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A Macrophage Cell Model for Selective Metalloproteinase Inhibitor Design

Faith E. Jacobsen,[a] Matthew W. Buczynski,[a, b] Edward A. Dennis,*[a, b] and Seth M. Cohen*[a]

The desire to inhibit zinc-dependent matrix metalloproteinases (MMPs) has, over the course of the last 30 years, led to the development of a plethora of MMP inhibitors that bind directly to the active-site metal. With one exception, all of these drugs have failed in clinical trials, due to many factors, including an apparent lack of specificity for MMPs. To address the question of whether these inhibitors are selective for MMPs in a biological setting, a cell-based screening method is presented to compare the relative activities of zinc, heme iron, and non-heme iron enzymes in the presence of these compounds using the RAW264.7 macrophage cell line. We screened nine different zinc-binding groups (ZBGs), four established MMP inhibitors (MMPis), and two novel MMP inhibitors developed in our laboratory to determine their selectivities against five different metalloenzymes. Using this model, we identified two nitrogen donor compounds—2,2′-dipyridylamine (DPA) and triazacyclononane (TACN)—as the most selective ZBGs for zinc metalloenzyme inhibitor development. We also demonstrated that the model could predict known nonspecific interactions of some of the most commonly used MMPis, and could also give cross-reactivity information for newly developed MMPis. This work demonstrates the utility of cell-based assays in both the design and the screening of novel metalloenzyme inhibitors.

One of the main targets for metalloenzyme inhibition over the last 30 years has been matrix metalloproteinases (MMPs).[1–6] MMPs comprise a family of calcium(II)- and zinc(II)-dependent hydrolytic enzymes involved in the maintenance of the extracellular matrix components.[7–10] Constitutively active MMPs facilitate nerve and bone growth, endometrial cycling, wound healing, and angiogenesis;[7,8] however, MMPs are also associated with chronic inflammatory diseases, cardiomyopathy, and cancer metastasis.[3,11–13] Development of potent and selective MMP inhibitors (MMPis) thus has therapeutic potential in the treatment of a number of human diseases.[1–6] Presently, the FDA has only approved one compound that inhibits MMP activity: the broad-spectrum inhibitor doxycycline[14] (under the commercial name Periostat) used for the treatment of periodontal disease.[14] However, the mechanism of MMP inhibition by doxycycline has not been fully elucidated,[15] and it is known also to have numerous interactions with other metalloenzymes.[16–18] Furthermore, hundreds of other MMPis that show excellent potency in vitro, only to exhibit several problematic side-effects in clinical trials, have been developed over the past 30 years.[5,19–21] A significant number of these effects have been attributed to a lack of selectivity. In particular, the hydroxamic acid zinc-binding group (ZBG) that is most often utilized in MMPis, and other metalloproteinase inhibitors, is not selective for the zinc(II) ion over other biologically relevant metal ions, such as iron.[22] To this end, several efforts have been made to identify more zinc-selective ZBGs.[23–25]

Too often, potential therapeutics are tested in vitro and in cellular assays against MMPs, only for a lack of efficacy and side-effects caused by their lack of selectivity to be discovered in animal models or in clinical trials. A number of studies have reported screening techniques that identify cross-reactivity between MMP isoforms;[26–28] however, they do not systematically test whether MMPis can have an effect on non-MMP, off-target enzymes. To overcome the limitations of previous in vitro screening approaches, we present here a robust assay system, using the macrophage as a model, that can determine the efficacies of inhibitors against MMPs alongside a number of other relevant metalloenzymes, which serve as markers of cross-reactivity. For these studies we selected the RAW264.7 murine macrophage cell line, which has been used extensively as a model of inflammation,[29–33] has a wealth of publicly accessible information available from the LIPID MAPS consortium (http://www.LipidMaps.org), and, in contrast to human monocyte cell lines such as U937 and THP-1, does not require differentiation into adherent macrophages.[34,35]

Acute inflammation serves as an important component of the innate immune system defense against bacterial infection and entails the activity of a number of important metalloenzymes (Figure 1). In response to pathogenic stimuli, macrophage cells upregulate and release pro-MMPs into the extracellular space. In addition, arachidonic acid (AA) released by activated macrophage cells is converted into bioactive mediators by a variety of cyclooxgenase (COX) and lipoxygenase (LO) en-

[a] *F. E. Jacobsen,[+] M. W. Buczynski,[+] Prof. E. A. Dennis, Prof. S. M. Cohen*
Department of Chemistry and Biochemistry
University of California in San Diego
La Jolla, CA 92093-0358 (USA)
Fax: (+1) 858-534-7390; (+1) 858-822-5598
E-mail: edennis@ucsd.edu
scohen@ucsd.edu

[b] *M. W. Buczynski,[+] Prof. E. A. Dennis*
Department of Pharmacology, University of California in San Diego
La Jolla, CA 92093-0601 (USA)

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: Experimental details on eicosanoid assays, MMP-3(ΔC) expression and purification, metal removal and activity assays, kinetic analysis of ZBG inhibition.

