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A Macrophage Cell Model for Selective Metalloprotein Inhibitor Design

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Abstract

The desire to inhibit zinc-dependent matrix metalloproteinases (MMPs) has lead to the development of a plethora of MMP inhibitors over the course of the last 30 years that bind directly to the active site metal. With the exception of one inhibitor, 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 (MMPi), and two novel MMP inhibitors developed in our laboratory to determine their selectivity 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 non-specific interactions of some of the most commonly used MMPi, as well as give cross-reactivity information for newly developed MMPi. This work demonstrates the utility of cell-based assays in both the design and screening of novel metalloenzyme inhibitors.

One of the main targets for metalloenzyme inhibition over the last thirty years has been matrix metalloproteinases (MMPs).[1–6] MMPs comprise a family of calcium(II)-and zinc(II)-dependant 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] Thus, the development of potent and selective MMP inhibitors (MMPi) 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 to have numerous interactions with other metalloenzymes.[16–18] Furthermore, hundreds of other MMPi have been developed over the past thirty years that show excellent potency in vitro, only to exhibit several problematic

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Supporting Information Available. Experimental details on eicosanoid assays, MMP-3(ΔC) expression and purification, metal removal and activity assays, kinetic analysis of ZBG inhibition, Table S1, and Figures S1, S2, and S3. This material is available free of charge via the Internet.

side-effects in clinical trials.[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 MMPi, and other metalloprotein 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 to discover a lack of efficacy and side-effects in animal models or in clinical trials caused by their lack of selectivity. A number of studies have reported screening techniques which identify cross-reactivity between MMP isoforms:[26–28] however, they do not systematically test whether MMPi can have an effect on non-MMP, off-target enzymes. To overcome the limitations of previous in vitro screening approaches, a robust assay system using the macrophage as a model is presented that can determine the efficacy 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 (LipidMaps.org), and, in contrast to human monocyte cells 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 cyclooxygenase (COX) and lipoxygenase (LO) enzymes.[29] The pro-inflammatory 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 MMPi on each metal-dependent pathway.

Using the RAW264.7 cell-line, the metalloenzyme selectivity of nine different ZBGs has 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 (Figure 2).[39] 3-Hydroxy-2-methyl-4H-pyran-4-one (maltol), 3-hydroxy-2-methyl-4H-pyran-4-thione (thiomaltol), 1-hydroxypyridin-2(1H)-one (1,2-HOPO), and 1-hydroxypyridin-2(1H)-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 triazocyclonane (TACN) also inhibit MMPs better when compared to AHA and are expected to be more selective for binding zinc(II) relative to the aforementioned chelating groups (Figure 2).[24]

In addition, the RAW264.7 cell-line model has been used to identify off-target interactions of a variety of known MMPi. For example, GM6001 (Figure 2) is a potent inhibitor of MMPs, but has also been shown to inhibit the zinc(II) enzyme TACE.[40,41] Doxycycline and minocycline (Figure 2), which are broad-spectrum MMP inhibitors, are also known to blunt the activity of iNOS.[42] Overall, six MMPi were evaluated in the macrophage model, including four commercially available MMPi (Figure 2) and two potent MMPi (Figure 2) developed in our laboratory[43] whose interactions with other metalloenzymes have not been

previously characterized. 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 activity of compounds on a variety of metalloenzymes simultaneously. While we have applied this model towards the design of selective MMPi, this screening method 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 to analyze the specificity of not only MMPi, but also TACE inhibitors, COX inhibitors, iNOS inhibitors, 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 examining metalloprotein activity, the toxicity of the different ZBGs was determined. Cell viability was assessed by the release of lactate dehydrogenase (LDH) from Kdo₂-Lipid A (KDO) stimulated macrophage cells in the presence of 100 μ M of each ZBG for 24 hours. LDH is a stable, cytosolic enzyme that is released upon cell death, and the concentration of LDH in the extracellular media correlates with cell death. The results are shown in Figure S1 and were confirmed visually using 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 μ M. 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 toxicity at 100 μ M. The high toxicity of OP at 100 μ M 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.

