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A cell-based assay that targets methionine aminopeptidase in a physiologically relevant environment

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Abstract

Methionine aminopeptidase (MetAP) is a promising target for the development of novel antibiotics. However, many potent inhibitors of the purified enzyme failed to show significant antibacterial activity. It is uncertain which divalent metal MetAP uses as its native cofactor in bacterial cells. Herein, we describe a cell-based assay that monitors the hydrolysis of a fluorogenic substrate by overexpressed MetAP in permeabilized *E. coli* cells and its validation with a set of MetAP inhibitors. This cell-based assay is applicable to those cellular targets with poorly defined native cofactor, increasing the chances of identifying inhibitors that can inhibit the cellular target.

Cell-based assays have become powerful tools in identifying biologically active small molecules. Unlike biochemical assays with well-defined molecular targets, most of the current cell-based assays focus on pathways by monitoring reporter genes or phenotypic variations.[1] We describe here a unique cell-based assay with *Escherichia coli* methionine aminopeptidase (MetAP) as the specific cellular target and its validation with a set of MetAP inhibitors by comparing the inhibition of the purified enzyme, the same enzyme in a cellular environment, and bacterial growth. This type of cell-based assay is critically important for molecular targets, such as MetAP, whose native cofactor is not clearly defined.

MetAP plays a critical role in protein maturation by catalyzing the removal of the N-terminal initiator methionine from nascent proteins.[2] MetAP is a ubiquitous enzyme found in both prokaryotes and eukaryotes, and the lethal effect of MetAP gene deletion has been reported for *E. coli*,[3] *Salmonella typhimurium*,[4] and *Saccharomyces cerevisiae*.[5] Therefore, MetAP is an appealing target for the development of broad-spectrum antibacterial and antifungal drugs with a novel mechanism of action.[6]

Divalent metal ions directly participate in the MetAP-catalyzed N-terminal methionine excision of proteins and peptides. The purified MetAP apoenzyme can be reproducibly activated by a number of divalent metal ions, such as Co(II), Mn(II), Ni(II), and Fe(II).[7, 8] Several X-ray structures of MetAP depict the catalytic site as a shallow and mostly hydrophobic pocket with two metal ions situated at the bottom in a dinuclear arrangement.[9] The structures also indicate that most of the inhibitors reported to date interact directly with the catalytic metal cofactor, taking advantage of strong interactions with the metal ions.[10-13] However, many MetAP inhibitors with high potency on the purified enzyme failed to show significant

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antibacterial activity.[12, 14, 15] This discrepancy is likely due to the difference in the metal cofactor utilized in biochemical assays for inhibitor screening and characterization and that used by MetAP in bacterial cells. Most *in vitro* MetAP assays make use of Co(II) or Mn(II) as the cofactor, as they are relatively stable and are consistently among the best activators of the enzyme. However, MetAP inhibitors with high potency against the Co(II)-form or Mn(II)-form may not inhibit MetAP in other metalloforms.[10] We have recently reported that Fe(II) is the physiologically relevant metal used by MetAP in *E. coli* cells, and probably in other bacteria such as *Bacillus*.[16, 17]

In situations when the native cofactor is not well defined, the best way is to evaluate the inhibitory effect of compounds on the target in a cellular environment where the native cofactor is functioning. We established the cellular MetAP activity assay by overexpressing *E. coli* MetAP in *E. coli* cells and making the *E. coli* cells permeable to substrates and inhibitors by Ca(II) treatment.[16] *E. coli* MetAP was overexpressed in *E. coli* BL21(DE3) cells.[8] With the enzymatically active MetAP inside live *E. coli* cells, we monitored the hydrolysis of a fluorogenic substrate, methionyl aminomethylcoumarin (Met-AMC), by fluorescence (ex/em 360/460 nm) as the indication of cell permeability, when we varied the concentration of Ca(II) in Tris buffer. We found that the rate of hydrolysis reached maximum at around 200 μM of $CaCl_2$ and maintained at that level to 10 mM of $CaCl_2$. Therefore, we choose the concentration of Ca(II) to be 5 mM for optimal cell permeability in our cellular MetAP activity assay.