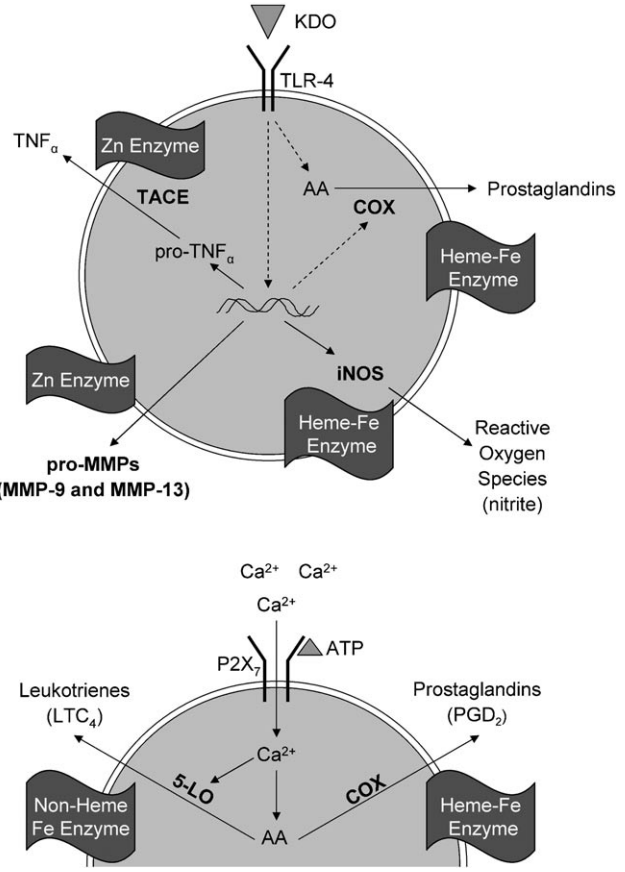


Figure 1. Overview of RAW264.7 macrophage activation. KDO (Kdo_2-Lipid A) is recognized by TLR-4 (toll-like receptor 4), resulting in upregulation of COX, TNF_α, iNOS, and pro-MMPs. ATP is recognized by purinergic receptors, including $P2X_7$, causing an influx of extracellular Ca^{2+}, which results in AA release and 5-lipoxygenase (5-LO) activation. The outcome is an increase in LTC_4 (leukotriene C_4) due to 5-LO and PGD_2 (prostaglandin D_2) due to COX. Metalloenzymes are flagged in dark gray, describing the type of metalloenzyme; the specific metabolites measured in this study are shown in parenthesis.

zymes.[29] The proinflammatory cytokine tumor necrosis factor α (TNF_α) is transformed from its upregulated pro-form to its activated state by TNF_α converting enzyme (TACE), also known as ADAM17.[36,37] Finally, macrophages upregulate inducible nitric oxide synthase (iNOS), which produces reactive oxygen species that kill bacteria.[38] Each of the aforementioned processes is metal-dependent: COX and iNOS are heme iron enzymes, LOs are non-heme iron enzymes, and TACE and MMPs are zinc-dependent metalloenzymes. The RAW264.7 cell line can recapitulate each of these hallmarks of acute inflammatory processes, and provides a relevant model system for examining the effects of MMPis on each metal-dependent pathway.

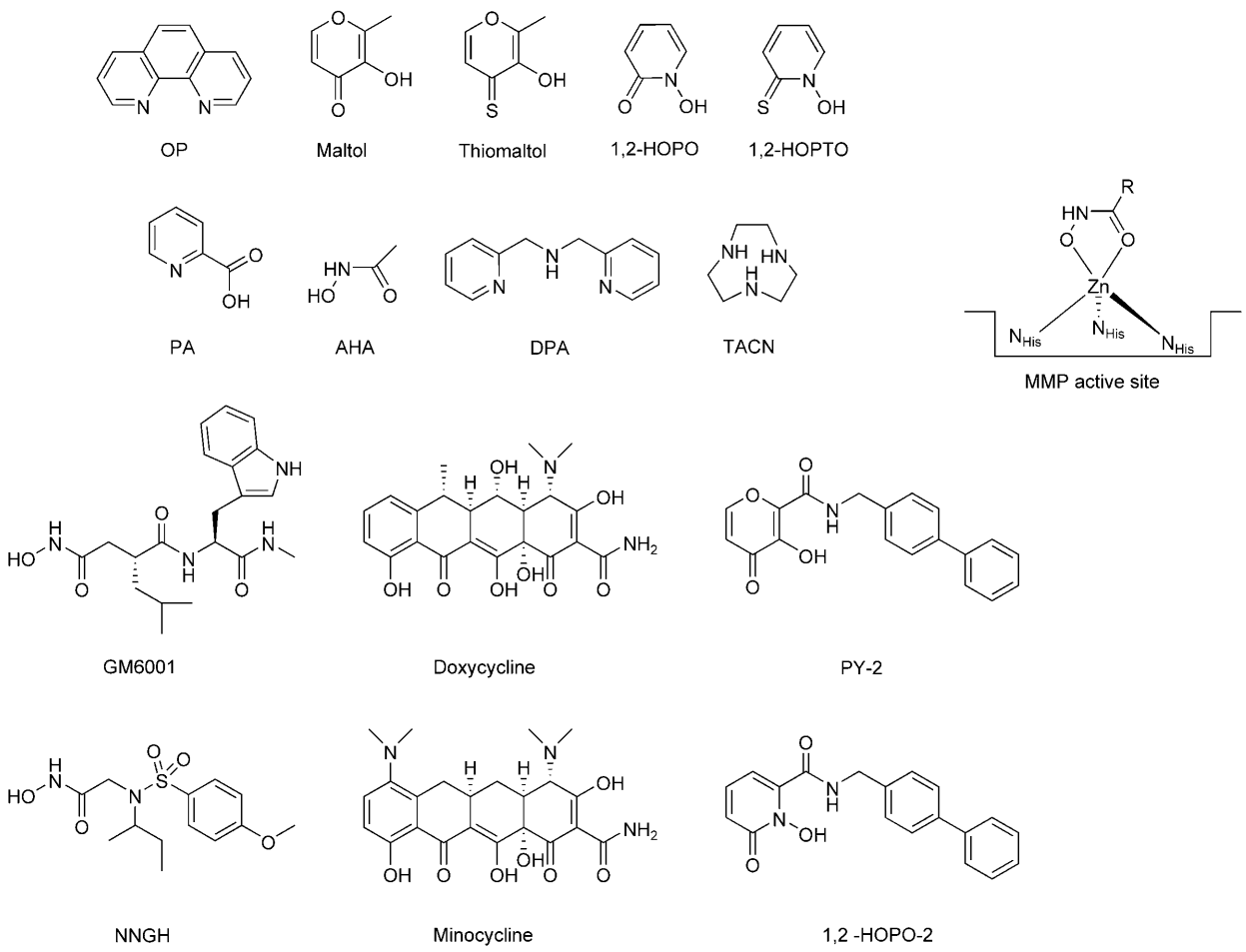
By using the RAW264.7 cell line, the metalloenzyme selectivities of nine different ZBGs have been examined. *o*-Phenanthroline (OP) is a common metal chelator that is known to remove the catalytic zinc(II) ion from the MMP active site (Scheme 1).[39] 3-Hydroxy-2-methyl-4*H*-pyran-4-one (maltol), 3-hydroxy-2-methyl-4*H*-pyran-4-thione (thiomaltol), 1-hydroxypyridin-2(1*H*)-one (1,2-HOPO), and 1-hydroxypyridine-2(1*H*)-thione (1,2-HOPTO) are chelators that have demonstrated greater MMP inhibition than simple hydroxamates such as acetohydroxamic acid (AHA).[25] Picolinic acid (PA), 2,2′-dipyridylamine (DPA), and triazacyclononane (TACN) also inhibit MMPs better than AHA and are expected to be more selective for binding zinc(II) than the aforementioned chelating groups (Scheme 1).[24]