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 metalloproteins by various ZBGs in a biological setting using 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 activity of different metalloenzymes. Stimulating the P2X₇ purinergic receptor with ATP generates an influx of extracellular Ca²⁺. Within minutes, this Ca²⁺ influx transiently activates both the cytosolic phospholipase A₂ (cPLA₂) to release AA from membrane phospholipids and the non-heme iron enzyme 5-LO to process AA to form bioactive leukotriene C₄ (LTC₄). [29] AA can also be acted upon by the constitutively expressed heme iron enzyme COX to make prostaglandin D₂ (PGD₂). 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 cPLA₂ activity over a 24 hour period.[29] Over this period, KDO activates the transcription factor NF- κ B, allowing for the upregulation of COX, iNOS, pro-TNF α , pro-MMP-9, and pro-MMP-13 (Figure S2, LipidMaps.org).[46] Pro-TNF α is cleaved by membrane associated TACE[36] to the soluble signaling protein TNF α . Using these two stimulation pathways, the activity of five different metalloenzymes could be examined in the presence of different ZBGs. In the sections below, the activity of different ZBGs against zinc-dependent enzymes is 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,

LipidMaps.org).[46] In this study, cells were stimulated with KDO in the presence of 100 μ M ZBG. 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 extra-cellular 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 extra-cellular media. This is confirmed by experiments showing no activity in the extra-cellular media when AMPA is excluded from the assay (data not shown).

At 100 μ M, DPA and TACN inhibited MMP activity greater than 90%. The sulfur-containing ligands, 1,2-HOPTO and thiomaltol also inhibited the MMP activity, but to a lesser extent (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 3.

Inhibition of TACE by ZBGs—TACE activity was measured by the relative amount of TNF_α in the extracellular media following KDO stimulation. Because TACE is a zinc-dependent enzyme, it was anticipated that some ZBGs would inhibit TNF_α release. Indeed, DPA and TACN are both inhibitors of activity (Figure 3), with 100 μ M DPA inhibiting TNF_α release by 60%. Other ZBGs including PA, maltol, and thiomaltol inhibited release by approximately 25%, 30%, and 40%, respectively. All other ZBGs inhibited TNF_α production by less than 20%, including 1,2-HOPTO. 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 activity of 5-LO and COX, the presence of their AA products were analyzed following 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 using LC-MS/MS methodology that has been previously described.[29] It is important to note that these metabolites could also be measured, if adequate mass spectrometers are unavailable, using commercially available ELISA assays (Cayman Chemicals, Ann Arbor, MI). Zileuton[51,52] and indomethacin[53] were used as a positive control 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 μ M, none of the ZBGs examined showed any significant inhibition of 5-LO (Figure 4). A potential explanation for this finding is that the concentration of these chelators was simply too low to effect 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-HOPTO, by 75% and 50%, respectively (Figure 4). 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, using 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). Similar to COX, the ZBGs that caused the most significant decrease in nitrite product were sulfur-containing ligands (Figure 5). Thiomaltol inhibited 80% and 1,2-HOPTO inhibited 75% of nitrite production. The only

other ZBG to significantly affect nitrite concentration was DPA, which inhibited iNOS activity by 40%.

Metalloenzyme Inhibition by Complete MMPi—In addition to testing ZBGs, the macrophage model system was used to determine the activity of several full length inhibitors. Six known MMPi were examined: GM6001, NNGH, doxycycline, minocycline, PY-2, and 1,2-HOPO-2 (Figure 2). GM6001 and NNGH are commercially available, broad spectrum, nanomolar MMPi.[6] Doxycycline and minocycline are tetracycline-based, broad spectrum MMP inhibitors,[15,57] which exhibit rather weak potency in vitro (150 μ M and 3500 μ M for MMP-3, respectively);[58] however, doxycycline is the only FDA approved drug for MMP inhibition.[14] PY-2 and 1,2-HOPO-2 are MMPi 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 possesses submicromolar inhibition of MMP-2 as well.[43] RAW264.7 macrophage cells were incubated with each MMPi at concentrations near or greater than their most potent reported IC₅₀ values (GM6001, NNGH, PY-2 and 1,2-HOPO-2 were applied at 5 μ M; doxycycline and minocycline at 100 μ M) and enzymatic assays were performed as described above. The summary of the data is shown in Figure 6.

