Published in final edited form as:

J Med Chem. 2021 May 13; 64(9): 5874–5885. doi:10.1021/acs.jmedchem.1c00038.

Highly Efficient Activatable MRI Probe to Sense Myeloperoxidase Activity

Cuihua Wang,

Institute for Innovation in Imaging, Department of Radiology, Massachusetts General Hospital, Charlestown, Massachusetts 02129, United States; Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

David Cheng,

Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia

Negin Jalali Motlagh,

Institute for Innovation in Imaging, Department of Radiology, Massachusetts General Hospital, Charlestown, Massachusetts 02129, United States; Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Enrico G. Kuellenberg,

Institute for Innovation in Imaging, Department of Radiology, Massachusetts General Hospital, Charlestown, Massachusetts 02129, United States; Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Gregory R. Wojtkiewicz,

Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Stephen P. Schmidt,

Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Roland Stocker,

Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia; Heart Research Institute, Newton, NSW 2042, Australia

John W. Chen

Institute for Innovation in Imaging, Department of Radiology, Massachusetts General Hospital, Charlestown, Massachusetts 02129, United States; Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, United States

Corresponding Author: John W. Chen, jwchen@mgh.harvard.edu.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00038>.

High-resolution LC-MS of heMAMP; in vitro relaxivity measurement experiments; cytotoxicity study; biodistribution and retention; and in vivo imaging and molecular docking experiments (PDF)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00038>

The authors declare the following competing financial interest(s): John W. Chen and Cuihua Wang are inventors of a patent application based on the technology reported in this manuscript.

Abstract

Myeloperoxidase (MPO) is a key component of innate immunity but can damage tissues when secreted abnormally. We developed a new generation of a highly efficient MPO-activatable MRI probe (heMAMP) to report MPO activity. heMAMP has improved Gd stability compared to bis-5-HT-Gd-DTPA (MPO-Gd) and demonstrates no significant cytotoxicity. Importantly, heMAMP is more efficiently activated by MPO compared to MPO-Gd, 5HT-DOTA(Gd), and 5HT-DOTAGA-Gd. Molecular docking simulations revealed that heMAMP has increased rigidity via hydrogen bonding intramolecularly and improved binding affinity to the active site of MPO. In animals with subcutaneous inflammation, activated heMAMP showed a 2–3-fold increased contrast-to-noise ratio (CNR) compared to activated MPO-Gd and 4–10 times higher CNR compared to conventional DOTA-Gd. This increased efficacy was further confirmed in a model of unstable atherosclerotic plaque where heMAMP demonstrated a comparable signal increase and responsiveness to MPO inhibition at a 3-fold lower dosage compared to MPO-Gd, further underscoring heMAMP as a potential translational candidate.

Graphical Abstract

1. INTRODUCTION

Myeloperoxidase (MPO) is a proinflammatory and oxidative enzyme expressed in neutrophils and M1-type microglia and macrophages, but not by anti-inflammatory M2-type microglia and macrophages.^{1–3} MPO is a key player in many diseases, including atherosclerosis,⁴ vasculitis,⁵ stroke,⁶ Parkinson's disease,⁷ Alzheimer's disease,^{8,9} and multiple sclerosis,^{10,11} and is emerging as an important diagnostic and therapeutic target.^{12–15} Imaging agents targeting MPO or its reactive oxidative products, especially hypochlorous acid (HOCl), for various imaging modalities such as fluorescence, positron emission tomography, and magnetic resonance imaging have been intensively investigated,^{16–24} which have provided powerful tools to better understand the roles MPO and its reactive oxidative species play in pathological conditions. In particular, the MRI agent bis-5-HT-Gd-DTPA (MPO-Gd)¹⁷ targeting MPO had been found to be specific at detecting inflammation and track treatment effects in animal models of experimental autoimmune encephalomyelitis (EAE),^{25,26} atherosclerosis,^{15,27} stroke,^{6,28} myocardial infarction,^{29,30} vasculitis,⁵ and to differentiate nonalcoholic steatohepatitis from steatosis.³¹

However, there have been safety concerns about gadolinium-based contrast agents (GBCAs) used in MRI, especially linear GBCAs, in the development of nephrogenic systemic fibrosis (NSF) in renal failure patients since GBCAs are primarily excreted through the kidneys and prolonged blood half-life of GBCAs was found in these patients.^{32,33} Recent studies also revealed that gadolinium deposition following administration of GBCAs was also found in

neural tissues in patients and animal models with normal renal function.^{34–36} Most cases of nephrogenic systemic fibrosis and gadolinium deposition in neural tissues were associated with the release of gadolinium from the use of GBCAs containing linear chelators, such as gadodiamide (Omniscan), gadopentetate dimeglumine (Gd-DTPA or Magnevist), and OptiMARK. Compared with linear GBCAs, macrocyclic GBCAs bind gadolinium tighter, thereby decreasing the likelihood of the gadolinium ion dissociating from the chelating molecule. Consistent with this, administration of macrocyclic GBCAs such as gadoterate meglumine (Dotarem)^{36,37} and gadoteridol (Prohance)³⁸ has little to no observed tissue deposition of gadolinium. Although to date there has been no scientific evidence showing that gadolinium deposition in the brain causes neurotoxicity, the safety of GBCAs has drawn great attention. In 2017, the European Medicine Agency (EMA) suspended the use of linear GBCAs with the exception of liver imaging with MultiHance and Primovist and intraarticular imaging with Magnevist.³⁹ In Japan, macrocyclic GBCAs are the primary choice and linear GBCAs are used only for patients with a history of adverse events with macrocyclic agents.⁴⁰ As such, the U.S. Food and Drug Administration (FDA) has issued a statement stating that linear GBCAs are more retained and retained for longer compared to macrocyclic GBCAs.⁴¹ Since MPO-Gd contains the linear chelate DTPA (Figure 1), the safety concerns limited its potential for translation. Furthermore, although MPO-Gd showed high specificity to MPO, the in vivo signal increase after activation was only about 2-fold, limiting its sensitivity. Therefore, a more efficient and safer MRI agent is needed for translation to more sensitively detect MPO activity. We report here the next generation of a highly efficient MPO-activatable MRI probe (heMAMP) that not only possesses higher stability and safety profiles, but also 4–10× increased signal after activation to improve the efficacy in detecting MPO activity and inflammation in vitro and in vivo.

2. RESULTS AND DISCUSSION

2.1. Design and Synthesis of heMAMP.

To improve the ability of the chelate to bind gadolinium tightly while retaining the specificity of the agent to detect MPO activity, we chose DOTA as a chelating ligand due to its thermodynamic and kinetic stability compared to the linear chelates and other macrocyclic-based chelates.⁴² We chose two 5-hydroxyindole units as MPO-activatable moieties to enhance retention as 5-hydroxyindole-based compounds are substrates of MPO and can be oxidized by MPO to form radicals in the presence of hydrogen peroxide through one-electron transfer.¹⁷ These resultant radicals after leaving the active site of MPO either undergo self-oligomerization to form oligomers/polymers or covalently bind to nearby proteins containing indolic or phenolic amino acid residues such as tyrosine,^{17,43} which facilitates the retention of the agent at the site of inflammation. The two amide bonds formed between the two 5-hydroxyindole units increased the rigidity of heMAMP (Figure 1).

The chemical synthesis of heMAMP is shown in Schemes 1 and 2. Intermediate 1 was synthesized through the bromination of glutamic acid, followed by protection with *tert*-butanol.⁴⁴ The synthesis of intermediate 4 began with the Boc-protection of 5-hydroxytryptophan to give compound 2, which subsequently underwent coupling with serotonin mediated by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) hydrochloride and

hydroxybenzotriazole (HOBt) to give compound **3**. Deprotection of compound **3** with trifluoroacetic acid provided intermediate **4** (Scheme 1). Compound **5** was reacted with intermediate **1** under basic conditions, followed by deprotection of the obtained compound **6** with trifluoroacetic acid to provide compound **7**. The synthesis of heMAMP was achieved by chelating compound **7** with gadolinium chloride and subsequent EDC coupling of the obtained compound **8** with intermediate **4** (Scheme 2). The characterization of heMAMP was performed using high-resolution mass spectrometry (HRMS) (Figure S1).