In addition, the RAW264.7 cell line model has been used to identify off-target interactions of a variety of known MMPis. For example, GM6001 is a potent inhibitor of MMPs, but has also been shown to inhibit the zinc(II) enzyme TACE.[40,41] Doxycycline and minocycline, which are broad-spectrum MMP inhibitors, are also known to blunt the activity of iNOS.[42] Overall, six MMPis—including four commercially available MMPis and two potent MMPis (Scheme 1) developed in our laboratory,[43] the interactions of which with other metalloenzymes had not previously been characterized—were evaluated in the macrophage model. The results show that the macrophage cell model is predictive of the off-target interactions that have been reported in the literature. The findings presented here show that a cell-based model can be used to examine the activities of compounds on a variety of metalloenzymes simultaneously. While we have applied this model towards the design of selective MMPis, this screening method also provides a useful tool for determining the specificity of a molecular fragment or complete inhibitor for any of the metalloenzymes assayed in this model. Thus, the screening method developed here represents a powerful tool for analysis of the specificities not only of MMPis, but also of TACE inhibitors, COX inhibitors, iNOS inhibitors, and 5-lipoxygenase (5-LO) inhibitors, as well as dual COX and 5-LO inhibitors.[44]

Results

Cell viability in the presence of ZBGs

Prior to examination of metalloenzyme activity, the toxicities of the different ZBGs were determined. Cell viabilities were assessed through the release of lactate dehydrogenase (LDH) from Kdo_2-Lipid A (KDO) stimulated macrophage cells in the presence of each ZBG (100 μM) over 24 h. LDH is a stable, cytosolic enzyme that is released upon cell death, and the concentration of LDH in the extracellular medium correlates with cell death. The results are shown in Figure S1 in the Supporting Information and were confirmed visually with Trypan Blue Dye. RAW264.7 cells proved to be greater than 90% viable in the presence of all the ZBGs tested except OP, which killed approximately 50% of the cells at 100 μM. The results were consistent with previous toxicity studies on cardiac fibroblasts,[45] which showed that maltol, thiomaltol, 1,2-HOPO, and 1,2-HOPTO demonstrated low toxicities at 100 μM. The high toxicity of OP at 100 μM makes it difficult to determine whether a decrease in enzymatic activity (as measured in the assays described below) is due to inhibition by OP or simply results from an overall increase in cell death. Because of this complication the data obtained from OP (vide supra) are not interpreted in detail.



Scheme 1. Chelators (top) and MMPis (bottom) evaluated in a macrophage-based model of metalloenzyme activity. Diagram depiciting how hydroxamate-based MMPis (GM6001, NNGH) bind to the MMP active site (right).

Metalloenzyme activity in RAW264.7 cells

Knowing that metal chelators have different thermodynamic affinities for metal ions in vitro,[24] we sought to evaluate the inhibition of several metalloenzymes by various ZBGs in a biological setting with a RAW264.7 macrophage cell model. As shown in Figure 1, two stimulation scenarios, using either ATP or KDO, were examined in order to probe the activities of different metalloenzymes. Stimulating the $P2X_7$ purinergic receptor with ATP generates an influx of extracellular Ca^{2+}. Within minutes, this Ca^{2+} influx transiently activates both the cytosolic phospholipase A_2 ($cPLA_2$) to release AA from membrane phospholipids and the non-heme iron enzyme 5-LO to process AA to form bioactive leukotriene C_4 (LTC_4).[29] AA can also be acted upon by the constitutively expressed heme iron enzyme COX to make prostaglandin D_2 (PGD_2). Additionally, the bacterial membrane component KDO, a specific lipopolysaccharide, was used to stimulate the toll-like receptor 4 (TLR-4) on the macrophages, which induces sustained $cPLA_2$ activity over a 24 h period.[29] Over this period, KDO activates the transcription factor NF-κB, allowing for the upregulation of COX, iNOS, pro-TNF_α, pro-MMP-9, and pro-MMP-13 (Figure S2, http://www.LipidMaps.org).[46] Pro-TNF_α is cleaved by membrane-associated TACE[36] to the soluble signaling protein TNF_α. Through these two stimulation pathways, the activities of five different metalloenzymes in the presence of different ZBGs could be examined. In the sections below, the activities of different ZBGs against zinc-dependent enzymes are described, followed by the results against iron-dependent enzymes.

Inhibition of MMPs by ZBGs: Previous work has shown that TLR-4-stimulated RAW264.7 cells primarily induce pro-MMP-9 and pro-MMP-13 expression (Figure S2, http://www.LipidMaps.org).[46] In this study, cells were stimulated with KDO in the presence of ZBG (100 μM). As macrophages in culture have not been shown to activate their own pro-MMP[47–49] and thus have no basal level of MMP activity in the extracellular media (data not shown), the pro-MMP was activated with *p*-aminophenylmercuric acetate (AMPA).[50] While the MMP fluorescent substrate can also be cleaved by TACE, this protein is cell-membrane-associated[36] and would not be present in an assay of the extracellular media. This is confirmed by experiments showing no activity in the extracellular media when AMPA is excluded from the assay (data not shown).