Both NNGH and GM6001 showed potent inhibition of MMPs. GM6001 also caused a reduction in TNF $_{\alpha}$, indicating inhibition of zinc-dependent TACE. The presence of NNGH leads to a decrease in LTC₄, the metabolite of 5-LO. Doxycycline shows minimal inhibition of MMPs at 100 μ M, but there is a marked increase in LTC₄ and PGD₂ metabolites, with a concomitant decrease in nitrite production. Minocycline does not affect LTC₄ and PGD₂ levels as strongly, but does show a decrease in nitrite similar to that observed with doxycycline. PY-2 does not appear to effect 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 rapidly screen metal chelating fragments or complete enzyme inhibitors against many metalloenzymes simultaneously, to determine the metalloenzyme selectivity of a compound in a biological setting. To this end, the ZBGs and MMPi shown in Figure 2 were examined.

A number of new ZBGs have been developed in recent years to address some of the in vivo problems associated with hydroxamic acids.[5,22–25,59,60] Many have been shown in vitro to have improved potency against MMPs when compared to a simple hydroxamic acid such as AHA (Figure 2).[25] Some of these ZBGs have been developed into complete MMPi that show semi-selective 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, hydroxypyrrone inhibitors have been shown to inhibit iron-dependent soybean lipoxygenase in vitro.[24,61] In the macrophage model presented here, 100 μ M 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 μ M in the cell based assay. In the case of MMPs, the lack of inhibition is likely due to the relatively low affinity of these compounds for the enzymes, as these ZBGs have IC₅₀ values of >100 μ M in vitro,[24,25] and in the 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 3). Thus, under the present 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 a stronger affinity for zinc(II) than their oxygen-only analogues (maltol and 1,2-HOPO, respectively, Figure 2). [25,45] Dialysis experiments demonstrate that thiomaltol has an intermediate mode of inhibition that includes both removal of the zinc(II) from MMP and formation of a ternary complex inside the 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 3). 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 3). Interestingly, thiomaltol and 1,2-HOPTO were extremely potent inhibitors of heme-iron enzymes. Both compounds inhibited COX activity by 70% (Figure 4), and iNOS activity by more than 80% (Figure 5). This strongly suggests that MMPi using these moieties could show cross reactivity with heme enzymes. Without appropriately designed substituents (i.e. 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 examining the mode of inhibition, DPA inhibited MMPs using 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 3) and 50% of TACE activity (Figure 3). This makes these two chelators the most potent ZBGs against the zinc(II) metalloenzymes based on 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 5), 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 to develop selective MMP and/or TACE inhibitors. With an appropriate backbone to further increase the potency and specificity for these targets, DPA and TACN provide an excellent starting point for creating 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 MMPi: 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 to also 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 6), 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 the clinical setting. However, to the best of our knowledge, NNGH has not yet undergone clinical trials.

The tetracycline compounds, doxycycline and minocycline, both showed a modest decrease in MMP activity, but did not effect 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 LTC₄ and the COX metabolite PGD₂; furthermore, AA levels were found to be increased by 5-fold (data not shown). This is consistent with work done by Attur et al., who showed that doxycycline increased PGE₂ production by lipopolysaccharide stimulated RAW264.7 cells.[62] Our data further demonstrates an increase 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 nature of these compounds in vivo and confirm that this model can accurately predict in a simple assay known off-target interactions for a variety of MMPi.

Two recently developed MMPi based on hydroxypyrrone (maltol) and hydroxypyridinone (1,2-HOPO) ZBGs, PY-2 and 1,2-HOPO-2, were analyzed to determine how MMPi using non-hydroxamate ZBGs interacted with the different metalloenzymes. At 5 μ M, neither compound was found to significantly reduce MMP activity in the macrophage model (Figure 6). However, RAW264.7 cells primarily express MMP-9 and MMP-13 (Figure S2, 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] Thus the observation that PY-2 and 1,2-HOPO-2 do not exhibit significant MMP inhibition in this assay confirms the isoform specificity of these MMPi as determined by in vitro experiments. PY-2 did not significantly inhibit TACE, 5-LO, COX, or iNOS at 5 μ M, 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 considering the similar overall structure of the two MMPi and this difference in activity was not recapitulated by the ZBGs alone (Figure 4, Figure 5). This finding points to the significance of the ZBG in designing MMPi, 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 to avoid such pitfalls prior to more advanced development (i.e. clinical) of these or other metalloenzyme inhibitors.

