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Methoxyphenol derivatives as reversible inhibitors of myeloperoxidase as potential antiatherosclerotic agents

Premkumar Jayaraj³, Chandrakala A Narasimhulu², Andrei Maiseyeu⁴, Rekha Durairaj⁴, Shashidhar Rao⁵, Sanjay Rajagopalan⁴, Sampath Parthasarathy² & Rajagopal Desikan^{*1,2,3}

¹ Carmel Biosciences, 3562 Habersham at Northlake, Building J, Suite A, Tucker, GA 30084, USA

² Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32827, USA

³ Department of Chemistry, School of Advanced Science, Vellore Institute of Technology, Vellore 632014, India

⁴ Cardiovascular Research Institute, Case Western Reserve University, School of Medicine, 10900 Euclid Ave, Cleveland, OH 44106, USA

⁵ Department of Chemistry & Chemical Biology, Rutgers University, Piscataway, NJ 08554, USA

* Author for correspondence: Fax: +1 91 416 224 3092; rajagopal.desikan@vit.ac.in

Aim: To evaluate new chemical entities, based on ferulic acid scaffolds, as reversible myeloperoxidase inhibitors (MPOI). **Methodology & results:** *In silico* docking studies are performed with MPO protein as a target for several ferulic acid analogs followed by multiple *in vitro* assays to validate this approach. Two lead compounds **2a** and **3** are identified with optimum docking and IC₅₀ values: -7.95 kcal/mol, 0.9 μ M and -8.35 kcal/mol, 8.5 μ M, respectively. These MPOIs are able to inhibit oxidation of high-density lipoprotein and further promoted functionality of high-density lipoprotein. **Conclusion:** Lead analogs are potent MPOIs that exert specific effects on MPO-mediated oxidation as well as inflammatory pathways. It also acts as promoters of cholesterol efflux that sheds light on pharmacological approach in atherosclerosis treatment.

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Neutrophils, monocytes and macrophages are the major source of myeloperoxidase (MPO) hemoprotein expressed in high levels. Formation of highly reactive chemical intermediates is mediated by MPO (Compounds I and II) that can promote further oxidation [1,2]. It is well known that enzymes such as MPO, lipoxygenase and enzyme cofactor NADPH promote the conversion of low-density lipoprotein (LDL) into proatherogenic forms within the atheroma [3]. A role of this enzyme in the oxidation of apolipoprotein A-1 of high-density lipoprotein (HDL) has also been recognized [4]. On the basis of structure, MPO is considered as lysosomal hemoprotein, generated during the process of myeloid variation route, which contains predominantly neutrophil primary granules also referred as azurophilic granules [5–7]. In monocytes and tissue macrophages, MPO is expressed in at much lower concentration [8–10]. Microbicidal role of MPO is characterized by both presence and absence of oxygen-mediated mechanisms governed by specific enzymes through several reactive oxygen species [11]. Specific indication pointed out that this hemoprotein primarily contributes in ending respiratory burst by streamlining the functionality of NADH oxidase [12]. It requires in-depth investigation to establish a link on the role of oxidative stress in the functionalities of other proteins (enzymes).

MPO has been clearly shown to play a role in the disease manifestation, however, treatment option to regulate this protein is either nonexistence or in preclinical level [13]. However, an oral MPO inhibitor (MPOI), AZD4831, is currently under clinical trial for hemodynamic effects of an MPOI with exercise in heart failure with preserved ejection fraction [14]. With the nonexistence of this oxidative protein, the activity of other MPO-free bactericidal methodologies are expected to spontaneously start. Due to this alternate mechanism, a specific connection between the role of neutrophils functionality and its straightforward reaction with MPO require detailed study. Nonspecific

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anti-MPO serum inhibits the extracellular functionality of this protein, however, the specific part of MPO in regulating neutrophil activity is carefully evaluated when a more specific neutrophil-permeable MPOI is available for in-depth investigation. Basil (also known as Tulsi) extract and its components were found to inhibit the oxidation of lipoproteins by MPO [15]. Recently, a triazolopyrimidine reversible inhibitor of MPO was identified adopting high-throughput screening methodology [16]. By utilizing specific types of mechanism studies, it is possible to identify nonreactive reversible MPOI. 1-([6-Aminopyridin-3-yl] methyl)-3-(4-bromophenyl) urea has been studied as irreversible MPOI [17]. Existence of methoxyphenol in many natural products is considered as a boon for medicinal chemistry. It offers unique protection against many diseases largely by antioxidant and anti-inflammatory mechanisms. Previously, we reported a novel pharmacological inhibitor of MPO in a mouse atherosclerotic model wherein we have shown that a small molecule, INV-315 can effectively inhibit MPO both *in vitro* and *in vivo* condition and it was shown to reduce the atherosclerotic plaque burden considerably [18]. Based on this result and upon searching the literature, utility of methoxyphenol as MPOI has not been fully explored though a patent was filed by the author [19] and this is first in kind that methoxyphenol is used for inhibiting complex oxidative enzyme. The Lipinski rule of five was used to evaluate the drug-like structure of the proposed scaffolds for validating our approach. We consider this approach to be unique since many natural phenols are highly safe and biocompatible and they are also potential antioxidants.