The cell-based assay was carried out on 384-well plates containing 12 serial concentrations for each inhibitor, and the highest final concentration in the assay was 1 mM. The freshly prepared suspension of cells containing the recombinant MetAP enzyme and the substrate Met-AMC were dispensed to the microplate by a Multidrop Combi reagent dispenser (Thermo Scientific, Waltham, MA). The final assay volume was 80 μl with 150 μM Met-AMC, 5 mM $CaCl_2$, and 50 mM Tris (pH 7.5). The plates were sealed, and production of the fluorescent aminomethylcoumarin was monitored via fluorescence using a SpectraMax Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA). Readings were taken at 2 min intervals for several hours at room temperature. IC_{50} values were calculated from the rate of substrate hydrolysis in the 2-4 h time range by non-linear regression curve fitting of percent inhibitions as a function of inhibitor concentrations.

To validate the cell-based MetAP assay, we tested a set of 31 MetAP inhibitors for their inhibition of the cellular MetAP activity and correlated the inhibition with the previously published inhibition of purified MetAP enzyme in the Fe(II)-form and inhibition of bacterial growth.[10, 11] Using the Ca(II) treated cells, IC_{50} values were obtained for all the compounds as presented in Table S1 (supplementary material) and illustrated in Fig 1A. The results from the cellular assay underscore the importance of the catechol group (such as in **1**), as decreased potency was observed for compounds with blocked dihydroxyls by methylation, or when the hydroxyl moieties are replaced by other functional groups such as amino, nitro, acetylamido, and aminocarbonyl groups (**2, 5-8**). The presence of the contiguous hydroxyl pair also plays a significant role in inhibition, as exclusion of one of the hydroxyl groups or their spatial separation resulted in no inhibition (**3-4**). While all the thiazole derivatives with intact catechol moiety (**1, 9-12**) displayed inhibition of intracellular Met-AMC hydrolysis, most thiophene derivatives (**13-27**) showed improved potency. Compounds **22** and **23** are exceptions, presumably due to the presence of bulkier groups or the amide moiety.

As an added advantage of the cell-based assay, these time-course plots can provide information on the time-dependent permeability of the inhibitors. Upon incubation of the Ca(II) treated cells with Met-AMC, the hydrolytic reaction continued steadily for over 8 h. (Fig. 2A). Most of the compounds tested gained access into the cells without noticeable delay, as the Met-AMC hydrolysis under different inhibitor concentrations resulted in linear product formation (Figure

2A). When the cellular assay was allowed to proceed for several hours, we noticed that compound **1** inhibited in a time-dependent fashion (Fig. 2B). This effect was more noticeable when the IC_{50} values were calculated from the slope of the curve at every 20 min intervals (Fig. 2C). The inhibitory potency increased over time, leveling off after 400 min, at which point the IC_{50} for **1** was 23 μM. If **1** were to be calculate in the same manner as with the other compounds (using the 2-4 h time range), the IC_{50} value for **1** would be 100 μM instead.[16]

The calculated IC_{50} values obtained by the cell-based assay were compared with the IC_{50} values previously obtained from purified enzyme preparations and bacterial growth assays (Fig. 1).[11] In general, there is correlation between the data from the cell-based assay and that from purified *E. coli* MetAP. Comparable among all three different types of assays, derivatives containing a thiophene coupled to the catechol (**13-27**) generally display the highest potency. Values from the cell-based assay correlate with those from the assay using the purified enzyme, because Fe(II) was used in the purified enzyme assay and Fe(II) is the likely the native cofactor in the cell-based assay.[16, 17] It is interesting that compounds **2-8** did not inhibit either the purified enzyme or the cellular enzyme potently, but they affected cell growth considerably. This may indicate that these compounds hamper cell growth by a different mechanism other than MetAP inhibition. Inhibitors that are highly potent and selective towards the purified MetAP in the Mn(II)-form (**28-29)** or in the Co(II)-form (**30-31)** displayed no inhibition of the Met-AMC hydrolysis in the cellular assay. Consistently, they showed weak inhibition of the purified MetAP in the Fe(II)-form and they did not halt bacterial growth.