A general, cell-based method to screen the effect 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-HOPO, inhibited not only MMPs, but also the heme-iron enzymes COX and iNOS. In addition to isolated ZBGs, full length MMPi were examined, including: the hydroxamate inhibitors GM6001 and NNGH, the tetracycline inhibitors doxycycline and minocycline, and the hydroxypyrrone and hydroxypyridinone inhibitors PY-2 and 1,2-HOPO-2. The macrophage screen correctly predicted the off-target inhibition that is known for the hydroxamate and tetracycline MMPi. The hydroxypyridinone MMPi 1,2-HOPO-2 reduced heme-iron dependant COX and iNOS activity, whereas its hydroxypyrrone analogue, PY-2, did not cause any non-specific inhibition. Overall, we identified TACN and DPA as excellent ZBGs for the development of potent, selective zinc(II) metalloenzyme inhibitors, and show that through a simple, cell-based experiment the potential limitations of new full length inhibitors can be evaluated.

METHODS

Materials

Maltol, PA, 1,2-HOPO, 1,2-HOPO-2, OP, TACN, DPA, doxycycline, minocycline, and ATP were obtained from Sigma-Aldrich (St. Louis, MO). 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 according to literature methods.[43,63] RAW264.7 murine macrophages were purchased from American Type Culture Collection (ATCC, Manassas, VA). LC grade solvents were purchased from EMD Biosciences. Synergy C18 reverse phase HPLC column 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). Kdo₂-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 (Madison, WI). All other reagents were reagent grade or better. UV-Visible spectra were recorded using a Perkin-Elmer Lambda 25 spectrophotometer. Metal content was determined using 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 has been 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 using the 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 10% fetal bovine serum and 100 units/mL penicillin/streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. 5×10^5 cells were plated in 24-well culture plates in 0.5 mL of media and allowed to adhere for 24 h. The media was replaced with 0.5 mL of serum free media, incubated for 1 h, and stimulated with either 2 mM ATP for 10 min, or with 100 ng/mL KDO for 24 h. Following stimulation, the media was removed and utilized in subsequent assays. All ZBGs and inhibitors were added 30 min prior to stimulation.

Sample Preparation for Short-term (ATP) Stimulation

Following ATP stimulation, the entire 0.5 mL of media was removed and supplemented with 50 μ L of internal standards (200 pg/ μ L of d₄-PGD₂, d₅-LTC₄, and d₈-AA in EtOH) and extracted for PGD₂ and LTC₄ analysis by SPE as previously described.[29,30] The samples were reconstituted in 50 μ L of LC solvent A (water-acetonitrile-acetic acid (70:30:0.02; v/v/v)) for LC-MS/MS analysis.

Sample Preparation for Long-term (KDO) Stimulation

Following KDO stimulation, the entire 0.5 mL of media was removed and supplemented with 50 μ L of internal standards (200 pg/ μ L of d₄-PGD₂, d₅-LTC₄, and d₈-AA in EtOH). The sample was then divided as follows: 100 μ L was extracted for PGD₂ analysis as previously described,[29,30] 50 μ L was analyzed for TNF α , 50 μ L was analyzed for nitrite levels, 80 μ L was analyzed for MMP activity, and 50 μ L 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 using the LDH release assay according to the manufacturer's protocol. Typically, 50 μ L of macrophage cell supernatant 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, thawed, and media was removed. To determine the amount of LDH released at 100% viability, media was removed from unfrozen cells. The viability of cells incubated with inhibitors was determined relative to these two endpoints.