2.2. Stability of heMAMP.

2.2.1. Transmetalation with Zn^{2+} and Ca^{2+} .—We compared the kinetic stability of heMAMP in the presence of $\text{Zn}^{2+}/\text{Ca}^{2+}$ with MPO-Gd since both are major competing ions for gadolinium and are present at high concentrations in physiological conditions.^{17,45} The transmetalation between $\text{Zn}^{2+}/\text{Ca}^{2+}$ and Gd^{3+} results in relaxation change due to the formation of the precipitate GdPO_4 . T_1 relaxation rates (R_1) were monitored as previously described at different time points.¹⁷ We used two indices: a long-term index, defined as the ratio of R_1 at 72 h to the initial R_1 value, measures the degree that transmetalation occurs by 72 h, and a ratio index, defined as the time required to lose 20% of the initial R_1 value, reports the rate at which transmetalation occurs. For both indices, the larger the number is, the more stable the chelate is at preventing transmetalation. There were no R_1 changes over 95 h for heMAMP in the presence of $\text{Zn}^{2+}/\text{Ca}^{2+}$ and the long-term index were >0.97 for Zn^{2+} and >0.96 for Ca^{2+} , and the ratio index >5000 min ($t = 72\text{--}95$ h). Both indices of heMAMP are substantially higher than that of MPO-Gd (Table 1), indicating high stability of heMAMP, consistent with a previous study on the kinetic stability of macrocyclic GBCAs.⁴⁶

2.2.2. Plasma Stability.—We next evaluated the stability of heMAMP under physiological conditions. heMAMP was incubated with plasma at 37 °C for 3 h and the relaxation time was monitored at 0, 30, 60, 90, 120, and 180 min. heMAMP demonstrated no significant T_1 changes over 3 h (Figure 2a), further validating the stability of heMAMP.

2.3. Cytotoxicity of heMAMP.

We evaluated the cytotoxicity of heMAMP with RAW 264.7 cells and Hek 293 cells using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. Both RAW 264.7 and HEK 293 cells (1.5×10^4 per well) were incubated with solutions of heMAMP at concentrations of 0, 0.1, 0.5, 1, 2, and 5 mM in DMEM containing 10% FBS for 16 h at 37 °C. The MTT assay showed that no significant cytotoxicity was found up to 5 mM for both RAW 264.7 and HEK 293 cells (Figure 2b), which is well above the expected in vivo concentration (sub- μM).

2.4. Relaxivity and In Vitro Reactivity of heMAMP with MPO.

The relaxivity (r_1) of heMAMP was $5.6 \text{ mM}^{-1} \text{ s}^{-1}$ at 0.47 T (PBS, 40 °C) (Figure S2), which is slightly higher than $4.3 \text{ mM}^{-1} \text{ s}^{-1}$, determined previously for MPO-Gd under the same conditions.⁴⁷ In the presence of MPO and H_2O_2 , both heMAMP and MPO-Gd can be oxidized to form oligomers/polymers, which will shorten the T_1 relaxation time and subsequently increase the relaxivity. To compare the reactivities of heMAMP and MPO-Gd

with MPO, we incubated heMAMP or MPO-Gd (0.5 mM) in PBS (2.7 mM of potassium chloride and 137 mM of sodium chloride as the chloride source) with MPO, and glucose oxidase (GOX) and glucose as the H₂O₂ donor.⁴⁸ We observed a >3-fold greater *R1* ratio change compared to that of MPO-Gd over 3 h (Figure 2c), indicating a substantially more effective activation of heMAMP compared with MPO-Gd.

In addition to self-oligomerization, “activated” heMAMP by MPO can covalently bind to nearby proteins that have accessible indolic or phenolic amino acids. The binding to proteins greatly increases the relaxivity of activated heMAMP by slowing down the rotational dynamics and makes it possible for heMAMP retention and prolonged enhancement at the site of inflammation. We evaluated the capability of heMAMP and MPO-Gd to bind to bovine serum albumin (BSA) after activation by MPO. In the presence of BSA, MPO-Gd demonstrated a modest 29% improvement in *T1* shortening (34–63%). However, heMAMP showed a 41% improvement with BSA (80–121%) (Figure 2d). Overall, MPO-mediated activation of heMAMP demonstrated an approximately 1.5-fold higher protein binding efficacy (41 vs 29%) in the presence of BSA compared to MPO-Gd. These data further confirmed that heMAMP is more responsive to MPO than MPO-Gd.

2.5. Biodistribution and Retention of heMAMP.

The biodistribution and retention of heMAMP were evaluated using a mouse model of paw inflammation induced by complete Freund’s adjuvant (CFA). Major organs, including forepaws with CFA and PBS, were collected at 3 h and day 7 after the administration of heMAMP, and the amounts of gadolinium were detected with inductively coupled plasma mass spectrometry (ICP-MS). As shown in Figures 2e and S3, heMAMP was mostly excreted by the kidneys with urine, and liver and kidneys are the major organs containing heMAMP. The forepaw with CFA demonstrated over four times higher amount of gadolinium compared to the control side with PBS at 3 h. At day 7, most of the gadolinium from heMAMP was eliminated, except for liver, kidneys, and spleen containing decreased heMAMP, and the bone retention slightly increased (Figure 2e). We evaluated the blood half-life of heMAMP by detecting the concentrations of gadolinium in blood samples using ICP-MS with a two-phase decay model. The results illustrated that the fast-phase blood half-life of heMAMP is 1.2 min and the slow-phase blood half-life is 31.7 min (Figure 2f).

2.6. MR Imaging of Subcutaneous Inflammation.

We validated the efficacy of heMAMP in a mouse model of subcutaneous inflammation induced by CFA. Twenty-four hours after injection of a CFA emulsion into the shoulder of mice, imaging agents were injected via the tail vein and the animals were imaged. Figure 3a–d illustrates images obtained precontrast and 60 min after administration of heMAMP, DOTA-Gd, and MPO-Gd in wild-type mice and for heMAMP in MPO-deficient (*Mpo*^{−/−}) mice, respectively. In wild-type mice, the contrast-to-noise ratio (CNR) observed with heMAMP increased over 60 min, while that seen with DOTA-Gd decreased rapidly over time (Figure 3e), demonstrating that heMAMP but not DOTA-Gd was retained in the inflamed tissue, likely as a result of activation of the former by inflammation-associated MPO activity and subsequent binding to protein residues as previously demonstrated for MPO-Gd¹⁷ and above for heMAMP (Figure 2d). In contrast, heMAMP MR imaging in

Mpo^{-/-} mice with the injury showed a minimal signal increase (Figure 3c,e), validating the in vivo specificity for MPO with heMAMP in this model. heMAMP demonstrated a 4-fold increase in the activation ratio ($AR = \text{CNR}_{(\text{post-contrast})} / \text{CNR}_{(\text{first post-contrast})}$)²⁸ compared with ~2-fold increase seen with MPO-Gd (Figure 3f). The higher enhancement and activation ratio of heMAMP prompted us to explore the efficacy of heMAMP at a lower dose. When the dosage of heMAMP was reduced from 0.3 to 0.1 mmol/kg, we observed a similar CNR from heMAMP compared to that of MPO-Gd at 0.3 mmol/kg (Figures 3e,f and S4). Taken together, these findings confirmed the higher sensitivity and efficacy of heMAMP compared with MPO-Gd for MPO activity.

2.7. Imaging of Unstable Atherosclerotic Plaque and Intervention with MPO Inhibitor AZM198.

MPO activity has been previously reported to be elevated in unstable compared with stable plaques in the tandem stenosis (TS) mouse model of plaque instability, as assessed by both MPO-Gd MRI and LC-MS/MS determination of the conversion of hydroethidine to 2-chloroethidium.¹⁵ In the current study, we validated the ability of heMAMP to image MPO activity in unstable plaque and compared it with MPO-Gd. The imaging was performed 7 weeks after the TS surgery, as done previously,¹⁵ administering 0.3 mmol/kg of MPO-Gd or 0.1 mmol/kg of heMAMP via the tail vein, given that these two doses from MPO-Gd and heMAMP demonstrated comparable signal enhancement in the study of CFA-induced inflammation (Figure 3e,f). As shown in Figure 4a,b,d, signal enhancement in unstable plaque (arrows) was comparable for heMAMP and MPO-Gd (CNR 17.8 vs 15.0, $P = 0.37$), though the dosage of heMAMP was 3-fold lower, further confirming the efficacy of heMAMP for detecting MPO activity.

To confirm that MR imaging of heMAMP can reflect MPO activity, we treated TS mice with the specific MPO inhibitor AZM198¹⁵ 1 week post-TS surgery and subjected the mice to MRI after an additional 6 week treatment period (a total of 7 weeks post-TS). Following the administration of heMAMP, the CNRs for stable and unstable plaques increased at 30 min post-contrast administration, but thereafter (60 min) decreased in mice treated with AZM198 but not in control animals (Figure S5). As a result, the CNR in the region corresponding to unstable plaque decreased by ~50% in AZM198-treated mice compared with that seen in control mice (Figure 4c,e), indicating effective inhibition of MPO activity by AZM198, while CNR in the stable plaque region between the control and AZM-treated mice showed no difference (Figure S6).

In the previous studies, we and other investigators have demonstrated that MPO-Gd showed about a 2-fold signal increase in multiple disease models, including Matrigel embedded with human MPO,^{17,43} atherosclerosis in rabbits²⁷ and mice,¹⁵ nonalcoholic steatohepatitis (NASH),³¹ and a 4-fold increase in myocardial infarction.²⁹ Two activatable analogues, 5HT-DOTA(Gd) and 5HT-DOTAGA-Gd, revealed a similar degree of increase to MPO-Gd in a Matrigel implant experiment and a model of lung inflammation, respectively.^{18,43} In the current study, heMAMP demonstrated >2 times higher CNR increase upon activation compared with MPO-Gd and comparable signal increase with one-third of the dosage of MPO-Gd in mouse models of CFA-induced inflammation and atherosclerosis (Figures S4

and 4d). Therefore, heMAMP is about 2–3 times more responsive to MPO activity after activation compared to MPO-Gd and other analogues, and 4–10 times higher signal increase compared to unactivated heMAMP and nonactivatable imaging agents (e.g., DOTA-Gd) (Figure 3e).