Materials & methods

Materials

All starting materials and reagents were purchased from Sigma Chemicals (MO, USA); primers and cell culture reagents were purchased from Invitrogen (CA, USA); Radioactive material was purchased from American Radiolabeled Chemicals, Inc., (MO, USA); Commercial MPO enzyme was purchased from Sigma-Aldrich, (MO, USA); *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and dimethylaminopyridine were purchased from Alfa Aesar Chemicals (Kandel, Germany); CDCl₃ was purchased from Merck, Mumbai, India). Solvents were purchased from SD Fine Chemicals India Ltd, (Vellore, India). For column chromatographic purification of synthesized compound, 60–100 mesh silica gel was purchased from SD Fine Chemicals India Ltd, and was used accordingly. Human MPO (lot 080M1579, 50 U/mg), hydrogen peroxide, tyrosine, methyl β-cyclodextrin were purchased from Sigma. Lympholyte-poly was purchased from Cedarlane Laboratories (ON, Canada). J774A.1 cell was purchased from American-Type Culture Collection (ATCC), and cell culture media were purchased from Invitrogen. Human plasma was obtained from American Red Cross Society.

Methods

Molecular docking

An AutoDock-1.5.6 docking tool was used for molecular-modeling studies [20] using protein–heme complex from the RCSB-PDB (RCSB Protein Data Bank – <https://www.rcsb.org>) crystal structure entry with the PDB ID: 3F9P. The original x-ray crystal structure resolution is 2.93 Å. This protein was employed as input structure for protein preparation. Out of four chains, A, B, C and D; chains A and C were considered for docking while the crystal symmetry-related B and D chains were removed. Hydrogen atoms were added to the MPO–heme followed by the addition of Gasteiger and Kollman charges. It may be noted that the PDB entry did not contain any crystallographic water molecules in the protein–heme complex. The ligands were drawn using Chemdraw-3D-15.0 and the grid box were prepared to cover active site pocket. Then, the grid run was performed followed by docking protocol was evaluated. After the completion of docking procedure, critical analysis of various parameters were performed for protein–ligand complex. The analyzed complex was visualized using Discovery Studio Visualizer-2019-R2 and the images were taken in 2D and 3D format. None of the MPO structures in the PDB contained a natural ligand and we have created lead entities based on the methoxyphenol pharmacophore-building block.

Chemical synthesis of MPOI

The chemical synthesis of proposed compounds was achieved by adopting two different chemical routes in order to synthesize compounds in high yield and purity. Accordingly, for the first method, we used various coupling reagents to chemically link *O*-methoxyphenol to create new chemical entities. By way of the second method, we synthesized acid chloride from ferulic acid (FrA), which would then react with proline/pipecolinic acid to create new derivatives of FrA. In a typical reaction using coupling reagents, a dry 100-ml round-bottom flask, the reactants were dissolved in anhydrous dichloromethane followed by dimethylaminopyridine and *N*-(3-dimethylaminopropyl)-*N'*-

ethylcarbodiimide hydrochloride added over a period of 45 min maintaining the reaction mixture under inert atmosphere. The resulting reaction mixture was stirred overnight, and thin layer chromatography (TLC) was used to monitor the reaction for completion. The crude reaction mixture was purified with column chromatography, the purified compound was taken in a 100-ml round-bottom flask with 10% ethanolic potassium hydroxide and refluxed for 3 h followed by evaporation of solvent under reduced pressure and the crude compound was treated with water. The aqueous solution was acidified with dilute hydrochloric acid until pH reached acidic. The organic compound was extracted in dichloromethane and was washed with brine solution followed by drying over anhydrous Mg_2SO_4 and organic layer was concentrated under reduced pressure. The resultant crude product was purified with column chromatography. The purified compounds were characterized with proton and carbon NMR spectra and molecular mass was identified using high-resolution mass spectrometer. In the case of the second method, acid chloride was prepared using thionyl chloride followed by base-catalyzed addition of acid chloride to proline/pipecolic acid. The resultant crude mixture was purified by column chromatography and compared with an authentic sample.

Biological experimental section

Cell culture

J774.1 cells were obtained from ATCC and maintained according to supplier's instructions. Briefly, J774.1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose, 10% fetal bovine serum (FBS), 1.5 gm/l sodium bicarbonate, 1% l-glutamine, 1% sodium pyruvate and 1% penicillin–streptomycin.

Isolation of human polymorphonuclear cells

Blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes from consented healthy volunteers, was layered on top of an equal volume of lympholyte-poly, a density gradient polymorphonuclear granulocytes separation media. It was centrifuged at 500x g for 35 min. Resulting preparations were 98% pure and more than 95% neutrophils were viable as measured by trypan blue.

