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Pyridinylquinazolines Selectively Inhibit Human Methionine Aminopeptidase-1 in Cells

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Abstract

Methionine aminopeptidases (MetAPs) which remove the initiator methionine from nascent peptides are essential in all organisms. While MetAP2 has been demonstrated to be a therapeutic

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Supporting Information Available: Supplemental figures (S1–S7), a supplemental table and experimental procedures for immunoblotting, expression and purification of *HsMetAP1*, *tHsMetAP1*, *HsMetAP2* and *BcProIP*, determination of the activity of *BcProIP* and the coupling condition, determination of the EC₅₀ or IC₅₀ values of inhibitors, construction of *HsMetAP1* expression vector, lentivirus production, transduction of human primary endothelial cells, crystallization of *tHsMetAP1* in complex with **11j**, data collection and processing, and structure determination and refinement are available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions: All authors were involved in designing experiments and interpreting data. F.Z. carried out most of the biological experiments and S.B. synthesized most of the new compounds for this study. F.Z., S.B.G. and J.O.L. wrote the manuscript. S.B. and Y.L.C. synthesized the pyridinylquinazolines. K.T. and P.C. contributed the natural sponge-derived bengamide analogues. D.J.M. contributed compound **1**. F.Z. and X.C. performed the *in vitro* MetAP assays for the commercially available compounds. F.Z. and B.A.N. performed the initiator methionine release assay. F.Z. and J.S.S. verified the activities of freshly prepared compound **1** independently. S.B.G., M.S.M. and F.Z. performed the crystallographic analysis.

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target for inhibiting angiogenesis in mammals, MetAP1 seems to be vital for cell proliferation. Our earlier efforts identified two structural classes of human MetAP1 (*HsMetAP1*)-selective inhibitors (**1–4**). But all of them failed to inhibit cellular *HsMetAP1*. Using Mn(II) or Zn(II) to activate *HsMetAP1*, we found that **1–4** could only effectively inhibit purified *HsMetAP1* in the presence of physiologically unachievable concentrations of Co(II). In an effort to seek Co(II)-independent inhibitors, a novel structural class containing a 2-(pyridin-2-yl)quinazoline core has been discovered. Many compounds in this class potently and selectively inhibited *HsMetAP1* without Co(II). Subsequently, we demonstrated that **11j**, an auxiliary metal-dependent inhibitor, effectively inhibited *HsMetAP1* in primary cells. This is the first report that an *HsMetAP1*-selective inhibitor is effective against its target in cells.

Introduction

Almost all cellular protein syntheses begin with either an initiator *N*-formylmethionine (ifMet) or an initiator methionine (iMet)¹. The ifMet or iMet in a significant number of proteins (e.g. about 60% in *Escherichia coli*²) will be proteolytically and irreversibly removed through N-terminal methionine excision (NME). In archaea and eukaryotes, NME is catalyzed by methionine aminopeptidases (MetAPs), a family of evolutionarily conserved metalloproteases.³ In the NME of eubacteria, mitochondria and plastids, MetAPs remove iMet after ifMet is converted to iMet by peptide deformylases.⁴ MetAPs utilize one or two divalent metal ions⁵ to catalyze the removal of iMet, and they only cleave iMet from nascent peptides in which the N-terminal P1 residues are methionine and the side chains of the P1' residues are small and uncharged (Ala, Gly, Pro, Ser, Cys, Thr or Val).^{6,7} Amino acid residues at the P2' position and beyond also contribute to the efficiency of cleavage.^{6c,7}

MetAPs can be classified into type 1 and type 2. When compared with type 1 MetAPs, type 2 enzymes contain a characteristic insertion (~60 a.a.) in their catalytic domain.³ Only one type of MetAP, either type 1 or type 2, can be found in a prokaryotic cell. Archaea contain type 2 MetAPs,^{5b} and eubacteria contain type 1 MetAPs.⁸ However, all eukaryotic genomes studied to date encode at least a cytosolic type 1 MetAP (MetAP1) and a cytosolic type 2 MetAP (MetAP2). Extensions at the N-terminus of the catalytic domain of eukaryotic cytosolic MetAPs distinguish them from their prokaryotic counterparts.^{9,10} The sequence identity between type 1 and type 2 MetAPs is very limited,^{9,10} however their catalytic domains adopt the same pita-bread fold,³ with both metal binding residues and the shape of methionine-binding pocket highly conserved.^{3,11}

NME accounts for the major source of N-terminal diversity in mature proteins.¹² NME is involved in the creation of N-degrons for the N-end rule pathway.¹² NME also prepares the nascent peptides for the subsequent N- α -acetylation which facilitates protein folding and protein-protein interaction, and dictates protein half-life.^{13,14} In addition, NME is a prerequisite for N-terminal myristoylation which contributes to the membrane localization and the function of some important proteins.¹⁵ Furthermore, there is a family of enzymes that require NME to expose a mature N-terminal residue other than methionine for catalytic activity.¹⁶

NME is an essential cellular process. *Mycoplasma genitalium* contains the smallest genome for cellular growth in pure medium, and its 382 essential genes include one (*map*) that encodes a MetAP.¹⁷ The disruption of the single *map* gene in *Escherichia coli* or *Salmonella typhimurium* resulted in lethality.⁸ In *Saccharomyces cerevisiae*, deletion of either *map1* or *map2* gene generated a slow growth phenotype, while the deletion of both became lethal.⁹ Cytosolic MetAP1 and MetAP2s are functionally interchangeable in *Arabidopsis thaliana*, but a complete blockade of NME halted the development of the plant after germination.¹⁸ The disturbance of MetAP2 in animals generated organism-dependent and tissue-specific

defects in development.¹⁹ When either human MetAP1 (*HsMetAP1*) or MetAP2 (*HsMetAP2*) was downregulated by small-interfering RNA (siRNA) in human umbilical vein endothelial cells (HUVEC), the growth of these primary cells were greatly inhibited.²⁰ Furthermore, it has been shown that the downregulation of MetAP1 by siRNA duplexes slowed the cell cycle progression of synchronized HeLa cells.²¹ Bengamides (Figure 1A), isolated from Jaspidae marine sponges, display a broad antitumor activity *in vitro*^{22,23} and *in vivo*²⁴. An extensive proteome analysis revealed that bengamides inhibit both *HsMetAP1* and *HsMetAP2*.²⁵ But unfortunately, the clinical study of a synthetic bengamide analogue LAF-389 in patients with advanced cancer was terminated due to its cardiovascular toxicity,²⁶ which might be caused by the simultaneous inhibition of *HsMetAP1* and *HsMetAP2*.

Fumagillin and its synthetic analogue TNP-470 (also called AGM-1470) (Figure 1A) were revealed as potent inhibitors of angiogenesis more than two decades ago.^{27,28} Later, we and others found that fumagillin and TNP-470 bind to MetAP2 covalently,²⁹ leading to an irreversible inhibition.^{30,31} Since the decreased enzymatic activity of MetAP2 preferentially affects the proliferation of endothelial cells,^{32,33} TNP-470 has been shown to inhibit angiogenesis and suppress the growth of solid tumors in various animal models.^{34,35} Fumagillin analogues also showed efficacy in the treatment of arthritis,³⁶ nutritionally induced obesity³⁷ and pulmonary hypertension³⁸. In addition, because of the selectivity of fumagillin and its analogues for type 2 MetAP enzymes, they were widely used as tools to study the functions of MetAP2 in higher eukaryotes.^{18,39}

In contrast to vigorous research on *HsMetAP2*, the understanding of the physiological functions of *HsMetAP1* is very limited and the efficacy of anti-MetAP1 therapy in the treatment of neoplasia remains unknown, largely due to the lack of MetAP1-selective inhibitors that are active in cells. Peptidyl hydroxamic acids and barbiturate-based compounds were shown to inhibit purified *HsMetAP1* *in vitro*.^{40,41} However, their selectivity for *HsMetAP1* was either low or undetermined, and their activities in cells remain unclear. Pyridine-2-carboxamides can potently inhibit the activities of purified type 1 MetAPs from bacteria, yeast and human.^{21,42,43} Later, Hu et al. reported that several analogues in this class had great selectivity for *HsMetAP1* over *HsMetAP2*, and 3-tert-butoxycarbonylaminopyridine-2-carboxylic acid thiazole-2-ylamide (**1**, Figure 1A) diminished the activity of *HsMetAP1* in HeLa cells.²¹ In addition to pyridine-2-carboxamides, 2-pyridin-2-ylpyrimidines were uncovered as a novel class of inhibitors of human MetAPs.⁴⁴ Hu et al. showed that these pyridinylpyrimidines potently inhibited both *HsMetAPs*, and 5-chloro-6-methyl-*N*-(2-phenylethyl)-2-pyridin-2-ylpyrimidine-4-amine (**2**, Figure 1A) could inhibit *HsMetAPs* in HeLa cells as well.⁴⁴ Recently, we communicated our systematic medicinal chemistry effort at improving the selectivity and potency of pyridinylpyrimidine class of *HsMetAP1* inhibitors (e.g. **3** and **4**, Figure 1A).⁴⁵

The X-ray crystal structures of the N-terminally truncated *HsMetAP1* in complex with Co(II) and MetAP1 inhibitors (PDB codes 2NQ6, 2G6P and 4HXX) indicated that instead of the two cobalt ions (M1 and M2) chelated at the bottom of the active site, each of **1–3** requires on an auxiliary cobalt ion (M3) to bind *HsMetAP1* (Figure 1B).^{21,44,45} Besides **1–3**, four structurally distinct inhibitors of bacterial type 1 MetAP have been uncovered independently by three research groups. Crystallographic analysis demonstrated that all four inhibitors mainly depended on the chelation of an auxiliary Co(II) or Mn(II) to inhibit two eubacterial MetAPs.⁴⁶ Being reminiscent of the cases in *HsMetAP1*, both bacterial type 1 MetAPs contacted the auxiliary metal atom solely through an imidazole nitrogen from His178 (equivalent to His310 in *HsMetAP1*)^{46a} or His79 (equivalent to His212 in *HsMetAP1*)^{46b–d}. In living cells, the concentration of free transition metals (especially cobalt) are believed to be several orders of magnitude lower than the concentrations at

which transition metals were routinely used in the *in vitro* MetAP assays and crystallography. Given that the ternary complexes of MetAP-Co(II)-inhibitor may not exist in cells,^{46b} it is not difficult to understand why several Co(II)-dependent inhibitors that were very potent *in vitro*, lack antibacterial activities *in vivo*.^{42,46b}

In the present work, we carefully assessed the cellular activities of the previously developed MetAP1-selective inhibitors using the NME of 14-3-3 γ protein as a readout. We found that 14-3-3 γ is a shared substrate between *Hs*MetAP1 and *Hs*MetAP2 in HUVEC, and the complete NME of 14-3-3 γ relies on the activities of both enzymes. Surprisingly, pyridine-2-carboxamide **1** and pyridinylpyrimidines **2–4** failed to inhibit the cellular activity of either *Hs*MetAPs in HUVEC appreciably at the highest tolerated concentrations (just below the severe cytotoxicity threshold). In addition, the reported inhibition of *Hs*MetAP1 by **1** and **2** in HeLa cells^{21,44} could not be reproduced, using either previously synthesized or freshly synthesized compounds. To assess the relevance of metal ions, we applied two *in vitro* assays to determine the potency of **1–4** and two chelating agents (thiabendazole and 2,2'-bipyridine) against purified *Hs*MetAP1 in the presence of Co(II), Mn(II) or Zn(II). As expected, **1–4**, as well as thiabendazole and 2,2'-bipyridine, could only inhibit *Hs*MetAP1 potently with the auxiliary Co(II). In the course of the SAR studies, a novel class of *Hs*MetAP1 inhibitors containing a 2-(pyridin-2-yl)quinazoline emerged, which served as a new lead for additional SAR studies. One analogue, **11j**, strongly inhibited the activity of MetAP1 in human cells. Besides its superior inhibitory potency and selectivity for *Hs*MetAP1, **11j** and related analogues also exhibited rarely seen “relaxed metal selectivity”, which appears to be a key feature for a successful inhibition of MetAP1 in cells.⁴⁷ Finally, we solved the X-ray crystal structure of **11j** in complex with Co(II) and N-terminally truncated *Hs*MetAP1. To the best of our knowledge, this is the first report of a small molecule inhibitor which can potently and selectively inhibit MetAP1 in human cells. The present work also provided compelling evidence that the auxiliary metal-mediated inhibitor identified *in vitro* can be equally effective against the same target in cells.

Chemistry

The syntheses of 2-(pyridin-2-yl)quinazoline derivatives (**7a**, **10a–10c**, **11a–11m** and **13–15**) with various C4 side chains and C5' substituents were carried out as shown in Scheme 1. Appropriately substituted 2-cyanopyridines (**5**) were treated with sodium in methanol and methyl pyridine-2-carboximidate generated *in situ* was condensed with anthranilic acid to yield 3,4-dihydroquinazolin-4-one (**6**). 4-Chloroquinazoline (**7**) synthesized by dehydrohalogenation of quinazolin-4-one (**6**) with phosphoryl chloride, served as a starting material for all the quinazoline derivatives discussed in this work. Thus, 4-chloroquinazoline (**7**) was treated with various amines (including **9**) while refluxing in 1,4-dioxane with *N,N*-diisopropylethylamine (*i*Pr₂NEt) as the base to afford the final products (**10a–10c**, **11a–11m**) and **12**. Quinazoline **11a** was obtained by reducing the corresponding acetophenone with sodium borohydride. Derivatives **11c** and **11e** were the saponification products from **11b** and **11d**, respectively. In the cases of **13–15**, the product after the chloride displacement reaction was 4-(piperazin-1-yl)-2-(pyridin-2-yl)quinazoline (**12**) which was converted to the desired final products (**13–15**) by amide coupling with appropriate acids.

Results and Discussion

The retention of the initiator methionine in 14-3-3 γ is a cellular indicator for the inhibition of *Hs*MetAP1 and/or *Hs*MetAP2

In primary (HUVEC) and transformed (H1299) human endothelial cells, inhibition of the enzymatic activity of *Hs*MetAP2 by a type 2-selective MetAP inhibitor (fumagillin) or siRNA mediated down-regulation of the protein level of *Hs*MetAP2 both led to strong

signals of unprocessed 14-3-3 γ protein with intact iMet in the immunoblot.²⁵ Therefore, 14-3-3 γ protein is a substrate of at least MetAP2 in human endothelial cells. But whether 14-3-3 γ is also a substrate of MetAP1 in primary human cells remained unclear.

To identify a human cell line in which the iMet retention in 14-3-3 γ caused by MetAP inhibition can be readily detected by immunoblot, we treated one primary (HUVEC) and five neoplastic cell lines (Jurkat E6-1, HeLa, HEK293T, PC-3 and MCF-7) with either a nonselective MetAP inhibitor (bengamide A/B) or a type 2-selective MetAP inhibitor (TNP-470) (Figure 2). In vehicle (dimethyl sulfoxide, DMSO) treated HUVEC, the unprocessed form of 14-3-3 γ could barely be detected by a monoclonal antibody raised against a peptide from the unprocessed N-terminus of 14-3-3 γ (Figure 2A). On the contrary, unprocessed 14-3-3 γ could readily be detected in the vehicle controls of all five neoplastic cell lines (especially in Jurkat E6-1, HeLa and HEK293T) (Figure 2). In HUVEC, after 24-hour treatments, the ratio of unprocessed 14-3-3 γ versus total 14-3-3 γ was increased 8-fold and 4.5-fold by bengamide A and TNP-470, respectively (Figure 2A), in agreement with previous observations.²⁵ The accumulation of unprocessed 14-3-3 γ in HUVEC had maximal responses to bengamide A and TNP-470 at concentrations around 10 nM and 1 nM, respectively (data not shown). Nevertheless in all five neoplastic cell lines, the treatment with 100 nM bengamide A/B merely increased the ratio of unprocessed versus total 14-3-3 γ by no more than 2.6 fold, while the treatment with 100 nM TNP-470 increased the ratio by no more than 1.6 fold (Figure 2). For this reason, we chose HUVEC as the optimal cell type for the detection of MetAP inhibition instead of HeLa cells.

It was noticeable that 100 nM bengamide A/B induced larger increases in the ratio of unprocessed versus total 14-3-3 γ than 100 nM TNP-470 in HUVEC, insinuating that *HsMetAP2* might not be the only human MetAP responsible for the NME of 14-3-3 γ . To test this possibility, siRNA knockdown of *HsMetAP1* or *HsMetAP2* was performed in HUVEC (Figure 3). In comparison with the scrambled siRNA control, 20 nM MetAP2 siRNA oligo decreased the protein level of *HsMetAP2* about 90% and brought a 7-fold increase in the ratio of unprocessed versus total 14-3-3 γ , in agreement with the earlier report in the literature²⁵. With a 30% decrease in the protein level of *HsMetAP1* upon siRNA treatment, there was a 3.2-fold increase in the ratio of unprocessed versus total 14-3-3 γ . Under the same conditions, there was no obvious change in the protein level of either *HsMetAP2* or 14-3-3 γ . Moreover, when a higher concentration (100 nM) of the siRNA oligo was used, an 80% reduction of *HsMetAP1* protein led to an increase in the ratio by nearly 5-fold. These pieces of evidence indicated that 14-3-3 γ is also a substrate of *HsMetAP1*, and the NME in 14-3-3 γ requires the activities of both *HsMetAPs*.

The first ten amino acid residues of nascent human 14-3-3 γ are Met-Val-Asp-Arg-Glu-Gln-Leu-Val-Gln-Lys. Both *HsMetAP1* and *HsMetAP2* disfavored large P1' (Val and Thr) and acidic P2' residues (Asp and Glu),⁷ marking 14-3-3 γ a slow substrate for either *HsMetAPs*. Purified *HsMetAP2* was more active than purified *HsMetAP1* towards several Met-Val peptides *in vitro*.^{7,32} However, a Met-Val peptide derived from the N-terminal sequence of human hemoglobin was a better substrate for *HsMetAP1* than *HsMetAP2* *in vitro*.³² It is thus possible that both *HsMetAPs* are required for the complete NME in some if not all Met-Val substrates.