2.8. Molecular Docking Simulations Reveal Improved Binding Affinity of heMAMP to MPO.

To explore why heMAMP is more effective than MPO-Gd at detecting MPO activity, we carried out a molecular docking study.⁴⁹ Unlike MPO inhibitors that block the active site, the 5-hydroxyindole moieties of heMAMP are MPO substrates that undergo one-electron transfer with Compound I in the MPO catalytic cycle to form radicals and do not remain at the active site.¹⁷ The MPO–cyanide (MPO–CN) complex has been suggested as a useful surrogate to study compound I, which is short-lived since both compound I and the MPO–CN complex contain six-coordinated low-spin $s = 1$ iron centers; therefore, the cocrystal structure of the MPO–CN complex from human MPO–cyanide–thiocyanate (MPO–CN–SCN, PDB database: 1DNW) was chosen as a model to perform the docking experiment.^{14, 50, 51} Because the parameters for Gd are not present in AutoDock Vina, we substituted iron (Fe) for Gd in the docking simulation, as was done in a prior study.⁵² Besides the same charges shared by iron and gadolinium, the crystal and computational structures of Fe–DOTA and Fe–DTPA complexes found that iron coordinates with the four nitrogens and carboxylic groups from DTPA and DOTA scaffolds as gadolinium does, and is “buried” inside the complex;^{53, 54} therefore, the replacement of Gd with Fe should not significantly affect the overall docking results. Indeed, the docking result demonstrated that the chelating part from either heMAMP or MPO-Gd is located outside of the active site and makes very limited contributions to the interaction between the agent and the active site of MPO (Figure 5a,b). The optimal conformation of heMAMP binding to MPO revealed hydrogen-bond formation between the two amide bonds in the linker, which constrains the rotation of heMAMP by forming a five-membered ring (Figure 5a), increasing the agent’s rigidity. Importantly, the binding affinities generated from 20 modes of heMAMP to MPO from docking simulations are overall much lower than those of MPO-Gd (49.0–39.8 vs 41.4–33.1 kJ/mol, Table S1), which could explain the higher efficacy of heMAMP when activated by MPO. Visualization of MPO-Gd and heMAMP binding to MPO showed that for both agents, one of the 5-hydroxyindole moieties is parallel to the MPO heme forming π – π stacking interaction, consistent with the previous docking study of serotonin and MPO.⁵¹ However, in heMAMP, the hydroxy group pointing to the heme center forms a hydrogen bond with cyanide (in purple and blue) and the imine group electrostatically interacts with the carbonyl group from the heme propionate ($\text{NH}\cdots\text{O}=\text{C}$, 3.7 Å). The other 5-hydroxyindole moiety is perpendicular to the heme with the imine forming two hydrogen bonds with Pro¹⁰¹ and Thr¹⁰⁰, respectively (Figure 5b). In contrast, for MPO-Gd, one of the 5-hydroxyindole moieties interacts with the MPO heme without the electrostatic interaction, while the other 5-hydroxyindole moiety only forms one hydrogen-bond with Phe¹⁴⁷ (Figure 5b). These differences resulted in improved binding affinity of heMAMP compared to that of MPO-Gd.

3. CONCLUSIONS

Detection and differentiation of detrimental from beneficial inflammation is challenging but essential, given the different roles they play in disease pathogenesis. MPO has been found to be an important marker for damaging inflammation.^{55,56} In this study, we developed a new generation of activatable macrocyclic gadolinium-based MPO-specific agent, heMAMP, which is not only significantly more stable than the linear MPO-Gd agent,¹⁷ but also up to 3-fold more responsive to MPO activity compared to prior MPO-sensing MRI agents. We explored the stability and activity of heMAMP after MPO activation, evaluated the biodistribution, retention, and blood half-life of heMAMP, and validated the efficacy and specificity both in vitro and in vivo in CFA-induced subcutaneous inflammation and in the tandem stenosis model of atherosclerotic plaque instability. We also identified the mechanism for the improvement seen in heMAMP over MPO-Gd through molecular docking experiments. We anticipate that heMAMP will be a much more potent in vivo reporter to detect and map damaging inflammation in many diseases, including multiple sclerosis, nonalcoholic steatohepatitis, rheumatoid arthritis, and atherosclerosis at comparable dosage, and similar efficacy at lower dosages. These findings strongly suggest that heMAMP is a potential translational candidate to improve the diagnosis and treatment monitoring of many inflammatory diseases. In addition to the improved in vitro stability of heMAMP using thermodynamically stable DOTA chelate, the in vivo biosafety is important for the translation of heMAMP. We anticipate performing the in vivo toxicology tests for heMAMP as part of the investigational new drug (IND) application in the near future.

4. EXPERIMENTAL SECTION

4.1. Materials and Methods.

All chemicals were obtained from Sigma Chemical Co. unless otherwise stated. 5-Hydroxy-L-tryptophan was obtained from Chem-Impex International Inc. (Wood Dale, IL). DO3A-*tert*-butyl ester was purchased from Macrocyclics Inc. (Plano, TX) and serotonin was purchased from TCI America (Portland, OR). Glucose oxidase was obtained from Affymetrix (Santa Clara, CA). Myeloperoxidase was obtained from Lee Biosolutions (St. Louis, MO). All animal experiments were approved and in compliance with the Institutional Animal Care and Use Committee at Massachusetts General Hospital and the Animal Ethics Committees of the Garvan Institute of Medical Research/St. Vincent's Hospital and the University of New South Wales. High-resolution mass spectrometry was performed with Thermo Scientific TM Q-Exactive Plus Ultimate 3000 HPLC flow injection analysis. ¹H NMR and ¹³C NMR were conducted with Bruker Ascend TM 400. Inductively coupled plasma mass spectrometry (ICP-MS) was conducted on an Agilent 8800-QQQ system. Liquid chromatography-mass spectrometry (LC-MS) was conducted with Waters 2545 HPLC equipped with a 2998 diode array detector, a Water 3100 ESI-MS module, using an XTerra MS C18 μ m, 4.6 $\times$ 50 mm² column at a flow rate of 5 mL/min with a linear gradient (5% of acetonitrile with 0.1% formic acid to 100% of water with 0.1% formic acid, 5–100%).

4.2. Synthesis of heMAMP.

Synthesis of intermediate **1**:^{44,57} A solution of sodium nitrite (0.9 g, 13 mmol) in water (5 mL) was added dropwise to a mixture of L-glutamic acid (1.47 g, 10 mmol) and sodium bromide (3.8 g, 37 mmol) in 0.75 M hydrogen bromide (30 mL) at -15°C for over 30 min. The reaction mixture was stirred for another 2 h at the same temperature, and then conc. sulfuric acid (1 mL, 95–98%) was slowly added to the solution, followed by extraction with diethyl ether (30 mL $\times$ 3). The combined organic phase was washed with brine, dried over Na_2SO_4 , and evaporated to give the desired brominating compound, which was used in the next step without further purification. A mixture of magnesium sulfate (5 g) and conc. sulfuric acid (0.5 mL) in dichloromethane (5 mL) was stirred at room temperature for 2 h, then the above compound and *tert*-butanol (3 g, 40 mmol) were added and stirred for another 20 h. The reaction mixture was filtered to remove the salt and extracted with dichloromethane (20 mL $\times$ 3). The combined organic layer was washed with brine, dried over Na_2SO_4 , evaporated, and subjected to flash chromatography (5:1 of hexane/ethyl acetate) to give compound **1** as a yellow oil (45% for two steps). ^1H NMR (500 MHz, CDCl_3) δ 4.23 (dd, 1H), 2.51 (m, 2H), 2.29 (m, 2H), 1.48 (s, 9H), 1.43 (s, 9H); ^{13}C NMR (125 MHz, CDCl_3) δ 172.7, 168.4, 82.8, 82.6, 46.8, 32.9, 30.0, 28.1, 27.9. LCMS found m/z (ES+): 325.3 (M + H).