Isolation of plasma lipoproteins & their modification

Lipoproteins (HDL and LDL) were isolated from EDTA-treated plasma using a Sorvall T-8100 rotor and Sorvall WX ultra 90 ultracentrifuges at 84,000 r.p.m for 3 h at 4°C as previously described [21]. A two-step density gradient isolation was used with density being adjusted to 1.21 in the first spin and the isolated lipoprotein band was respun overlaying with 0.15 M NaCl. The isolated HDL and LDL were dialyzed against phosphate-buffered saline (PBS) containing 0.3-mM EDTA at 4°C for 6 h. The concentrations of lipoproteins (HDL and LDL) were determined using Bio-Rad DC protein assay. The purity of HDL was assessed by 20% SDS-PAGE gel and stained with Bio-Rad Coomassie and isolated lipoproteins were used within 48h. LDL was acetylated with acetic anhydride over purified by dialysis against PBS over 6 h [22].

Tagging of ac-LDL with 3 H-cholesterol was performed as described in the literature [23]. 3 H-cholesterol in ethanol [$1\alpha 2\alpha(n)-3\text{H}$]-cholesterol (American Radiolabeled Chemicals, Inc.) and ac-LDL were incubated with DMEM for 24 h at 37°C. This stock reaction mixture was diluted to obtain a working solution that contained 10 µg protein/ml of ac-LDL and 0.5 µCi/ml 3 H-cholesterol in DMEM and used for the experiments.

Oxidation of HDL

Freshly isolated HDL from human plasma was diluted with PBS containing 200 µg/ml HDL, 0.1 U/ml MPO enzyme, 50 µM H_2O_2 , 10 µM tyrosine and different concentrations of the inhibitors at room temperature. The formation of conjugated dienes was measured at 234 nm using Beckman DU800 Spectrophotometer [24]. Copper oxidation of HDL was carried similarly by using 200 µg/ml HDL in PBS containing 5-µM copper sulfate.

Thiobarbituric acid-reactive substances assay

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content in the MPO-oxidized HDL samples by thiobarbituric acid-reactive substances assay as described previously [25]. MPO-modified HDL in the presence and absence of drug was mixed with 0.3 ml of 6N HCl and 1 ml of 0.67% thiobarbituric acid. After heating at 100°C for 10 min, the product's absorbance was read at 532 nm. Tetramethoxypropane served as a standard for preparing the calibration curve. Results were expressed as percent increase in MDA content.

MPO assay

MPO activity was determined by the ability to oxidize 3,5,3',5'-tetramethylbenzidine (TMB) [26]. Freshly isolated human neutrophils were washed twice with 0.9% NaCl and the pellet was suspended in Hank's balanced salt solution. 1×10^5 cells were placed in 500- μ l Hank's balanced salt solution buffer and incubated with different concentrations of drug for an hour at 37°C. Then, the cells were lysed by sonication for 10 min on ice and centrifuged for 10 min at 10,000 r.p.m. The lysates (100 μ l) were assayed using TMB/H₂O₂ mixture as previously described. The samples were mixed well and allowed to incubate for 5 min prior to the addition of 2N-H₂SO₄ to quench the reaction. Absorbance was read at 450 nm.

Cholesterol efflux assays

Reverse cholesterol efflux was studied in J774 A.1 cells 30–33, 5×10^5 cells were plated in a 24-well plate. The day after cell plating, cells were cholesterol loaded for 24 h with 0.5 μ Ci/ml of [³H]-cholesterol [1 α 2 α (n)-3H]-cholesterol-labeled acetylated LDL in serum-free medium containing high glucose and BSA (0.2%). Cells were washed with PBS twice and equilibrated with serum-free medium containing high glucose and BSA (0.2%) for an hour. Cells were washed twice and incubated with control HDL or MPO-modified HDL or copper-oxidized HDL in the presence and absence of different concentrations of inhibitors samples for 4h. Cholesterol efflux was determined after measuring radioactivity in cells and media and expressed as: %Efflux = 100 %*([counts in media]/[counts in media + counts in cells]) [27,40].

DNA synthesis & real-time-PCR reaction

Total RNA was extracted from cells in a 12-well tissue culture plates using the Absolutely RNA Miniprep Kit (Stratagene, CA, USA) according to the manufacturer's protocol, and treated with DNase I to remove residual contaminating DNA. The total RNA (1 μ g) was reverse transcribed and cDNA was amplified by PCR using high-capacity cDNA Reverse Transcription Kit from Applied Biosystems. The sequences of the primers are listed in Supplementary Table 1.