A pyridine-2-carboxamide derivative and type 1-selective pyridinylpyrimidines failed to inhibit *HsMetAP1* in cells

Hu et al. showed that two cobalt-dependent MetAP1 inhibitors, pyridine-2-carboxamide **1** and pyridinylpyrimidine **2**, inhibited the enzymatic activity of *HsMetAP1* in HeLa cells, indicated by the enhanced signals of N-terminal unprocessed 14-3-3 γ protein in the immunoblots.^{21,44} To investigate whether our recently identified MetAP1-selective

inhibitors based on pyridinylpyrimidine could target *HsMetAP1* inside cells, we treated HUVEC with **2–4** for 24 hours, and then examined the iMet status of 14-3-3 γ by immunoblot with a monoclonal antibody²⁵ specific for the unprocessed form of 14-3-3 γ (Figure 4A). When HUVEC were treated with 0.1 μ M TNP-470 (a cytostatic dose without cytotoxic effects⁴⁸), compared to the vehicle (DMSO) controls, the ratios of unprocessed 14-3-3 γ versus total 14-3-3 γ increased 4.3-fold and 5.9-fold (Figure 4A). However, when HUVEC were treated with escalating doses of **4** or **3**, no increase could be observed in the ratios of unprocessed versus total 14-3-3 γ at the doses up to 20 μ M for **4** and up to 4 μ M for **3** (Figure 4A, left). However, compound **2** which was reported to potently inhibit *HsMetAPs* at the concentrations from 1 μ M to 10 μ M in HeLa cells,⁴⁴ merely raised the ratios of unprocessed versus total 14-3-3 γ by no more than 2.1-fold at the concentrations from 0.1 μ M to 90 μ M in HUVEC (Figure 4A, right). More surprisingly, compound **1** which was reported by Hu et al. to potently inhibit *HsMetAP1* at the concentrations of 1 μ M and 10 μ M in HeLa cells,²¹ failed to increase the ratio of unprocessed versus total 14-3-3 γ in HUVEC, HeLa and human dermal fibroblast-adult (HDFa) cells at the concentrations of 0.1 μ M, 1 μ M and 10 μ M (Figure S1). In order to address this issue comprehensively, we resynthesized pyridine-2-carboxamide **1** following the same synthetic route as previously described,²¹ characterized it fully and found it was indistinguishable from the previously synthesized sample in terms of both *in vitro* MetAP enzymatic assays and cell proliferation assays (Table 1). Unfortunately, the freshly prepared **1** at the same concentrations still could not increase the ratio of unprocessed versus total 14-3-3 γ in HUVEC, HeLa and HDFa cells (Figure 4B and Figure S1B).

The accumulation of unprocessed MetAP substrates relies on not only the inhibition of cellular MetAPs, but also the continuous production of nascent peptides by ribosomes. Therefore, a MetAP inhibitor which is capable of inhibiting translation may not increase the ratio of unprocessed versus total 14-3-3 γ in cells. To investigate this possibility, we treated HUVEC with the combination of TNP-470 and one of our pyridinylpyrimidines. When 0.1 μ M TNP-470 was applied together with 20 μ M compound **4**, the ratio of unprocessed versus total 14-3-3 γ became 2.5 times higher than the ratio in the vehicle control, and was 5 times higher than the ratio in HUVEC treated with 20 μ M compound **4** alone (Figure 4A, left). A 3.5-fold increase in the ratio has been observed when TNP-470 was supplemented to the cells treated with 30 μ M compound **2** (Figure 4A, right). A similar result for compound **1** was obtained in HUVEC as well (Figure S1A). Therefore, 10 μ M **1**, 20 μ M **2** or 30 μ M **4** did not completely inhibit translation, and compounds **1–4** could not effectively inhibit either *HsMetAPs* in HUVEC at the highest concentrations which did not lead to severe cytotoxicity.

It has been observed that small molecule inhibitors with high potency against purified type 1 MetAPs failed to inhibit the corresponding enzymes in cells. Because the single MetAP (*EcMetAP*) is essential in *Escherichia coli*,^{8a} inhibition of *EcMetAP* slowed down the growth of bacterial cells.⁴⁹ Within the past decade, three distinct classes of MetAP inhibitors have been reported which potently inhibited purified *EcMetAP in vitro*, but none were active *in vivo*. First, several pyridine-2-carboxamides were reported for their potent inhibition of *EcMetAP* (0.15 μ M) with IC₅₀ values between 0.13 μ M and 0.33 μ M *in vitro*.⁴² However, the minimum inhibitory concentrations (MICs) of these compounds against an enteropathogenic *Escherichia coli* strain were 1,527 to 3,338 times higher than their IC₅₀ values against purified *EcMetAP*.⁴² In an independent study, thiabendazole, a fungicide and parasiticide (Figure 1A), was found to be inactive against the growth of *Escherichia coli* even at 1 mM, a concentration 2,500 times higher than the K_i value of thiabendazole against purified *EcMetAP*.^{46b} More recently, Chai et al. showed that a thiazol-2-yl-oxalamide derivative⁵⁰ failed to halt the growth of three *Escherichia coli* strains at 1 mM, a concentration almost 15,000 times higher than this compound's IC₅₀ value

against Co(II)-loaded *EcMetAP* *in vitro*.⁴⁷ Intriguingly, the crystallographic analyses demonstrated that all of these inhibitors depend on an auxiliary Co(II) to interact with an imidazole nitrogen from His79 in the active site of *EcMetAP*.^{46b,47} Historically, the lack of activity in cells for these inhibitors has been attributed to the assumed poor pharmacokinetic properties,⁴² the identity of the physiologically relevant metal in the active site of *EcMetAP*,⁴² or the limited availability of free Co(II) in cells.^{46b}

The capabilities of our pyridinylpyrimidine derivatives (**2–4**) to affect cell proliferation were measured using a 30-hour [³H]-thymidine incorporation assay (Table 1). No solubility problems were seen for any compounds at their highest doses tested (90 μ M for **2**, 40 μ M for **3** and 40 μ M for **4**). Compound **2** inhibited the proliferation of HUVEC and HDFa cells with IC₅₀ values of 11 μ M and 3.6 μ M, respectively. Although these values are 58 and 19 times higher than the IC₅₀ value of **2** against purified *HsMetAP1*, they are comparable to the reported IC₅₀ value (9.6 μ M) of **2** against the proliferation of HeLa cells.⁴⁴ The proliferation of HUVEC, HDFa and HeLa cells were inhibited by **4** with IC₅₀ values of 4.3 μ M, 1.9 μ M and 10 μ M, respectively. Compound **3** was the most potent at inhibiting the proliferation of HDFa and HeLa cells with IC₅₀ values below 1 μ M. Compound **2** at 30 μ M and compound **4** at 20 μ M concentrations inhibited more than 90% of HUVEC proliferation within 30 hours. Compound **3** at 10 μ M exhibited severe cytotoxicity as most cells were floating after 24 hours. Since compounds **1–4** could not diminish the activity of any *HsMetAP* effectively in cells, the inhibition of cell proliferation is likely to be due to unknown off-target effects. Unless their antiproliferative effects were mediated by changes in the property of plasma membrane or transmembrane signaling transduction, compounds **1–4** should have no pharmacokinetic problems to stay inside the cells.

The active site of *HsMetAP1* is shallow and mostly hydrophobic.¹¹ At the bottom of the active site, two divalent transition metals (M1 and M2) are coordinated by five highly conserved residues (one His, two Glu and two Asp) (Figure 1B). M1 adopts distorted trigonal bipyramidal coordination geometry and is coordinated by one His, two Glu, one Asp and one water (W4), whereas M2 adopts distorted octahedral geometry and is coordinated by one shared Glu, one shared Asp, one unique Asp and two water molecules (W4 and W5). The three crystal structures of N-terminally truncated *HsMetAP1* in complex with Co(II) and an inhibitor revealed that compounds **1–3** all utilize an auxiliary Co(II) (M3) to bind to the active site of *HsMetAP1* (Figure 1B).^{21,44,45} In addition, the pyridinylpyrimidine core of **4** also binds *HsMetAP1* in a similar way (personal communication with Dr. Anthony Addlagatta). It is likely that MetAP inhibitors which directly bind the active site metal(s) must inhibit cellular MetAPs in their physiologically relevant metalloforms to be active in cells. However, the M3 utilized by the aforementioned compounds was not one of the “regular” metals (M1 and M2) in the active site. This auxiliary metal only contacted the enzyme through an imidazole nitrogen from a catalytically important histidine (His212). It is known that the identity of M1 and M2 in active site did not result in significant changes to the MetAP structures.⁵⁰ Moreover, the structures of *HsMetAP1* containing 1–3 transition metal ions were essentially identical.¹¹ Therefore, if the physiologically relevant metals in the active site of *HsMetAP1* are not Co(II), the inhibitory potency of auxiliary metal-dependent inhibitors in cells should not be crippled.

Like many other metalloenzymes, activation of purified MetAP *in vitro* requires relatively high concentrations of transition metal ions. For example, the assays to measure the activity of purified MetAP *in vitro* often require the supplementation of 1–100 μ M transition metals. Furthermore, to obtain crystals of MetAP holo enzymes in complex with inhibitors, the final concentrations of transition metals used in the experiments could easily exceed 1 mM. But due in part to their cytotoxicity, transition metals are tightly regulated in living cells.⁵¹

Co(II) is the optimal cofactor to active the enzymatic activities of most MetAPs *in vitro*. As a result, Co(II) has been widely used in various MetAP assays and most X-ray crystal structures of MetAP enzymes have been obtained with Co(II). However, except for the Co(II) tightly coordinated by the corrin ring in vitamin B12, cobalt is relatively rare and it is not known to be stringently required for the biological function of any cytosolic enzyme.⁵² Consequently, if a small molecule inhibitor strictly requires an auxiliary Co(II) to bind to and inhibit a MetAP, it will have little chance to be effective in cells due to the inaccessibility of the free metal ion.

HsMetAP1 can be activated not only by Co(II), but also by Mn(II) and Zn(II)

Before we investigated whether auxiliary Co(II)-mediated HsMetAP1 inhibitors can utilize any other metal to bind the cellular enzyme, it was deemed critical to determine the effects of other metals on HsMetAP1. It has been previously reported that Co(II), Mn(II), Fe(II), Zn(II) and Ni(II) can all activate bacterial type 1 MetAP *in vitro*.⁵³ When a tripeptide (Met-Ala-Ser) was used as the substrate and L-amino acid oxidase (LAO) and horseradish peroxidase (HRP) were used as the coupling enzymes, recombinant HsMetAP1 was activated by Co(II) and Mn(II), but inhibited by Zn(II), Ni(II) and Cu(II).⁵⁴ However, when Met-SCH₂CO-Phe was used as the substrate, both Co (II) and Zn(II) activated purified recombinant HsMetAP1, while Mn(II) and Ni(II) did not.⁴³ When Met-Pro-*p*-nitroanilide was used as the substrate and *Bacillus coagulans* proline iminopeptidase (BcProIP) was used as the coupling enzyme, HsMetAP1 was activated by Co(II) and a very high concentration (10 mM) of Zn(II), but inhibited by Mn(II) and Ni(II).¹¹ We employed the assay with Met-Pro-*p*-nitroanilide as the substrate and BcProIP as the coupling enzyme to further assess the effects of different metal ions on HsMetAP1 (Figure 5A). Full-length HsMetAP1 was expressed as a GST-fusion protein in *Escherichia coli* after IPTG induction. Upon purification, the N-terminal GST tag was cleaved by PreScission^{TM} protease. Purified recombinant HsMetAP1 displayed a basal activity about 10 $\mu\text{M}\cdot\text{min}^{-1}\cdot\mu\text{M}^{-1}$ (Figure 5), which was close to the previously reported values.^{43,54} It is well known that one or two divalent metal ions are required to activate each molecule of MetAP *in vitro*. The activities of all MetAPs tested could be inhibited by metal chelators such as EDTA. Thus, the basal activity observed in the HsMetAP1 assays must come from the co-purified metal(s) in the active site. As shown in Figure 5A, Co(II) was the best cofactor to activate HsMetAP1. In agreement with the literature⁴³, HsMetAP1 was most active (about 45 $\mu\text{M}\cdot\text{min}^{-1}\cdot\mu\text{M}^{-1}$) with Co(II). However, the highest enzymatic activity was found in the presence of 10 μM Co(II), instead of 100 μM Co(II) reported previously⁵⁴ which later was used by several other groups^{11,21,41,44}. Excess metals are known to inhibit metallopeptidases,⁵⁵ thus it is not surprising that higher concentrations of Co(II) decreased MetAP activity. Mn(II) was also an activator of HsMetAP1 (Figure 5A). On the contrary, Cu(II), Ni(II) and Zn(II) inhibited HsMetAP1 at the concentrations of 10 μM and above. Two physiologically abundant divalent metals, Mg(II) and Ca(II), did not affect the activity of HsMetAP1 over a wide concentration range (from 0.1 nM to 1 mM).

Because BcProIP was used as the coupling enzyme in the assay, all compounds and metal ions that inhibited the readout of the assay were counter screened against BcProIP. As negative controls, Co(II) and Mn(II) did not inhibit BcProIP at concentrations up to 1 mM (Figure S2). It has been previously pointed out that 500 μM ZnCl₂ abrogated the activity of BcProIP.⁵⁶ Indeed, 1 μM Zn(II) decreased the activity of BcProIP by about 45% and 10 μM Zn(II) led to a complete inhibition (Figure S2). In addition, more than 95% of the ProIP activity was inhibited by Cu(II) at 0.1 μM . In contrast, 100 μM Ni(II) only reduced the activity of BcProIP by less than 20%. Based on the fact that the amount of BcProIP routinely used in our MetAP assay (15 U/ml) was at least 100 times higher than the amount

required to saturate the coupling reaction, there is no doubt that under the assay conditions, Ni(II) is an inhibitor of *HsMetAP1*.

The incompatibility of Zn(II) and the *BcProIP*-coupled MetAP assay prompted us to turn to another assay to measure MetAP activity in the presence of Zn(II). When we used LAO and HRP as the coupling enzymes and Met-Ala-Ser as the substrate,^{6d} the basal activity of purified recombinant *HsMetAP1* was close to $10 \mu\text{M}\cdot\text{min}^{-1}\cdot\mu\text{M}^{-1}$ (Figure 5B). In this LAO and HRP-coupled MetAP assay, $10 \mu\text{M}$ Co(II) and $1 \mu\text{M}$ Zn(II) increased the activity of *HsMetAP1* to $56 \mu\text{M}\cdot\text{min}^{-1}\cdot\mu\text{M}^{-1}$ and $19 \mu\text{M}\cdot\text{min}^{-1}\cdot\mu\text{M}^{-1}$, respectively (Figure 5B). Both $0.1 \mu\text{M}$ and $10 \mu\text{M}$ Zn(II) consistently activated *HsMetAP1* as well. Moreover, at concentrations higher than $1 \mu\text{M}$, Cu(II) began to inhibit *HsMetAP1* in the LAO and HRP-coupled *HsMetAP1* assay, similar to its effect in the *BcProIP*-coupled assay. In summary, we demonstrated that Co(II), Mn(II) and Zn(II) can activate *HsMetAP1 in vitro*.

Type 1-selective MetAP inhibitors 1–4 are selectively dependent on Co(II) for their inhibition of *HsMetAP1 in vitro*

As both Zn(II) and Mn(II) can activate purified *HsMetAP1 in vitro* and these two metals are more abundant and more stable than Co(II) in living cells,^{51,52} we measured the IC_{50} (or EC_{50}) values of the pyridine-2-carboxamide derivative **1** and pyridinylpyrimidine derivatives **2–4** against *HsMetAP1* supplemented with either Zn(II) or Mn(II) (Table 1 and Figure S3). When $10 \mu\text{M}$ Co(II) in the MetAP assay was replaced by $1 \mu\text{M}$ Zn(II), a 3.7-fold increase in the EC_{50} value of **2** was observed. The inhibition of *HsMetAP1* by **2** reached a plateau at 65% even at concentrations up to $100 \mu\text{M}$. The IC_{50} values of compounds **1**, **3** and **4** against *HsMetAP1* were greater than $40 \mu\text{M}$, $30 \mu\text{M}$ and $100 \mu\text{M}$, respectively. Similarly, when Co(II) in the MetAP assay was replaced by the same concentration of Mn(II), the inhibitory potency of **1–4** were also greatly compromised. The EC_{50} value of **2** for the inhibition of *HsMetAP1* was increased about 80 times from $0.19 \mu\text{M}$ with Co(II) to $15 \mu\text{M}$ with Mn(II), and the maximal level of inhibition induced by **2** was merely 68% at the concentrations from 10 nM to $100 \mu\text{M}$. The IC_{50} values of **1**, **3** and **4** against *HsMetAP1* were greater than $100 \mu\text{M}$, $100 \mu\text{M}$ and $200 \mu\text{M}$, respectively. The “metal selectivity” of compound **1–4** (Table 1) was consistent with the known preference of *N*, *N*-donor ligands to coordinate soft metals like Co(II).⁴⁷ Thus, the inhibition of purified *HsMetAP1* by compound **1**, **3** and **4** was strictly Co(II)-dependent, and in the absence of Co(II), compound **2** could not inhibit purified *HsMetAP1* effectively. Their high activities against the Co(II) form of *HsMetAP1 in vitro* mainly come from the stability of their complexes with Co(II).