Synthesis of intermediate **4**:²⁰ A solution of di-*tert*-butyl dicarbonate (784 mg, 3.6 mmol) in tetrahydrofuran (4 mL) was added to a solution of 5-hydroxy-L-tryptophan (5-HTP, 660 mg, 3.0 mmol) and potassium carbonate (1.37 g, 10.0 mmol) in water (8 mL). The reaction mixture was stirred at room temperature for 2 h and then neutralized to pH 2–3 by adding 1 M hydrogen chloride solution. After evaporating to remove the solvent, the solution was extracted with ethyl acetate (20 mL $\times$ 3), the organic phase was washed with brine (10 mL $\times$ 3), dried over anhydrous Na_2SO_4 , and evaporated to give compound **2** without further purification. To a solution of compound **2** (385 mg, 1.2 mmol) in dimethylformamide (5 mL) were added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (280 mg, 1.4 mmol), subsequently hydroxybenzotriazole (216 mg, 1.4 mmol), and the mixture was stirred for 10 min. Then, a solution of serotonin (220 mg, 1.0 mmol) in dimethylformamide (4 mL) was added to the above mixture. The reaction mixture was stirred for another 2 h. The reaction mixture was extracted with ethyl acetate (10 mL $\times$ 3), the organic layer was washed with brine (10 mL $\times$ 3), dried over anhydrous Na_2SO_4 , and evaporated. The residue underwent flash chromatography (ethyl acetate as an eluent) to give compound **3** as a white solid (390 mg) with a yield of 82%. ^1H NMR (500 MHz, DMSO) δ 10.45 (s, 2H), 8.56 (s, 1H), 8.54 (s, 1H), 7.89 (t, 1H), 7.10 (d, 1H), 7.09 (d, 1H), 7.0 (m, 2H), 6.88 (d, 1H), 6.83 (d, 1H), 6.67 (d, 1H), 6.57 (m, 2H), 4.12 (m, 1H), 3.26 (m, 2H), 2.96 (dd, 1H), 2.79 (dd, 1H), 2.66 (m, 2H), 1.32 (s, 9H); ^{13}C NMR (125 MHz, DMSO) δ 171.8, 155.1, 150.15, 150.13, 130.8, 130.6, 128.1, 127.8, 123.9, 123.0, 111.6, 111.4, 111.2, 111.1, 110.7, 109.3, 102.5, 102.2, 77.9, 55.0, 40.1, 28.2, 28.0, 25.2; LCMS found m/z : 479.4 (M + H). Compound **3** (300 mg) was added to a solution of 10% trifluoroacetic acid in dichloromethane (4 mL), and the reaction mixture was stirred for 5 h at room temperature. Then, the reaction mixture was evaporated under reduced pressure to remove the solvent and subjected to preparative high-performance liquid chromatography to give the desired intermediate **4** (63% of yield). ^1H NMR (500 MHz, DMSO) δ 10.70 (d, 1H), 10.50 (d, 1H), 8.57 (m, 3H), 8.05 (m, 2H),

7.14 (dd, 2H), 7.09 (d, 1H), 6.98 (dd, 2H), 6.81 (d, 1H), 6.62 (dd, 2H), 3.87 (dt, 1H), 3.34 (m, 2H), 3.10 (dd, 1H), 2.98 (dd, 1H), 2.68 (m, 2H); ^{13}C NMR (125 MHz, DMSO) δ 168.3, 150.4, 150.2, 130.9, 130.8, 127.8, 127.7, 125.2, 123.2, 111.7, 111.5, 111.2, 110.4, 106.0, 102.6, 102.1, 94.8, 52.8, 39.9, 27.6, 25.0. LCMS found m/z : 379.5 (M + H).

Synthesis of compound **6**: Potassium carbonate (210 mg, 1.5 mmol) was added to a solution of DO3A-*t*Bu-ester **5** (770 mg, 1.5 mmol) and compound **1** (480 mg, 1.5 mmol) in acetonitrile (5 mL), and the reaction mixture was heated under reflux for 24 h. After removing the solvent under reduced pressure, the residue was dissolved in dichloromethane and filtered. The flash chromatography column with gradient elution of dichloromethane to 5% methanol in dichloromethane gave the desired compound **6** as a pale yellow solid with a yield of 78%. ^1H NMR (500 MHz, DMSO) 3.48–1.90 (m, 27 H), 1.40 (s, 45 H); ^{13}C NMR (125 MHz, DMSO) δ 174.8, 173.1, 173.0, 172.3, 82.5, 82.0, 81.9, 80.6, 60.0, 55.9 (2), 55.6, 52.7, 52.5, 48.6, 48.1, 47.3, 44.3, 32.8, 28.2, 28.0, 27.9. LCMS found m/z : 757.7 (M + H), 779.6 (M – H + Na).

Synthesis of compound **8**: The synthesis of intermediate **8** was performed following a published literature.⁵⁸ A solution of compound **6** (250 mg, 0.33 mmol) in a mixture of trifluoroacetic acid and dichloromethane (4/2 mL) was stirred for 18 h. The solvent was removed under reduced pressure and the above process was repeated until the deprotection was complete (monitored by LC-MS, m/z (ES⁺): 477.4 (M + H)) to obtain compound **7** without further purification. Gadolinium chloride hexahydrate (135 mg, 0.36 mmol) was added to a solution of compound **7** in water (10 mL). The pH of the solution was adjusted to 5.5–6.0 by adding 1 M sodium hydroxide solution until it was steady. The reaction mixture was heated to 50 °C overnight. After cooling down, the reaction mixture was adjusted to pH ~ 10.9 by adding 1 M hydrogen chloride, stirred for 40 min, and then centrifuged. The supernatant was adjusted to pH 6.5 and lyophilized to give compound **8** as a white solid. LCMS found m/z (ES[–]): 630.2 (M), 652.1 (M – H + Na).

Synthesis of heMAMP. To a solution of compound **8** (125 mg, 0.2 mmol) and triethylamine (56 μL , 0.4 mmol) in dimethyl sulfoxide (3 mL) were added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (57 mg, 0.3 mmol) and hydroxybenzotriazole (40 mg, 0.3 mmol). After 20 min, a solution of compound **4** (76 mg, 0.2 mmol) in dimethyl sulfoxide was added to the above reaction. The reaction mixture was stirred for 1 h at ambient temperature and then subjected to preparative high-performance liquid chromatography to give the desired heMAMP (25%) as a white powder. LCMS found m/z (ES⁺): 992.5 (M + H); HRMS: 992.2795 (M + H, cal. $\text{C}_{40}\text{H}_{49}\text{GdN}_8\text{O}_{12}$, 992.2793) as shown in Figure S1.

4.3. Stability, Reactivity and Cytotoxicity of heMAMP.

4.3.1. Transmetalation with Zn^{2+} and Ca^{2+} .—heMAMP (1 mM) was incubated with ZnCl_2 (2.5 mM) and CaCl_2 (2.5 mM) in PBS (sodium phosphate dibasic 10.1 mM, potassium phosphate monobasic, 1.76 mM, potassium chloride, 2.7 mM, sodium chloride, 137 mM) at 40 °C, respectively, and the relaxation time was measured for 95 h using an inversion-recovery pulse sequence on the Bruker Minispec (Bruker Analytics, North Billerica, MA) at 0.47 T (20 MHz) at 40 °C. The stability of heMAMP was evaluated by the

ratio index ($R1(t)/R1(t=0) = 0.8$) and the long-term index ($R1_{(72h)}/R1_{(t=0)}$) and compared with MPO-Gd.¹⁷

4.3.2. Plasma Stability.—heMAMP (2.5 μL of 10 mM in DMSO) and plasma (97.5 μL) were incubated at 40 °C. The relaxation time was measured at 0, 30, 30, 60, 90, and 180 min as described above ($n = 3$).

4.3.3. Measurement of Relaxivity of heMAMP.—The relaxation time of heMAMP in PBS at concentrations of 0.1, 0.2, 0.33, 0.5, 0.6, 0.75, and 1 mM was measured as described above at 40 °C. The $1/T1$ -values were plotted against heMAMP concentrations and fitted with linear regression. The slope value of the linear function is defined as the relaxivity of heMAMP ($n = 3$).⁵⁹

4.3.4. In Vitro Activity of heMAMP after Activation by MPO.—A solution of heMAMP or MPO-Gd in PBS that contains potassium chloride (2.7 mM) and sodium chloride (137 mM) as the chloride source (0.5 mM, 150 μL total volume) was incubated with glucose (6 μL , 1 M), GOX (4 μL , 1 mg/mL, 100 units/mg protein, Sigma-Aldrich, MA), and MPO (10 μL , 2 mg/mL, 1080 units/mg protein, Lee Biosolutions, MO) at 40 °C. The reaction was stopped by adding sodium azide (1 μL , 250 mg/mL) and the T1 relaxation time was measured at 0 (before MPO added), 1, 2, 5, 10, 30, 60, 120, and 180 min as above at 40 °C. The $R1$ ratio was expressed as $(R1(t) - R1(t=0))/R1(t=0)$ ($n = 3$).

4.3.5. Binding to Proteins after Activation by MPO.—A solution of heMAMP or MPO-Gd (0.5 mM) in PBS (150 μL total volume) with or without BSA was incubated with glucose (6 μL , 1 M), GOX (4 μL , 1 mg/mL), and MPO (10 μL , 2 mg/mL) at 40 °C for 1 h. The relaxation time was measured as described above ($n = 3$).