J774 cells were used to determine the reverse cholesterol transport (RCT)-related genes in presence and absence of the different concentrations of MPOI compounds Mouse *ABCA1*, *ABCG1*, *SRB1*, *GAPDH* genes expression were quantified by real-time PCR using the Roche Light Cycler 480 detection system. Amplification was detected with SYBR Green as a fluorogenic probe specific for dsDNA by using a Power SYBR Green PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). The genes amplification was normalized to the GAPDH for mouse or β -Actin for human housekeeping gene corresponding (Δ Ct). Relative copy number (RCN) and fold change were determined. RCN was calculated as follows: $RCN = E^{-\Delta Ct} \times 100$. E is the efficiency ($2 = 100\%$ efficiency) and $\Delta Ct = Ct(\text{target}) - Ct(\text{reference})$. Fold change was calculated as $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct = \Delta Ct(\text{experimental cell group}) - \Delta Ct(\text{untreated cells})$. Duplicate samples were analyzed in each experiment [28].

Antioxidant assay

Briefly, 0.1-mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was prepared and 1 ml of this solution was added to 3 ml of the solution of lead compound at different concentrations (5, 15, 20, 30, 50, 75 and 100 μ M). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm. By spectroscopically, monitoring the color change will provide clue on antioxidant properties of the drug. Similar study was performed using the 2,2'-azinobis(ethyl benzthiozoline 6-sulfonic acid) (ABTS) method of antioxidant study to scavenge ABTS⁺ radical cation to identify antioxidant potentials of the drugs. Briefly, ABTS⁺ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12–16 h before use. ABTS⁺ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After the addition of lead compound at different concentration (10, 20, 50 and 75 μ M) to a required volume of diluted ABTS⁺ solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. To compare the efficiency, ascorbic acid was used as a positive control. Measurement was also done in the absence of inhibitor [29].

Nonsuicide inhibition

Irreversible inhibitors are suicidal compounds. They react with the active site of the enzyme-forming stable covalent bonds. These bonds may be formed between the inhibitor and the heme group or between the inhibitor and

Table 1. Ligand along with their predicted affinity (ΔG_{score} [kcal/mol]) toward myeloperoxidase and their best docking score with specific interactions (PDB code: 3F9P).

Ligand	Binding energy ΔG_{score} (kcal/mol)	Ligand–receptor interactions
1a*	-6.07	GLU:102, GLU:116; PHE:147, PHE:366, PHE:407; ARG:239
1b*	-5.81	PHE:99, PHE:146, PHE:147; THR:100; ARG:239
1c	Ligand similar to 1a	
1d	Ligand similar to 1b	
2a	-6.85	PHE:147, PHE:366, PHE:407; ARG:239; MET:411
2b	-5.43	GLU:102; LEU:216; ASP:218; PRO:220; PHE:407; VAL:410
3	-6.29	ARG:239, ARG:333; PHE:407; MET:411
4	-5.37	ASP:218, ARG:219; PRO:220; ARG:239; VAL:410
5	-6.03	PRO:220; PHE:366; VAL:410; MET:411; LEU:415
6	Ligand similar to 5	

Bold terms indicates the activity of the compounds.
PDB: Protein data bank.

the amino acids located beside the heme group. Two families of compounds were characterized as irreversible inhibitors including aminobenzoic acid hydrazide and thioxanthenes. It seems that all of MPO reversible inhibitors are competitive inhibitors. They react reversibly with the active site of the enzyme. The reversible nature of inhibitor was determined by performing two simple experiments, viz. the UV–visible spectroscopic method and TLC method.

By UV–visible spectrophotometer

Typically, the reaction mixture contains 100 mU human MPO (Sigma-Aldrich), 2 μmol s H_2O_2 , 8 μmol s of TMB and varying concentrations of the inhibitor. The final volume is made to 1 ml with 50 mM sodium acetate buffer at pH 5.6. The reaction is induced by adding MPO. Wavelength scan between 200 and 700 nm will detect any products and will be reflected in the spectrum. Products that are generated will easily be identified by comparing with a spectrum without MPO. This experiment is carried out with two controls, one without MPO enzyme and another with dead MPO enzyme.

By TLC method

Initially suitable mobile phase (eluant) was identified to determine the precise R_f value for each inhibitor on a precoated UV active silica gel plate (EMD, TLC silica gel 60 F254). MPO-mediated reaction was carried out by the reaction mixture contains 100 mU human MPO (Sigma-Aldrich), 2 μmol s H_2O_2 , 8 μmol s of TMB and varying concentrations of the inhibitor. The final volume is made to 1 ml with 50-mM sodium acetate buffer at pH 5.6. The reaction is induced by adding MPO. The organic compound present in the mixture is extracted with minimum amount of organic solvent and concentrated followed loaded on a precoated TLC plate and eluted with a known mobile phase. Appearance of any new products apart from the inhibitors will be determined either by exposing the plate to UV light or iodine vapor to get a bright spot on the TLC.

Statistics

The data are representative of three or more separate, independent experiments. The Student's *t*-test was used to determine the significance of the differences between treated and nontreated groups. A *p*-value of less than 0.05 was considered to indicate a statistically significant difference.