It is critically important that appropriate assay conditions are used during the screening process in order to obtain inhibitors that can act on the cellular target. The biochemical assay using the purified MetAP enzyme in the Fe(II)-form is predicative for inhibition of the cellular MetAP activity and inhibition of bacterial growth, because the native metal has been correctly assigned.[16] However, the native cofactor for a cellular target is often not well defined. In those situations, the cell-based assay, exemplified by the cell-based MetAP assay, places the target protein with its native cofactor in a cellular environment, increasing the chances of identifying inhibitors that can inhibit the cellular target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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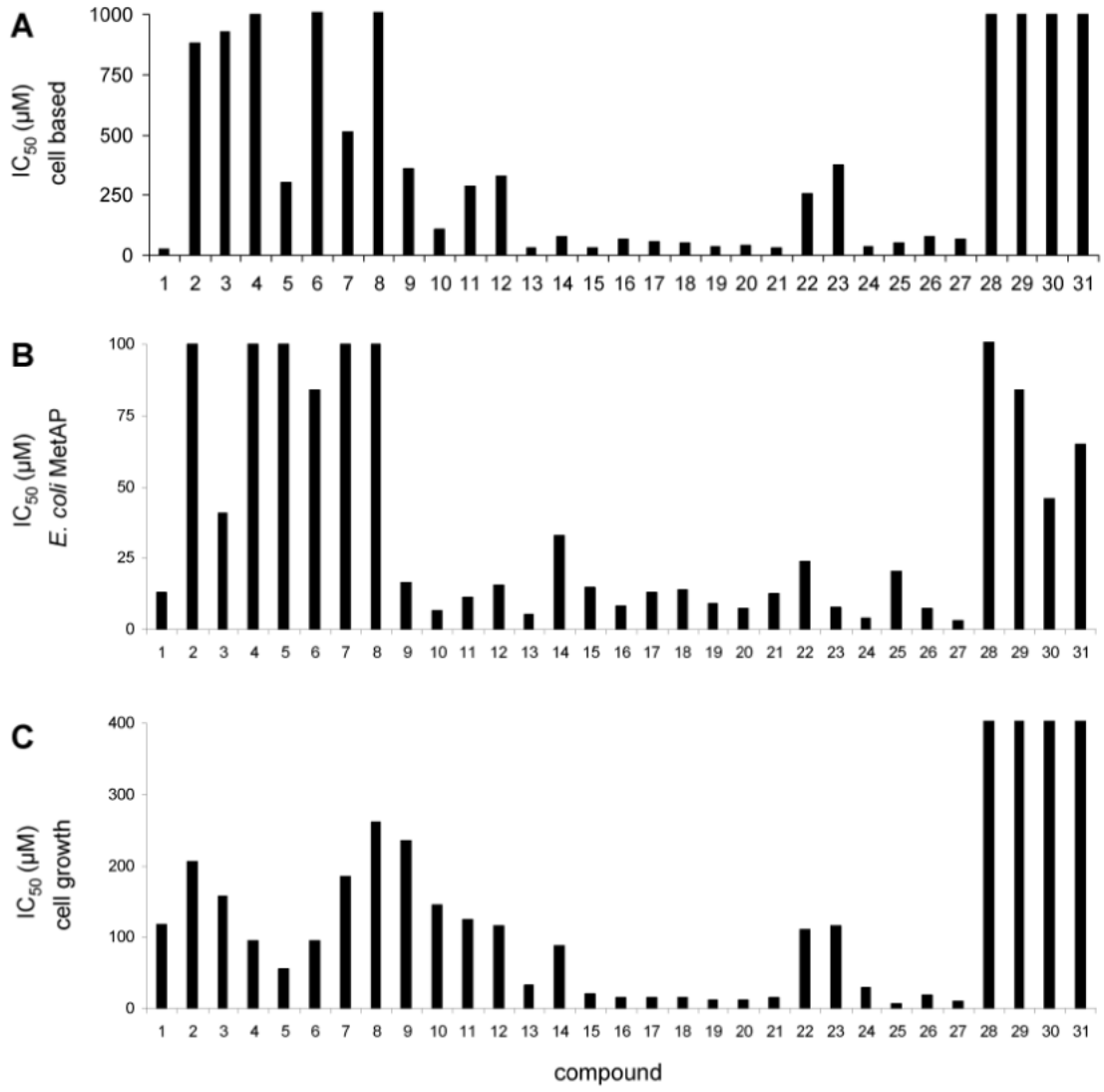

Figure 1.