The tight regulation of cobalt ion likely offers a plausible explanation for why inhibitors **1–4** do not work in cells. However, it is conceivable that one of the two less tightly bound metal ions in the active site of MetAP1 may become available to facilitate the formation of an inhibitor-enzyme complex. It is well known that the first transition metal ion binds MetAP much more tightly than the second. For example, Co(II) binds *EcMetAP* with a K_d of $0.3 \mu\text{M}$ at the first site (most likely to be the M1 site) and with a K_d of 2.5 mM at the second site (most likely to be the M2 site).^{5a} More than 3 orders of magnitude difference in the binding affinities raised a possibility that auxiliary metal-dependent inhibitors might competitively chelate a Co(II) directly from the active site of MetAP. Although Co(II) remains the best cofactor to activate nearly all MetAPs *in vitro*, it is uncertain whether Co(II) is the physiologically relevant cofactor of MetAPs in cells. Rather than Co(II), Fe(II) was reported as the native cofactor of *EcMetAP*,⁴⁹ and Zn(II) is believed to be the native cofactor of the MetAP1 in *Saccharomyces cerevisiae*.⁵² Moreover, in humans the physiologically relevant cofactor of MetAP2 turned out to be Mn(II).⁵⁴ The utilization of physiologically abundant metals other than Co(II) could also enable metal-dependent

inhibitors to form complexes with MetAP in cells. It has been demonstrated that a quinolinyl sulfonamide derivative inhibited *EcMetAP* *in vivo* at a sublethal concentration, and the addition of extra 1 mM Mg(II) or 100 μ M Ca(II) moderately increased the potency of this compound to inhibit the Mn(II) form of *EcMetAP*.⁴⁷ However, when we activated purified *HsMetAP1* with 1 μ M Zn(II), and supplemented with extra Mg(II) or Ca(II) to form a ternary complex of *HsMetAP1* with an auxiliary metal ion, none of the compounds **1-4** registered a significant improvement in the inhibitory activities (Figure S4A). Thus compounds **1-4** appear to stringently require an auxiliary Co(II) for their tight binding to *HsMetAP1*, hence function as inhibitors of *HsMetAP1*. This perhaps is the reason for the lack of inhibitory activities of these compounds against *HsMetAP1* in cells.

Pyridinylquinazoline derivatives are novel *HsMetAP1*-selective inhibitors with relaxed metal selectivity

For MetAP inhibitors to work *in vivo*, it is vital that they effectively bind MetAPs in their physiologically relevant metalloforms.⁴⁹ For an inhibitor which mainly relies on an auxiliary metal to inhibit MetAP, we reasoned that its ability to use one of the physiologically abundant metal ions could be a solution. It has been reported that the NME of recombinant glutathione-S-transferase in *E. coli* was inhibited by an auxiliary metal-mediated inhibitor.⁴⁷ In addition to Co(II), this inhibitor could also utilize Mn(II) and Fe(II) to inhibit *EcMetAP* effectively *in vitro*.⁴⁷ In retrospect, this was the first hint that the relaxed metal selectivity of an auxiliary metal-mediated MetAP inhibitor is the key for its activity in cells.

Since both pyridine-2-carboxamides and pyridinylpyrimidines have stringent metal requirement for Co(II) and lack cellular activity, we began to search for novel auxiliary metal-mediated MetAP inhibitors with both good selectivity over *HsMetAP2* and relaxed metal selectivity for the inhibition of *HsMetAP1*. We tested six 2-pyridin-2-ylquinazoline derivatives (**7a**, **10a-10c**, **16** and **17**) and three 2-pyridin-2-ylquinoline derivatives (**18-20**) that contain new structural scaffolds (Table 2). Seven of the nine compounds displayed submicromolar IC₅₀ values against *HsMetAP1* in the presence of 10 μ M Co(II). None of them showed appreciable inhibition of *HsMetAP2* at concentrations below 100 μ M. In comparison to compound **2**, **10a** contains the same C-4 side chain (shown as the “R” group in Table 2) and 2-pyridinyl group and is about five fold less potent against *HsMetAP1* than **2**. Unlike **2**, **10a** could completely inhibit *HsMetAP1* in the presence of 10 μ M Mn(II). In addition, the IC₅₀ value of **10a** against *HsMetAP1* with Mn(II) was 24 μ M, smaller than that of **2** (30 μ M; the EC₅₀ value of **2** was 14 μ M). Thus, the 2-(pyridin-2-yl)quinazolines displayed more relaxed metal selectivity than the 2-(pyridin-2-yl)pyrimidines. When the C4 side chain was extended, the resultant **10b** completely lost the inhibitory activity against *HsMetAP1* in the presence of Mn(II), while its potency against *HsMetAP1* with Co(II) was retained. Changing the phenyl group at the end of the C4 side chain of **10a** to a 3-1H-isoindole led to 7.8-fold and 4.5-fold increases in the potency of **10c** against *HsMetAP1* with Co(II) and Mn(II), respectively. Swapping the long C4 side chain of **10c** for a shorter and more rigid *N*-piperidinyl group afforded **16** with similar potency. Interestingly, when a 4-morpholinyl group was installed on the 2-(pyridin-2-yl)quinazoline core, **17** became 19 times more potent against *HsMetAP1* with Co(II) compared with the lead **10a**. But the IC₅₀ value of **17** against *HsMetAP1* with Mn(II) was not improved. Furthermore, when the large C4 side chain was replaced by a chloro substitution, **7a** completely lost the inhibitory activity against *HsMetAP1* in the absence of Co(II). At last, in contrast to **16** and **17**, all three 2-(pyridin-2-yl)quinoline derivatives (**18-20**) had no effect on Mn(II)-bound *HsMetAP1* (Table 2). Therefore, the 2-(pyridin-2-yl)quinazoline core is necessary but not sufficient for the relaxed metal selectivity of our novel *HsMetAP1*-selective inhibitors.

Based on the structure of **16**, sixteen more 2-(pyridin-2-yl)quinazoline derivatives were synthesized. After analyzing the inhibitory potencies of these compounds against both *HsMetAP1* and *HsMetAP2* (Table 3), three SAR trends became evident. (1) The 4-piperazin-1-yl-2-(pyridin-2-yl)quinazoline core seemed to guarantee considerable selectivity for *HsMetAP1* over *HsMetAP2*. Except **11e** and **11l**, the other fourteen derivatives all had submicromolar IC₅₀ values against *HsMetAP1* with Co(II), while their IC₅₀ values against *HsMetAP2* were above 100 μM. (2) In N4'' side chains (R2 groups in Table 3) of eleven derivatives (**11a–11j**, and **11m**), various substituents at the *para*-position of the phenyl group were well tolerated thus retained the inhibitory potency against *HsMetAP1* in the presence of Mn(II), with two exceptions (**11c** and **11e**). Both **11c** and **11e** contained a free carboxyl group at the very end of the C4 side chains. The IC₅₀ values of both compounds against *HsMetAP1* with Mn(II) were higher than 100 μM. Neutralization of the free carboxyl group via the formation of an ethyl ester immediately restored the potency against *HsMetAP1* with either Co(II) or Mn(II), as seen in **11b** and **11d**. (3) Unlike the trend in 2-(pyridin-2-yl)pyrimidine based MetAP1 inhibitors,⁴⁵ the installation of halogen substituents at C5' position of **11j** failed to enhance the potency against *HsMetAP1*. To the contrary, compared to **11j**, **11k** with a chloro substituent and **11l** with a bromo substituent were actually less potent against *HsMetAP1* with either Co(II) or Mn(II).

As shown in Table 3 and Figure S3D, the EC₅₀ value of **11j** (5.1 μM) for *HsMetAP1* with 10 μM Mn(II) was 51-fold higher than its IC₅₀ value (0.10 μM) against *HsMetAP1* with 10 μM Co(II). However, in the presence of Mn(II), 10 μM and 100 μM **11j** managed to inhibit about 62% and 86% activity of purified *HsMetAP1*, respectively. Because Zn(II) could activate *HsMetAP1*, we also determined the EC₅₀ value of **11j** for the inhibition of *HsMetAP1* in the presence of 1 μM Zn(II) (Table 1 and Figure S3D). In the presence of Zn(II), **11j** had an EC₅₀ value about 1.1 μM and it inhibited about 84% activity of purified *HsMetAP1* at a concentration of 100 μM. Interestingly, the IC₅₀ value of **11j** (0.13 μM) against *HsMetAP1* with both 10 μM Mn(II) and 10 μM Co(II) was much closer to its IC₅₀ value (0.10 μM) against *HsMetAP1* with Co(II) alone than its IC₅₀ value (5.1 μM) against *HsMetAP1* with Mn(II) alone (Figure S3D). This indicates that **11j** prefers to utilize Co(II) to inhibit *HsMetAP1* over Mn(II).

To investigate whether physiologically abundant divalent metals could be utilized by **11j** to inhibit *HsMetAP1*, 0.15 μM purified *HsMetAP1* was activated by 10 μM Mn(II). The *in vitro* enzymatic activity of *HsMetAP1* in the presence of 1 μM **11j** was monitored after the supplementation of extra divalent metals to the reaction (Figure S4B). Mg(II) and Ca(II) are both relatively abundant in cells and they did not dramatically affect the activity of *HsMetAP1* at the concentrations from 0.1 nM to 1 mM *in vitro* (Figure 5). As shown in Figure S4B, **11j**'s inhibitory potency against *HsMetAP1* was not significantly enhanced by 10–1000 μM MgCl₂. The highest concentration (1 mM) of CaCl₂ only increased the potency of **11j** by less than 8% (*p*-value = 0.036). Meanwhile, despite the fact that it would further activate *HsMetAP1*, 10 μM Co(II) improved the potency of 1 μM **11j** by 80% (*p*-value < 0.0001). Therefore, in contrast to the previously reported quinolinyl sulfonamide based *EcMetAP* inhibitor (**21**) whose potency was increased by both Mg(II) and Ca(II),⁴⁷ the relaxed metal selectivity of **11j** was only limited to some divalent transition metals.

A 4-piperazin-1-yl-2-(pyridin-2-yl)quinazoline derivative (**11j**) inhibits *HsMetAP1* in cells

To determine whether **11j** could eventually inhibit the activity of *HsMetAP1* inside the cells, we determined its effect on the NME of 14-3-3γ protein by immunoblot in two primary cell lines (Figure 6). As a positive control, a 24-hour treatment with TNP-470 in HUVEC increased the ratio of unprocessed 14-3-3γ versus total 14-3-3γ more than 6 times. A dose-dependent inhibition of the NME of 14-3-3γ by **11j** was observed (Figure 6A, left). **11j**

started to inhibit MetAP activity at a concentration of 0.4 μM , and a 7.1-fold increase in the ratio was achieved at the highest concentration. The siRNA mediated down-regulation of *HsMetAP1* and *HsMetAP2* proved that 14-3-3 γ was also a shared substrate of both *HsMetAPs* in normal HDFa (data not shown). After 24-hour incubation, TNP-470 and bengamide G caused 3.4-fold and 7.0-fold increases, respectively, in the ratio of unprocessed versus total 14-3-3 γ in HDFa cells (Figure 6A, right). Reminiscent of the observation in HUVEC, **11j** dose-dependently inhibited the NME of 14-3-3 γ in HDFa cells. Therefore, **11j** brought appreciable increases in the ratio of unprocessed versus total 14-3-3 γ in both human primary cell lines at single-digit micromolar concentrations, which are very close to its IC_{50} values for the inhibition of cell proliferation as well as its EC_{50} values against *HsMetAP1* bound to Zn(II) or Mn(II) *in vitro* (Table 1).

4-(1*H*-1,3-benzodiazol-2-yl)-1,3-thiazole (thiabendazole), a compound frequently used as a fungicide and parasiticide, has been reported as a potent inhibitor of *EcMetAP* with a K_i value of 0.4 μM .^{46b} *EcMetAP* is essential for the growth of *E. coli*.^{8a} But thiabendazole was inactive against the proliferation of this bacteria at concentrations up to 1 mM.^{46b} As shown in Table 1, thiabendazole inhibited 0.15 μM *HsMetAP1* in the presence of Co(II) with an EC_{50} value of 3.4 μM . In the absence of Co(II), thiabendazole failed to inhibit the activity of *HsMetAP1* at concentrations up to 250 μM (Figure S3E). The X-ray crystal structure of thiabendazole in complex with *EcMetAP* and Co(II) (PDB code 1YVM) revealed that thiabendazole mainly depended on an auxiliary Co(II) to inhibit *EcMetAP*.^{46b} Although thiabendazole was reported as a vascular disrupting agent and it could disrupt the tube formation in cultured HUVEC,⁵⁷ this compound did not show any appreciable inhibition of the proliferation of HUVEC, as well as HDFa cells, at concentrations up to 250 μM (Table 1). If the relaxed metal selectivity is crucial for the cellular activities of auxiliary metal-mediated MetAP inhibitors, an auxiliary Co(II)-dependent inhibitor may fail to inhibit cellular *HsMetAPs*. Indeed, 3–250 μM thiabendazole did not affect the NME of 14-3-3 γ in two human primary cell lines (Figure 6B). 2, 2'-bipyridine is a cell-permeable bidentate chelating agent which can form complexes with many transition metals, and its structure is closely related to the 2-(pyridin-2-yl)pyrimidine core of compound **2–4**. 2,2'-bipyridine inhibited purified *HsMetAP1* with Co(II) as potently as **2–4**, but it inhibited the activity of *HsMetAP1* in the absence of Co(II) by no more than 30% at 100 μM (Table 1 and Figure S3F). As shown in Figure 6B, the ability of 2,2'-bipyridine to block the NME of 14-3-3 γ in cells was at least 30 times lower than **11j**. Together, these results demonstrate that among similar pharmacophores of pyridinylpyrimidine, pyridinylquinoline, pyridinylquinazoline, benzodiazolylthiazole and 2,2'-bipyridine, only the 2-(pyridin-2-yl)quinazoline core offers the relaxed metal selectivity to auxiliary metal-mediated *HsMetAP1* inhibitors.

Accumulation of N-terminal unprocessed MetAP substrates in cells depends on not only the inhibition of cellular MetAP(s), but also the active translation producing nascent peptides. In theory, accumulation of iMet 14-3-3 γ caused by any MetAP inhibitor should be time-dependent. To test this hypothesis, we determined the time-course of the iMet processing of 14-3-3 γ in HUVEC treated with **11j**. Cell lysates were collected 1–7 hours after the addition of 10 μM **11j** to the cell culture. With no appreciable change in the levels of total 14-3-3 γ , the level of unprocessed 14-3-3 γ increased in a time-dependent manner (Figure S5A). To assess the importance of translation in the accumulation of unprocessed 14-3-3 γ , we treated HUVEC with cycloheximide prior to the addition of MetAP inhibitors. Cycloheximide is known to inhibit the elongation step of eukaryotic translation through binding to a pocket in the E-site of the 60S ribosomal subunit.⁵⁸ In the absence of cycloheximide, all MetAP inhibitors (bengamide A, TNP-470 and **11j**) resulted in elevated levels of unprocessed 14-3-3 γ (Figure S5B, left). But in the presence of cycloheximide, none of the MetAP inhibitors could generate a detectable signal of unprocessed 14-3-3 γ . Therefore, the

accumulation of N-terminal unprocessed 14-3-3 γ induced by **11j** relied on active translation as expected.

To determine whether the elevated level of the iMet unprocessed 14-3-3 γ caused by **11j** can be counteracted by upregulated MetAP activity in cells, *HsMetAP1* was ectopically expressed in HUVEC via a construct transduced by lentivirus. Lentiviruses containing either an empty pLenti6m vector (control virus) or the coding sequence of full-length *HsMetAP1* (overexpression virus) were produced in human embryonic kidney 293T (HEK293T) cells. Concentrated lentiviruses were used to transduce HUVEC. The transduced cells were pooled to preclude the possible clonal differences in response to **11j**. As shown in Figure S6, the protein level of *HsMetAP1* was dramatically upregulated in HUVEC infected by the overexpression virus. In HUVEC infected by the control virus, the signals of endogenous *HsMetAP1* were barely visible as the exposure time was greatly decreased to avoid saturation. While there was no obvious change in the levels of total 14-3-3 γ , compared with the HUVEC transduced by the control virus, the ectopic expression of *HsMetAP1* decreased the ratio of unprocessed 14-3-3 γ versus total 14-3-3 γ up to 40% in the cells treated with **11j**. Interestingly, in the absence of **11j**, *HsMetAP1* overexpression reduced the background level of unprocessed 14-3-3 γ as well.

Human thioredoxin-like protein 1 (TXNL1) was previously shown to be a specific substrate of *HsMetAP2* in HEK293T cells.⁷ However, this conclusion was based on the unrepeatably finding of *HsMetAP1* inhibition by compound **1** in human cells.²¹ To reinvestigate the role of *HsMetAP1* in the removal of the iMet of TXNL1, HEK293T cells expressing C-terminally Flag-tagged BTF3L4 or TXNL1 were treated with vehicle (0.2% DMSO), 10 μ M **11j**, 100 nM TNP-470, or the combination of the latter two, and half an hour later, newly synthesized proteins were labeled by [³⁵S]-Met. The BTF3L4 protein has MNQEKLA at its N-terminus. Therefore, it should not be a substrate of any MetAP. TXNL1 contains an N-terminal sequence of MVGVKPV. An oligopeptide (MVGVKPY) sharing the first six a.a. residues with TXNL1 was preferably processed by *HsMetAP2* *in vitro*.⁷ BTF3L4-3 $\times$ Flag or TXNL1-3 $\times$ Flag was immunoprecipitated from the whole cell extract using anti-Flag-conjugated resin. The unprocessed iMet at the N-terminus of partially purified TXNL1-3 $\times$ Flag was released by incubation with both purified recombinant *HsMetAP1* and *HsMetAP2* (Figure 7). The resin containing TXNL1-3 $\times$ Flag purified from the TNP-470 treated cells released 2.2 times more [³⁵S]-Met into the solution than the resin from the vehicle control, recapitulating earlier findings⁷. Surprisingly, unlike compound **1**,⁷ the *HsMetAP1*-selective **11j** increased the release of [³⁵S]-Met as well (40 $\pm$ 17% more than the vehicle). 100 nM of a covalent inhibitor in fumagillin family was believed to be high enough to abolish the enzymatic activity of cellular *HsMetAP2*.²⁰ In addition, [³⁵S]-Met released from the immunoprecipitated TXNL1-3 $\times$ Flag in the TNP-470 and **11j** co-treatment group significantly more abundant than the group treated only with TNP-470 (2.20 fold versus 2.73 fold of the vehicle control, p = 0.03). As expected, for BTF3L4-3 $\times$ Flag, there was no appreciable increase in its iMet retention after the treatment of **11j**, TNP-470 or a combination of two (Figure 7). Together, these results suggest that TXNL1 serves as a substrate of both *HsMetAP1* and *HsMetAP2*, similar to 14-3-3 γ .