Results & discussion

Molecular modeling

The docking study was carried out with proposed MPOI to investigate the possible binding site on MPO(3F9P) and the binding energy of synthesized inhibitors were found to be ranging from -5.37 to -6.85 kcal/mol. The docking score for the top-ranked poses of the docked ligands (**1a** through **6**) are provided in Table 1. Two potent compounds **2a** and **3** have shown the best binding energy based on its docking score. The correlation between the docking score and observed IC_{50} values suggested moderate correlation (correlation factor $r^2 = 0.58$). The

Table 2A. Evaluation of drug likeness of compounds **2a** and **3** with respect to the Lipinski's rule.

Lipinski property	Compound 2a	Compound 3
M _w (500) (for nonionized derivatives)	305.1	314.1
HBD-5	2	1
HBA-10	3	5
Clog P (<5) LogP	+1.87 1.39 ± 0.47 [‡] 1.70 ± 0.49 [§] 1.41 ± 0.94 [¶]	+3.32 3.12 ± 0.47 [‡] 3.01 ± 0.49 [§] 2.60 ± 0.73 [¶]

[‡]By Crippen's fragmentation.
[§]By Viswanathan's fragmentation.
[¶]By Broto's method.
 HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; M_w: Molecular weight.

binding modes of all docking compounds in the active site of MPO structure are illustrated in Supplementary Figure 1A–G. These illustrations demonstrated their top-ranked docked poses that have docking scores (binding energy) of (**1a**: -6.07), (**1b**: -5.81), (**2a**: -6.85), (**2b**: -5.43), (**3**: -6.29), (**4**: -5.37), (**5**: -6.03) kcal/mol. The most potent compound **2a**-binding interactions as: PHE:147, PHE:366, PHE:407; ARG:239; and MET:411. On the other hand, the compound **3**-binding interactions are ARG:239, ARG:333; PHE:407 and MET:411 are active site residues for MPO-heme protein. Based on the binding energy of docked molecules in the MPO active site pocket, selected compounds were synthesized utilizing feasible synthetic methods. With the outcome of preclinical results, the lead molecules could be considered as potential inhibitors of MPO.

Chemical synthesis

FrA (3-methoxy-4-hydroxyl cinnamic acid) is an abundant phenolic phytochemical, related to transcinnamic acid found in plant cell wall components that have been shown to exert potent antioxidant functions including MPO-mediated reactions [30,31]. We thus posited that this may represent a scaffold that can be further modified to potentially increase the affinity of the ligands leading to greater inhibition of MPO.

The optimized compounds **1a**, **1b**, **1c**, **1d** and **2a**, **2b**, **5**, **6** have an amide bond connectivity with FrA and compounds **3** and **4** have ester bond connectivity with FrA. In general, amide and ester bonds have more biological relevance and several of commercial drugs hold these two types of chemical bonds. In order to achieve cost-effective chemical synthesis with high yield and purity, we tried two chemical methodologies. For ester bond formation between acid and alcoholic functional groups, we used various coupling reagents, however, this method has limitation due to cost of the reagent and extrachemical steps that will be part of synthesizing final molecules. On the other hand, the acid chloride method using thionyl chloride has yielded final products but with reduced yield and purity.

The proposed analogs of FrA were synthesized using different experimental conditions like change in solvents or base or temperature with two different methodologies, which are shown in Supplementary Table 2A & B.

Compounds **1a**, **1b**, **1c**, **1d**, **2a**, **2b**, **3**, **4**, **5**, **6** were synthesized with feasible chemical routes as shown in Supplementary Figures 2 & 3. These compounds were fully characterized by NMR spectroscopic technique (Bruker Instrument 400, 600 MHz); the data and figure provided in Supplementary Figure 4 as an MS technique (Micromass QTOF II model) the data and figure shown in Supplementary Figure 5. The complete synthetic procedure and spectral data are also provided in the Supplementary data.

Drug likeness

Evaluation of the drug likeness of FrA derivatives with respect to the Lipinski's rule was assessed using ChemDraw Professional 15.0 to increase the success rate of developing MPOI for advanced preclinical development for cardiovascular disease (CVD). The calculations concerning hydrogen bond donor and hydrogen bond acceptor groups were based on the number of hydrogen bonds that could be formed under physiological condition. The results from the calculations revealed that the lead derivatives of FrA fulfilled the Lipinski's rule since none of the compounds violated rules. The Lipinski prediction of lead compounds was summarized in Table 2A.

Figure 1. Effect of myeloperoxidase inhibitors on high-density lipoprotein and lipid peroxidation. (A & B) Oxidative modification of high-density lipoprotein by myeloperoxidase is inhibited in the presence of **3** and **2a**. **(C)** Thiobarbituric acid-reactive substance formation is inhibited by **3** and **2a**. '612'>. TBARS: Thiobarbituric acid-reactive substance.

In vitro MPO Inhibition

The biological activity of FrA derivatives was assessed by the inhibition of the MPO activity in a cell-free system using commercially available enzyme as reported in the literature [32]. The IC_{50} value of inhibitors was determined by plotting absorption of resulting solutions at 650 nm against inhibitor concentrations. The IC_{50} values of all synthesized compounds are presented in Table 2B. The compounds **1a** and **2a** were structurally similar analogs and have comparable IC_{50} values. In order to keep the structural diversity for efficacy testing, two lead compounds **2a** (IC_{50} = 0.9 μ M) and **3** (IC_{50} = 8.5 μ M) were chosen for further studies since **1a** and **3** have been shown to inhibit MPO under comparable concentration.

