Type I methionine aminopeptidase from *Saccharomyces cerevisiae* is a potential target for antifungal drug screening[1]

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ABSTRACT

AIM: To screen antifungal drug candidates using *in vitro* and *in vivo* assays based on type I methionine aminopeptidase from *Saccharomyces cerevisiae* (*Sc*MetAP1). **METHODS:** A colorimetric assay suitable for high throughput screening (HTS) using recombinant *Sc*MetAP1 protein expressed in *Escherichia coli* was established for antifungal lead discovery. A series of pyridine-2-carboxylic acid derivatives were characterized and a chemical library of 12 800 pure organic compounds was screened with the *in vitro Sc*MetAP1 assay. Active compounds from the *in vitro* assay were further evaluated by a growth inhibition assay on yeast strain with deletion of *Sc*MetAP1 gene *map1* in comparison with the wild-type yeast strain and the yeast strain with deletion of type II enzyme (*Sc*MetAP2) gene *map2*. **RESULTS:** Active *Sc*MetAP1 inhibitors were identified from HTS. Some of the pyridine-2-carboxylic acid derivatives (compound 2 and 3) had selective inhibition of the growth of *map2* deletion yeast and weak inhibition on wild-type yeast growth, while no inhibition on *map1* deletion yeast. **CONCLUSION:** *Sc*MetAP1 is a novel potential target for developing antifungal drugs. The *in vitro* and *in vivo Sc*MetAP1 assays can serve as tools in discovering antifungal drug candidates.

INTRODUCTION

Methionine aminopeptidase (MetAP), existing in both prokaryotic and eukaryotic cells, plays an important role in removing the first methionine from nascent polypeptides[1,2]. Up to now, two major isoforms of MetAP (type I and type II MetAPs) have been identified[3]. Eubacteria has only type I MetAP, and archaea has only type II MetAP, while eukaryotic cells contain both types of MetAPs[1]. MetAPs are organized in a similar 'pita-bread' conformation as characterized by several available X-ray structures, and they are highly conservative in catalytic domain with respect to five amino acid residues coordinating two cobalt ions[4-6]. MetAPs have been considered as Co^{2+}-enzymes since MetAPs can be reproducibly activated by Co^{2+} *in vitro*, although recent studies showed other divalent metal ions such as Zn^{2+}, Fe^{2+}, Mn^{2+}, and Ni^{2+} could also activate MetAPs[7-12].

MetAP has important physiological function. Deletion of the unique MetAP gene from *Escherichia coli*[13] or *Salmonella typhimurium*[14] is lethal, demonstrating

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the essential role of MetAP in bacteria. In *Saccharomyces cerevisiae*, disrupting either type I or type II MetAP renders a slow growth phenotype, and removal of both is nonviable[15]. Therefore, MetAPs are potential targets for developing antibacterial and antifungal drugs[16]. The natural product fumagillin selectively inhibited type II MetAPs and killed yeast which lacking type I enzyme *Sc*MetAP1, and it did not affect the growth of wild-type and *Sc*MetAP2 deletion strain[17], suggesting dual inhibitors for both type I and type II enzymes is required for antifungal application. However, *in vivo* experiments showed that *Sc*MetAP1 in yeast played a bigger role in N-terminal methionine removal and in methionine metabolism than *Sc*MetAP2[18,19], indicating the development of *Sc*MetAP1 inhibitors is more important for therapeutic application.

Although the therapeutic potentials have been recognized, there are few inhibitors against MetAPs reported in literatures. Fumagillin and its analogues are specific inhibitors for type II MetAPs with covalent modification of the enzyme[17,20]. The substrate-like inhibitor AHHpA-Ala-Leu-Val-Phe-OMe inhibited the *E coli* type I enzyme *Ec*MetAP1 with a reported IC_{50} at 5 μmol/L[21], and we recently discovered pyridine-2-carboxylic acid derivatives which showed potent inhibition against type I enzymes *Ec*MetAP1 and *Sc*MetAP1 [22]. In this study, we prepared apo-*Sc*MetAP1 and used it in high throughput screening (HTS). The *Sc*MetAP1 inhibitors were further tested on yeast growth with wild-type, *Sc*MetAP1 lacking or *Sc*MetAP2 lacking yeast strains. The information presented here would help in designing functional inhibitors against MetAP1 that were effective *in vivo* for therapeutic applications.

