target for the discovery of anti-angiogenic agents. These results do support the hypothesis that manganese (Mn^{2+}) rather than cobalt (Co^{2+}) may be the physiologically relevant divalent

cation for MetAP-2 cellular activity [20] and furthermore provide a plausible explanation for the lack of cellular activity observed with the compounds which we identified as MetAP-2 inhibitors using the Co^{2+}-activated MetAP-2 enzyme. It is likely that a reversible inhibitor of high potency will be required to produce suppression of angiogenesis comparable to that seen with fumagillin or TNP-470, since these compounds covalently inactivate both the Co^{2+} and Mn^{2+} forms of MetAP-2.

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