4.3.6. Cytotoxicity MTT Assay.—The cytotoxicity of heMAMP was evaluated using RAW 264.7 (Passage 8, from the cell core of Center for Systems Biology at Massachusetts General Hospital, Boston) and HEK 293 cells (passage 3, from Vector Production and Development Core at Massachusetts General Hospital, Boston) and measured using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction assay, as previously described (triplicate for each cell line).¹⁷ Then, 1.5×10^4 cells per well in a 96-well plate were cultured at 37 °C for 16 h in solutions of heMAMP in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermal Fisher) containing 10% fetal bovine serum (FBS, Thermal Fisher) and 5% DMSO at concentrations of 0, 0.1, 0.5, 1, 2, and 5 mM. Following culture, the cells were treated with 100 μL of 0.5 mg/mL MTT (Sigma-Aldrich) for 2 h at 37 °C before 100 μL of DMSO was added to each well, the plates were incubated overnight at 37 °C, and optical density was measured at 570 nm using a microplate reader (Tecan Safire2, Tecan, Mannedorf, Switzerland).

4.4. Biodistribution and Retention of heMAMP.

A mouse model of complete Freund's adjuvant (CFA)-induced paw inflammation was performed to evaluate the biodistribution and retention of heMAMP. Six to ten weeks old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were injected with an

emulsion of CFA/PBS (40 μ L, 1:1) on the dorsal side of one forepaw and PBS (40 μ L) on the other forepaw as the control under isoflurane anesthesia. After 24 h, heMAMP (0.1 mmol/kg mice) was administered intravenously through the tail vein. The major organs, including forepaws, were harvested at 3 h and day 7 after the administration of heMAMP ($n = 3$ for each time point). The samples were prepared by weighed, treated with nitric acid, and incubated overnight at 37° C. Dysprosium (10 ppb) was added to the samples as the internal standard. The samples were subjected to inductively coupled plasma mass spectrometry (ICP-MS) to determine the retention of gadolinium. Calibration standards were prepared by diluting certified dysprosium standards at $C = 1000$ mg/L. Seven standard solutions in the range of 0.1–400 ppb were used for high concentrations of gadolinium and five standard solutions in the range of 0.1–50 for lower ppb concentrations.

4.5. Blood Half-Life of heMAMP.

heMAMP (0.1 mmol/kg mice) was administered intravenously through the tail vein to 6–10 weeks old C57BL/6J mice ($n = 5$). Blood samples were taken before and at different time points after the administration of heMAMP (0.1 mmol/kg) and centrifuged at 15 000 rpm for 15 min to obtain the plasma. The plasma samples were treated with nitric acid (70%, 200 μ L) and incubated overnight at 37 °C. The above samples were subjected to the ICP-MS as described above to determine the clearance rate of heMAMP from the blood with a two-phase decay model.

4.6. MR Imaging of CFA-Induced Subcutaneous Inflammation.

Six to ten weeks old female C57BL/6J mice and MPO-deficient mice were used for this experiment. The mice were injected subcutaneously with CFA emulsion (40 μ L, 1:1) on one forearm under isoflurane anesthesia. PBS (40 μ L) was injected on the other side as control. After 24 h, gadolinium agents (heMAMP in PBS containing 10% DMSO and 20% *N,N*-dimethylacetamide or MPO-Gd in PBS containing 5% DMSO or Dotarem in PBS) were administered through tail vein injection. Due to the faster metabolic rate of rodents compared to humans,⁶⁰ we had empirically determined that a dose of 0.3 mmol/kg (three times of the human dose of 0.1 mmol/kg) demonstrate enhancement characteristics similar to that of humans at 0.1 mmol/kg on our scanner for Gd-based agents. A lower dosage of heMAMP at 0.1 mmol/kg was explored as well due to its higher activation ratio and efficacy. The mice were imaged at 0, 15, 30, 45, and 60 min using serial T1 rapid acquisition with relaxation enhancement (RARE) sequence (TR: 935.77 ms, TE: 13.59 ms, averages: 12, rare factor: 4, $256 \times 256 \times 28$ matrix size, $0.156 \times 0.156 \times 1$ mm³ voxel size) with chemical fat suppression using a Hermitian pulse shape with an 8.253 ms pulse and 701.19 Hz bandwidth 3.5 ppm down from the water peak and respiratory gating on a 4.7 T small animal MR scanner (Bruker, Cambridge, MA) with a 3 cm quadrature volume coil (Rapid MR International, Germany). Regions of interest (ROI) were drawn manually and the contrast-to-noise ratios (CNRs) were calculated by an experienced radiologist blinded to the identity of the imaging agent used and the strain of the mice.

4.7. MR Imaging of Tandem Stenosis (TS) and Intervention with AZM198.

Male apolipoprotein E gene-deficient (Apoe^{-/-}) mice, obtained originally from the Animal Resource Center in Western Australia, were bred in the BioCORE facility at the Victor

Chang Cardiac Research Institute. Mice (6 weeks of age) were fed Western Diet (WD) containing 22% fat and 0.15% cholesterol (SF00–219, Specialty Feeds, Western Australia) for a total of 13 weeks. Six weeks after the commencement of WD, tandem stenosis (TS) was introduced into the mice, as previously described.¹⁵ Briefly, male *Apoe*^{−/−} mice were anesthetized with 4% isoflurane. The right common carotid artery was dissected from circumferential connective tissues. Two stenoses were placed by tying a 6–0 blue-braided polyester fiber suture (TICRON 0.7 Metric) around the exposed artery, with the distal stenosis 1 mm from the carotid artery bifurcation and the proximal stenosis 3 mm from the distal stenosis. Blood flow was measured before and after the addition of each ligature using a perivascular flow module (Transonic, TS420) and a 0.7 mm perivascular flow probe (Transonic MA0.7PSB). Flow for each ligature in the TS was defined as 70% of baseline flow after the addition of the distal ligature and 20% of baseline flow after the addition of the proximal ligature. Alterations in the flow predispose the right carotid artery to developing an atherosclerotic plaque with an unstable phenotype in the segment proximal to the proximal suture, characterized by consistent thinning of the fibrous cap, abundant inflammatory cells, occasional neovessels, cap disruption, and intraplaque hemorrhage, as well as luminal thrombus with fibrin and platelets depositions.⁶¹ By comparison, the atheroma in the brachiocephalic trunk contains a thick cap and abundance of collagen, which are the features of a stable plaque phenotype.¹⁵

Before, and at 1, 2, 4, and 7 weeks following TS surgery, isoflurane-anesthetized mice were imaged in the prone position using a 9.4 T Bruker Biospec 94/20 Avance III system (Bruker, Ettlingen, Germany) with a 35 mm quadrature radiofrequency coil and respiratory-gated image acquisition as described previously before and after intravenous administration of 0.3 mmol/kg of MPO-Gd via a tail vein catheter.¹⁵ A T1-weighted fast spin echo (TurboRARE, T1-TSE) was acquired with the following parameters: TR: 1500 ms, TE: 8.5 ms, ETL: 8, slice thickness: 1 mm, FOV: 20 × 20 mm², matrix size: 192 × 192, and in-plane resolution: 104 × 104 μm². This T1-TSE protocol was then repeated in a scan series covering a period of 1 h following contrast injection to assess contrast agent inflow and retention. OsiriX (Version 10.0.2, Pixmeo, Switzerland) was used for image analysis. In T1-TSE images, separate regions of interest were assigned to the vessel wall, skeletal muscle (reference), and background (air). The contrast-to-noise ratio (CNR) was calculated as follows: $CNR = (SI_{\text{vessel wall}} - SI_{\text{skeletal muscle}}) / SD_{\text{background}}$. The mean CNRs of three consecutive slices in plaque with unstable phenotype and corresponding segments of the left carotid artery (plaque-free), in addition to 1–2 consecutive slices in the brachiocephalic trunk (stable plaque), were calculated. Segmental enhancement attributable to MPO was assessed by calculating the $CNR = CNR_{\text{post-contrast}} - CNR_{\text{pre-contrast}}$.

For pharmacological inhibition of MPO, AZM198 (AstraZeneca, Sweden) was administered by incorporation into WD at a daily dose of 500 μmol/kg bodyweight based on an average daily food consumption of ~3.7 g per mouse, as described previously.¹⁵ Treatment with AZM198 commenced 1 week post-TS surgery and continued for 6 weeks until MRI at 7 weeks post-TS surgery, before and after intravenous administration of 0.1 mmol/kg of heMAMP as per the protocol described above.

4.8. Molecular Docking Experiments.

Molecular docking of MPO with heMAMP and MPO-Gd was performed with AutoDock Vina⁴⁹ and visualized by PyMol. Gadolinium (Gd) was replaced with iron (Fe) for the docking since the parameters of Gd are not included in AutoDock Vina, as was done in a prior study.⁵² Dimer of chains A and C with cyanide of human MPO–cyanide–thiocyanate (PDB database: 1DNW) was selected as a surrogate of MPO compound I for docking.⁵⁰ Twenty docking conformations were generated for each agent.