Comparison of IC_{50} values from the cell-based MetAP assay (**A**), the biochemical assay using the purified *E. coli* MetAP in the Fe(II)-form (**B**), and the cell growth assay using *E. coli* AS19 strain (**C**). Data from **C** were scaled to a maximum value of 400 μM, although **28**-**31** showed no inhibition up to 1 mM. Data from **B** and **C** obtained from references [11] and [16].

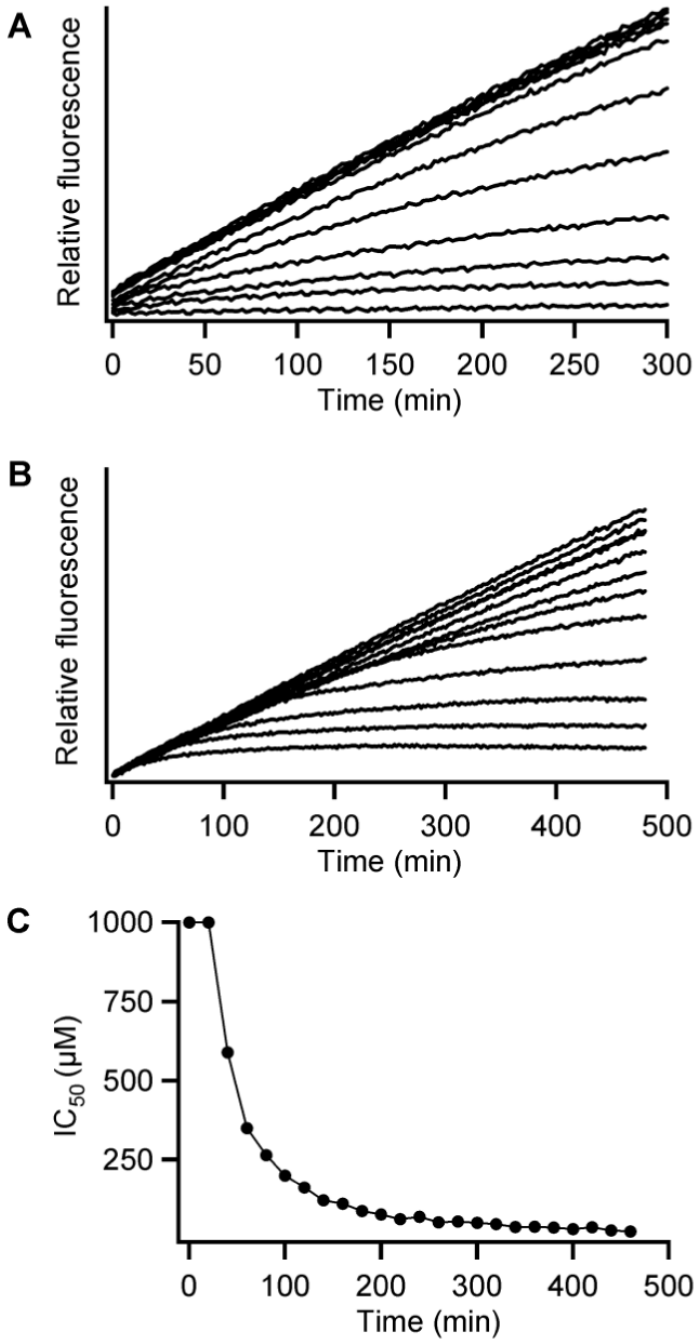

Figure 2.

(**A**) Intracellular hydrolysis of the fluorogenic substrate Met-AMC in the presence of various concentrations of **16**. (**B**) Time-dependent inhibition of intracellular Met-AMC hydrolysis at several concentrations of **1**. (**C**) IC_{50} values corresponding to **1** obtained at various time points using data from Fig. 1B.