The X-ray crystal structure of N-terminally truncated *HsMetAP1* in complex with **11j**

The crystal structure of N-terminally truncated (Δ 1–80) human MetAP1 (*tHsMetAP1*) in complex with Co(II) and **11j** was determined at a resolution of 2.09 Å (Table 4). The structure revealed the presence of three Co(II) in the active site: Co1 and Co2 found in all members of the MetAP1 family and Co3 coordinating a quinazoline nitrogen (N1) and the pyridine nitrogen (N1') of **11j** (Figure 8A). The octahedral coordination of Co3 is completed by His212 and three water molecules. Similar coordination has been described

for the structures of *tHsMetAP1* in complex with **2** (PDB code 2G6P) or **3** (PDB code 4HXX) in the presence of Co(II) (Figure 8B).^{44,45} The 2-pyridinyl group of **11j** occupies a hydrophobic pocket formed by Pro192, Tyr195, Phe198, Cys203, Phe309 and Trp353 (Figure 8C). This pocket is the binding site for the side chain of the iMet residue when a substrate polypeptide is being processed by MetAP and it was occupied by an acetate ion in the structure of holoenzyme of *tHsMetAP1* (PDB code 2B3K).¹¹ The relative positions of the 2-pyridinyl group in the methionine side chain binding pocket are similar for **2** and **11j** (Figure 8B). The distances from C5' of the pyridine ring to C1 and C4 of the phenyl group in Phe198 are 3.87 Å and 4.64 Å, respectively, in 2G6P; and the distances are 4.02 Å and 5.70 Å, respectively, in 4IU6. The quinazoline part of **11j** displays a tight stacking with the imidazole ring of His310. The pyridine ring and the quinazoline ring of **11j** are nearly coplanar. The C4 side chain of **11j** is projected out towards the opening of the active site cavity and the α -helix 125-133. In the C4 side chain of **11j**, the 4-methoxyphenyl group forms a stacking interaction with the side chain of Tyr196, and the terminal methoxy group may participate in an indirect ionic interaction with Gln129, through a water molecule.

From a structural perspective, there seems to be two critical elements contributing to the interactions between **11j** and *tHsMetAP1*. The first element is the 2-(pyridin-2-yl)quinazoline part of **11j**, which despite its simplicity is able to (1) perfectly coordinate the third metal; (2) occupy the hydrophobic binding cavity for the substrate; and (3) stack with the conserved His310 at the same time (Figure 8A). The second element is the 4-methoxyphenyl group in the C4 side chain of **11j** which stacks with the side chain of Tyr196. The adequate spacing between the pyridinylquinazoline core and the 4-methoxyphenyl group is achieved by using a piperazine ring as a linker. Piperazine provides enough flexibility without an onerous increase in degrees of freedom. Various additions to the C4 side chain of **11j** beyond the phenyl group failed to significantly improve the inhibitory potency against purified *HsMetAP1*, as evidenced by **11a–11i** and **11m** (Table 3). This may be partially due to the fact that the 4-methoxyphenyl group of **11j** is very close to the surface of *tHsMetAP1*, since at the end of the C4 side chain which points towards the aqueous solution, it would be quite difficult to introduce any new interaction between the inhibitor and the enzyme. To further increase the interactions between an inhibitor and *HsMetAP1*, one option is to simultaneously increase the length and the flexibility of the C4 side chain, but at the expense of losing the stacking interaction with Tyr196. The chloro group at C5' position of **3** improved its potency against *HsMetAP1* with Co(II).⁴⁵ If **11k** and **11l** adopt the same binding mode as **3** binds to *HsMetAP1* with Co(II) (Figure 8B), to accommodate the C5' substituents, the 2-(pyridin-2-yl)quinazoline moiety of **11k** and **11l** may need to move up to 1 Å out of the hydrophobic pocket for methionine side chain. Unlike the flexible C4 side chain of **3**, the C4 side chains of both **11k** and **11l** contain relatively rigid piperazine ring and 4-methoxyphenyl group. Therefore, the stacking interaction of the 4-methoxyphenyl group to the side chain of Tyr196 and the potential indirect ionic interaction between the terminal methoxy group and Gln129 are very likely to be disrupted because of the halogen substituents in **11k** and **11l**. This may help to explain why the introduction of chloro and bromo groups at C5' position decreased the activities of **11k** and **11l** against *HsMetAP1* with Co(II) or Mn(II).

The crystal structure of *tHsMetAP1* in complex with **11j** was superimposed on the crystal structure of *HsMetAP2* in complex with L-methionine (PDB code 1KQ9⁵⁹) (Figure 9). Given the present conformation, it is obvious that the piperazine ring in the C4 side chain of **11j** suffers a severe clash with the side chain of Tyr444 if **11j** were to bind the active site of *HsMetAP2*. This steric clash may help to explain why 4-piperazin-1-yl-2-(pyridin-2-yl)quinazoline derivatives (**11a–11m** and **13–15**) have great selectivity for *HsMetAP1* over *HsMetAP2*.

With four crystal structures of Co(II)-bound *HsMetAP1* in complex with auxiliary metal-dependent inhibitors in hands, we analyzed the angle formed from the two coordinating atoms of an inhibitor to the auxiliary metal ion and the distances from the two coordinating atoms to the auxiliary metal ion (Table S1 and Figure S7). It was speculated that the small angle (76.0°) formed from the two coordinating nitrogen atoms of a quinolinyln sulfonamide based *EcMetAP* inhibitor to the auxiliary Mn(II) and the long distances (2.32, 2.22 Å) from the two nitrogen atoms of the inhibitor to the auxiliary Mn(II) might provide the structural basis to the relaxed metalloform selectivity of that inhibitor.⁴⁷ However, we do not think that the coordinating geometry of an auxiliary metal is a reliable indicator for the relaxed metal selectivity of a MetAP inhibitor.

Conclusion

In this work, we demonstrated that 14-3-3 γ is a shared substrate between MetAP1 and MetAP2 in HUVEC. The complete excision of the iMet in 14-3-3 γ relies on the activities of both *HsMetAP1* and *HsMetAP2*. Using the retention of the iMet in 14-3-3 γ as an indicator of cellular MetAP inhibition, we found that one pyridine-2-carboxamide derivative (**1**) and two pyridinylpyrimidines (**3–4**) do not inhibit any *HsMetAP* in cells, and the pyridinylpyrimidine derivative **2** cannot potentially decrease the cellular activities of *HsMetAPs*. Purified recombinant *HsMetAP1* can be reliably activated by not only Co(II), but also Mn(II) and Zn(II). In the absence of Co(II), compounds **1–4**, thiabendazole and 2,2'-bipyridine no longer can inhibit purified *HsMetAP1* potently *in vitro*. In an effort to seek *HsMetAP1*-selective inhibitors with relaxed metal selectivity, we succeeded in finding a novel class of inhibitors containing a 2-(pyridin-2-yl)quinazoline. Many compounds in this class can potently and selectively inhibit *HsMetAP1* with relaxed metalloform selectivity. Among them, a 4-piperazin-1-yl-2-(pyridin-2-yl)quinazoline derivative **11j** can utilize at least Co(II), Zn(II) and Mn(II) to potently inhibit *HsMetAP1* *in vitro*, without affecting *HsMetAP2*. In addition, **11j** can dose-, time- and translation-dependently induce the accumulation of the N-terminal unprocessed form of 14-3-3 γ in human primary cells, and this accumulation can be partially reversed by ectopic expression of *HsMetAP1*. Lastly, crystallographic analysis confirmed that **11j** is an auxiliary metal-mediated MetAP inhibitor. **11j** may serve as a useful molecular probe to facilitate the understanding of the physiological functions of *HsMetAP1*.

Experimental Section

General

Unless stated otherwise, all non-aqueous reactions were carried out under ambient atmosphere in oven-dried glassware. Indicated reaction temperatures refer to those of the reaction bath, while room temperature (rt) is noted as 25 °C. All the solvents were of reagent grade purchased from Fisher Scientific or VWR and they were used as received. Commercially available starting materials and reagents were purchased from Acros, Aldrich, or TCI America and were used as received.

Analytical thin layer chromatography (TLC) was performed on Analtech Uniplates (silica gel HLF, W/UV254, 250 μ m). Visualization was achieved using 254 nm UV light, or additionally by staining with iodine, or ceric ammonium molybdate stain. Crude products were purified by air-flashed column chromatography over silica gel (0.06–0.2 mm, 60 Å, from Acros) using indicated eluents. Melting points were recorded on a Mel-Temp II apparatus and are uncorrected. NMR data were collected on a Varian Unity-400 (400 MHz ¹H, 100.6 MHz ¹³C), Inova-500 (500 MHz ¹H, 125 MHz ¹³C), or Bruker-Spectrospin 400 MHz spectrometers. Chemical shifts are reported in ppm (δ) with the solvent resonance or 0.1% tetramethylsilane contained in the deuterated solvent as the internal reference. Data are

reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, m = multiplet, br = broad, app = apparent, exch = exchangeable), coupling constants (J, reported in Hertz, Hz), and number of protons. Low resolution mass spectra were acquired on a Thermo-Finnigan MAT, LCQ Classic ESI-mass spectrometer or Voyager DE-STR, MALDI-TOF (Applied Biosystems) instruments. The MALDI-samples were prepared by mixing the droplets of the sample solutions in chloroform or methanol and 2,5-dihydroxybenzoic acid solution in acetone, where the latter served as the matrix. Data are reported in the form m/z (molecular ion).

If needed, products were purified using a JASCO HPLC fitted with a semi-preparative VyDAC C18 column (7 micron, 10 × 250 mm). A mixture of water, acetonitrile, and 2-propanol was used as a mobile phase while eluting at a flow rate of 3 mL/min. Samples were analyzed for purity on a JASCO HPLC equipped with a Phenomenex Luna C18 column (3 μm, 4.6 × 150 mm). The mobile phase set at a flow rate of 1.0 mL/min was programmed for a gradient elution starting from a 90:5:5 mixture of H₂O—MeCN—MeOH to 50:45:5 over six minutes. Thereafter, the gradient was ramped up to 10:80:10 H₂O—MeCN—MeOH over five minutes, maintained with the same gradient for four more minutes and was reverted back to the starting gradient of 90:5:5 H₂O—MeCN—MeOH; making the whole run sixteen minutes long. Detection was achieved using JASCO MD-2010 photo-diode array detector while monitoring the absorption at 254, 275, and 296 nm wavelengths. Purity of the final compounds was determined to be >95%, using a 10 μL injection (approximately 1 mM in acetonitrile) with quantitation by area under the curve (AUC) at 254, 275, and 296 nm (JASCO Diode Array Detector). The retention time (tR) is reported in minutes with the AUC given in parentheses.

Commercially available 2-(pyridin-2-yl)quinazolines **16** (CAS# 57768-72-0) and **17** (CAS# 836652-57-4) were purchased from Ambinter (Orléans, France), and 2-(pyridin-2-yl)quinoline **18** (CAS# 633199-21-5), **19** (CAS# 133698-98-3), and **20** (CAS# 156094-96-1) were purchased from Maybridge (Cambridge, United Kingdom), Sigma-Aldrich (St. Louis, MO) and Ambinter, respectively. The masses of the five compounds were confirmed by ESI-MS (Agilent Technologies 6120 Quadrupole LC/MS system). Alternatively, quinazolines **16** and **17** may also be synthesized using the general procedure described below and the syntheses of commercially available quinolines **18**, **19**, and **20** have also been described in the literature.⁶⁰

Bengamides A, B, and G were provided by Dr. Philip Crews (UC Santa Cruz). Fumagillin, thiabendazole, 2,2'-bipyridine, 2-(pyridin-2-yl)pyrimidines **2** and **4** were acquired from commercial sources. TNP-470 was prepared starting from the commercially available fumagillin analogous to the procedure described by Arico-Muendel et al.⁶¹ The synthesis of pyridinylpyrimidine **3** has been communicated recently.⁴⁵

Synthesis of t-butyl 2-(thiazol-2-ylcarbamoyle)pyridin-3-ylcarbamate (**1**)

The synthesis of compound **1** followed the previously described procedure.²¹ The characterization of the final product matched with the literature report.²¹

General Procedure for the Synthesis of 2-(pyridin-2-yl)quinazolin-4-amine derivatives

According to the procedure described by Flanagan et al.⁶² suitably substituted cyanopyridine was treated with sodium metal in methanol and refluxed in methanol with anthranilic acid after an hour of stirring at room temperature. Quinazolinone **6** was collected by filtering and cooled reaction mixture and a second crop was obtained after letting the mother liquor stand overnight. Quinazolinone **6** was pure by NMR and was directly converted to the 4-chloroquinazoline (**7**) by refluxing with diethylaniline and phosphorus oxychloride in

benzene for 4 h. Diethylaniline was distilled off under high vacuum and the crude product was washed with saturated NaHCO₃ and the concentrated reaction mixture was purified by column chromatography over silica gel (eluent: hexanes and then neat DCM). The chloro group in **7** was replaced with various amines by heating at 110 °C overnight. Substituted quinazolines **8** were purified by column chromatography over silica gel.

4-Chloro-2-(pyridin-2-yl)quinazoline (**7a**)⁶²

Yield: 69% (over two steps). ¹H NMR (400 MHz, CDCl₃): δ 8.91 (d, *J* = 4.5 Hz, 1H), 8.63 (d, *J* = 7.5 Hz, 1H), 8.31 (m, 2H), 7.99 (dt, *J* = 8.5, 1.6 Hz, 1H), 7.89 (dt, *J* = 8.1, 1.7 Hz, 1H), 7.74 (t, *J* = 8.1 Hz, 1H), 7.41 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 163.33, 158.91, 154.01, 151.82, 150.46, 137.21, 135.14, 129.66, 129.19, 125.85, 125.22, 124.43, 123.09. MALDI-TOF: *m/z* 243 (M+H)⁺, 265 (M+Na)⁺.

N-Phenethyl-2-(pyridin-2-yl)quinazolin-4-amine (**10a**)⁶³

Yield: 72%. *t*_R: 8.440 min. (98.6%). ¹H NMR (400 MHz, CDCl₃): δ 8.81 (ddd, *J* = 8.4, 1.6, & 0.8 Hz, 1H), 8.06 (d, *J* = 8.0 Hz, 1H), 8.63 (d, *J* = 8.4 Hz, 1H), 7.84 (ddd, *J* = 8.4, 7.6, & 1.6 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 7.34 (m, 2H), 7.66 (t, *J* = 7.6 Hz, 1H), 7.29 (m, 2H), 7.23 (m, 2H), 6.47 (br t, 1H), 3.99 (dt, *J* = 12.4, 6.8 Hz, 2H), 3.02 (t, *J* = 7.6 Hz, 2H). ¹³C NMR (125 MHz, CD₃OD): δ 161.85, 160.65, 157.00, 150.78, 150.28, 141.02, 138.55, 134.11, 130.04, 129.57, 128.77, 127.51, 127.37, 126.13, 125.25, 123.33, 115.71, 44.06, 36.43. MALDI-TOF: *m/z* 327 (M+H)⁺, 349 (M+Na)⁺.

N¹-Phenyl-N²-[2-(pyridin-2-yl)quinazolin-4-yl]ethane-1,2-diamine (**10b**)

Yield: 82%. *t*_R: 8.840 min. (95.1%). ¹H NMR (400 MHz, acetone-d₆): δ 8.83 (m, 1H), 8.6 (app d, *J* = 8.4 Hz, 1H), 8.37 (m, 1H), 7.93 (m, 3H), 7.51 (m, 2H), 7.13 (m, 2H), 6.73 (m, 2H), 6.55 (t, *J* = 7.6 Hz, 1H), 4.13 (br s, 2H), 3.71 (m, 4H). ¹³C NMR (125 MHz, CD₃CN): δ 164.32, 156.88, 155.02, 150.45, 149.31, 148.43, 137.26, 132.93, 128.25, 122.58, 121.29, 120.89, 118.91, 112.58, 48.77, 47.83. MALDI-TOF: *m/z* 342 (M+H)⁺, 364 (M+Na)⁺.

N-[2-(1*H*-indol-3-yl)ethyl]-2-(pyridin-2-yl)quinazolin-4-amine (**10c**)

Yield: 88%. *t*_R: 8.520 min. (96.9%). ¹H NMR (500 MHz, methanol-d₄): δ 8.63 (d, *J* = 4.3 Hz, 1H), 8.23 (d, *J* = 7.8 Hz, 1H), 8.00 (d, *J* = 8.4 Hz, 1H), 7.85–7.78 (m, 2H), 7.69 (td, *J* = 7.7 & 0.9 Hz, 1H), 7.56 (dt, *J* = 8.0 & 0.9 Hz, 1H), 7.45–7.38 (m, 2H), 7.22 (dt, *J* = 8.2 & 0.9 Hz, 1H), 7.02–6.98 (m, 2H), 6.88 (td, *J* = 6.7 & 0.9, 1H), 3.99 (t, *J* = 7.2 Hz, 2H), 3.27 (m, 2H), 3.13 (t, *J* = 7.4 Hz, 2H). ¹³C NMR (125 MHz, CD₃OD): δ 162.77, 160.47, 155.97, 151.04, 149.11, 139.43, 138.98, 135.44, 129.70, 128.65, 127.78, 127.40, 126.08, 124.43, 123.19, 120.42, 120.30, 116.07, 114.29, 113.13, 44.38, 27.04. MALDI-TOF: *m/z* 366 (M+H)⁺, 388 (M+Na)⁺.