MMP Activity Assay

MMP activity of KDO stimulated cell media was analyzed using a fluorescence substrate assay on a Bio-Tek FLX 800. 80 μ L of cell media was incubated at 37°C with 20 μ L of assay buffer (50 mM MES, 10 mM CaCl_2 , 0.05% Brij-35, 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 ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 485 \text{ nm}$) 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 using a fluorometric assay. Typically, 50 μ L of KDO-stimulated cell media was diluted 1:50 in PBS, and 50 μ L of the diluted solution 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 using 23 pg/mL to 1500 pg/mL mouse TNF α standard. Results are reported as relative release to cells without inhibitors.

PGD $_2$ and LTC $_4$ Eicosanoid Production Assay

The activity of COX and 5-LO were determined by measuring the levels of PGD $_2$ and LTC $_4$, respectively, by using a previously published LC-MS/MS methodology.[29,30]

Nitrite Production Assay

The activity of iNOS was determined by the measuring the amount of nitrite in KDO stimulated cell media. Nitric oxide is readily oxidized into nitrite, which can be measured using the Griess assay.[56] Following the manufacturer's protocol, 50 μ L of KDO stimulated media was analyzed using a colorimetric assay. Briefly, to the cell media was added sulfanilamide, which reacts with nitrite to form a diazonium salt. *N*-1-Naphthylethylenediamine dihydrochloride was then added, which reacts with the diazonium salt to form a colored azo compound that was detected by absorbance at 550 nm on a Bio-Tek ELX808 absorbance microplate reader. Results are reported as relative release to cells without inhibitors.

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GLOSSARY

AHA	aceto-hydroxamic acid
AA	arachidonic acid
AMPA	<i>p</i> -aminophenylmercuric acetate
COX	cyclooxygenase
cPLA₂	cytosolic phospholipase A ₂
DPA	2,2'-dipyridylamine
ELISA	enzyme-linked immunosorbent assay
maltol	3-hydroxy-2-methyl-4 <i>H</i> -pyran-4-one
thiomaltol	3-hydroxy-2-methyl-4 <i>H</i> -pyran-4-thione
1,2-HOPO	1-hydroxypyridin-2(1 <i>H</i>)-one
1,2-HOPTO	1-hydroxypyridin-2(1 <i>H</i>)-thione
ICP-OES	inductively coupled plasma optical emission spectrometer
iNOS	inducible nitric oxide synthase
KDO	Kdo ₂ -Lipid A
LDH	lactate dehydrogenase
LTC₄	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
PGD₂	prostaglandin D ₂
TLR-4	Toll-like receptor 4
TACN	triazocyclonane
TNF_α	tumor necrosis factor α
TACE	TNF _α converting enzyme
ZBG	zinc-binding group

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Figure 1.

Overview of RAW264.7 macrophage activation. KDO (Kdo₂-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 P2X₇, causing an influx of extracellular Ca²⁺ which results in AA release and 5-lipoxygenase (5-LO) activation. The outcome is an increase of LTC₄ (leukotriene C₄) by 5-LO and PGD₂ (prostaglandin D₂) by COX. Metalloenzymes are flagged in green, describing the type of metalloenzyme; the specific metabolites measured in this study are shown in parenthesis.

Figure 2. Chelators (top) and MMPi (bottom, in box) evaluated in a macrophage-based model of metalloenzyme activity. Diagram depicting how hydroxamate-based MMPi (GM6001, NNGH) bind to the MMP active site (right of box).

Figure 3. Inhibition of zinc-dependent enzymes MMPs and TACE by different ZBGs. A) MMP activity and B) release of TNF α from KDO stimulated RAW264.7 cells in the presence of 100 μ M of each ZBG.

Figure 4. Inhibition of 5-LO and COX by different ZBGs. A) Production of the LTC₄ and B) PGD₂ from ATP stimulated RAW264.7 cells in the presence of 100 μ M of each ZBG.

Figure 5.
Inhibition of iNOS by different ZBGs. Production of nitrite from KDO stimulated RAW264.7 cells in the presence of 100 μ M of each ZBG.

Figure 6. Metalloenzyme inhibition profile of RAW264.7 cells in the presence of GM6001 (5 μ M), NNGH (5 μ M), PY-2 (5 μ M), and 1,2-HOPO-2 (5 μ M), doxycycline (100 μ M), and minocycline (100 μ M).