MATERIALS AND METHODS

Materials and instruments Expression vector pGEX-KG was a generous gift from Prof Kun-liang GUAN at University of Michigan (USA)[23]. Yeast strain W303-1A (*MATa ade2-1 can1-100 ura3-1 leu2-3, 112 trp-1 his3-11, 15*) contains wild-type *Sc*MetAP1 and *Sc*MetAP2. Null *Sc*MetAP1 (Δ*map1*) and null *Sc*MetAP2 (Δ*map2*) strains are isogenic haploid derivatives of W303-1A containing an additional *map1*::*HIS3* or *map2*::*URA3* gene disruption, respectively[24,25]. These series of yeast strains were generous gifts from Prof Yie-hwa CHANG at St Louis University.

Chromogenic thiopeptolide substrate Met-S-Gly-Phe[26], inhibitors (3*R*)-amino-(2*S*)-hydroxyheptanoic acid (AHHpA)[22] and pyridine-2-carboxylic acid derivatives were synthesized in this laboratory. The 12 800 compounds were all small organic compounds with unrelated and diverse chemical structures and purchased from SPECS/Bio SPECS (Rijswijk, The Netherlands) with purity higher than 90 %.

Continuous kinetic monitoring of enzyme activity was performed on SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA) for UV absorption and controlled by the Softmax software. Liquid handling for random screening was carried out on the ORCA automation system with a Biomek 2000 liquid handling workstation from Beckman Coulter (Fullerton, CA) and with the Hydro 96 semi-automated 96-channel pipettor from Robbins (Sunnyvale, CA). Protein purification was carried out on an Akta FPLC system with UV and conductivity monitors from Amersham Biosciences (Piscataway, NJ).

Preparation of *Sc*MetAP1 Full-length *Sc*MetAP1 gene was obtained by direct PCR amplification from the total genomic DNAs of the *S cerevisiae* strain *YPH499* with the forward primer (G*GAATTC*TAAT GAGCACTGCAACTACAAC) with *EcoR* I site (*italic*) and the reverse primer (*GTCGAC*TTACTATTTAATTC-TCTGTCTTGG) with *Sal* I site (*italic*). The PCR fragment was then cloned into the *EcoR* I and *Sal* I sites of pGEX-KG vector for expressing glutathione *S*-transferase fusion *Sc*MetAP1 protein (GST-*Sc*MetAP1) in strain BL21(DE3)pLysS under the control of a *Tac* promoter.

Protein expression and purification were carried out as described in literature with slight modifications[27,28]. *E coli* cells bearing plasmid were cultured in 500 mL of LB medium in the presence of ampicillin (100 mg/L) with shaking at 37 ºC. When OD_{600} reached 0.6-0.8, the expression of GST-*Sc*MetAP1 was induced by adding isopropyl thil-β-*D*-galactoside (IPTG) to 0.25 mmol/L and allowed to continue shaking at 22 ºC for another 14 h. Cells (0.81 g cell paste) from the 500 mL culture were resuspended in 50 mL PBS (NaCl 140 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 1.8 mmol/L) with 0.1 % Triton X-100 (Bio-Rad) and sonicated on ice for 2 min. After centrifugation at 16 000×*g* for 15 min twice, the filtered supernatant was loaded to a 5 mL Glutathione Sepharose 4B column (Pharmacia) that previously equilibrated with 50 mL PBS. The loaded column was washed with 200 mL PBS and the bound active protein was eluted with glutathione 10 mmol/L, Tris 50 mmol/L, pH 8.0. The elution was then exchanged

to Tris 50 mmol/L, pH 8.0, NaCl 150 mmol/L, $CaCl_2$ 2.5 mmol/L buffer for thrombin cleavage[23]. After incubated with thrombin (0.3 kU/L, Sigma) at 4 ºC for 16-20 h, the mixture was loaded to 5 mL Glutathione Sepharose 4B column again to remove free GST. To make an apo-enzyme, *Sc*MetAP1 were incubated with EDTA 5 mmol/L overnight, and exchanged into HEPES 50 mmol/L, pH 7.5, KCl 150 mmol/L, which has been treated with Chelex resin (Bio-Rad) to remove adventurous metal ions. Apo-*Sc*MetAP1 (3.78 mg) showed a single band on 12 % SDS-PAGE gel by Coomassie Brilliant Blue staining.