1-(4-{4-[2-(Pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}phenyl)ethanol (**11a**)

This was obtained by reducing the corresponding ketone using sodium borohydride in methanol. Yield: 93%. *t*_R: 7.373 min. (97.6%). ¹H NMR (400 MHz, CDCl₃): δ 8.87 (d, *J* = 4 Hz, 1H), 8.60 (dt, *J* = 6.0 & 2.8 Hz, 1H), 8.21 (d, *J* = 8.4 Hz, 1H), 7.97 (m, 2H), 7.82 (m, 2H), 7.49 (m, 2H), 7.40 (m, 1H), 7.00 (m, 2H), 4.86 (q, *J* = 6.4 Hz, 1H), 4.04 (m, 4H), 3.44 (m, 4H), 1.49 (d, *J* = 6.0 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 157.40, 151.42, 143.87, 137.72, 134.78, 126.69, 124.09, 116.40, 85.84, 50.01, 49.40, 17.33. MALDI-TOF: *m/z* 412 (M+H)⁺, 434 (M+Na)⁺.

Ethyl 4-{4-[2-(pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}benzoate (11b)

Yield: 78%. t_R : 8.000 min. (96.1%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.25 (d, J = 1.6 Hz, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.29 (m, 4H), 6.82 (m, 3H), 6.23 (m, 2H), 4.17 (q, J = 4.6 Hz, 2H), 3.70 (m, 4H), 3.57 (m, 4H), 0.93 (t, J = 4.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.86, 155.47, 152.02, 150.37, 148.88, 136.98, 133.76, 131.28, 129.03, 127.48, 124.21, 123.59, 122.13, 113.78, 60.52, 49.37, 47.81, 14.60. MALDI-TOF: m/z 440 (M+H) $^+$.

4-{4-[2-(pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}benzoic acid (11c)

This benzoic acid was obtained after performing a saponification reaction on the ethyl ester. Yield: 89%. t_R : 10.013 min. (96.3%). ^1H NMR (400 MHz, $CDCl_3$): δ 11.02 (br s, 1H), 8.77 (d, J = 1.6 Hz, 1H), 8.59 (dd, J = 7.6 & 1.5 Hz, 1H), 8.27 (dd, J = 7.5 & 1.5 Hz, 1H), 8.11 (m, 2H), 7.85 (m, 4H), 7.67 (m, 1H), 7.59 (dt, J = 7.8 & 1.2 Hz, 2H), 3.79 (m, 4H), 3.65 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD): δ 171.42, 165.85, 164.56, 151.96, 151.04, 150.69, 139.76, 136.85, 135.94, 133.60, 129.36, 128.48, 128.18, 124.14, 123.94, 122.36, 108.39, 52.88, 50.48. MALDI-TOF: m/z 411 (M) $^+$, 434(M+Na) $^+$.

Ethyl 2-(4-{4-[2-(Pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}phenoxy)acetate (11d)

The amine used in the final step was ethyl 2-[4-(piperazin-1-yl)phenoxy]acetate. Yield: 72%. t_R : 8.560 min. (95.5%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.64 (d, J = 2.6 Hz, 1H), 8.57 (t, J = 8.0 Hz, 1H), 8.33 (d, J = 7.6 Hz, 1H), 7.89 (m, 4H), 7.49 (m, 1H), 6.95 (m, 4H), 4.58 (s, 2H), 4.25 (q, J = 7.2 Hz, 2H), 4.01 (m, 4H), 3.44 (m, 4H), 1.29 (t, J = 6.8 Hz, 3H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 161.59, 153.03, 150.28, 148.89, 148.53, 137.70, 134.76, 128.19, 127.47, 126.92, 126.43, 122.15, 118.46, 115.60, 66.26, 61.50, 50.60, 50.03, 14.35. MALDI-TOF: m/z 470 (M+H) $^+$, 508 (M+K) $^+$.

2-(4-{4-[2-(Pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}phenoxy)acetic acid (11e)

This was prepared by alkylation of SB11882B with ethyl bromoacetate followed by alkaline hydrolysis. Yield: 66% (over two steps). t_R : 10.027 min. (98.2%). ^1H NMR (400 MHz, $CDCl_3$): δ 11.05 (br s, 1H), 8.67 (d, J = 4.8 Hz, 1H), 8.58 (app d, J = 8.0 Hz, 1H), 8.36 (app d, J = 8.4 Hz, 1H), 7.90 (m, 3H), 7.80 (m, 4H), 7.04 (m, 2H), 6.85 (m, 2H), 4.62 (s, 2H), 3.66 (m, 4H), 3.10 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.80, 154.85, 149.00, 148.71, 137.75, 135.42, 134.89, 128.24, 127.58, 127.00, 126.49, 123.20, 122.57, 120.92, 119.89, 115.80, 65.77, 51.91, 49.11. MALDI-TOF: m/z 441 (M) $^+$.

4-{4-[2-(Pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}phenol (11f)

In the final step, displacement reaction was performed using 1-{4-[(tert-butyl)dimethylsilyl]oxy}phenyl]piperazine and the silyl group was deprotected using 1 M tetrabutylammonium fluoride in THF. Yield: 76% (over two steps). t_R : 6.720 min. (96.5%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.79 (s, 1H), 8.58 (app d, J = 6.8 Hz, 1H), 8.10 (app d, J = 7.2 Hz, 1H), 7.90 (m, 1H), 7.87 (m, 1H), 7.73 (m, 1H), 7.41 (m, 2H), 6.79 (m, 4H), 4.02 (m, 4H), 3.18 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.06, 158.17, 155.50, 152.63, 151.78, 149.69, 144.60, 137.33, 132.94, 129.59, 125.94, 125.03, 124.85, 124.26, 119.11, 116.34, 115.76, 51.33, 49.92. MALDI-TOF: m/z 385 (M+H) $^+$, 407 (M+Na) $^+$.

4-[4-(4-Nitrophenyl)piperazin-1-yl]-2-(pyridin-2-yl)quinazoline (11g)

Yield: 81%. t_R : 8.400 min. (99.1%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.87 (d, J = 4.4 Hz, 1H), 8.57 (t, J = 10.4 Hz, 1H), 8.19 (m, 2H), 7.97 (t, J = 10.3 Hz, 1H), 7.81 (m, 2H), 7.51 (m, 1H), 7.38 (m, 1H), 7.27 (m, 1H), 6.85 (m, 2H), 4.08 (m, 4H), 3.70 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.91, 158.48, 155.76, 154.68, 153.06, 150.35, 138.97, 136.97,

133.05, 130.35, 126.20, 124.70, 124.61, 124.06, 115.83, 112.72, 49.12, 46.78. MALDI-TOF: m/z 413 (M+H)⁺, 435 (M+Na)⁺.

1-(4-{4-[2-(Pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}phenyl)methanone (11h)

Yield: 81%. t_R : 7.773 min. (98.3%). ¹H NMR (400 MHz, CDCl₃): δ 8.87 (d, J = 4.0 Hz, 1H), 8.58 (d, J = 7.6 Hz, 1H), 8.21 (d, J = 8.4 Hz, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.91 (app d, J = 9.2 Hz, 2H), 7.83 (app t, J = 8.0 Hz, 1H), 7.76 (app t, J = 8.2 Hz, 1H), 7.50 (app t, J = 8.0 Hz, 1H), 7.37 (m, 1H), 6.91 (app d, J = 8.8 Hz, 2H), 4.05 (m, 4H), 3.63 (m, 4H), 2.53 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 196.76, 165.05, 158.48, 155.80, 153.97, 153.03, 150.31, 136.93, 132.94, 130.64, 130.27, 128.16, 126.10, 124.64, 124.60, 124.06, 115.89, 113.54, 49.40, 47.23, 26.38. MALDI-TOF: m/z 410 (M+H)⁺.

4-{4-[4-(Allyloxy)phenyl]piperazin-1-yl}-2-(pyridin-2-yl)quinazoline (11i)

This was prepared by displacement of the the chloro group on **7a** using a known 4-allyloxyphenylpiperazin which in turn was prepared in two steps starting from 4-hydroxypiperazine. Yield: 65% (over 3 steps). t_R : 9.987 min. (96.4%). ¹H NMR (400 MHz, CDCl₃): δ 8.65 (d, J = 1.2 Hz, 1H), 8.61 (d, J = 3.6 Hz, 1H), 8.36 (d, J = 3.5 Hz, 1H), 7.93 (m, 2H), 7.81 (m, 2H), 7.53 (m, 3H), 6.84 (m, 2H), 5.93 (m, 1H), 5.25 (dd, J = 16.1 & 10 Hz, 2H), 3.58 (m, 4H), 3.45 (m, 4H), 3.01 (m, 2H). ¹³C NMR (125 MHz, CD₃CN): δ 162.42, 154.48, 151.05, 150.66, 150.54, 150.09, 139.65, 136.25, 135.75, 129.50, 128.86, 128.20, 127.86, 124.20, 123.32, 120.00, 116.92, 70.43, 52.24, 51.91. MALDI-TOF: m/z 424 (M+H)⁺, 462 (M+K)⁺.

4-[4-(4-Methoxyphenyl)piperazin-1-yl]-2-(pyridin-2-yl)quinazoline (11j)

Yield: 90%. t_R : 10.133 min. (98.9%). ¹H NMR (400 MHz, CDCl₃): δ 8.89 (m, 1H), 8.6 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 7.98 (d, J = 7.6 Hz, 1H), 7.86 (m, 1H), 7.8 (m, 1H), 7.5 (m, 1H), 7.39 (m, 1H), 7.01 (m, 2H), 6.9 (m, 2H), 4.05 (m, 4H), 3.79 (s, 3H), 3.34 (m, 4H). ¹³C NMR (100 MHz, CD₃OD): δ 166.43, 159.50, 156.77, 156.07, 153.71, 150.66, 146.98, 138.95, 134.55, 129.83, 127.53, 126.79, 126.66, 125.58, 120.08, 116.97, 115.74, 56.23, 52.29, 51.14. MALDI-TOF: m/z 398 (M+H)⁺, 420 (M+Na)⁺.

2-(5-Chloropyridin-2-yl)-4-[4-(4-methoxyphenyl)piperazin-1-yl]quinazoline (11k)

This was synthesized starting from 4-chloro-2-cyanopyridine. Yield: 47% (over 3 steps). t_R : 9.000 min. (98.7%). ¹H NMR (400 MHz, acetone-d₆): δ 8.74 (d, J = 2.8 Hz, 1H), 8.62 (d, J = 8.8 Hz, 1H), 8.18 (d, J = 8.8 Hz, 1H), 8.01 (d, J = 2.8 Hz, 1H), 7.99 (m, 1H), 7.86 (app t, J = 7.2 Hz, 1H), 7.60 (app t, J = 7.2 Hz, 1H), 7.03 (app d, J = 9.2 Hz, 2H), 6.88 (app d, J = 9.2 Hz, 2H), 4.06 (m, 4H), 3.75 (s, 3H), 3.64 (m, 4H). ¹³C NMR (125 MHz, CD₃OD): δ 166.43, 158.51, 156.12, 149.38, 146.93, 138.39, 134.50, 129.72, 127.53, 126.70, 126.32, 120.11, 115.65, 56.10, 52.34, 51.07. MALDI-TOF: m/z 433 (M+H)⁺, 455 (M+Na)⁺, 494 (M+K)⁺.

2-(5-Bromopyridin-2-yl)-4-[4-(4-methoxyphenyl)piperazin-1-yl]quinazoline (11l)

This was synthesized starting from 4-bromo-2-cyanopyridine. Yield: 53% (over 3 steps). t_R : 9.147 min. (98.6%). ¹H NMR (400 MHz, acetone-d₆): δ 8.84 (d, J = 2.4 Hz, 1H), 8.55 (d, J = 8.8 Hz, 1H), 8.16 (m, 2H), 7.99 (dd, J = 7.2 & 2.3 Hz, 1H), 7.86 (dt, J = 8.8 & 2.4 Hz, 1H), 7.60 (dt, J = 8.8 & 2.4 Hz, 1H), 7.02 (dd, J = 7.2 & 2.4 Hz, 2H), 6.88 (dd, J = 6.8 & 2.0 Hz, 2H), 4.06 (m, 2H), 3.75 (s, 3H), 3.36 (m, 4H). ¹³C NMR (125 MHz, CD₃OD): δ 166.72, 157.12, 151.38, 145.91, 136.31, 134.03, 129.67, 127.11, 126.51, 126.02, 119.66, 115.19, 62.64, 51.91, 50.62. MALDI-TOF: m/z 476 (M+H)⁺, 500 (M+Na)⁺.

4-[4-(3,4-Dichlorophenyl)piperazin-1-yl]-2-(pyridin-2-yl)quinazoline (11m)

Yield: 91%. t_R : 9.987 min. (99.0%). ^1H NMR (500 MHz, $CDCl_3$): δ 3.32 (m, 4H), 3.91 (m, 4H), 6.68 (dd, J = 9, 2.9 Hz, 1H), 6.92 (d, J = 2.84 Hz, 1H), 7.21 (d, J = 8.3 Hz, 1H), 7.30 (ddd, J = 7.3, 4.7, & 1.2 Hz, 1H), 7.41 (td, J = 8.6 & 1.2 Hz, 1H), 7.70 (td, J = 8.4 & 1.4 Hz, 1H), 7.78 (ddd, J = 8.27, 7.7, & 1.8 Hz, 1H), 7.86 (dd, J = 8.4, & 0.9 Hz), 8.16 (d, J = 8.5 Hz, 1H), 8.51 (pair of t's, J = 1.0 Hz, 1H), 8.82 (dq, J = 4.7 & 0.8 Hz, ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.01, 158.41, 155.77, 152.94, 150.45, 136.84, 132.95, 132.85, 130.65, 130.17, 126.04, 124.63, 124.03, 122.59, 117.34, 115.45, 49.48, 48.56. MALDI-TOF: m/z 437 (M+H)⁺.

4-[4-(4-Methylbenzenesulfonyl)piperazin-1-yl]-2-(pyridin-2-yl)quinazoline (13)

Yield: 77%. t_R : 8.547 min. (98.4%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.83 (m, 1H), 8.55 (d, J = 8.2 Hz, 1H), 8.21 (d, J = 8.2 Hz, 1H), 7.84 (m, 1H), 7.63 (m, 4H), 7.29 (m, 4H), 3.97 (m, 4H), 3.21 (m, 4H), 2.41 (s, 3H). ^{13}C NMR (125 MHz, CD_3CN): δ 166.36, 159.74, 157.16, 153.85, 150.94, 145.75, 145.55, 138.17, 134.44, 133.98, 133.84, 131.28, 129.26, 127.49, 126.55, 125.26, 116.97, 51.40, 50.37, 47.61, 47.34, 21.99. MALDI-TOF: m/z 446 (M+H)⁺, 468 (M+Na)⁺.

N-[4-(2S)-1-Oxo-3-phenyl-1-{4-[2-(pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}propan-2-yl]acetamide (14)

This was prepared by coupling Ac-Phe-OH to 4-(piperazin-1-yl)-2-(pyridin-2-yl)quinazoline using HBTU and DIEA. Yield: 67%. t_R : 7.080 min. (98.8%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.89 (d, J = 4.0 Hz, 1H), 8.79 (d, J = 8.0 Hz, 1H), 8.40 (d, J = 8.4 Hz, 1H), 8.36 (d, J = 8.4 Hz, 1H), 8.25 (t, J = 6.0 Hz, 1H), 8.14 (t, J = 7.2 Hz, 1H), 7.84 (m, 2H), 7.50 (d, J = 7.6 Hz, 1H), 7.28 (m, 5H), 7.21 (m, 1H), 5.11 (dt, J = 8.0 & 6.8 Hz, 1H), 3.90 (m, 4H), 3.70 (m, 4H), 2.93 (d, J = 6.8 Hz, 2H), 1.91 (s, 3H). ^{13}C NMR (125 MHz, CD_3CN): δ 171.83, 170.74, 164.59, 153.90, 151.04, 148.20, 140.30, 138.62, 137.75, 130.97, 129.84, 128.96, 128.23, 127.18, 125.38, 113.84, 51.65, 45.29, 42.19, 39.67, 23.25. MALDI-TOF: m/z 481 (M+H)⁺, 503 (M+Na)⁺.

N-[4-(2-Oxo-2-{4-[2-(pyridin-2-yl)quinazolin-4-yl]piperazin-1-yl}ethyl)thiazol-2-yl]acetamide (15)

This was prepared by coupling 2-(2-acetamidothiazol-4-yl)acetic acid to 4-(piperazin-1-yl)-2-(pyridin-2-yl)quinazoline using HBTU and DIEA. Yield: 71%. t_R : 7.160 min. (95.9%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.58 (d, J = 4.4 Hz, 1H), 8.67 (d, J = 7.6 Hz, 1H), 8.26 (d, J = 8.4 Hz, 1H), 8.12 (m, 1H), 8.01 (t, J = 6.8 Hz, 1H), 7.73 (m, 3H), 6.87 (m, 2H), 4.87 (s, 2H), 3.65 (m, 4H), 3.31 (m, 4H), 2.18 (s, 3H). ^{13}C NMR (125 MHz, CD_3CN): δ 169.87, 164.67, 159.28, 153.93, 151.04, 148.24, 146.27, 142.25, 140.28, 137.73, 130.00, 129.90, 128.96, 125.39, 121.59, 113.86, 111.33, 45.99, 42.25, 37.76, 23.58. MALDI-TOF: m/z 474 (M+H)⁺, 496 (M+Na)⁺.