In vitro MPO-mediated oxidation of HDL

MPO-mediated oxidation of HDL has been considered one of the major mechanisms responsible for HDL oxidation *in vivo* [33–35]. To further evaluate efficiency of synthesized inhibitors, we performed *in vitro* MPO-mediated oxidation of HDL in the presence of two lead compounds **2a** and **3**. HDL oxidation in the system consisting of MPO, hydrogen peroxide and tyrosine (Ox-HDL-sys), which has been extensively used as an *in vitro* system for HDL oxidation [34,36,37]. We performed series of oxidation reactions using freshly isolated HDL from human plasma. First, concentration of HDL, hydrogen peroxide, tyrosine as well as enzyme was optimized on the samples without inhibitors. Second, concentrations of lead inhibitors were optimized using 0.01–10 μ M of both **2a** and **3**. We found that high concentrations of **3** had no inhibitory effect on the formation of conjugated dienes in Ox-HDL-sys suggesting the efficacy of compounds even at lower conc. and at higher conc. losing its efficacy might be due to auto-oxidation; while **2a** leads to increase in lag time (oxidation was inhibited) in the inhibitor response fashion. This auto-oxidation may be explained due to presence of MPO, which contains Fe^{2+} (heme); it can initiate free-radical-mediated oxidative product formation. This results in the generation of peroxides of compounds **2a** and **3**, resulting in the propagation of peroxy radicals. Subsequently, these peroxy radicals are converted into modified neutral molecule via the process of radical termination reaction [38]. Representative oxidation curves are shown on Figure 1A and '9053'>B. HDL alone without the treatment with MPO served as a control. For further studies, we chose two concentrations (0.1 and 1 μ M) of both compounds as they exhibited pronounced and reproducible effects in HDL oxidation assays. When oxidation of HDL was mediated by the addition of copper sulfate rather than by MPO, there was an increase in lag time for both compounds with more evident effects in case of **3**, suggesting an apparent antioxidant effect.

Table 2B. IC₅₀ values of synthesized compounds.

Entry	Structure	IC ₅₀ (μM)
1a [‡]		14.0
1b [‡]		45.0
1c		42.0
1d		39.0
2a		0.9
2b		75.0
3		8.5
4		25.0
5 [‡]		40.0

[‡]Optically active S-proline was used to prepare ferulyl derivative.

Table 2B. IC₅₀ values of synthesized compounds (cont.).

Entry	Structure	IC ₅₀ (μM)
6		52.0

†Optically active S-proline was used to prepare ferulyl derivative.

Figure 2. Myeloperoxidase inhibition in human granulocytes. (A & B) Compound concentration-dependent inhibition of myeloperoxidase by **3** and **2a** in human granulocytes. MPO: Myeloperoxidase.

Assessment on lipid peroxidation

MDA colorimetric assay is the most commonly used technique to quantify the lipid peroxidation [25]. We assessed the formation of oxidized products in Ox-HDL-sys in the presence of two concentrations of **3** and **2a**. MDA content was measured after incubation of Ox-HDL with **3** or **2a** for 3 h and compared with that of nontreated HDL. The percent of increase in MDA content in Ox-HDL-sys without inhibitor was compared with that of **2a** and **3** at concentrations 0.1 and 1 μM. There was highly significant ($p < 0.01$) reduction in MDA content after treatment with 0.1 μM of **3**. In the line with our data shown in Figure 1C on conjugated dienes formation, higher concentration of **3** had no effect on MDA content. Both concentration of **2a** showed similar inhibition capacity and were able to significantly reduce MDA formation.

Inhibition of human leukocyte-derived MPO

We then proceeded to evaluate the performance of two lead compounds in the cell-based assays to determine whether **3** and/or **2a** are able to inhibit human leukocyte-derived MPO. Using density centrifugation, we isolated polymorphonuclear cells (granulocytes) from human blood of healthy volunteers [39]. Freshly isolated granulocytes were incubated with (range: 0.01–1000 μM) **3** and **2a**, followed by MPO activity determination with TMB. Initial MPO activity was determined in the control sample incubated with vehicle alone and considered as 100% activity. Logarithmic compound concentration–response inhibition curves were then obtained and *in vitro* IC₅₀ values were calculated from the best curve fit (Figure 2A & B). **3** was found to have *in vitro* IC₅₀ = 8.5 μM, while **2a** was almost two-times more active with IC₅₀ = 0.9 μM.