At 100 μM, DPA and TACN inhibited MMP activity by more than 90%. The sulfur-containing ligands 1,2-HOPTO and thiomaltol also inhibited the MMP activity, but to lesser extents

(80% and 45%, respectively). The model hydroxamic acid, AHA, inhibited 35% of the expressed MMP activity in the cells, while the other ZBGs (maltol, 1,2-HOPO, PA) inhibited less than 20% of the MMP activity. The MMP assay results are summarized in Figure 2.

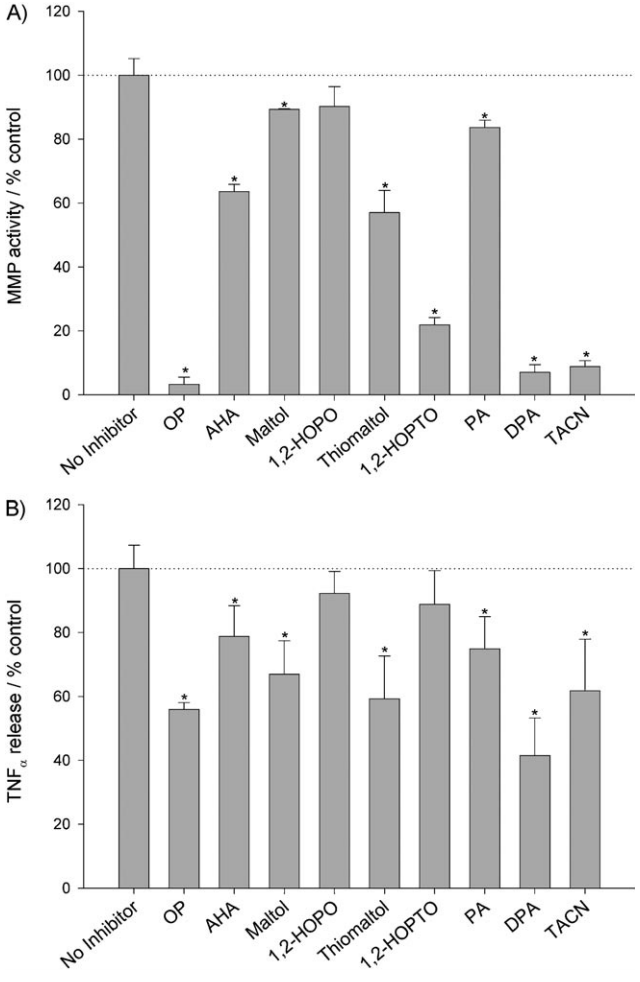


Figure 2. Inhibition of zinc-dependent enzymes MMPs and TACE by different ZBGs. A) MMP activity and B) release of TNF$_\alpha$ from KDO-stimulated RAW264.7 cells in the presence of each ZBG (100 μM).

Inhibition of TACE by ZBGs: TACE activity was measured through the relative amount of TNF$_\alpha$ in the extracellular medium following KDO stimulation. Because TACE is a zinc-dependent enzyme, it was anticipated that some ZBGs would inhibit TNF$_\alpha$ release. Indeed, DPA and TACN are both inhibitors of activity (Figure 2), with 100 μM DPA inhibiting TNF$_\alpha$ release by 60%. Other ZBGs including PA, maltol, and thiomaltol inhibited release by approximately 25%, 30%, and 40%, respectively. All other ZBGs, including 1,2-HOPTO, inhibited TNF$_\alpha$ production by less than 20%. The low inhibition by 1,2-HOPTO was surprising, as this chelator is a potent ZBG against MMP-3 in vitro,[25,45] but does not appear to inhibit the zinc-dependent TACE in this model.

Inhibition of 5-LO and COX by ZBGs: To determine the activities of 5-LO and COX, the presence of their AA products was analyzed after stimulation with ATP. The activity of 5-LO was measured by the production of LTC_4; the activity of COX was monitored by the production of PGD_2. Both metabolites were monitored simultaneously by the previously described LC-MS/MS methodology.[29] It is important to note that these metabolites could also be measured, if suitable mass spectrometers were unavailable, by commercially available ELISA assays (Cayman Chemicals, Ann Arbor, MI, USA). Zileuton[51,52] and indomethacin[53] were used as positive controls for 5-LO and COX inhibition, respectively (data not shown). The levels of AA, the substrate for both 5-LO and COX, were also examined and determined to be independent of the presence/absence of the ZBGs (data not shown). This confirms that the ZBGs did not affect the availability of AA in the assay.

At 100 μM, none of the ZBGs examined showed any significant inhibition of 5-LO (Figure 3). A potential explanation for this finding is that the concentrations of these chelators were simply too low to affect lipoxygenase activity. In addition, 5-LO is localized in the nuclear envelope,[54,55] potentially making it more difficult for the ZBGs to gain access to this enzyme. In contrast, the activity of COX decreased noticeably in the presence of the sulfur-containing ZBGs—thiomaltol and 1,2-