Materials

30% acrylamide/bis solution (37.5:1), ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) for SDS-PAGE was purchased from Bio-Rad Laboratories (Hercules, CA). $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $NiCl_2 \cdot 6H_2O$ and $CuCl_2 \cdot 2H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO). $CaCl_2 \cdot 2H_2O$ was purchased from Fisher Scientific (Hampton, NH). $MgCl_2 \cdot 6H_2O$ was purchased from Avantor Performance Materials (Center Valley, PA). Restriction enzymes were purchased from New England BioLabs (Ipswich, MA).

Cell Lines and Cell Culture

Human umbilical vein endothelial cells (HUVEC) (Lonza, Allendale, NJ) were grown in endothelial cell growth medium-2 (EGM-2) using the EGM-2 bullet kit (Lonza) per manufacturer's instructions. Normal human fibroblasts-adult (HDFa) (Lifeline Cell Technology, Frederick, MD) were grown in FibroLife-S2 cell growth medium using the FibroLife-S2 LifeFactors kit (Lifeline Cell Technology) per manufacturer's instructions. The phenotypes of HUVEC and normal HDFa were verified by the manufacturers based on morphological observation. Jurkat E6-1 human acute T cell leukemia lymphocytes (ATCC TIB-152, Manassas, Virginia), PC-3 human prostate adenocarcinoma epithelial cells (ATCC CRL-1435) and MCF-7 human breast adenocarcinoma epithelial cells (ATCC HTB-22) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 100 units/mL penicillin plus 100 μ g/mL streptomycin (Life Technologies). HeLa human cervical adenocarcinoma epithelial cells (ATCC CCL-2) and HEK293T human embryonic kidney epithelial cells expressing SV40 T antigen (ATCC CRL-11268) were grown in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Life Technologies), supplemented with 10% FBS and 100 units/mL penicillin plus 100 μ g/mL streptomycin. The genotypes of the five transformed cell lines were verified by ATCC. All cell lines were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. High Five (BTI-TN-5B1-4) cells (Life Technologies) used to overexpress human MetAP2 were grown in Grace's insect cell medium (Life Technologies) supplemented with 10% FBS and were maintained in a nonhumidified incubator at 27°C as per manufacturer's instructions.

Primary and Secondary Antibodies

Mouse anti-human unprocessed 14-3-3 γ (iMet 14-3-3 γ) monoclonal antibody (mAb) (NB100-407) was purchased from Novus Biologicals (Littleton, CO). Rabbit anti-human total 14-3-3 γ mAb (5522) was purchased from Cell Signaling (Danvers, MA). Mouse anti-human MetAP1 mAb (MAB3537) was purchased from R&D Systems (Minneapolis, MN). Mouse anti-human MetAP2 mAb was generated as described previously.²¹ Mouse anti-human α -tubulin mAb (sc-5286), goat anti-human GAPDH pAb (sc-20357) and goat anti-mouse IgM antibody conjugated with Horseradish Peroxidase (HRP) (sc-2064) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human β -actin (JLA20) mAb was purchased from Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa (Iowa City, IA). Sheep anti-mouse IgG antibody conjugated HRP (NA931V) and donkey anti-rabbit IgG antibody conjugated with HRP (NA934V) was purchased from GE Healthcare (Pittsburgh, PA).

Transfection of HUVEC with siRNAs against MetAP1 and MetAP2

A duplex oligonucleotides encoding siRNA against human MetAP1, a siRNA against human MetAP2 and a scrambled control siRNA were purchased from Qiagen (Valencia, CA). The sequences of the sense strands of the siRNAs were as follows: MetAP1 siRNA, 5'-GGCCAGUGCCAAGUUAUUAU-dTdT-3', targeting the bases 290-308 in the ORF of MetAP1 mRNA; MetAP2 siRNA, 5'-GACUGUUCACGCAAGUUAU-dTdT-3', targeting the bases 604-622 in the ORF of MetAP2 mRNA; and the scrambled control siRNA, 5'-UUCUCCGAACGUGUCACGU-dTdT-3'. 7.5×10^4 HUVEC were seed into one well of 6-well plates with 2 mL EGM-2 growth medium. Cells were allowed to adhere at 37°C overnight. Next day, cells were transfected with scrambled control siRNA (20 and 100 nM), MetAP1 siRNA (20 and 100 nM) or MetAP2 siRNA (20 nM) diluted in serum free EBM-2 basal medium (Lonza) using either HiPerFect (Qiagen) or *TransIT*-TKO (Mirus Bio, Madison, WI) transfection reagent per manufacturer's instructions. 48 hours after transfection, cell lysates were prepared and boiled in 1 $\times$ Laemmli buffer for immunoblot analysis.

[³H]-Thymidine Incorporation Assay

To determine the IC₅₀ values of the compounds against cell proliferation, seven different concentrations and one vehicle (DMSO) control were used for each compound. HUVEC (2,500 cells/well), HeLa cells (3,000 cells/well) or HDFa (2,000 cells/well) were seeded into 96-well plates with 190 μ L corresponding growth medium. Cells were allowed to adhere at 37°C overnight. Next day, serial dilutions of the compounds in vehicle (DMSO) or vehicle itself were diluted 50 times with the growth medium before they were added to the cells. Final concentration of DMSO never exceeded 0.1%. The cells were treated with DMSO or the compounds for 24 hours. Cells were pulsed with 1 μ Ci [³H]-thymidine (5 Ci per mmol) (American Radiolabeled Chemicals, St. Louis, MO) for 6 hours, and then treated with trypsin-EDTA (Life Technologies). The suspended cells were harvested onto Filtermat A glass fiber filters (PerkinElmer, Waltham, MA) and washed at least 7 times with dH₂O using a Harvester 96 cell harvester (Tomtec, Hamden, CT). The glass fiber filters were dried in a ventilated hood overnight. Next day, the [³H]-thymidine counts on the glass fiber filters were measured using a MicroBeta plate reader (PerkinElmer) with Betaplate Scint Wallac HiSafe cocktail (PerkinElmer).

ProIP-coupled MetAP Activity Assay

This assay was performed as described.⁴⁵ In brief, *HsMetAP1* (0.15 μ M) or *HsMetAP2* (0.1 μ M) was incubated with either vehicle (1% DMSO) or test compounds in MetAP assay buffer (100 mM NaCl, 40 mM HEPES, pH 7.5) containing 15 U/mL *BcProIP* and 10 μ M CoCl₂ or MnCl₂ at room temperature for 20 minutes. The enzymatic reaction was initiated by the addition of 600 μ M Met-Pro-*pNA*. Absorbance at 405 nm ($\epsilon = 1.06 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) was recorded every 30 seconds within the first 30 minutes of reaction by the FLUOstar OPTIMA microplate reader. The initial rate was then calculated from the early linear portion (usually between 2 and 15 minutes after the reaction started) of the curve when absorbance values were plotted versus the reaction time. In addition, to rule out the possibility that any inhibitor found by the *BcProIP*-coupled MetAP assay might work simply because it inhibited the coupling enzyme, compounds showing inhibitory effects were counter screened against 0.1 U/mL *BcProIP*, 600 μ M Pro-*pNA* and 10 μ M CoCl₂ or MnCl₂ in the MetAP assay buffer.

LAO and HRP-coupled MetAP Activity Assay

This assay was performed as described.⁵⁴ MetAP activity was measured by continuously monitoring the production of free L-methionine with L-amino acid oxidase (LAO, from Sigma) and horseradish peroxidase (HRP, from Sigma). The lyophilized powder of HRP was reconstituted in MetAP assay buffer to 5 mg/mL. The substrate Met-Ala-Ser (Sigma) and *o*-dianisidine (3,3'-dimethoxybenzidine, from Sigma) were dissolved in ddH₂O. The test compounds were dissolved in 100% DMSO. The total reaction volume was 100 μ L and the reaction was carried out in 96-well plates at room temperature. 0.15 μ M *HsMetAP1* was incubated with either vehicle (DMSO) or test compounds in MetAP assay buffer containing 1 U/mL LAO, 0.1 mg/mL HRP, 0.2 mg/mL *o*-dianisidine and 1 μ M ZnCl₂ at room temperature for 20 minutes. The enzymatic reaction was initiated by the addition of 600 μ M Met-Ala-Ser. Absorbance at 450 nm ($\epsilon = 1.53 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) was recorded every 30 seconds within the first 30 minutes of reaction by the FLUOstar OPTIMA microplate reader. After subtracting the background hydrolysis determined in the absence of MetAP, the net increases in the absorbance were plotted versus the reaction time and the slope of the early linear portion (usually between 2 minutes and 20 minutes after the reaction started) of the curve was determined by the function "linear regression analysis" in GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The initial rate was then calculated from the slope.

Metal Activation Assay

All buffer solutions, ddH₂O (used to dissolve and dilute metals), substrates and enzymes (except *HsMetAP1*) in the metal activation assays were pretreated with Chelex 100 resin (Bio-Rad). Except (1) the indicated concentration of hydrochloride salts of divalent metals were used in the metal activation assays, instead of fixed concentrations of CoCl₂, MnCl₂ or ZnCl₂ and (2) there was no incubation with vehicle or compounds, anything else in the metal activation assays were the same as ProIP-coupled or LAO and HRP-coupled MetAP activity assays. The initial rate was calculated from the slope of the early linear portion of the enzymatic reaction curve and expressed as $\mu\text{mol/L}$ of methionine release per minute per $\mu\text{mol/L}$ of human MetAP1.

Initiator Methionine Release Assay

This assay was performed as described.⁵⁴ In brief, HEK293T cells were transiently transfected with Superfect transfection reagent (Qiagen) and the appropriate plasmid DNA (p3×FLAG-CMV-14-BTF3L4 or p3×FLAG-CMV-14-TXNL1). 19.5 hours after transfection, cells were treated with either vehicle (DMSO), **11j**, TNP-470 or the combination of the latter two. 30 minutes later, the cells were incubated with 0.2 mCi of [³⁵S]-methionine (PerkinElmer) for 4 hours. BTF3L4-3×Flag or TXNL1-3×Flag in the whole cell extract of HEK293T cells was immunoprecipitated by anti-Flag-conjugated resin (Sigma), washed extensively, resuspended in MetAP assay buffer and split into two equal aliquots (A and B). A was incubated with 50 μL mixture of recombinant *HsMetAP1* and *HsMetAP2* (5 μM each) in MetAP assay buffer and B was incubated with the buffer only. After incubation overnight (~16 h) at room temperature, the [³⁵S]-methionine released from the resin into the solution was determined by scintillation counting (scintillation fluid, from PerkinElmer) on a 1450 MicroBeta apparatus (Wallac) for both A and B. The percentage of [³⁵S]-methionine release was calculated from the ratio of counts in the assay buffer to the sum of these counts and the counts from the corresponding resin sample. The percentage of [³⁵S]-methionine release in B was subtracted from that in A, and the resulting numbers in inhibitor-treated groups were normalized to the number in the corresponding vehicle control.

Statistical Analysis

The statistical significance of the difference between two experimental groups was determined by two-tailed unpaired Student's *t*-test using GraphPad Prism 5 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kozak M. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *J Microbiol Rev.* 1983; 47:1–45. [PubMed: 6343825]

2. Waller JP. The NH₂-terminal residue of the proteins from cell-free extract of *E. coli*. *J Mol Biol.* 1963; 7:483–496. [PubMed: 14079588]
3. Bradshaw RA, Brickey WW, Walker KW. N-terminal processing: the methionine aminopeptidase and N α -acetyl transferase families. *Trends in Biochem Sci.* 1998; 23:263–267. [PubMed: 9697417]
4. (a) Meinnel T, Mechulam Y, Blanquet S. Methionine as translation start signal: A review of the enzymes of the pathway in *Escherichia coli*. *Biochimie.* 1993; 75:1061–1075. [PubMed: 8199241] (b) Solbiati J, Chapman-Smith A, Miller JL, Miller CG, Cronan JE Jr. Processing of the N termini of nascent polypeptide chains requires deformylation prior to methionine removal. *J Mol Biol.* 1999; 290:607–614. [PubMed: 10395817] (c) Bingel-Erlenmeyer R, Kohler R, Kramer G, Sandkci A, Antolice S, Maier T, Schaffitzel C, Wiedmann B, Bukau B, Ban N. A peptide deformylase-ribosome complex reveals mechanism of nascent chain processing. *Nature.* 2008; 452:108–111. [PubMed: 18288106]
5. (a) D'souza VM, Bennett B, Copik AJ, Holz RC. Divalent metal binding properties of the methionyl aminopeptidase from *Escherichia coli*. *Biochemistry.* 2000; 39:3817–3826. [PubMed: 10736182] (b) D'souza VM, Swierczek SI, Cosper NJ, Meng L, Ruebush S, Copik AJ, Scott RA, Holz RC. Kinetic and structural characterization of manganese(II)-loaded methionyl aminopeptidases. *Biochemistry.* 2002; 41:13096–13105. [PubMed: 12390038] (c) Ye QZ, Xie SX, Ma ZQ, Huang M, Hanzlik RP. Structural basis of catalysis by monometalated methionine aminopeptidase. *Proc Natl Acad Sci U S A.* 2006; 103:9470–9475. [PubMed: 16769889] (d) Chai SC, Ye QZ. Analysis of the stoichiometric metal activation of methionine aminopeptidase. *BMC Biochem.* 2009; 10:32. [PubMed: 20017927] (e) Chai SC, Lu JP, Ye QZ. Determination of binding affinity of metal cofactor to the active site of methionine aminopeptidase based on quantitation of functional enzyme. *Anal Biochem.* 2009; 395:263–264. [PubMed: 19712663]
6. (a) Flinta C, Persson B, Jornvall H, von Heijne G. Sequence determinants of cytosolic N-terminal protein processing. *Eur J Biochem.* 1986; 154:193–196. [PubMed: 3080313] (b) Hirel PH, Schmitter JM, Dessen P, Fayat G, Blanquet S. Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci USA.* 1989; 86:8247–8251. [PubMed: 2682640] (c) Chang YH, Teicher U, Smith JA. Purification and characterization of a methionine aminopeptidase from *Saccharomyces cerevisiae*. *J Biol Chem.* 1990; 265:19892–19897. [PubMed: 2246265] (d) Yang G, Kirkpatrick RB, Ho T, Zhang GF, Liang PH, Johanson KO, Casper DJ, Doyle ML, Marino JP Jr, Thompson SK, Chen W, Tew DG, Meek TD. Steady-state kinetic characterization of substrates and metal-ion specificities of the full-length and N-terminally truncated recombinant human methionine aminopeptidases (type 2). *Biochemistry.* 2001; 40:10645–10654. [PubMed: 11524009] (e) Frottin F, Martinez A, Peynot P, Mitra S, Holz RC, Giglione C, Meinnel T. The proteomics of N-terminal methionine cleavage. *Mol Cell Proteomics.* 2006; 5:2336–2349. [PubMed: 16963780]
7. Xiao Q, Zhang F, Nacev BA, Liu JO, Pei D. Protein N-terminal processing: substrate specificity of *Escherichia coli* and human methionine aminopeptidases. *Biochemistry.* 2010; 49:5588–5599. [PubMed: 20521764]
8. (a) Chang SY, McGary EC, Chang S. Methionine aminopeptidase gene of *Escherichia coli* is essential for cell growth. *J Bacteriol.* 1989; 171:4071–4072. [PubMed: 2544569] (b) Miller CG, Kukral AM, Miller JL, Movva NR. *pepM* is an essential gene in *Salmonella typhimurium*. *J Bacteriol.* 1989; 171:5215–5217. [PubMed: 2670909]
9. Li X, Chang YH. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. *Proc Natl Acad Sci U S A.* 1995; 92:12357–12361. [PubMed: 8618900]
10. Arfin SM, Kendall RL, Hall L, Weaver LH, Stewart AE, Matthews BW, Bradshaw RA. Eukaryotic methionyl aminopeptidases: two classes of cobalt-dependent enzymes. *Proc Natl Acad Sci U S A.* 1995; 92:7714–7718. [PubMed: 7644482]
11. Addlagatta A, Hu X, Liu JO, Matthews BW. Structural basis for the functional differences between type I and type II human methionine aminopeptidases. *Biochemistry.* 2005; 44:14741–14749. [PubMed: 16274222]
12. Sriram SM, Kim BY, Kwon YT. The N-end rule pathway: emerging functions and molecular principles of substrate recognition. *Nat Rev Mol Cell Biol.* 2011; 12:735–747. [PubMed: 22016057]