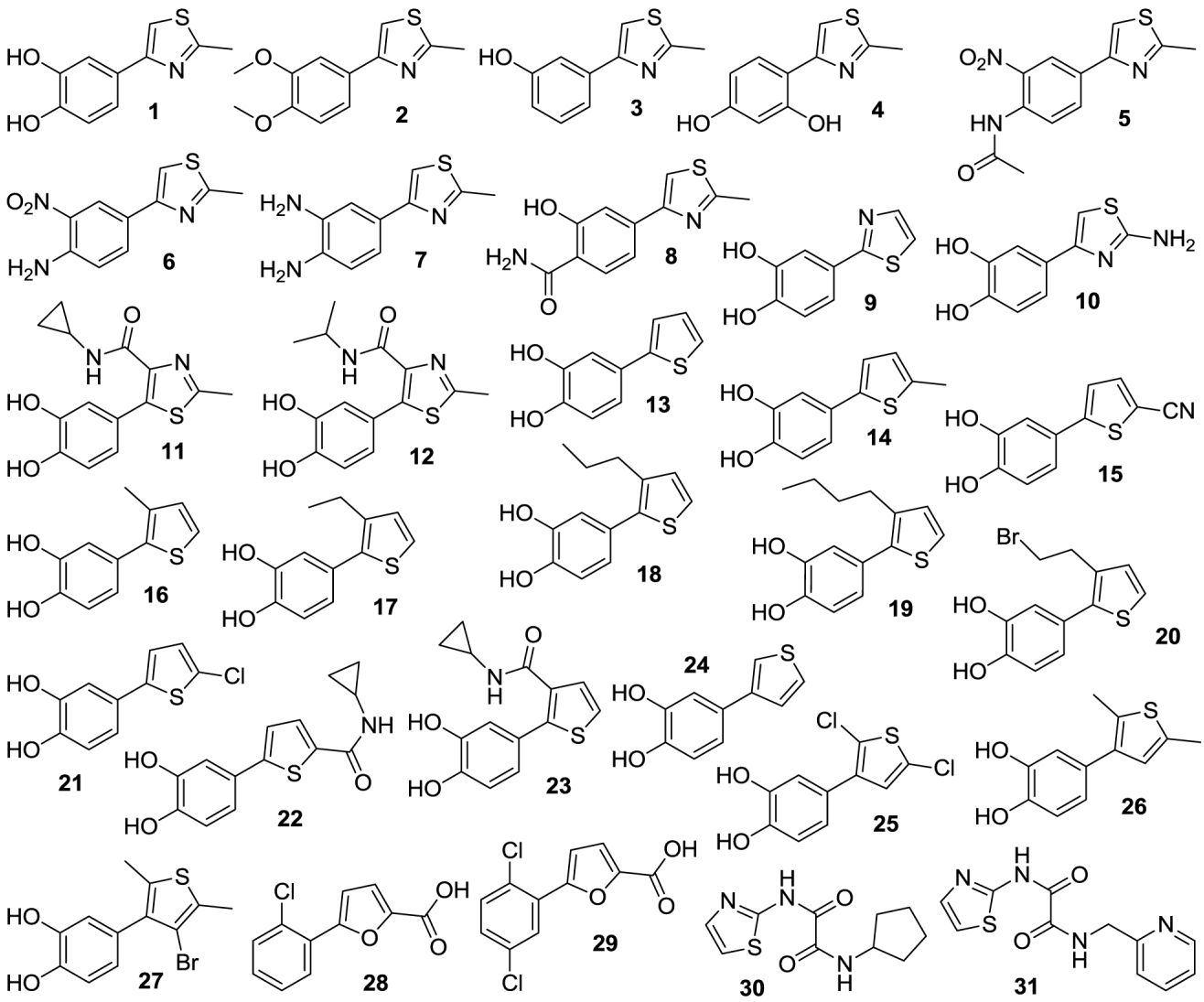

Chart 1.
MetAP inhibitors used for the assay validation