Protein concentration determination and metal analysis Protein concentration was determined by the Bradford method with BSA as the standard. Metal analysis of the apo-*Sc*MetAP1 solution was carried out by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (performed by Shanghai Institute of Measurement and Testing Technology).

***Sc*MetAP1 activity assays** The *Sc*MetAP1 activity was detected by a colorimetric assay with the thiopeptolide Met-S-Gly-Phe as substrate as described previously[26] with modifications for reading on microplates. The assay was usually performed at room temperature on a 96-well clear polystyrene microplate. In metal activation experiments, the assay mixture in each well containing MOPS 50 mmol/L, pH 7.0, DTNB 1 mmol/L, Met-S-Gly-Phe 100 μmol/L, apo-*Sc*MetAP1 0.84 μmol/L and various amounts of divalent metal ions ($CoCl_2$, $MnCl_2$, or $ZnCl_2$). The hydrolysis of Met-S-Gly-Phe was monitored continuously by change of UV absorbance at 412 nm, and the initial rate of the hydrolysis was determined using the early linear region of the enzymatic reaction curve. The rate for background hydrolysis of the thiopeptolide was subtracted.

***Sc*MetAP1 inhibitors screening** A total of 12 800 pure organic compounds were used for HTS against Co^{2+}-*Sc*MetAP1 with the aforementioned colorimetric assay. The test compounds were solubilized in Me_2SO at 1 g/L, and then distributed 2 μL on the daughter plates. The Me_2SO (2 μL) as the test vehicle was distributed in eight blank cells. The enzymatic assay was carried out on 96-well clear polystyrene plates in a 100 μL volume in each well and immediately before the assay, apo-*Sc*MetAP1 2.1 μmol/L was mixed with $CoCl_2$ 250 μmol/L. An assay mixture (78 μL) containing MOPS 50 mmol/L, pH 7.0, DTNB 1 mmol/L, Met-S-Gly-Phe 100 μmol/L, was added to each well with Robbins liquid-handling system, and then added 20 μL of the Co^{2+} activated *Sc*MetAP1 (final concentrations: *Sc*MetAP1 0.42 μmol/L and $CoCl_2$ 50 μmol/L). The hydrolysis of Met-S-Gly-Phe was monitored on SpectraMax 340 microplate reader at 412 nm for 1 min at room temperature.

Kinetic characterization of metal-substituted *Sc*MetAP1s and their inhibitors Metal-substituted *Sc*MetAP1s were characterized by determining their kinetic parameters k_{cat}, K_m, and k_{cat}/K_m values using the chromogenic substrate Met-S-Gly-Phe. A 100 μL assay mixture containing MOPS 50 mmol/L, pH 7.0, DTNB 1 mmol/L, apo-*Sc*MetAP1 0.42 μmol/L, $CoCl_2$ 50 μmol/L, $MnCl_2$ 50 μmol/L or $ZnCl_2$ 30 μmol/L and Met-S-Gly-Phe in two fold dilutions up to 5 mmol/L was used to obtain the k_{cat}, K_m, and k_{cat}/K_m values. The k_{cat} and K_m values of the metal-substituted *Sc*MetAP1s were derived from a non-linear regression fitting of the curve in the plot of the initial rates *vs* the substrate concentrations, using the Michaelis-Menton equation: $v=V_{max}\cdot[S]/(K_m+[S])$, where v is the initial rate, V_{max} is the maximum rate, and [S] is the substrate concentration.