13. Meinnel T, Peynot P, Giglione C. Processed N-termini of mature proteins in higher eukaryotes and their major contribution to dynamic proteomics. *Biochimie*. 2005; 87:701–712. [PubMed: 16054524]
14. Hwang CS, Shemorry A, Varshavsky A. N-terminal acetylation of cellular proteins creates specific degradation signals. *Science*. 2010; 327:973–977. [PubMed: 20110468]
15. (a) Hu X, Dang Y, Tenney K, Crews P, Tsai CW, Sixt KM, Cole PA, Liu JO. Regulation of c-Src nonreceptor tyrosine kinase activity by bengamide A through inhibition of methionine aminopeptidases. *Chem Biol*. 2007; 14:764–74. [PubMed: 17656313] (b) Carr SA, Biemann K, Shoji S, Parmelee DC, Titani K. n-Tetradecanoyl is the NH₂-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. *Proc Natl Acad Sci U S A*. 1982; 79:6128–6131. [PubMed: 6959104] (c) Buss JE, Mumby SM, Casey PJ, Gilman AG, Sefton BM. Myristoylated α subunits of guanine nucleotide-binding regulatory proteins. *Proc Natl Acad Sci U S A*. 1987; 84:7493–7497. [PubMed: 3118369]
16. (a) Dummitt B, Micka WS, Chang YH. Yeast glutamine-fructose-6-phosphate aminotransferase (Gfa1) requires methionine aminopeptidase activity for proper function. *J Biol Chem*. 2005; 280:14356–14360. [PubMed: 15699032] (b) Löwe J, Stock D, Jap B, Zwickl P, Baumeister W, Huber R. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science*. 1995; 268:533–539. [PubMed: 7725097]
17. Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA 3rd, Smith HO, Venter JC. Essential genes of a minimal bacterium. *Proc Natl Acad Sci U S A*. 2006; 103:425–430. [PubMed: 16407165]
18. Ross S, Giglione C, Pierre M, Espagne C, Meinnel T. Functional and developmental impact of cytosolic protein N-terminal methionine excision in *Arabidopsis*. *Plant Physiol*. 2005; 137:623–637. [PubMed: 15681659]
19. (a) Boxem M, Tsai CW, Zhang Y, Saito RM, Liu JO. The *C. elegans* methionine aminopeptidase 2 analog *map-2* is required for germ cell proliferation. *FEBS Lett*. 2004; 576:245–250. [PubMed: 15474045] (b) Cutforth T, Gaul U. A methionine aminopeptidase and putative regulator of translation initiation is required for cell growth and patterning in *Drosophila*. *Mech Dev*. 1999; 82:23–28. [PubMed: 10354468] (c) Zhang Y, Yeh JR, Mara A, Ju R, Hines JF, Cirone P, Griesbach HL, Schneider I, Slusarski DC, Holley SA, Crews CM. A chemical and genetic approach to the mode of action of fumagillin. *Chem Biol*. 2006; 13:1001–1009. [PubMed: 16984890] (d) Ma AC, Fung TK, Lin RH, Chung MI, Yang D, Ekker SC, Leung AY. Methionine aminopeptidase 2 is required for HSC initiation and proliferation. *Blood*. 2011; 118:5448–5457. [PubMed: 21937698] (e) Wernert N, Stanjek A, Kiriakidis S, Hügel A, Jha HC, Mazitschek R, Giannis A. Inhibition of Angiogenesis In Vivo by *ets-1* Antisense Oligonucleotides-Inhibition of Ets-1 Transcription Factor Expression by the Antibiotic Fumagillin. *Angew Chem Int Ed Engl*. 1999; 38:3228–3231. [PubMed: 10556911] (f) Yeh JR, Ju R, Brdlik CM, Zhang W, Zhang Y, Matyskiela ME, Shotwell JD, Crews CM. Targeted gene disruption of methionine aminopeptidase 2 results in an embryonic gastrulation defect and endothelial cell growth arrest. *Proc Natl Acad Sci U S A*. 2006; 103:10379–10384. [PubMed: 16790550]
20. Bernier SG, Taghizadeh N, Thompson CD, Westlin WF, Hannig G. Methionine aminopeptidases type I and type II are essential to control cell proliferation. *J Cell Biochem*. 2005; 95:1191–1203. [PubMed: 15962312]
21. Hu X, Addlagatta A, Lu J, Matthews BW, Liu JO. Elucidation of the function of type 1 human methionine aminopeptidase during cell cycle progression. *Proc Natl Acad Sci U S A*. 2006; 103:18148–18153. [PubMed: 17114291]
22. Quinoa E, Adamczeski M, Bakus GJ, Crews P. Bengamides, heterocyclic anthelmintics from a *Jaspidae* marine sponge. *J Org Chem*. 1986; 51:4494–4497.
23. Thale Z, Kinder FR, Bair KW, Bontempo J, Czuchta AM, Versace RW, Phillips PE, Sanders ML, Wattanasin S, Crews P. Bengamides revisited: new structures and antitumor studies. *J Org Chem*. 2001; 66:1733–1741. [PubMed: 11262120]
24. Kinder FR Jr, Versace RW, Bair KW, Bontempo JM, Cesarz D, Chen S, Crews P, Czuchta AM, Jagoe CT, Mou Y, Nemzek R, Phillips PE, Tran LD, Wang RM, Weltchek S, Zabudoff S. Synthesis and antitumor activity of ester-modified analogues of bengamide B. *J Med Chem*. 2001; 44:3692–3699. [PubMed: 11606134]

25. Towbin H, Bair KW, DeCaprio JA, Eck MJ, Kim S, Kinder FR, Morollo A, Mueller DR, Schindler P, Song HK, van Oostrum J, Versace RW, Voshol H, Wood J, Zabudoff S, Phillips PE. Proteomics-based target identification: bengamides as a new class of methionine aminopeptidase inhibitors. *J Biol Chem*. 2003; 278:52964–52971. [PubMed: 14534293]
26. Dumez H, Gall H, Capdeville R, Dutreix C, van Oosterom AT, Giaccone G. A phase I and pharmacokinetic study of LAF389 administered to patients with advanced cancer. *Anticancer Drugs*. 2007; 18:219–225. [PubMed: 17159608]
27. Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature*. 1990; 348:555–557. [PubMed: 1701033]
28. Kusaka M, Sudo K, Fujita T, Marui S, Itoh F, Ingber D, Folkman J. Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem Biophys Res Commun*. 1991; 174:1070–1076. [PubMed: 1705118]
29. (a) Griffith EC, Su Z, Turk BE, Chen S, Chang YH, Wu Z, Biemann K, Liu JO. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. *Chem Biol*. 1997; 4:461–471. [PubMed: 9224570] (b) Sin N, Meng L, Wang MQ, Wen JJ, Bornmann WG, Crews CM. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc Natl Acad Sci U S A*. 1997; 94:6099–6103. [PubMed: 9177176]
30. Griffith EC, Su Z, Niwayama S, Ramsay CA, Chang YH, Liu JO. Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc Natl Acad Sci U S A*. 1998; 95:15183–15188. [PubMed: 9860943]
31. Liu S, Widom J, Kemp CW, Crews CM, Clardy J. Structure of human methionine aminopeptidase-2 complexed with fumagillin. *Science*. 1998; 282:1324–1327. [PubMed: 9812898]
32. Turk BE, Griffith EC, Wolf S, Biemann K, Chang YH, Liu JO. Selective inhibition of amino-terminal methionine processing by TNP-470 and ovalicin in endothelial cells. *Chem Biol*. 1999; 6:823–833. [PubMed: 10574784]
33. Wang J, Lou P, Henkin J. Selective inhibition of endothelial cell proliferation by fumagillin is not due to differential expression of methionine aminopeptidases. *J Cell Biochem*. 2000; 77:465–473. [PubMed: 10760954]
34. Gervaz P, Fontollet C. Therapeutic potential of the anti-angiogenesis drug TNP-470. *Int J Exp Pathol*. 1998; 79:359–362. [PubMed: 10319017]
35. Kruger EA, Figg WD. TNP-470: an angiogenesis inhibitor in clinical development for cancer. *Expert Opin Investig Drugs*. 2000; 9:1383–1396.
36. Brahn E, Schoettler N, Lee S, Banquerigo ML. Involution of collagen-induced arthritis with an angiogenesis inhibitor, PPI-2458. *J Pharmacol Exp Ther*. 2009; 329:615–624. [PubMed: 19218530]
37. Lijnen HR, Frederix L, Van Hoef B. Fumagillin reduces adipose tissue formation in murine models of nutritionally induced obesity. *Obesity*. 2010; 18:2241–2246. [PubMed: 20094042]
38. Kass DJ, Rattigan E, Kahloon R, Loh K, Yu L, Savir A, Markowski M, Saqi A, Rajkumar R, Ahmad F, Champion HC. Early treatment with fumagillin, an inhibitor of methionine aminopeptidase-2, prevents Pulmonary Hypertension in monocrotaline-injured rats. *PLoS One*. 2012; 7:e35388. [PubMed: 22509410]
39. (a) Zhang Y, Griffith EC, Sage J, Jacks T, Liu JO. Cell cycle inhibition by the anti-angiogenic agent TNP-470 is mediated by p53 and p21WAF1/CIP1. *Proc Natl Acad Sci U S A*. 2000; 97:6427–6432. [PubMed: 10841547] (b) Yeh JR, Mohan R, Crews CM. The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. *Proc Natl Acad Sci U S A*. 2000; 97:12782–12787. [PubMed: 11070090] (c) Zhang Y, Yeh JR, Mara A, Ju R, Hines JF, Cirone P, Griesbach HL, Schneider I, Slusarski DC, Holley SA, Crews CM. A chemical and genetic approach to the mode of action of fumagillin. *Chem Biol*. 2006; 13:1001–1009. [PubMed: 16984890] (d) Sundberg TB, Darricarrere N, Cirone P, Li X, McDonald L, Mei X, Westlake CJ, Slusarski DC, Beynon RJ, Crews CM. Disruption of Wnt planar cell polarity signaling by aberrant accumulation of the MetAP-2 substrate Rab37. *Chem Biol*. 2011; 18:1300–1311. [PubMed: 22035799] (e) Yeh JR, Ju R, Brdlik CM, Zhang W, Zhang Y, Matyskiela ME, Shotwell JD, Crews

- CM. Targeted gene disruption of methionine aminopeptidase 2 results in an embryonic gastrulation defect and endothelial cell growth arrest. *Proc Natl Acad Sci U S A*. 2006; 103:10379–10384. [PubMed: 16790550] (f) Ma AC, Fung TK, Lin RH, Chung MI, Yang D, Ekker SC, Leung AY. Methionine aminopeptidase 2 is required for HSC initiation and proliferation. *Blood*. 2011; 118:5448–5457. [PubMed: 21937698]
40. Hu X, Zhu J, Srivathsan S, Pei D. Peptidyl hydroxamic acids as methionine aminopeptidase inhibitors. *Bioorg Med Chem Lett*. 2004; 14:77–79. [PubMed: 14684302]
  41. Haldar MK, Scott MD, Sule N, Srivastava DK, Mallik S. Synthesis of barbiturate-based methionine aminopeptidase-I inhibitors. *Bioorg Med Chem Lett*. 2008; 18:2373–2376. [PubMed: 18343108]
  42. Luo QL, Li JY, Liu ZY, Chen LL, Li J, Qian Z, Shen Q, Li Y, Lushington GH, Ye QZ, Nan FJ. Discovery and structural modification of inhibitors of methionine aminopeptidases from *Escherichia coli* and *Saccharomyces cerevisiae*. *J Med Chem*. 2003; 46:2631–2640. [PubMed: 12801227]
  43. Li JY, Chen LL, Cui YM, Luo QL, Gu M, Nan FJ, Ye QZ. Characterization of full length and truncated type I human methionine aminopeptidases expressed from *Escherichia coli*. *Biochemistry*. 2004; 43:7892–7898. [PubMed: 15196033]
  44. Hu X, Addlagatta A, Matthews BW, Liu JO. Identification of pyridinylpyrimidines as inhibitors of human methionine aminopeptidases. *Angew Chem Int Ed Engl*. 2006; 45:3772–3775. [PubMed: 16724298]
  45. Zhang P, Yang X, Zhang F, Gabelli SB, Wang R, Zhang Y, Bhat S, Chen X, Furlani M, Amzel LM, Liu JO, Ma D. Pyridinylpyrimidines selectively inhibit human methionine aminopeptidase-*Bioorg. Med Chem*. 2013 Online early access. 10.1016/j.bmc.2013.02.023
  46. (a) Douangamath A, Dale GE, D'Arcy A, Almstetter M, Eckl R, Frutos-Hoener A, Henkel B, Illgen K, Nerdinger S, Schulz H, Mac Sweeney A, Thormann M, Trembl A, Pierau S, Wadman S, Oefner C. Crystal structures of *Staphylococcus aureus* methionine aminopeptidase complexed with keto heterocycle and aminoketone inhibitors reveal the formation of a tetrahedral intermediate. *J Med Chem*. 2004; 47:1325–1328. [PubMed: 14998322] (b) Schiffmann R, Heine A, Klebe G, Klein CD. Metal ions as cofactors for the binding of inhibitors to methionine aminopeptidase: a critical view of the relevance of in vitro metalloenzyme assays. *Angew Chem Int Ed Engl*. 2005; 44:3620–3623. [PubMed: 15880695] (c) Xie SX, Huang WJ, Ma ZQ, Huang M, Hanzlik RP, Ye QZ. Structural analysis of metalloform-selective inhibition of methionine aminopeptidase. *Acta Crystallogr, Sect D*. 2006; 62:425–432. [PubMed: 16552144] (d) Huang M, Xie SX, Ma ZQ, Hanzlik RP, Ye QZ. Metal mediated inhibition of methionine aminopeptidase by quinolinyl sulfonamides. *Biochem Biophys Res Commun*. 2006; 339:506–513. [PubMed: 16300729]
  47. Chai SC, Ye QZ. Metal-mediated inhibition is a viable approach for inhibiting cellular methionine aminopeptidase. *Bioorg Med Chem Lett*. 2009; 19:6862–6864. [PubMed: 19889537]
  48. Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, Ingber D, Folkman J. Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). *Br J Cancer*. 1994; 69:212–216. [PubMed: 8297716]
  49. Chai SC, Wang WL, Ye QZ. FE(II) is the native cofactor for *Escherichia coli* methionine aminopeptidase. *J Biol Chem*. 2008; 283:26879–26885. [PubMed: 18669631]
  50. Ye QZ, Xie SX, Huang M, Huang WJ, Lu JP, Ma ZQ. Metalloform-selective inhibitors of *Escherichia coli* methionine aminopeptidase and X-ray structure of a Mn(II)-form enzyme complexed with an inhibitor. *J Am Chem Soc*. 2004; 126:13940–13941. [PubMed: 15506752]
  51. Finney LA, O'Halloran TV. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science*. 2003; 300:931–936. [PubMed: 12738850]
  52. Walker KW, Bradshaw RA. Yeast methionine aminopeptidase I can utilize either Zn²⁺ or Co²⁺ as a cofactor: a case of mistaken identity? *Protein Sci*. 1998; 7:2684–2687. [PubMed: 9865965]
  53. (a) D'souza VM, Holz RC. The methionyl aminopeptidase from *Escherichia coli* can function as an iron(II) enzyme. *Biochemistry*. 1999; 38:11079–11085. [PubMed: 10460163] (b) Lu JP, Ye QZ. Expression and characterization of *Mycobacterium tuberculosis* methionine aminopeptidase type 1a. *Bioorg Med Chem Lett*. 2010; 20:2776–2779. [PubMed: 20363127] (c) Lu JP, Chai SC, Ye QZ. Catalysis and inhibition of *Mycobacterium tuberculosis* methionine aminopeptidase. *J Med Chem*. 2010; 53:1329–1337. [PubMed: 20038112]

54. Wang J, Sheppard GS, Lou P, Kawai M, Park C, Egan DA, Schneider A, Bouska J, Lesniewski R, Henkin J. Physiologically relevant metal cofactor for methionine aminopeptidase-2 is manganese. *Biochemistry*. 2003; 42:5035–5042. [PubMed: 12718546]
55. Holland DR, Hausrath AC, Juers D, Matthews BW. Structural analysis of zinc substitutions in the active site of thermolysin. *Protein Sci*. 1995; 4:1955–1965. [PubMed: 8535232]
56. Kitazono A, Yoshimoto T, Tsuru D. Cloning, sequencing, and high expression of the proline iminopeptidase gene from *Bacillus coagulans*. *J Bacteriol*. 1992; 174:7919–7925. [PubMed: 1459939]
57. Cha HJ, Byrom M, Mead PE, Ellington AD, Wallingford JB, Marcotte EM. Evolutionarily repurposed networks reveal the well-known antifungal drug thiabendazole to be a novel vascular disrupting agent. *PLoS Biol*. 2012; 10:e1001379. [PubMed: 22927795]
58. Schneider-Poetsch T, Ju J, Eyler DE, Dang Y, Bhat S, Merrick WC, Green R, Shen B, Liu JO. Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol*. 2010; 6:209–217. [PubMed: 20118940]
59. Nonato MC, Widom J, Clardy J. Human methionine aminopeptidase type 2 in complex with L- and D-methionine. *Bioorg Med Chem Lett*. 2006; 16:2580–2583. [PubMed: 16540317]
60. Mokrosz JL, Duszynska B, Charakchieva-Minol S, Bojarski AJ, Mokrosz MJ, Wydra RL, Janda L, Strekowski L. Structure-activity relationship studies of CNS agents. Part 29. N-Methylpiperazino-substituted derivatives of quinazoline, phthalazine and quinoline as novel $\alpha 1$, 5-HT_{1A} and 5-HT_{2A} receptor ligands. *Eur J Med Chem*. 1996; 31:973–980.
61. Arico-Muendel CC, Benjamin DR, Caiazzo TM, Centrella PA, Contonio BD, Cook CM, Doyle EG, Hannig G, Labenski MT, Searle LL, Lind K, Morgan BA, Olson G, Paradise CL, Self C, Skinner SR, Sluboski B, Svendsen JL, Thompson CD, Westlin W, White KF. Carbamate analogues of fumagillin as potent, targeted inhibitors of methionine aminopeptidase-2. *J Med Chem*. 2009; 52:8047–8056. [PubMed: 19929003]
62. Flanagan SP, Goddard R, Guiry PJ. The preparation and resolution of 2-(2-pyridyl)- and 2-(2-pyrazinyl)-Quinazolinap and their application in palladium-catalysed allylic substitution. *Tetrahedron*. 2005; 61:9808–9821.
63. Lee NK, Lee JW, Lee S, Im GJ, Han HY, Kim TK, Kim YH, Kwak WJ, Kim SW, Ha J. Quinazoline derivatives for the treatment and prevention of diabetes and obesity. *PCT Int Appl*. WO 2006/071095.