4.9. Statistical Analysis.

All numeric data were first analyzed for normality using the Shapiro–Wilk normality test, with significance determined subsequently using the appropriate parametric or nonparametric test. Dynamic *RI* ratio differences over time between heMAMP and MPO-Gd were determined by the Kolmogorov–Smirnov test. Differences in CNR between TS Apoe^{−/−} fed WD ± AZM198 were assessed using the Kruskal–Wallis test followed by the Mann–Whitney rank sum test or Welch’s *t*-test where appropriate. All statistical analyses were performed with GraphPad Prism version 8.01 for Mac (GraphPad Software, La Jolla California) and individual data are shown with mean ± SEM. *P*-values < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Small Molecule Mass Spectrometry from Harvard University for analysis of the final compound. This study was supported in part by grants from the NIH (R01 NS103998 and K25 HL150305), the National Multiple Sclerosis Society, the National Health and Medical Research Council (NHMRC) of Australia, the Australian Research Council (Discovery Project 170101453 R.S.), and a Bridging Grant from the Australian Academy of Technology and Engineering. We also acknowledge infrastructure support from New South Wales Health. R.S. was supported by an NHMRC Senior Principal Research Fellowship and C.W. was supported by a Research Scientist Award from the American Society of Neuroradiology (ASNR).

ABBREVIATIONS

CFA	complete Freund’s adjuvant
CNR	contrast-to-noise ratio
GBCAs	gadolinium-based contrast agents
EAE	experimental autoimmune encephalomyelitis
heMAMP	highly efficient myeloperoxidase-activatable MRI probe
HRMS	high-resolution mass spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
MPO	myeloperoxidase

NASH	nonalcoholic steatohepatitis
TS	tandem stenosis

REFERENCES

- (1). Swirski FK; Wildgruber M; Ueno T; Figueiredo JL; Panizzi P; Iwamoto Y; Zhang E; Stone JR; Rodriguez E; Chen JW; Pittet MJ; Weissleder R; Nahrendorf M Myeloperoxidase-rich Ly-6C⁺ myeloid cells infiltrate allografts and contribute to an imaging signature of organ rejection in mice. *J. Clin. Invest.* 2010, 120, 2627–2634. [PubMed: 20577051]
- (2). Bradley PP; Christensen RD; Rothstein G Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 1982, 60, 618–622. [PubMed: 6286012]
- (3). Scholz W; Platzer B; Schumich A; Hoher B; Fritsch G; Knapp W; Strobl H Initial human myeloid/dendritic cell progenitors identified by absence of myeloperoxidase protein expression. *Exp. Hematol* 2004, 32, 270–276. [PubMed: 15003312]
- (4). Teng N; Maghazal GJ; Talib J; Rashid I; Lau AK; Stocker R The roles of myeloperoxidase in coronary artery disease and its potential implication in plaque rupture. *Redox Rep.* 2017, 22, 51–73. [PubMed: 27884085]
- (5). Su HS; Nahrendorf M; Panizzi P; Breckwoldt MO; Rodriguez E; Iwamoto Y; Aikawa E; Weissleder R; Chen JW Vasculitis: molecular imaging by targeting the inflammatory enzyme myeloperoxidase. *Radiology* 2012, 262, 181–190. [PubMed: 22084204]
- (6). Forghani R; Kim HJ; Wojtkiewicz GR; Bure L; Wu Y; Hayase M; Wei Y; Zheng Y; Moskowitz MA; Chen JW Myeloperoxidase propagates damage and is a potential therapeutic target for subacute stroke. *J. Cereb. Blood Flow Metab.* 2015, 35, 485–493. [PubMed: 25515211]
- (7). Choi DK; Pennathur S; Perier C; Tieu K; Teismann P; Wu DC; Jackson-Lewis V; Vila M; Vonsattel JP; Heinecke JW; Przedborski S Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice. *J. Neurosci.* 2005, 25, 6594–6600. [PubMed: 16014720]
- (8). Maki RA; Tyurin VA; Lyon RC; Hamilton RL; DeKosky ST; Kagan VE; Reynolds WF Aberrant expression of myeloperoxidase in astrocytes promotes phospholipid oxidation and memory deficits in a mouse model of Alzheimer disease. *J. Biol. Chem.* 2009, 284, 3158–3169. [PubMed: 19059911]
- (9). Tzikas S; Schlak D; Sopova K; Gatsiou A; Stakos D; Stamatelopoulos K; Stellos K; Laske C Increased myeloperoxidase plasma levels in patients with Alzheimer's disease. *J. Alzheimer's Dis.* 2014, 39, 557–564. [PubMed: 24217274]
- (10). Gray E; Thomas TL; Betmouni S; Scolding N; Love S Elevated myeloperoxidase activity in white matter in multiple sclerosis. *Neurosci. Lett.* 2008, 444, 195–198. [PubMed: 18723077]
- (11). Pulli B; Bure L; Wojtkiewicz GR; Iwamoto Y; Ali M; Li D; Schob S; Hsieh KL; Jacobs AH; Chen JW Multiple sclerosis: myeloperoxidase immunoradiology improves detection of acute and chronic disease in experimental model. *Radiology* 2015, 275, 480–489. [PubMed: 25494298]
- (12). Ruggeri RB; Buckbinder L; Bagley SW; Carpino PA; Conn EL; Dowling MS; Fernando DP; Jiao W; Kung DW; Orr ST; Qi Y; Rocke BN; Smith A; Warmus JS; Zhang Y; Bowles D; Widlicka DW; Eng H; Ryder T; Sharma R; Wolford A; Okerberg C; Walters K; Maurer TS; Zhang Y; Bonin PD; Spath SN; Xing G; Hepworth D; Ahn K; Kalgutkar AS Discovery of 2-(6-(5-Chloro-2-methoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl)acet amide (PF-06282999): A Highly Selective Mechanism-Based Myeloperoxidase Inhibitor for the Treatment of Cardiovascular Diseases. *J. Med. Chem.* 2015, 58, 8513–8528. [PubMed: 26509551]
- (13). Jucaite A; Svenningsson P; Rinne JO; Cselenyi Z; Varnas K; Johnstrom P; Amini N; Kirjavainen A; Helin S; Minkwitz M; Kugler AR; Posener JA; Budd S; Halldin C; Varrone A; Farde L Effect of the myeloperoxidase inhibitor AZD3241 on microglia: a PET study in Parkinson's disease. *Brain* 2015, 138, 2687–2700. [PubMed: 26137956]
- (14). Malle E; Furtmuller PG; Sattler W; Obinger C Myeloperoxidase: a target for new drug development? *Br. J. Pharmacol.* 2007, 152, 838–854. [PubMed: 17592500]