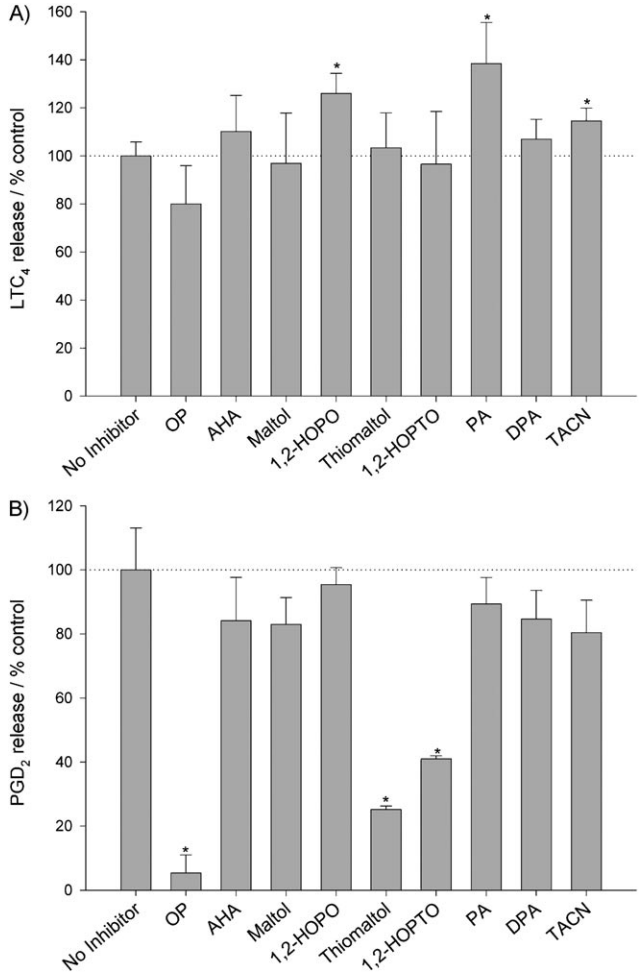


Figure 3. Inhibition of 5-LO and COX by different ZBGs. Production of A) LTC_4 and B) PGD_2 from ATP-stimulated RAW264.7 cells in the presence of each ZBG (100 μM).

HOPTO—by 75% and 50%, respectively (Figure 3). None of the other ZBGs showed greater than 20% inhibition of COX.

Inhibition of iNOS by ZBGs: The activity of iNOS was monitored by measuring one of its reactive oxygen products—nitrite—by the Griess reagent assay.[56] In a control experiment, all ZBGs were tested and shown to have no significant reactivity with the nitrite ion under standard assay conditions (data not shown). As with COX, the ZBGs that caused the most significant decreases in nitrite product were sulfur-containing ligands (Figure 4). Thiomaltol inhibited nitrite production by 80% and 1,2-HOPTO by 75%. The only other ZBG to affect nitrite concentration significantly was DPA, which inhibited iNOS activity by 40%.

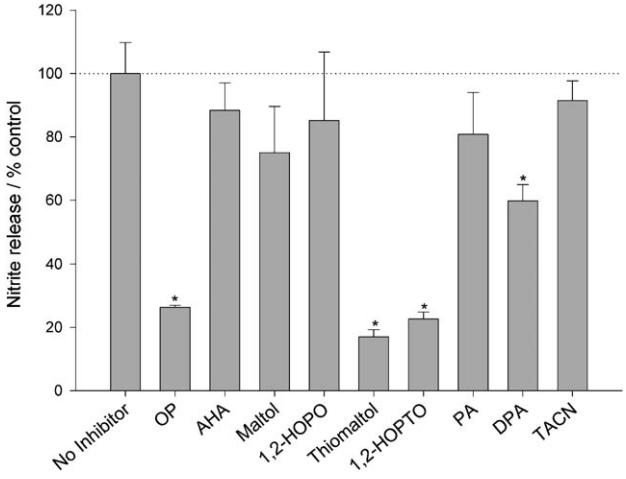


Figure 4. Inhibition of iNOS by different ZBGs. Production of nitrite from KDO-stimulated RAW264.7 cells in the presence of each ZBG (100 μM).

Metalloenzyme inhibition by complete MMPis: In addition to testing ZBGs, the macrophage model system was used to determine the activities of several full-length inhibitors. Six known MMPis were examined: GM6001, NNGH, doxycycline, minocycline, PY-2, and 1,2-HOPO-2 (Scheme 1). GM6001 and NNGH are commercially available, broad-spectrum, nanomolar MMPis.[6] Doxycycline and minocycline are tetracycline-based, broad-spectrum MMP inhibitors,[15,57] which exhibit rather weak potency in vitro (150 μM and 3500 μM, respectively, for MMP-3);[58] however, doxycycline is the only FDA-approved drug for MMP inhibition.[14] PY-2 and 1,2-HOPO-2 are MMPis that have been shown to be semi-selective, with submicromolar inhibition of MMP-3, MMP-8, and MMP-12.[43] 1,2-HOPO-2 also displays submicromolar inhibition of MMP-2.[43] RAW264.7 macrophage cells were incubated with each MMPi at concentrations near to or greater than their most potent reported IC_{50} values (GM6001, NNGH, PY-2, and 1,2-HOPO-2 were applied at 5 μM; doxycycline and minocycline at 100 μM), and enzymatic assays were performed as described above. A summary of the data is shown in Figure 5.

Both NNGH and GM6001 showed potent inhibition of MMPs. GM6001 also caused a reduction in TNF_{α}, indicating inhibition of zinc-dependent TACE. The presence of NNGH leads to a decrease in LTC_4, the metabolite of 5-LO. Doxycycline shows

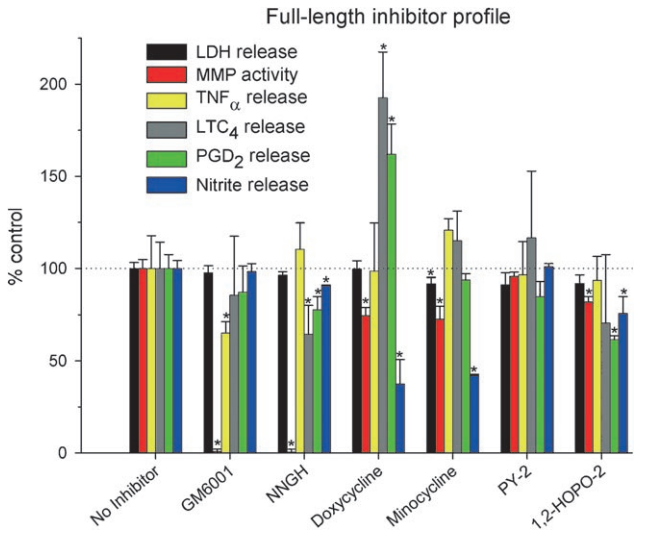


Figure 5. Metalloenzyme inhibition profile of RAW264.7 cells in the presence of GM6001 (5 μM), NNGH (5 μM), PY-2 (5 μM), 1,2-HOPO-2 (5 μM), doxycycline (100 μM), and minocycline (100 μM).

minimal inhibition of MMPs at 100 μM, but there is a marked increase in LTC_4 and PGD_2 metabolites, with a concomitant decrease in nitrite production. Minocycline does not affect LTC_4 and PGD_2 levels as strongly, but does show a decrease in nitrite similar to that observed with doxycycline. PY-2 does not appear to affect any of the enzymes examined in this assay, while 1,2-HOPO-2 appears to cross-inhibit the iron enzymes, slightly blunting the production of 5-LO, COX, and iNOS products.