The inhibitory activities of compounds were characterized by IC_{50} determinations at multiple inhibitor concentrations. The assay mixture in each well of a 96-well microplate containing MOPS 50 mmol/L, pH 7.0, DTNB 1 mmol/L, Met-S-Gly-Phe 100 μmol/L, and apo-*Sc*MetAP1 0.42 μmol/L with $CoCl_2$ 50 μmol/L plus compound at one of the several concentrations around its estimated IC_{50} value. The hydrolysis of Met-S-Gly-Phe was monitored continuously by change of absorbance at 412 nm, and IC_{50} was calculated from the non-linear curve fitting of percent inhibition (% inhibition) *vs* inhibitor concentration [I] by using the following equation: $\%\ \text{Inhibition}=100/\{1+(IC_{50}/[I])^k\}$, where k is the Hill coefficient.

Yeast growth inhibition assay Compounds dissolved in Me_2SO were spotted onto sterile filter disks with diameter 7 mm and then placed on YPD (1 % yeast extract, 2 % peptone, and 2 % glucose, 1 % agar) containing logarithmically growing yeast strains. After incubated at 30 ºC for 48 h, the diameters that yeast can not grow were measured, indicating the inhibitory effects of relevant compounds. Solvent Me_2SO was used as negative control, while amphotericine B (25 mg/L) and fumagillin (10 μmol/L) were both used as positive controls, in which amphotericine B showed inhibitory activity in all the three types of yeast strains (wild, Δ*map1* and Δ*map2*) and fumagillin selectively inhibited Δ*map1* yeast strain which only contains *Sc*MetAP2.

RESULTS

Preparation of *Sc*MetAP1 Soluble fusion protein GST-*Sc*MetAP1 was highly expressed in *E coli* BL21(DE3)pLysS cells, purified with affinity chromatography , and cleaved by thrombin. After flowing through affinity chromatography to remove free GST, the *Sc*MetAP1 exhibited a single band with an apparent molecular weight (MW) about 44 kDa on the SDS-PAGE gel (Fig 1). The concentration of the purified *Sc*MetAP1 protein was determined, and the yield of *Sc*MetAP1 was 3-4 mg from 500 mL LB culture. When the protein was purified without adding divalent metal ions and removing adventurous metals in the buffer, the purified protein showed a basal activity. This basal activity was reduced to an undetectable level by EDTA treatment. Metal analysis of the apo-*Sc*MetAP1 solution by ICP-AES demonstrated that each of metal ions Co, Mn, Ni, and Fe was <0.001 ppm (about 0.02 μmol/L), while the concentration of Zn was 0.007 ppm (about 0.1 μmol/L) for the 0.84 μmol/L protein solution which we used in the activation assay.

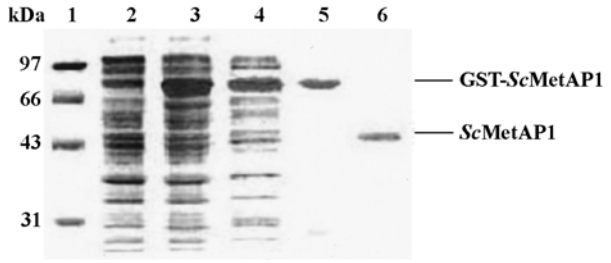

Fig 1. Expression and purification of *Sc*MetAP1. Lane 1, protein molecular mass marker; Lane 2, pre-induction control of total cell lysate; Lane 3, post-induction whole cell lysate; Lane 4, soluble fraction of cell lysate; Lane 5, after GST-affinity chromatography; Lane 6, after thrombin cleavage and remove free GST.