- (15). Rashid I; Maghzal GJ; Chen YC; Cheng D; Talib J; Newington D; Ren M; Vajandar SK; Searle A; Maluenda A; Lindstedt EL; Jabbour A; Kettle AJ; Bongers A; Power C; Michaelsson E; Peter K; Stocker R Myeloperoxidase is a potential molecular imaging and therapeutic target for the identification and stabilization of high-risk atherosclerotic plaque. *Eur. Heart J.* 2018, 39, 3301–3310. [PubMed: 30219874]
- (16). Chen JW; Pham W; Weissleder R; Bogdanov A Jr. Human myeloperoxidase: a potential target for molecular MR imaging in atherosclerosis. *Magn. Reson. Med.* 2004, 52, 1021–1028. [PubMed: 15508166]
- (17). Rodríguez E; Nilges M; Weissleder R; Chen JW Activatable magnetic resonance imaging agents for myeloperoxidase sensing: mechanism of activation, stability, and toxicity. *J. Am. Chem. Soc.* 2010, 132, 168–177. [PubMed: 19968300]
- (18). Rodríguez-Rodríguez A; Shuvaev S; Rotile N; Jones CM; Probst CK; Dos Santos Ferreira D; Graham ORK; Boros E; Knipe RS; Griffith JW; Tager AM; Bogdanov A Jr.; Caravan P Peroxidase Sensitive Amplifiable Probe for Molecular Magnetic Resonance Imaging of Pulmonary Inflammation. *ACS Sens.* 2019, 4, 2412–2419. [PubMed: 31397156]
- (19). Wang C; Keliher E; Zeller MWG; Wojtkiewicz GR; Aguirre AD; Buckbinder L; Kim HY; Chen J; Maresca K; Ahmed MS; Motlagh NJ; Nahrendorf M; Chen JW An activatable PET imaging radioprobe is a dynamic reporter of myeloperoxidase activity in vivo. *Proc. Natl. Acad. Sci. U.S.A* 2019, 116, 11966–11971. [PubMed: 31123149]
- (20). Wang C; Pulli B; Jalali Motlagh N; Li A; Wojtkiewicz GR; Schmidt SP; Wu Y; Zeller MWG; Chen JW A versatile imaging platform with fluorescence and CT imaging capabilities that detects myeloperoxidase activity and inflammation at different scales. *Theranostics* 2019, 9, 7525–7536. [PubMed: 31695784]
- (21). Hu JJ; Wong NK; Lu MY; Chen X; Ye S; Zhao AQ; Gao P; Yi-Tsun Kao R; Shen J; Yang D HKOCl-3: a fluorescent hypochlorous acid probe for live-cell and in vivo imaging and quantitative application in flow cytometry and a 96-well microplate assay. *Chem. Sci* 2016, 7, 2094–2099. [PubMed: 29899935]
- (22). Wei P; Yuan W; Xue F; Zhou W; Li R; Zhang D; Yi T Deformylation reaction-based probe for in vivo imaging of HOCl. *Chem. Sci.* 2018, 9, 495–501. [PubMed: 29619205]
- (23). Shepherd J; Hilderbrand SA; Waterman P; Heinecke JW; Weissleder R; Libby P A fluorescent probe for the detection of myeloperoxidase activity in atherosclerosis-associated macrophages. *Chem. Biol.* 2007, 14, 1221–1231. [PubMed: 18022561]
- (24). Liu L; Wei P; Yuan W; Liu Z; Xue F; Zhang X; Yi T Detecting Basal Myeloperoxidase Activity in Living Systems with a Near-Infrared Emissive “Turn-On” Probe. *Anal. Chem.* 2020, 92, 10971–10978. [PubMed: 32674562]
- (25). Chen JW; Breckwoldt MO; Aikawa E; Chiang G; Weissleder R Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis. *Brain* 2008, 131, 1123–1133. [PubMed: 18234693]
- (26). Forghani R; Wojtkiewicz GR; Zhang Y; Seeburg D; Bautz BR; Pulli B; Milewski AR; Atkinson WL; Iwamoto Y; Zhang ER; Etzrodt M; Rodriguez E; Robbins CS; Swirski FK; Weissleder R; Chen JW Demyelinating diseases: myeloperoxidase as an imaging biomarker and therapeutic target. *Radiology* 2012, 263, 451–460. [PubMed: 22438365]
- (27). Ronald JA; Chen JW; Chen Y; Hamilton AM; Rodriguez E; Reynolds F; Hegele RA; Rogers KA; Querol M; Bogdanov A; Weissleder R; Rutt BK Enzyme-sensitive magnetic resonance imaging targeting myeloperoxidase identifies active inflammation in experimental rabbit atherosclerotic plaques. *Circulation* 2009, 120, 592–599. [PubMed: 19652086]
- (28). Breckwoldt MO; Chen JW; Stangenberg L; Aikawa E; Rodriguez E; Qiu S; Moskowitz MA; Weissleder R Tracking the inflammatory response in stroke in vivo by sensing the enzyme myeloperoxidase. *Proc. Natl. Acad. Sci. U.S.A.* 2008, 105, 18584–18589. [PubMed: 19011099]
- (29). Nahrendorf M; Sosnovik D; Chen JW; Panizzi P; Figueiredo JL; Aikawa E; Libby P; Swirski FK; Weissleder R Activatable magnetic resonance imaging agent reports myeloperoxidase activity in healing infarcts and noninvasively detects the antiinflammatory effects of atorvastatin on ischemia-reperfusion injury. *Circulation* 2008, 117, 1153–1160. [PubMed: 18268141]
- (30). Ali M; Pulli B; Courties G; Tricot B; Sebas M; Iwamoto Y; Hilgendorf I; Schob S; Dong A; Zheng W; Skoura A; Kalgukar A; Cortes C; Ruggeri R; Swirski FK; Nahrendorf M; Buckbinder

- L; Chen JW Myeloperoxidase Inhibition Improves Ventricular Function and Remodeling After Experimental Myocardial Infarction. *JACC Basic Transl. Sci* 2016, 1, 633–643. [PubMed: 30167547]
- (31). Pulli B; Wojtkiewicz G; Iwamoto Y; Ali M; Zeller MW; Bure L; Wang C; Choi Y; Masia R; Guimaraes AR; Corey KE; Chen JW Molecular MR Imaging of Myeloperoxidase Distinguishes Steatosis from Steatohepatitis in Nonalcoholic Fatty Liver Disease. *Radiology* 2017, 284, 390–400. [PubMed: 28358240]
- (32). Grobner T Gadolinium—a specific trigger for the development of nephrogenic fibrosing dermopathy and nephrogenic systemic fibrosis? *Nephrol., Dial., Transplant* 2006, 21, 1104–1108. [PubMed: 16431890]
- (33). Marckmann P; Skov L; Rossen K; Dupont A; Damholt MB; Heaf JG; Thomsen HS Nephrogenic systemic fibrosis: suspected causative role of gadodiamide used for contrast-enhanced magnetic resonance imaging. *J. Am. Soc. Nephrol.* 2006, 17, 2359–2362. [PubMed: 16885403]
- (34). McDonald RJ; McDonald JS; Kallmes DF; Jentoft ME; Murray DL; Thielen KR; Williamson EE; Eckel LJ Intracranial Gadolinium Deposition after Contrast-enhanced MR Imaging. *Radiology* 2015, 275, 772–782. [PubMed: 25742194]
- (35). Kanda T; Fukusato T; Matsuda M; Toyoda K; Oba H; Kotoku J; Haruyama T; Kitajima K; Furui S Gadolinium-based Contrast Agent Accumulates in the Brain Even in Subjects without Severe Renal Dysfunction: Evaluation of Autopsy Brain Specimens with Inductively Coupled Plasma Mass Spectroscopy. *Radiology* 2015, 276, 228–232. [PubMed: 25942417]
- (36). Robert P; Lehericy S; Grand S; Violas X; Fretellier N; Idee JM; Ballet S; Corot C T1-Weighted Hypersignal in the Deep Cerebellar Nuclei After Repeated Administrations of Gadolinium-Based Contrast Agents in Healthy Rats: Difference Between Linear and Macrocyclic Agents. *Invest. Radiol* 2015, 50, 473–480. [PubMed: 26107651]
- (37). Radbruch A; Weberling LD; Kieslich PJ; Eidel O; Burth S; Kickingereeder P; Heiland S; Wick W; Schlemmer HP; Bendszus M Gadolinium retention in the dentate nucleus and globus pallidus is dependent on the class of contrast agent. *Radiology* 2015, 275, 783–791. [PubMed: 25848905]
- (38). Kanda T; Osawa M; Oba H; Toyoda K; Kotoku J; Haruyama T; Takeshita K; Furui S High Signal Intensity in Dentate Nucleus on Unenhanced T1-weighted MR Images: Association with Linear versus Macrocyclic Gadolinium Chelate Administration. *Radiology* 2015, 275, 803–809. [PubMed: 25633504]
- (39). PRAC Concludes Assessment of Gadolinium Agents Used in Body Scans and Recommends Regulatory Actions, Including Suspension for Some Marketing Authorisations, <https://www.ema.europa.eu/en/news/prac-concludes-assessment-gadolinium-agents-used-body-scans-recommends-regulatory-actions-including> (accessed Oct, 2017).
- (40). Kanda T The New Restrictions on the Use of Linear Gadolinium-based Contrast Agents in Japan. *Magn. Reson. Med. Sci* 2019, 18, 1–3. [PubMed: 29553066]
- (41). FDA Drug Safety Communication: FDA Warns that Gadolinium-Based Contrast Agents (GBCAs) are Retained in the Body; Requires New Class Warnings, <https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-warns-gadolinium-based-contrast-agents-gbcas-are-retained-body> (accessed May, 2018).
- (42). Port M; Idee JM; Medina C; Robic C; Sabatou M; Corot C Efficiency, thermodynamic and kinetic stability of marketed gadolinium chelates and their possible clinical consequences: a critical review. *BioMetals* 2008, 21, 469–490. [PubMed: 18344005]
- (43). Chen JW; Querol Sans M; Bogdanov A Jr.; Weissleder R Imaging of myeloperoxidase in mice by using novel amplifiable paramagnetic substrates. *Radiology* 2006, 240, 473–481. [PubMed: 16864673]
- (44). Moumne R; Lavielle S; Karoyan P Efficient synthesis of beta2-amino acid by homologation of alpha-amino acids involving the Reformatsky reaction and Mannich-type imminium electrophile. *J. Org. Chem.* 2006, 71, 3332–3334. [PubMed: 16599644]
- (45). Laurent S; Elst LV; Copoix F; Muller RN Stability of MRI paramagnetic contrast media: a proton relaxometric protocol for transmetallation assessment. *Invest. Radiol* 2001, 36, 115–122. [PubMed: 11224760]