Discussion

Many inhibitors that target metalloenzymes use chelating moieties that are not selective for the metal contained in the target protein, potentially leading to undesirable side effects in animal model and in clinical evaluations.[3] The findings described here show that a series of straightforward assays in a macrophage cell model can be used to screen metal-chelating fragments or complete enzyme inhibitors against many metalloenzymes rapidly and simultaneously, to determine the metalloenzyme selectivity of a compound in a biological setting. To this end, the ZBGs and MMPi shown in Scheme 1 were examined.

A number of new ZBGs have been developed in recent years in order to address some of the in vivo problems associated with hydroxamic acids.[5,22–25,59,60] Many have been shown to have improved in vitro potency against MMPs relative to simple hydroxamic acids such as AHA (Scheme 1).[25] Some of these ZBGs have been developed into complete MMPis that show semiselective inhibition against several MMP isoforms.[43] One drawback of many of these new ZBGs is that they do not selectively bind zinc(II) over other biologically relevant metal ions. In fact, hydroxypyrone inhibitors have been shown to inhibit iron-dependent soybean lipoxygenase in vitro.[24,61] In the macrophage model presented here, 100 μM of maltol, 1,2-HOPO, or PA showed little inhibition of MMP activity in

RAW264.7 cells. Similarly, none of these ZBGs inhibited iNOS, COX, or 5-LO at 100 μM in the cell-based assay. In the case of MMPs, the lack of inhibition is likely due to the relatively low affinities of these compounds for the enzymes, as these ZBGs have in vitro IC_{50} values of >100 μM,[24,25] and in MMP inhibition experiments maltol and 1,2-HOPO were readily removed by dialysis (Table S1), indicating very weak chelation to the active site zinc(II). Maltol and PA showed minimal in vivo inhibition of TACE, while 1,2-HOPO showed no significant inhibition (Figure 2). Thus, under these assay conditions, maltol, 1,2-HOPO, and PA did not stand out as particularly potent zinc(II) chelators.

The sulfur-containing ligands—thiomaltol and 1,2-HOPTO—demonstrate stronger affinities for zinc(II) than their oxygen-only analogues (maltol and 1,2-HOPO, respectively, Scheme 1).[25,45] Dialysis experiments demonstrate that thiomaltol has an intermediate mode of inhibition that includes both removal of the zinc(II) from, and formation of a ternary complex, inside the MMP protein active site (Table S1, Figure S3). These same experiments indicate that 1,2-HOPTO inhibited MMP by virtually complete removal of the active site zinc(II) ion. 1,2-HOPTO has a lower IC_{50} value than thiomaltol against MMP-3 in vitro,[25,45] and similarly was also more effective than thiomaltol at inhibiting MMP activity in RAW264.7 cells (Figure 2). In contrast, these chelators only weakly inhibited the zinc-dependent enzyme TACE in the cellular assay, to a degree similar to their oxygen-only counterparts (Figure 2). Interestingly, thiomaltol and 1,2-HOPTO were extremely potent inhibitors of heme iron enzymes. Both compounds inhibited COX activity by 70% (Figure 3) and iNOS activity by more than 80% (Figure 4). This strongly suggests that MMP is incorporating these moieties could show cross reactivity with heme enzymes. Without appropriately designed substituents (that is, a selective backbone moiety),[5,6] the use of thiomaltol and 1,2-HOPTO as binding groups for either zinc or heme iron enzymes should be approached with caution, as these chelators may act promiscuously in vivo.

Nitrogenous ligands such as DPA and TACN demonstrated the greatest promise as platforms for zinc(II)-dependent enzyme inhibitors. When the mode of inhibition was examined, DPA inhibited MMPs through a combination of both metal removal and the formation of a stable protein-metal-ligand ternary complex (Table S1, Figure S3). Consistent with in vitro results,[24] TACN and DPA were potent inhibitors of MMP and TACE in RAW264.7 cells, inhibiting ~90% of MMP activity (Figure 2) and 50% of TACE activity (Figure 2). This makes these two chelators the most potent ZBGs against the zinc(II) metalloenzymes on the basis of the results of the macrophage screening presented in this study. In contrast, neither DPA nor TACN significantly inhibited any of the iron enzymes examined. DPA showed some inhibition of iNOS (Figure 4), but had no effect on COX or 5-LO. TACN demonstrated no significant inhibition of any of these metalloenzymes. Given that both of these ZBGs inhibit zinc(II) enzymes in vitro[24] and show selective inhibition over other metalloenzymes in the macrophage model, these ZBGs stand out as excellent candidates for development of selective MMP and/or TACE inhibitors. With an appropriate backbone to increase the potency and specificity for these targets further, DPA and TACN provide an excellent starting point for the creation of potent and selective zinc(II)-dependent metalloenzyme inhibitors.

In addition to screening simple ZBGs, the macrophage model was used to identify potential off-target activity for four known MMPis: GM6001, NNGH, doxycycline, and minocycline. GM6001 completely inhibited MMP activity in RAW264.7 cells, while also blunting TNF_α production. This is not surprising, as GM6001 is known also to inhibit TACE.[40,41] NNGH also showed complete inhibition of MMPs, had no effect on TNF_α production, but blunted the activity of 5-LO, COX, and iNOS (Figure 5), indicating some degree of promiscuity of this compound between zinc- and iron-dependent enzymes. The hydroxamate ZBG employed by NNGH has a strong thermodynamic preference for binding iron(III) over zinc(II),[5,22] and this type of broad, albeit weak, inhibition of several metalloenzymes could contribute to potential side effects in a clinical setting. However, to the best of our knowledge, NNGH has not yet undergone clinical trials.