Abbreviations Used

BcProIP	<i>Bacillus coagulans</i> proline iminopeptidase
DMSO	dimethyl sulfoxide
EC₅₀	half maximal effective concentration
EcMetAP	<i>Escherichia coli</i> MetAP
HDFa	human dermal fibroblasts-adult
HRP	horseradish peroxidase
HsMetAP1	human cytosolic MetAP type 1
HsMetAP2	human cytosolic MetAP type 2
HUVEC	human umbilical vein endothelial cells
IC₅₀	half maximal inhibitory concentration (IC ₅₀ equals to EC ₅₀ when the bottom value of an inhibitory dose-response curve is zero)
ifMet	initiator <i>N</i> -formylmethionine
iMet	initiator methionine
K_i	dissociation constant for inhibitor binding

<i>map</i>	gene encoding prokaryotic methionine aminopeptidase
LAO	L-amino acid oxidase
<i>map1</i>	gene encoding MetAP1
<i>map2</i>	gene encoding MetAP2
MetAP	methionine aminopeptidase
MetAP1	eukaryotic cytosolic MetAP type 1
MetAP2	eukaryotic cytosolic MetAP type 2
NME	N-terminal methionine excision
SAR	structure-activity relationship
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small-interfering RNA

Figure 1.

(A) Structures of *HsMetAP* inhibitors. (B) A stereo view of the active site in the X-ray crystal structure of N-terminally truncated *HsMetAP1* in complex with Co(II) and compound **1** (PDB code 2NQ6²¹). Hot pink spheres are Co(II) ions. Surrounding residues are shown as sticks (carbon white), water molecules as small red spheres and metal interactions as dashed lines. Compound **1** shown as sticks (carbon green) largely depends on an auxiliary Co(II) (M3) to bind and inhibit *HsMetAP1*. For all non-carbon atoms, nitrogen is blue, oxygen is red and sulfur is yellow.

Figure 2.

Inhibition of iMet processing of 14-3-3 γ by MetAP inhibitors in human cell lines. Cells were treated with vehicle (DMSO), bengamide A, bengamide B or TNP-470 for 24 hours. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted (IB) with antibodies specific for β -actin, α -tubulin, total 14-3-3 γ or the iMet unprocessed form of 14-3-3 γ (iMet 14-3-3 γ). The levels of iMet 14-3-3 γ were quantified by densitometry and then normalized to total 14-3-3 γ . The IB of β -actin and α -tubulin served as loading controls. (A) HUVEC, human umbilical vein endothelial cells; Jurkat E6-1, T lymphocytes from human acute T cell leukemia; HeLa, epithelial cells from human cervical adenocarcinoma; and HEK293T, human embryonic kidney epithelial cells constitutively expressing SV40 T antigen; (B) PC-3, epithelial cells from human prostate adenocarcinoma; and MCF-7, epithelial cells from human breast adenocarcinoma.

Figure 3.

Downregulation of the protein level of either MetAP1 or MetAP2 inhibited the iMet processing of 14-3-3 γ in HUVEC. A scrambled control siRNA and siRNAs specific for MetAP1 or MetAP2 were introduced into HUVEC using either HiPerFect or TransIT-TKO transfection reagent. 48 hours after transfection, cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted (IB) with antibodies specific for α -tubulin, MetAP1, MetAP2, total 14-3-3 γ or iMet 14-3-3 γ . The levels of MetAP1 and MetAP2 were quantified by densitometry and then normalized to α -tubulin. The levels of iMet 14-3-3 γ were quantified by densitometry and then normalized to total 14-3-3 γ .

Figure 4.

Auxiliary metal-mediated human MetAP1 inhibitors **1–4** did not inhibit the iMet processing of 14-3-3γ in human primary cells. (A) The 2-(pyridin-2-yl)pyrimidine derivatives **2–4** failed to inhibit the NME of 14-3-3γ in HUVEC. (B) The pyridine-2-carboxamide derivative **1** (freshly prepared) failed to inhibit the NME of 14-3-3γ in HUVEC. Cells were treated with vehicle (DMSO), TNP-470, bengamide B or compound **1–4** for 24 hours. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted (IB) with antibodies specific for α-tubulin, total 14-3-3γ or iMet 14-3-3γ. The IB of α-tubulin served as a loading control. The levels of iMet 14-3-3γ were quantified by densitometry and then normalized to total 14-3-3γ.

Figure 5.

Activation of full-length human MetAP1 by divalent metal ions. The metal activation assays were carried out in a 100 μL mixture of (A) 150 nM MetAP1, 600 μM Met-Pro-*p*-nitroanilide, 15 U/mL *Bacillus coagulans* proline iminopeptidase or (B) 150 nM MetAP1, 600 μM Met-Ala-Ser, 1 U/mL L-amino acid oxidase (LAO), 0.1 mg/mL horseradish peroxidase, 0.2 mg/mL *o*-dianisidine (3,3'-dimethoxybenzidine) and one of the divalent metal ions in MetAP assay buffer. Initial rates are expressed as the mean (symbol) and standard deviation (error bar) of two (A) or three (B) determinations. Invisible error bars are included in the symbols.

Figure 6.

The iMet processing of 14-3-3 γ in human primary cells was inhibited by (A) **11j**, but not (B) thiabendazole. Cells were treated with vehicle (DMSO), bengamide G, TNP-470, **11j**, 2,2'-bipyridine or thiabendazole for 24 hours. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted (IB) with antibodies specific for β -actin, α -tubulin, total 14-3-3 γ or iMet 14-3-3 γ . The IB of β -actin and α -tubulin served as loading controls. The levels of iMet 14-3-3 γ were quantified by densitometry and then normalized to total 14-3-3 γ .

Figure 7.

Both **11j** and TNP-470 caused the retention of the initiator methionine of TXNL1 in HEK293T cells. BTF3L4 and TXNL1 contain the N-terminal sequences of MNQEKLA and MGVGKPV, respectively. HEK293T cells overexpressing C-terminally 3×Flag-tagged BTF3L4 and TXNL1 were treated with vehicle (0.2% DMSO), **11j** (10 μM), TNP-470 (100 nM), or a combination of **11j** and TNP-470, and subsequently pulsed with [³⁵S]-Met. 4.5 hours later, cells were lysed and the immunoprecipitated BTF3L4-3×Flag and TXNL1-3×Flag proteins were washed and aliquoted for [³⁵S]-Met scintillation counting after incubation with MetAP reaction buffer (buffer only) or after *in vitro* processing by purified HsMetAP1 and HsMetAP2 (MetAP1+2). The percentage of [³⁵S]-Met released from anti-Flag-conjugated resin was calculated. For each compound treatment, the percentage in the “buffer only” was subtracted from the percentage in “MetAP1+2”, and the resulted number was normalized to the vehicle control. Error bars are the standard deviation of three biological independent determinations. * *p* = 0.03 (unpaired two-tailed t test).

Figure 8.

The X-ray crystal structure of N-terminally truncated human MetAP1 in complex with **11j**. (A) and (C) *tHsMetAP1* in complex with **11j**, PDB code 4IU6 (orange). (B) and (D) A superimposition of 4IU6 to the crystal structures of *tHsMetAP1* with **2** (purple) and **3** (cyan) (PDB codes 2G6P⁴⁴ and 4HXX⁴⁵). A HEPES molecule (carbon purple) in 2G6P was from the buffer. The stereo views of the active site of *tHsMetAP1* in (A) and (B) show that all three compounds (sticks) mainly depend on an auxiliary Co(II) (Co3, green sphere) to bind the H212 residue (sticks) of *HsMetAP1*. Water molecules are shown as red spheres and metal interactions are shown as dashed lines. The surface-filling views of *tHsMetAP1* (surface colored gray) in (C) and (D) show the cavity in the active site which accommodates **11j**, **2** or **3**. The C4 side chains of the three compounds interact with different parts of the enzyme. For all non-carbon atoms, nitrogen is blue, oxygen is red, sulfur is yellow and chlorine is green.

Figure 9.

A superimposition of the crystal structures of N-terminally truncated human MetAP1 in complex with **11j** and human MetAP2 with L-methionine. *HsMetAP1* is shown as grey surface and **11j** is shown as orange sticks. The PDB code of *HsMetAP2* structure is 1KQ9⁶⁰. The backbone of *HsMetAP2* is shown as teal wires and ribbons, and the residues of *HsMetAP2* are shown as teal sticks. The numbering of the residues is based on *HsMetAP2*. The insertion (382–444) in the catalytic domain of *HsMetAP2* generates a tight entrance to the active site. In this superimposition, the Y444 of *HsMetAP2* clashed with **11j**. D442 and H339 on the other side of **11j** narrow the binding site as well. The L-methionine and the surface filling model of *HsMetAP2* are not shown for the sake of clarity. For all non-carbon atoms, nitrogen is blue and oxygen is red.

Scheme 1.

Reagents and conditions: (a) Na/MeOH, rt, then anthranilic acid, MeOH, 85 °C, 18 h; (b) PhNEt₂, POCl₃, 100 °C; (c) *i*-Pr₂NEt, 1,4-dioxane, 110 °C; (d) TBSCl/Et₃N/DCM; (e) Boc₂O/Et₃N/DCM; (f) allyl bromide, K₂CO₃, THF; then 5% TFA/DCM; (g) NaBH₄/MeOH; (h) 2 N NaOH/MeOH/THF; (i) (n-Bu)₄NF/THF; (j) p-TsCl/Et₃N/DCM; (k) aq. NaOH/THF, AcCl; (l) HBTU, *i*-Pr₂NEt, MeCN.

Table 1
Inhibition of Different Metalloforms of Purified Human MetAP1 and Cell Proliferation by Metal Coordinating Inhibitors

Cmpd	Inhibition of <i>His</i> MetAP1 ^a (EC ₅₀ , μ M)			Inhibition of Cell Proliferation ^b (EC ₅₀ , μ M)		
	Co(II)	Zn(II)	Mn(II)	HUVEC	HDFa	HeLa
1 (DJM) ^c	0.35 $\pm$ 0.10	>40	>100	2.0 $\pm$ 0.9 (B ^d = 10%)	0.79 $\pm$ 0.15	2.3 $\pm$ 0.4 (B ^d = 27%)
1 (JL) ^c	0.36 $\pm$ 0.10	>40	>100	1.5 $\pm$ 0.6 (B ^d = 11%)	0.65 $\pm$ 0.09	2.0 $\pm$ 0.6 (B ^d = 21%)
2	0.19 $\pm$ 0.04	0.71 $\pm$ 0.20 (B ^d = 35%)	15 $\pm$ 12 (B = 32%)	11 $\pm$ 2.1	3.6 $\pm$ 1.5	7.1 $\pm$ 0.9
3	0.43 $\pm$ 0.08	>30	>100	ND ^e	0.76 $\pm$ 0.11	0.97 $\pm$ 0.25
4	0.28 $\pm$ 0.05	>100	>200	4.3 $\pm$ 1.0	1.9 $\pm$ 0.29	10 $\pm$ 5.9
11j	0.10 $\pm$ 0.01	1.1 $\pm$ 0.3 (B ^d = 16%)	5.1 $\pm$ 0.8 (B ^d = 12%)	1.6 $\pm$ 0.4	1.9 $\pm$ 0.7	5.2 $\pm$ 1.2
Thiabenzazole	3.4 $\pm$ 0.4 (B ^d = 15%)	>250	>100	>250	>250	ND ^e
2,2'-Bipyridine	0.32 $\pm$ 0.07 (B ^d = 16%)	>100	>100	10 $\pm$ 1	10-30 ^f	ND ^e

^aEC₅₀ values are shown as mean $\pm$ SD of at least three determinations. 0.15 μ M purified recombinant *His*MetAP1 activated by 10 μ M CoCl₂, 1 μ M ZnCl₂ or 10 μ M MnCl₂ were used in the experiments. Representative inhibitory dose-response curves can be found in Figure S3.

^bEC₅₀ values were shown as mean $\pm$ SD of at least three experiments.

^cFor compound **1**, the batch "JL" was previously prepared [21] and the batch "DJM" was freshly prepared.

^dB: the bottom value of the inhibitory dose-response curve. It has been noted in parentheses only when B > 10%.

^eND, not determined.

^fThe inhibitory dose-response curves for 2,2'-bipyridine were extremely steep between 10 μ M and 30 μ M (the Hill slopes were around -14). 10 μ M 2,2'-bipyridine did not inhibit the cell proliferation of HDFa, but 30 μ M 2,2'-bipyridine inhibited 94 $\pm$ 2% of cell proliferation.

Table 2
Inhibition of Purified Human MetAPs by 2-(Pyridin-2-yl)Quinoline and 2-(Pyridin-2-yl)Quinazoline Derivatives

For 18-20

For other compounds

Cmpd	R	EC ₅₀ ^a (μM)		
		<i>HsMetAP1</i> Co(II)	<i>HsMetAP1</i> Mn(II)	<i>HsMetAP2</i> Mn(II)
7a	Cl	1.5 ± 0.2	>100	>100
10a	NHCH ₂ CH ₂ Ph	0.94 ± 0.12	24 ± 5	>100
10b	NHCH ₂ CH ₂ NHPh	1.6 ± 0.2	>100	>300
10c	NHCH ₂ CH ₂ -(3-1H-isoindole)	0.12 ± 0.02	5.3 ± 0.7 (B ^b = 19%)	>300
16	<i>N</i> -piperidinyI	0.11 ± 0.01	7.6 ± 1.3 (B ^b = 14%)	>100
17	4-morpholinyl	0.049 ± 0.004	41 ± 33	>100
18	<i>N</i> -piperidinyI	0.39 ± 0.08	>100	>100
19	4-morpholinyl	0.41 ± 0.09	>100	>100
20	4-methylpiperazin-1-yl	0.87 ± 0.43	>100	>100

^aEC₅₀ values are shown as mean ± SD of at least three determinations.

^bB: the bottom value of the inhibitory dose-response curve. It has been noted in parentheses only when B > 10%.

Table 3
Inhibition of Purified Human MetAPs by 4-(Piperazin-1-yl)-2-(Pyridin-2-yl)Quinazoline Derivatives

Cmpd	R1	R2	EC ₅₀ ^a (μM)			
			HsMetAP1 Co(II)	HsMetAP1 Mn(II)	HsMetAP2 Mn(II)	
11a	H	(4-CH[OH](CH ₃)Ph	0.073 ± 0.023	8.4 ± 1.6		>100
11b	H	(4-COOEt)Ph	0.094 ± 0.017	15 ± 6 (B ^b = 14%)		>100
11c	H	(4-COOH)Ph	0.90 ± 0.42	>100		>100
11d	H	(4-OCH ₂ COOEt)Ph	0.18 ± 0.03	11 ± 3		>100
11e	H	(4-OCH ₂ COOH)Ph	6.0 ± 3.0 (B ^b = 12%)	>400		>100
11f	H	(4-OH)Ph	0.094 ± 0.018	8.1 ± 2.6		>100
11g	H	(4-nitro)Ph	0.11 ± 0.2	3.7 ± 1.8		>100

Cmpd	R1	R2	EC ₅₀ ^a (μM)			
			H ₅ MetAP1	Co(II)	H ₅ MetAP1 Mn(II)	H ₅ MetAP2 Mn(II)
11h	H	(4-CHO)Ph	0.081 ± 0.014		4.8 ± 4.3	>100
11i	H	(4-allyloxy)Ph	0.59 ± 0.28		17 ± 6 (B ^b = 30%)	>100
11j	H	(4-OMe)Ph	0.10 ± 0.02		5.1 ± 0.7 (B ^b = 12%)	>100
11k	Cl	(4-OMe)Ph	0.85 ± 0.16		>100	>200
11l	Br	(4-OMe)Ph	1.4 ± 0.3		>100	>200
11m	H	(3,4-dichloro)Ph	0.39 ± 0.07		5.0 ± 3.3 (B ^b = 27%)	>100
13	H	SO ₂ -(4-Me)Ph	0.25 ± 0.04		8.0 ± 1.3 (B ^b = 36%)	>100

Cmpd	R1	R2	EC ₅₀ ^a (μM)			
			HsMetAP1	Co(II)	HsMetAP1 Mn(II)	HsMetAP2 Mn(II)
14	H		0.093 ± 0.017		3.4 ± 1.6	>100
15	H		0.12 ± 0.02		4.8 ± 1.1	>100

^aEC₅₀ values are shown as mean ± SD of at least three determinations.

^bB: the bottom value of the inhibitory dose-response curve. It has been noted in parentheses only when B > 10%.

Table 4
X-ray Data Collection and Refinement Statistics of the N-terminal Truncated ($\Delta 1-80$)
Human MetAP1 (tHsMetAP1) in Complex with 11j

tHsMetAP1 with Co(II) and 11j (PDB code 4IU6)	
Space group	$P2_1$
Cell dimensions	
a (Å)	47.3
b (Å)	77.3
c (Å)	47.8
β (deg)	91.5
X-ray data collection statistics	
X-ray Source	FR-E ⁺ /Raxis IV
Wavelength (Å)	1.54178
Resolution range (Å)	50.00-1.90
(HighRes shell)	(1.97-1.90)
Collected Reflections	74,840
Unique Reflections	24,981
I/σ	28.8 (3.3)
Completeness (%)	91.6 (59.7)
R_{merge} (%)	5.5 (27.6)
Refinement statistics	
R_{cryst} (%)	0.17 (0.25)
R_{free} (%)	0.22 (0.33)
R.m.s deviations	
Bond length (Å)	0.016
Angle (deg)	1.432
Monomer in ASU	1
Total Atoms	2,731
Protein atoms	2,411
Water molecules	276
Ligand	34
B -factor (Δ HsMetAP1) (Å ²)	29.1
B -factor (11j) (Å ²)	33.0
B -factor (H ₂ O) (Å ²)	39.7