- (46). Laurent S; Elst LV; Muller RN Comparative study of the physicochemical properties of six clinical low molecular weight gadolinium contrast agents. *Contrast Media Mol. Imaging* 2006, 1, 128–137. [PubMed: 17193689]
- (47). Querol M; Chen JW; Weissleder R; Bogdanov A Jr. DTPA-bisamide-based MR sensor agents for peroxidase imaging. *Org. Lett.* 2005, 7, 1719–1722. [PubMed: 15844889]
- (48). Dupuy C; Virion A; Ohayon R; Kaniewski J; Deme D; Pommier J Mechanism of hydrogen peroxide formation catalyzed by NADPH oxidase in thyroid plasma membrane. *J. Biol. Chem.* 1991, 266, 3739–3743. [PubMed: 1995628]
- (49). Trott O; Olson AJ AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 2010, 31, 455–461. [PubMed: 19499576]
- (50). Blair-Johnson M; Fiedler T; Fenna R Human myeloperoxidase: structure of a cyanide complex and its interaction with bromide and thiocyanate substrates at 1.9 Å resolution. *Biochemistry* 2001, 40, 13990–13997. [PubMed: 11705390]
- (51). Hallingbäck HR; Gabdoulline RR; Wade RC Comparison of the binding and reactivity of plant and mammalian peroxidases to indole derivatives by computational docking. *Biochemistry* 2006, 45, 2940–2950. [PubMed: 16503648]
- (52). Wu G; Gao XJ; Jang J; Gao X Fullerenes and their derivatives as inhibitors of tumor necrosis factor- α with highly promoted affinities. *J. Mol. Model.* 2016, 22, No. 161. [PubMed: 27316702]
- (53). Sillanpää AJ; Aksela R; Laasonen K Density functional complexation study of metal ions with (amino) polycarboxylic acid ligands. *Phys. Chem. Chem. Phys.* 2003, 5, 3382–3393.
- (54). Chang CA; Francesconi LC; Malley MF; Kumar K; Gougoutas JZ; Tweedle MF; Lee DW; Wilson LJ Synthesis, Characterization, and Crystal Structures of M(D03A) (M = Fe, Gd) and Na[M(DOTA)] (M = Fe, Y, Gd). *Inorg. Chem.* 1993, 32, 3501–3508.
- (55). Klebanoff SJ Myeloperoxidase: friend and foe. *J. Leukocyte Biol.* 2005, 77, 598–625. [PubMed: 15689384]
- (56). van der Veen BS; de Winther MP; Heeringa P Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. *Antioxid. Redox Signaling* 2009, 11, 2899–2937.
- (57). Yatendra KMP; Kaptan S; Kumar SD; Venkata RK Process for the Preparation of Esters of Piperazic Acid, WO Patent 20051226822005.
- (58). Kielar F; Tei L; Terreno E; Botta M Large relaxivity enhancement of paramagnetic lipid nanoparticles by restricting the local motions of the Gd(III) chelates. *J. Am. Chem. Soc.* 2010, 132, 7836–7837. [PubMed: 20481537]
- (59). Rohrer M; Bauer H; Mintonovitch J; Requardt M; Weinmann HJ Comparison of magnetic properties of MRI contrast media solutions at different magnetic field strengths. *Invest. Radiol* 2005, 40, 715–724. [PubMed: 16230904]
- (60). Perlman RL Mouse models of human disease: An evolutionary perspective. *Evol Med. Public Health* 2016, 2016, 170–176. [PubMed: 27121451]
- (61). Chen YC; Bui AV; Diesch J; Manasseh R; Hausding C; Rivera J; Haviv I; Agrotis A; Htun NM; Jowett J; Hagemeyer CE; Hannan RD; Bobik A; Peter K A novel mouse model of atherosclerotic plaque instability for drug testing and mechanistic/therapeutic discoveries using gene and microRNA expression profiling. *Circ. Res.* 2013, 113, 252–265. [PubMed: 23748430]

Figure 1.
Chemical structure of MPO-Gd and heMAMP.

Figure 2.

(a) Plasma stability. heMAMP was incubated with plasma at 37 °C up to 3 h and the relaxation time was monitored at 0, 30, 60, 90, 120, and 180 min. There was no significant relaxation time change over 3 h ($n = 3$, means with standard error of the mean (SEM)). (b) Cytotoxicity of heMAMP. RAW 264.7, and HEK 293 cells (1.5×10^4 cells per well) were incubated with heMAMP in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at different concentrations for 16 h at 37 °C. The MTT assay demonstrated no significant toxicity of heMAMP up to 5 mM for both cell lines ($n = 3$, means with SEM). (c) Dynamic R_1 ratios of heMAMP and MPO-Gd after activation by MPO. heMAMP demonstrated more efficient activation with >3 times higher R_1 ratio

compared with MPO-Gd when incubated with MPO, glucose oxidase (GOX), and glucose over 180 min at 40 °C ($n = 3$, $*p = 0.033$, Kolmogorov–Smirnov test). (d) Binding to bovine serum albumin (BSA) after activation by MPO. When incubated with MPO, GOX, glucose, and BSA for 1 h at 40 °C, heMAMP showed about 1.5-fold higher binding efficacy compared to MPO-Gd ($n = 3$). (e) Biodistribution and retention of heMAMP at 3 h and day 7 determined by inductively coupled plasma mass spectrometry (ICP-MS) using a mouse model of complete Freund's adjuvant (CFA)-induced paw inflammation ($n = 3$ for each time point). (f) Blood half-life of heMAMP determined by ICP-MS with the two-phase decay model. The fast-phase blood half-life of heMAMP is 1.2 min and the slow phase is 31.7 min ($n = 5$). A total of 0.1 mmol/kg of heMAMP was intravenously administered as shown in Figure 2e,f.

Figure 3.

MR imaging of CFA-induced subcutaneous inflammation. (a–d) Imaging from precontrast and at ~60 min for heMAMP, DOTA-Gd, and MPO-Gd in wild-type mice and heMAMP in *Mpo*^{-/-} mouse. (e) Normalized contrast-to-noise ratio (CNR) of the MR imaging over a time period of ~60 min (all at 0.3 mmol/kg dosage, except for 0.1 mmol/kg of heMAMP for wild-type mice as labeled). (f) Activation ratio of the MR imaging over a time period of ~60 min demonstrated an approximately 2-fold higher activation ratio for heMAMP compared to that of MPO-Gd at ~60 min, both of which are much higher than DOTA-Gd, and that of *Mpo*^{-/-} mouse (mouse CNR_{post-contrast} – CNR_{pre-contrast}, *n* = 3 for each group).

Figure 4.

MR imaging of unstable and stable plaque in TS mice and intervention with MPO inhibitor AZM198. (a–c) Representative MR imaging of MPO-Gd and heMAMP $\pm$ AZM198 treatment in the tandem stenosis model of atherosclerotic plaque instability. (d) Contrast-to-noise ratios (CNRs) of unstable plaque for MPO-Gd (0.3 mmol/kg) and heMAMP (0.1 mmol/kg) ($n = 3$ mice for each probe), showing no significant difference ($p = 0.368$, ns, not significant, determined by Mann-Whitney test). (e) CNRs of unstable plaque (brachiocephalic trunk) in control and AZM198-treated TS mice. Signal in the unstable plaque of AZM198-treated mice decreased $\sim 50\%$ compared to that of no drug control ($*p = 0.033$, determined by Welch's t -test). $\Delta\text{CNR} = \text{CNR}_{\text{post-contrast}} - \text{CNR}_{\text{pre-contrast}}$.

Figure 5. Molecular docking of heMAMP binding to MPO. (a) An intramolecular hydrogen bond formed between the two amide bonds increases the rigidity of heMAMP. (b) Representations of heMAMP (left) and MPO-Gd (right) binding to the MPO-CN complex using PyMol. Amino acid residues Q91, H95, and R239 (shown as lines) together with heme (in green sticks) form the active site of MPO. All of the other amino acid residues (as lines) shown are within 4 Å to heMAMP and MPO-Gd. The hydrogen bonds are shown as red dashes and the electrostatic interaction is shown as orange dashes.

Scheme 1. Synthesis of Intermediates 1 and 4^a

^aReagents and conditions: (a) NaNO₂, NaBr, 0.75 M HBr, -15 °C; (b) MgSO₄, H₂SO₄, *t*-BuOH, rt, 45% for two steps; (c) Boc₂O, K₂CO₃, tetrahydrofuran (THF)/H₂O (1/4), rt; (d) EDC.HCl, HOBt, dimethylformamide (DMF), rt, 82% for two steps; and (e) TFA/DCM (1/2), 63%.

Scheme 2. Synthesis of heMAMP^a

^aReagents and conditions: (a) K_2CO_3 , CH_3CN , reflux, 78%; (b) TFA/DCM, rt; (c) $GdCl_3$, pH 5.5, rt, then 50 °C overnight; and (d) EDC.HCl, HOBT, NEt_3 , dimethyl sulfoxide (DMSO), 25%.

Table 1.Stability of heMAMP: Transmetalation in the Presence of Zn^{2+} and Ca^{2+}

	$R1_{(t=72h)}/R1_{(t=0)}$		$t \text{ (min) for } R1_{(t)}/R1_{(t=0)} = 0.8$	
heMAMP	Zn^{2+}	>0.97	Zn^{2+}	>5000
	Ca^{2+}	>0.96	Ca^{2+}	>5000
MPO-Gd	Zn^{2+}	0.76 *	Zn^{2+}	2866 *
	Ca^{2+}	0.94	Ca^{2+}	4260

heMAMP (1 mM) or MPO-Gd (1 mM) was incubated with ZnCl_2 (2.5 mM) or CaCl_2 (2.5 mM) in phosphate-buffered saline (PBS) for 95 h at 40 °C and T1 relaxation time was measured at different time points at 0.47 T ($n = 3$).

* Taken from ref 17, other data are from this work.