The tetracycline compounds—doxycycline and minocycline—both showed modest decreases in MMP activity, but did not affect levels of TNF_α. Both tetracyclines decreased nitrite production, which is likely caused or exacerbated by a decrease in iNOS expression, a known off-target interaction of doxycycline.[42] Interestingly, doxycycline increased the production of the 5-LO metabolite LTC_4 and the COX metabolite PGD_2; furthermore, AA levels were found to be increased five-fold (data not shown). This is consistent with work done by Attur et al., who showed that doxycycline increased PGE_2 production by lipopolysaccharide-stimulated RAW264.7 cells.[62] Our data further demonstrate increases in levels of AA, suggesting that doxycycline may hyperactivate cPLA2 and exacerbate the inflammatory response.[29] In general, our findings in the macrophage model support the known pleiotropic natures of these compounds in vivo and confirm that this model can accurately predict known off-target interactions for a variety of MMPis in a simple assay.

Two recently developed MMPis based on hydroxypyrone (maltol) and hydroxypyridinone (1,2-HOPO) ZBGs—PY-2 and 1,2-HOPO-2—were analyzed to determine how MMPis using non-hydroxymate ZBGs interacted with the different metalloenzymes. At 5 μM, neither compound was found to reduce MMP activity in the macrophage model significantly (Figure 5). However, RAW264.7 cells primarily express MMP-9 and MMP-13 (Figure S2, http://www.LipidMaps.org), and while PY-2 and 1,2-HOPO-2 inhibit MMP-2, -3, -8, and -12 effectively, they do not significantly inhibit MMP-1, -7, -9, or -13.[43] The observation that PY-2 and 1,2-HOPO-2 do not exhibit significant MMP inhibition in this assay thus confirms the isoform specificity of these MMPis as determined by in vitro experiments. PY-2 did not significantly inhibit TACE, 5-LO, COX, or iNOS at 5 μM, demonstrating good selectivity against these enzymes. In contrast, 1,2-HOPO-2 did show some inhibition of COX and iNOS, indicating it may have significant off-target activity in vivo. The difference between PY-2 and 1,2-HOPO-2 with respect to the heme iron enzymes is quite striking in view of the similar over-

all structures of the two MMPIs, and this difference in activity was not recapitulated by the ZBGs alone (Figures 3 and 4). This finding points to the significance of the ZBG in MMPI design, and supports the hypothesis that small changes in the ZBG may have profound effects on the behavior of these compounds in vivo.[43,45] The results of these facile screening experiments may be useful for avoiding such pitfalls prior to more advanced development (that is, clinical) of these or other metalloenzyme inhibitors.

Conclusions

A general, cell-based method to screen the effects of compounds against a broad range of zinc- and iron-dependent enzymes has been presented. Two ZBGs—DPA and TACN—showed good selectivity, inhibiting the zinc(II) metalloenzymes MMP and TACE, while sparing the iron enzymes 5-LO, COX, and iNOS. Mixed oxygen-/sulfur-based ZBGs, such as thiomaltol and 1,2-HOPTO, inhibited not only MMPs, but also the heme iron enzymes COX and iNOS. In addition to isolated ZBGs, full-length MMPIs—including the hydroxamate inhibitors GM6001 and NNGH, the tetracycline inhibitors doxycycline and minocycline, and the hydroxypyrone and hydroxypyridinone inhibitors PY-2 and 1,2-HOPO-2—were examined. The macrophage screen correctly predicted the off-target inhibition that is known for the hydroxamate and tetracycline MMPIs. The hydroxypyridione MMPI 1,2-HOPO-2 reduced heme-iron-dependant COX and iNOS activity, whereas its hydroxypyrone analogue PY-2 did not cause any nonspecific inhibition. Overall, we identified TACN and DPA as excellent ZBGs for the development of potent, selective zinc(II) metalloenzyme inhibitors, and have shown that the potential limitations of new full-length inhibitors can be evaluated through a simple, cell-based experiment.

Experimental Section

Materials: Maltol, PA, 1,2-HOPO, 1,2-HOPTO, OP, TACN, DPA, doxycycline, minocycline, and ATP were obtained from Sigma–Aldrich. GM6001 (Illomastat), NNGH (*N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid), the Griess assay, and fluorogenic MMP Substrate III were purchased from Calbiochem (San Diego, CA). OmniMMP fluorogenic substrate was purchased from Biomol (Plymouth, PA). Thiomaltol, PY-2, and 1,2-HOPO-2 were prepared by literature methods.[43,63] RAW264.7 murine macrophages were purchased from the American Type Culture Collection (ATCC, Manassas, VA). LC grade solvents were purchased from EMD Biosciences. Synergy C18 reversed-phase HPLC columns and Strata-X solid-phase extraction columns were purchased from Phenomenex (Torrance, CA). Phosphate buffered saline (PBS) was purchased from VWR. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Kdo_2-Lipid A (KDO) was purchased from Avanti Polar Lipids (Alabaster, AL). All eicosanoids and indomethacin were purchased from Cayman Chemicals (Ann Arbor, MI). Slide-A-Lyzer dialysis cassettes were purchased from Pierce Biotechnologies (Rockford, IL). The TNF_α assay kit was purchased from R&D Systems (Minneapolis, MN). The CytoTox 96 Non-Radioactive Cytotoxicity Assay for measuring lactate dehydrogenase (LDH) activity was purchased from Promega. All other reagents were reagent grade or better. UV/Visible spectra were recorded with a Perkin–Elmer Lambda 25 spectrophotometer. Metal contents were determined with a Perkin–Elmer Optima 3000 DV inductively coupled plasma optical emission spectrometer (ICP-OES) located at the Analytical Facility at the Scripps Institute of Oceanography.

Data analysis: The data were normalized to values measured in uninhibited cells or media, unless explicitly described otherwise. Results were reported as mean $\pm$ standard deviation, and statistical analysis was performed by use of Student's *t*-test. The critical values for statistical significance were set at $\alpha = 0.05$, and *p* values meeting this threshold were denoted in the figures with an asterisk (*).