Activations of *Sc*MetAP1 by divalent metals Using the colorimetric activity assay, different divalent metal ions were added to test the stimulation of the EDTA-treated apo-*Sc*MetAP1 and to find the appropriate metals and the concentrations for *Sc*MetAP1 *in vitro* for high throughput screening. Along with the increasing amount of $CoCl_2$, $ZnCl_2$, or $MnCl_2$, the activity of apo-*Sc*MetAP1 increased accordingly (Fig 2). However, $CoCl_2$ started to show inhibition on *Sc*MetAP1 in a concentration above 62.5 μmol/L, $ZnCl_2$ started to show inhibition above 31.25 μmol/L, indicating that Zn^{2+} activation of apo-*Sc*MetAP1 was in a relatively narrow range

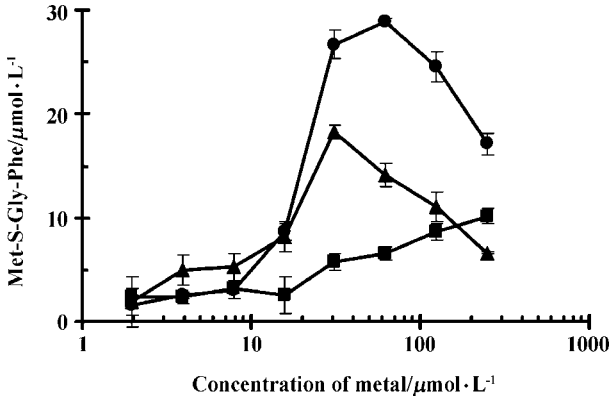

Fig 2. Activation of apo-*Sc*MetAP1 by divalent metals Co^{2+} (circles), Mn^{2+} (squares) and Zn^{2+} (triangles). The enzyme activity was monitored by the hydrolysis of Met-S-Gly-Phe in a continuous colorimetric assay with apo-*Sc*MetAP1 0.84 μmol/L.

compared with Co^{2+}. While Mn^{2+} had a broad concentration to active *Sc*MetAP1 and did not show the maximal activation in the range we examined (1.95 to 250 μmol/L). Other divalent metal ions tested, such as Ni^{2+}, Mg^{2+}, Ca^{2+}, Cd^{2+} or Cu^{2+}, failed to active the enzyme in the range examined. So Co^{2+}, Zn^{2+}, or Mn^{2+} substituted apo-*Sc*MetAP1s were further determined their efficiency for cleavage of the thiopeptolide substrate.

Determination of kinetic properties of metal substituted apo-*Sc*MetAP1 The kinetic constants for the metal-substituted enzymes were determined by hydrolyzing Met-S-Gly-Phe with the most favorable metals for *Sc*MetAP1. Co^{2+}-*Sc*MetAP1 was an efficient enzyme in cleaving the thiopeptolide substrate with k_{cat}/K_m, 2894 $L \cdot mol^{-1} \cdot s^{-1}$, Zn^{2+}-*Sc*MetAP1 showed a catalytically competent enzyme with about 60 % of Co^{2+}-*Sc*MetAP1 efficiency (k_{cat}/K_m, 1838 $L \cdot mol^{-1} \cdot s^{-1}$) and Mn^{2+}-*Sc*MetAP1 was less effective in hydrolyze the thiopeptolide and showed a catalytic activity with only about 30 % of Co^{2+}-*Sc*MetAP1 activity (k_{cat}/K_m, 985 $L \cdot mol^{-1} \cdot s^{-1}$) (Tab 1). The reduction of catalytic efficiency for Zn^{2+} or Mn^{2+} substituted enzymes mainly resulted from their K_m values significantly increased. These results, consistent with the previous observations that *Sc*MetAP1 could be activated by Co^{2+}, Mn^{2+}, or Zn^{2+} with an HPLC assay and octapeptide[7], showed that *Sc*MetAP1 can be reproducibly activated by Co^{2+} *in vitro* and the Co^{2+}-*Sc*MetAP1 enzyme showed the most efficient catalytic activity by using Met-S-Gly-Phe as substrate. The Co^{2+} activated *Sc*MetAP1 and the thiopeptolide assay could be used for high throughput

Tab 1. Kinetic constants for the metal-substituted *Sc*MetAP1s.

*Sc*MetAP1s	K_m (mmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m ($L \cdot mol^{-1} \cdot s^{-1}$)
Co^{2+}-*Sc*MetAP1	1.24±0.11	3.58±0.17	2895
Zn^{2+}-*Sc*MetAP1	6.75±0.38	12.8±0.56	1838
Mn^{2+}-*Sc*MetAP1	16.0±1.48	15.8±1.18	984

Assays with Met-S-Gly-Phe were carried out at room temperature with apo-*Sc*MetAP1 0.42 μmol/L and $CoCl_2$ 50 μmol/L, $ZnCl_2$ 30 μmol/L or $MnCl_2$ 50 μmol/L.

screening.