RCT assay

The capacity of HDL to promote cholesterol efflux from macrophages is an important initial step in the process of RCT [40–42]. Dysfunctional HDL (e.g., oxidized with MPO) has impaired cholesterol efflux and, as a result, fails to transport an excess of cholesterol from the cell. To investigate whether new MPO inhibitors **3** and **2a**

Figure 3. Reverse cholesterol transport assay. (A & B) The effect of high-density lipoprotein oxidation in the presence of **3** or **2a** on lipoprotein-induced cellular cholesterol efflux. HDL: High-density lipoprotein.

Figure 4. Effects of **3 and **2a** on mRNA expression levels of *ABCA1*, *ABCG1* and *SRB1* in J774 A.1 cells.**

have an effect on cholesterol efflux mediated by Ox-HDL, we performed RCT assays using radiolabeled (tritium, H^3) cholesterol probe [43,44]. Tritium-labeled cholesterol incorporated into Ac-LDL (Ac-LDL- H^3) was incubated with murine macrophage cell line (J774 A.1), followed by exposure to Ox-HDL with or without inhibitors for 4 h. Cholesterol efflux was calculated as: %Efflux = (radioactivity in media/total radioactivity [cell lysates + media])*100% (Figure 3A). Results were compared with HDL that was not oxidized. There was insignificant improvement in efflux with low concentration of **3** while 1 μM of **3** and both concentrations of **2a** improved the cholesterol efflux with high significance. Interestingly, cholesterol efflux with copper-oxidized HDL (Figure 3B) was slightly improved in the presence of 1 μM **3** but not 1 μM **2a**, however, the difference was not significant.

Effect of MPOI on cholesterol efflux gene expressions

We next investigated the effects of **3** and **2a** on the expression of key cholesterol transport genes: *ABCA1*, *ABCG1* and *SRB1*. Expression was analyzed in J774 A.1 cells treated with different concentrations of drugs for 4 h. As shown in Figure 4, both compounds did not have significant effects on mRNA expression at all tested concentrations, thus ruling out the possibility that drug-induced protein expression may contribute to increased cholesterol efflux.

Antioxidant assay

The DPPH radical absorbs at 517 nm and antioxidant activity can be determined by monitoring the decrease in this absorbance. Results were reported as percentage inhibition of free radical generated during the course of

Figure 5. 2,2-Diphenyl-1-picrylhydrazyl and 2,2'-azinobis(ethyl benzthiozoline 6-sulfonic acid) antioxidant assay with compounds **2a** and **3** with ascorbic acid as positive control and pure solvent as negative control.

reaction in presence of compounds **2a** and **3**. Similarly, ABTS antioxidant assay was performed without using any substrate. ABTS with an absorption maximum at 342 nm has high water solubility and chemical stability. Figure 5 provides the details of antioxidant assay using DPPH and ABTS radicals for two lead MPO inhibitors (**2a** and **3**). Our *in vitro* antioxidant study with lead compounds along with ascorbic acid as a positive control showed there is a correlation in antioxidant activity though the level of activity in comparison to known antioxidant, seems moderate in reactivity.

Nonsuicide inhibition

UV-visible spectroscopic method

MPO acts on a variety of substrates and oxidizes them, it is theoretically possible that our inhibitors were acting as substrates and competitively masking formation of product(s) from the added substrate. However, we concluded earlier that this chemistry was unlikely as the substrate was added several folds in excess. The spectroscopic method of evaluating reaction mixture failed to reveal any additional products in the presence of MPO. There was no colored product even when the reaction mixture contained only the inhibitors and not the TMB or Guaiacol substrates. As can be seen from Figure 6A & B, there is no indication of chemical transformation that could have occurred when our lead inhibitors can covalently link at the MPO active site, since UV-visible spectrum of lead inhibitors with and without MPO showed the same absorption bands.

TLC method

Formation of any product out of an inhibitor due to oxidation of MPO will be reflected on TLC by specific new spots when it is eluted with specific solvent systems (eluent). Based on this TLC experiment, we observed the lead inhibitor **2a**, when it was reacted with MPO protein in a suitable buffer, did not produce any characteristic oxidative products mediated MPO. As can be seen from TLC in Figure 6C, spots correspond to before and after MPO treatment appear at same R_f value indicating there was no specific product formed between inhibitor and MPO indicating the reversible nature of the inhibitor. Assuming the lead compounds inhibit MPO irreversibly (suicide inhibition), the nature of resultant product will be chemically modified. The modified structure can be easily observed in TLC when compared with the parent compound. A similar pattern of reactivity was observed when MPO was reacted with lead inhibitor **3** in the presence of protein substrate tetramethylbenzidine, as given in Figure 6C.