Cell culture and stimulation: The RAW264.7 murine macrophage cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and penicillin/streptomycin (100 units mL^{-1}) at 37 °C in a humidified 5% CO_2 atmosphere. Cells (5×10^5) were plated in 24-well culture plates in medium (0.5 mL) and were allowed to adhere for 24 h. The medium was replaced with serum-free medium (0.5 mL), and the system was incubated for 1 h and stimulated either with ATP (2 mM) for 10 min, or with KDO (100 ng mL^{-1}) for 24 h. After stimulation, the medium was removed and partitioned for use in all subsequent assays. All ZBGs and inhibitors were added 30 min prior to stimulation.

Sample preparation for short-term (ATP) stimulation: After ATP stimulation, the whole of the medium (0.5 mL) was removed and supplemented with internal standards (50 μL, 200 pg μL^{-1} of $[D_4]PGD_2$, $[D_5]LTC_4$, and $[D_8]AA$ in EtOH) and extracted for PGD_2 and LTC_4 analysis by SPE as previously described.[29,30] The samples were reconstituted in LC solvent A (water/acetonitrile/acetic acid 70:30:0.02, *v/v/v*, 50 μL) for LC-MS/MS analysis.

Sample preparation for long-term (KDO) stimulation: After KDO stimulation, the whole (0.5 mL) of the medium was removed and supplemented with internal standards (50 μL, 200 pg μL^{-1} of $[D_4]PGD_2$, $[D_5]LTC_4$, and $[D_8]AA$ in EtOH). The sample was then divided as follows: 100 μL was extracted for PGD_2 analysis as previously described,[29,30] 50 μL was analyzed for TNF_α, 50 μL was analyzed for nitrite levels, 80 μL was analyzed for MMP activity, and 50 μL was analyzed for LDH activity to determine cell viability. Samples were stored at −20 °C until analysis.

Lactate dehydrogenase release assay: Cell viability was assessed by the LDH release assay according to the manufacturer's protocol. Typically, macrophage cell supernatant (50 μL) was incubated for 30 min with a tetrazolium substrate that is converted by LDH activity (via NADH) into a red formazan product that was measured by absorbance at 490 nm on a Bio-Tek ELX808 absorbance microplate reader. To determine the amount of LDH released at 0% viability, cells were frozen at −80 °C for 1 h and then thawed, and medium was removed. To determine the amount of LDH released at 100% viability, medium was removed from unfrozen cells. The viability of cells incubated with inhibitors was determined relative to these two endpoints.

MMP activity assay: MMP activities of KDO-stimulated cell media were analyzed by a fluorescence substrate assay on a Bio-Tek FLX 800. Cell medium (80 μL) was incubated at 37 °C with assay buffer [20 μL, MES (50 mM), $CaCl_2$ (10 mM), Brij-35 (0.05%), pH 6.0] containing MMP Substrate III (final concentration in each well 400 μM) and *p*-aminophenylmercuric acetate (AMPA, final concentration in each well 1 mM). Upon substrate cleavage, the fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 485$ nm) of each well was measured

after 24 h at 37 °C and expressed as relative activity to cells without inhibitors.[25,45]

TNF_α release assay: TACE activity was determined by measuring the amount of TNF_α secretion by a fluorimetric assay. Typically, KDO-stimulated cell media (50 μL) was diluted in PBS (1:50), and the diluted solution (50 μL) was analyzed according to the manufacturer's protocol. The concentration of TNF_α was measured by absorbance at 450 nm on a Bio-Tek ELX808 absorbance microplate reader and compared to a standard curve established by use of a mouse TNF_α standard (23 pg mL^{-1} to 1500 pg mL^{-1}). Results are reported as relative release to cells without inhibitors.

PGD_2 and LTC_4 eicosanoid production assay: The activities of COX and 5-LO were determined by measurement of the levels of PGD_2 and LTC_4, respectively, by a previously published LC-MS/MS methodology.[29,30]

Nitrite production assay: The activity of iNOS was determined by measurement of the amount of nitrite in KDO-stimulated cell medium. Nitric oxide is readily oxidized into nitrite, which can be measured by the Griess assay.[56] By the manufacturer's protocol, KDO-stimulated media (50 μL) was analyzed by a colorimetric assay. Briefly, sulfanilamide was added to the cell medium (this reacts with nitrite to form a diazonium salt). *N*-1-Napthylethylenediamine dihydrochloride was then added (this reacts with the diazonium salt to form a colored azo compound detectable by absorbance at 550 nm on a Bio-Tek ELX808 absorbance microplate reader). Results are reported as relative release to cells without inhibitors.

Abbreviations

AHA: acetohydroxamic acid; AA: arachidonic acid; AMPA: *p*-aminophenylmercuric acetate; COX: cyclooxgenase; $cPLA_2$: cytosolic phospholipase A_2; DPA 2,2′-dipyridylamine; ELISA: enzyme-linked immunosorbent assay; maltol: 3-hydroxy-2-methyl-4*H*-pyran-4-one; thiomaltol: 3-hydroxy-2-methyl-4*H*-pyran-4-thione; 1,2-HOPO: 1-hydroxypyridine-2(1*H*)-thione; ICP-OES: inductively coupled plasma optical emission spectrometer; iNOS: inducible nitric oxide synthase; KDO: Kdo_2-Lipid A; LDH: lactate dehydrogenase; LTC_4: leukotriene C_4; LO: lipoxygenase; 5-LO: 5-lipoxygenase; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MMP: matrix metalloproteinase; MMPi: MMP inhibitor; NNGH: *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid; OP: *o*-phenanthroline; PBS: phosphate-buffered saline; PA: picolinic acid; PGD_2: prostaglandin D_2; TLR-4: Toll-like receptor 4; TACN: triazacyclononane; TNF_α: tumor necrosis factor α; TACE: TNF_α converting enzyme; ZBG: zinc-binding group.

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