High throughput screening for Co^{2+}-*Sc*MetAP1 inhibitors Compounds were solved in Me_2SO for high throughput screening, and the presence of 2 % Me_2SO in the HTS assay would not significantly affect the Co^{2+}-*Sc*MetAP1 activity with more than 80 % of the enzyme activity remained. In addition, Me_2SO solvent was used as negative control in this assay. The substrate-like bastatin-based inhibitor AHHpA-Ala-Leu-Val-Phe-OMe, with IC_{50} around 5 μmol/L, is one of the few specific inhibitors toward *Ec*MetAP1 as reported[2,25] and part of the structure, the AHHpA (Fig 3, compound **1**) moiety itself, was an inhibitor for *Ec*MetAP1[12]. We reported here that AHHpA (**1**) also had a moderate inhibitory activity against Co^{2+}-*Sc*MetAP1 with IC_{50} 16.54±1.69 μmol/L by using the thiopeptolide assay and was used as positive control in this HTS assay.

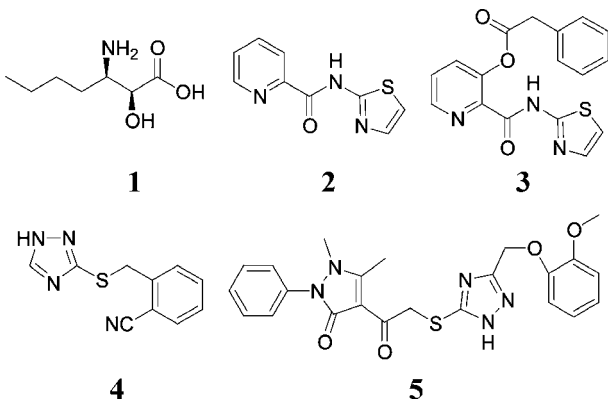

Fig 3. Structures of *Sc*MetAP1 inhibitors.

A random selected 12 800 organic compound library was tested by using the purified Co^{2+}-*Sc*MetAP1. All the compounds were initially screened at the concentrations of 20 μg/mL. A total of 83 compounds with larger than 60 % inhibition were obtained from the screening, and their inhibitory activities on *Sc*MetAP1 were further confirmed by determining the IC_{50} values at multiple concentrations.

Two compounds with pyridine-2-carboxylic acid moiety reported earlier[22] showed good inhibitory activity. Compound **2** (Fig 3), which was the lead compound for structure modifications and inhibited Co^{2+}-*Sc*MetAP1 with IC_{50} value at 7.10±0.36 μmol/L, and compound **3** (Fig 3), derived from compound **2**, was one of the best Co^{2+}-*Sc*MetAP1 inhibitors with IC_{50} at 0.46±0.11 μmol/L (Fig 4). Inhibitors with new structural types were also identified from the screening, such as compounds **4** and **5** with a 1,2,4-triazole moiety (Fig 3) with IC_{50} values at 0.66±0.05 μmol/L and 0.99±0.04 μmol/L, respectively. Similar compounds with the 1,2,4-triazole moiety has been reported as *Hs*MetAP2 inhibitor[29].

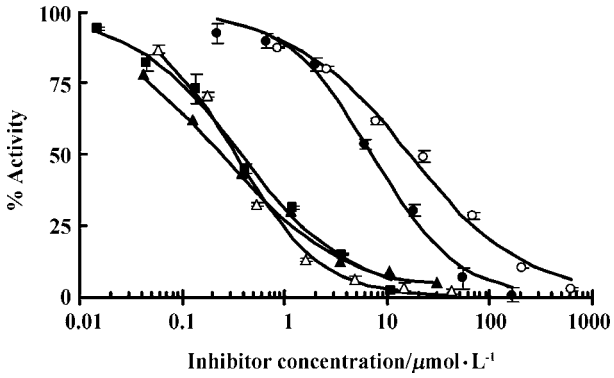

Fig 4. Inhibiton of *Sc*MetAP1 in the colorimetric assay with Met-S-Gly-Phe as the substrate in the presence of Co^{2+}. Inhibitors are 1 (open circles), 2 (filled circles), 3 (filled squares), 4 (filled triangles) and 5 (open triangles). X-axis: Inhibition concentration (μmol/L), Y-axis: Relative activity (%). The colorimetric assay include apo-*Sc*MetAP1 0.42 μmol/L; Met-S-Gly-Phe 100 μmol/L; $CoCl_2$ 50 μmol/L.

***In vivo* growth inhibition assay** Some of *Sc*MetAP1 inhibitors were further tested by an *in vivo* assay on yeast growth inhibition. In addition to wild-type yeast strain with two intact MetAPs (type I and type II)[14], mutant null MetAP1(Δ*map1*) and null MetAP2 (Δ*map2*) yeast strain which only contain *Sc*MetAP2 or *Sc*MetAP1[23,24] were also surveyed in this assay for further elucidating the sensitivity and selectivity of these compounds on *Sc*MetAP1. Amphotericine B used as the positive inhibitor can inhibit all the three yeast strains

with a similar inhibitory activity, and fumagillin markedly inhibited the growth of Δ*map1* strain as expected due to its selective inhibition on *Sc*MetAP2 (Tab 2).

Tab 2. Compounds *in vivo* inhibitory effects on wild-type, Δ*map1* and Δ*map2* yeast strains.

Compound	Diameter (mm) of yeast not growth		
	wt	Δ*map1*	Δ*map2*
1	7.56±0.37	7	7
2	9.16±1.00	7	9.38±0.85
3	12.41±1.70	7	14.72±2.15
4	7	7.57±0.76	7
5	7.56±0.37	7	7
Amphertericine B	13.35±1.37	13.08±1.29	12.69 ± 1.26
Fumagillin	7	37.34±4.16	7
Me_2SO	7	7	7

Sterile filter disks (the diameter is 7mm) impregnated with compounds **1** to **5** (10 mmol/L), fumagillin 10 μmol/L, or amphotericine B 25 μg/mL, or Me_2SO control were placed on wild-type strain W303-1A, a *Sc*MetAP1 deletion strain, *map1::HIS3*, or a *Sc*MetAP2 deletion strain, *map2::URA3* of *S. cerevisiae*. The diameters that fungal growth inhibited by compounds were measured after 48-h incubation.

The inhibitors (compounds **1** to **5**) for Co^{2+}-*Sc*MetAP1 behaved differently for the inhibitory effects on the three yeast strains. Compound **2** and compound **3** both inhibited the growth of Δ*map2* yeast strain, which depended on the functional *Sc*MetAP1 for viability. They also had weak inhibitory activities against wild-type yeast, in which the *Sc*MetAP1 was selectively inhibited (Tab 2 and Fig 5). While Δ*map1* yeast strain, which only has *Sc*MetAP2 to maintain its growth, was resistant to these two *Sc*MetAP1 inhibitors. These data indicated that pyridine-2-carboxylic acid derivatives showed antifungal activity by selectively inhibiting *Sc*MetAP1 *in vivo*, although in high concentrations. On the contrary, the *in vivo* inhibitory activities of AHHpA (**1**), compound **4** and **5** on wild-type, Δ*map2* as well as Δ*map1* yeast strains were almost undetectable, even though they inhibited *Sc*MetAP1 *in vitro*. It needs further studies to determine the differences between *in vitro* and *in vivo* inhibitory activities of these compounds.

DISCUSSION

*Sc*MetAP1 was expressed and purified from *E coli* with affinity chromagraphy and its activity was easily monitored by thiopeptolide substrate Met-S-Gly-Phe, which provided a convenient assay for further divalent metal activation investigations and large-scale random screening for inhibitor discovery. The results showed that divalent metals such as Co^{2+}, Zn^{2+}, and Mn^{2+} activated apo-*Sc*MetAP1 by thiopeptolide assay was consistent with the results by using octapeptide MSSHRWDW as substrate[7], which suggested that thiopeptolide substrate was an efficient and reliable substrate for monitoring *Sc*MetAP1 activity. However, we could not observe the *Sc*MetAP1 activity with Ni^{2+} under the assay, perhaps because the occupation by different metals may cause subtle change at the active site to deduce the change of specificity for substrate[12].

Although recently studies showed that several divalent metal ions, such as Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, and Ni^{2+} could activate MetAPs[7-12], metal substitution assay in this study indicated that Co^{2+} was the most fa-

Fig 5. *In vivo* inhibition of *Sc*MetAP1 activity. Sterile filter disks impregnated with 10 μL compound 2, 3 (10 mmol/L) or Me_2SO (anti-clockwise from the top) were placed on wild-type strain W303 1A (left), a *Sc*MetAP2 deletion strain, *map2::URA3* (middle), or a *Sc*MetAP1 deletion strain, *map1::HIS3 S cerevisiae* (right).

vorite metal for *Sc*MetAP1 activity with Met-S-Gly-Phe as substrate *in vitro*, so we selected Co^{2+}-*Sc*MetAP1 for the HTS assay. Through large scale screening, two groups of compounds with distinctive basal structures have been identified for Co^{2+}-*Sc*MetAP1 inhibitors. The one was compound **2** and **3** which contained pyridine-2-carboxylic acid structure with IC_{50} value around 7.10 μmol/L and 0.46 μmol/L, respectively. And the other was compound **4** and **5** which derived from a basal 1,2,4-triazoles structure with IC_{50} value less than 1 μmol/L.

Three types of yeast stains, named wild-type, *Sc*MetAP1 and *Sc*MetAP2 deletion strains, were all available to test the *in vivo* selectivity and sensitivity of these inhibitors described above. The pyridine-2-carboxylic acid derivatives inhibited the growth of wild-type and *Sc*MetAP2 deletion strains with selective inhibition on *Sc*MetAP1 *in vivo*, while no inhibitory effect on *Sc*MetAP1 deletion strain were performed in this case. The inhibition of wild-type yeast growth perhaps due to *Sc*MetAP1 plays a more significant physiological function than *Sc*MetAP2 *in vivo*[18,19], which at the same time indicated that *Sc*MetAP1 alone could be a potential target for antifungal drug discovery.

However, pyridine-2-carboxylic acid derivatives inhibited yeast growth only occurred at a high concentration such as 10 mmol/L, and no obvious *in vivo* inhibition could be observed when the concentration decreased to 1 mmol/L (data not shown). It seemed that there was little relationship between the *in vitro* inhibitory potencies for the Co^{2+}-*Sc*MetAP1 and the *in vivo* inhibition against the wild-type or *map2* deletion yeast strains. There are three main possibilities responsible for these differences. The first is the differences of these compounds in their ability of passing the cell wall, which we have not tested. The second is the diversity of these compounds in their solubility in the YPD culture medium, since these compounds could not be dissolved in water and all dissolved in Me_2SO for the tests. In addition, the *in vivo* physiological metal that located in the active site of MetAPs still remained to be defined and recent data suggested that MetAPs might have a metal other than cobalt at their active sites. We used Co^{2+}-*Sc*MetAP1 for screening and characterization of these inhibitors, and our previous data showed that the metal substitutions could bring on the changes in specificity toward substrates and inhibitors[12]. And we are in the process of assessing these and other inhibitors on the other metal substituted *Sc*MetAP1s with the hope of improving their *in vivo* potencies.

In summary, a large-scale screening assay for Co^{2+}-*Sc*MetAP1 was established and two groups of compounds were discovered. The pyridine-2-carboxylic acid derivatives selectively inhibited the growth of yeast and their *in vivo* target were demonstrated as *Sc*MetAP1, while the 1,2,4-triazoles derivatives showed no evident inhibitions on yeast growths. *Sc*MetAP1 is a potential target for antifungal drug screening, and inhibitors we discovered here need further modifications not only for the *in vitro* activity against *Sc*MetAP1 but also for the *in vivo* efficacy on antifungal function.

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