Discussion

Methoxyphenol is a building block for this study to develop as an antiatherosclerosis agent. It is well known in the literature that existence of methoxyphenol in many natural products is considered as a boon for drug development. Therefore, this study primarily focuses on *O*-methoxyphenol-based inhibitors mainly due to its unique property of protection against many diseases largely by antioxidant and anti-inflammatory mechanisms due to presence of

Figure 6. Myeloperoxidase (MPO) inhibition mechanism. (A & B) The UV pattern of MPO reaction of lead inhibitors in presence and absence of MPO substrate with compounds **2a** and **3**. **(C)** The thin layer chromatography pattern of MPO reaction of lead inhibitors in presence and absence of MPO substrate with compounds **2a** and **3**. MPO: Myeloperoxidase.

radical-scavenging phenolic group and methoxy group offers synergism in terms of therapeutic effects. Although several MPO inhibitors such as indole analogs, hydrazides derivatives, hydroxamic acid analogs and flufenamic acid are reported in the literature, none of the molecules reached the market as a drug for the availability to general public. Based on the literature, it was identified that aromatic hydroxy compounds seem to function well in terms deactivating MPO. An investigation of methoxyphenol derivatives to target CVD-mediated MPO has not been studied in the literature. The compounds **2a** and **3** can be considered as potential antiatherosclerotic agent and it may open up a platform to develop more active MPO inhibitor utilizing the *O*-methoxyphenol-type molecular structure

Structural–activity relationship

The identification of two lead compounds **2a** and **3** is based on the critical *in vitro* experiments wherein we performed functional group modification of lead molecules and studied their efficacy of modified resultant moieties. Modification of carboxylic acid in **2a** into ester **2b** has dramatically reduced the MPO inhibition value from 0.9 to 75 μ M. By reducing one carbon of **2a** (six-membered ring to five-membered ring) to **1a**, the MPO inhibition value increased to 4 μ M. Similar trend was observed when we tried to compare the results in the inhibition of human leukocyte-derived MPO (data not shown). The influence of stereo chemical center on MPO inhibitor was also studied. The effect of racemic and optical active form of **1a** with **1c** (optically active and racemic prolyl acid, respectively) and **1b** with **1d** (optically active and racemic prolyl methyl ester, respectively) was also investigation. Based on MPO inhibition value, it is evident that the influence of either chiral or achiral carbon does not make much difference. By substituting isoferulyl group in place of ferulyl moiety, there was a 25-fold decrease in the MPO inhibition indicating the importance of natural analog of methoxyphenol for better activity against MPO. Protection of phenolic –OH in the FrA moiety as methyl ester group has significantly reduced the MPO inhibition activity (data not shown). Based on these observations on the structural modification of lead molecules **2a** and **3**, we have selected two upper-lead for further preclinical study.

Extreme generation of MPO-mediated oxidants was shown to cause tissue injury in several diseases. This is very obvious when it is characterized by acute or chronic inflammation [6]. Inflammation is identified as the

primary reason for pathophysiological pathways in the manifestation and advancement of atherosclerosis and its atherothrombotic complexity. Therefore, there is an intense enduring attention to study about the precise mechanism of inflammatory intermediaries in cardiovascular disease. The concentration level of MPO in the circulatory system was demonstrated to forecast dangers for main adverse cardiac proceedings. The identification on the concentration of MPO-mediated halogenated products are specific biomarkers for disease manifestation, which received significant attention in the development of medicinally active inhibitors of MPO. Yet another reason is that the nonavailability of drug against MPO is mainly due to molecular flexibility, intricate redox reactivity and vague reaction pathways with which molecular scaffolds react with the enzyme. When appropriate physiological environment is maintained, the functional role of MPO in microbicide pathway is characterized by the existence of characteristic chemical scaffolds that induces the activity of this oxidative protein. Formation of Compound I and Compound II is attributed to a sequence of redox reactions that MPO is shown to undergo in physiological host medium. The halogenation cycle or peroxidation pathway is the main reaction of this oxidative protein when it is exposed to a chemically highly susceptible structural environment. The powerful oxidants that are produced due to reactivity of MPO are demonstrated to arbitrarily oxidize the biomolecules such as DNA, lipids and other proteins. This oxidation leads to the impairment of biomolecules completely and prevent their functional duties. Therefore, an appropriate MPO drug molecule must be a stable chemical entity and worthy substrate at a given concentration, and complications due to adverse reactions are maintained as few as possible. The structure-based computational molecular docking of FrA derivatives in the MPO active site pocket to develop molecular derivatives with highly efficacious inhibitory functionality against MPO. The proposed molecular scaffolds are designed in such a way that all the molecules could be docked within the active site pocket of this oxidative protein. The 3D structures of the FrA derivatives (**1a**, **1b**, **1c**, **1d**, **2a**, **2b**, **3–6**) used in the molecular docking studies were obtained by subjecting their 2D structures within the ligand preparation module of AutoDock. In cases of compounds containing the proline moiety, only the S configuration (corresponding to the natural amino acid) was considered. On the other hand, both R and S configurations of the stereo chemical center in the piperidine moiety were considered. It should be mentioned that generated structures have stereochemically acceptable bond lengths and bond angles, which do not vary during the docking simulations. The starting 3D structures are endowed with reasonable dihedral angles, which do vary during the flexible docking of ligands. The ligand structures thus prepared were docked into the prepared 3F9P protein–heme complex. Protein flexibility was not employed since the putative binding site was observed to be significantly larger than the most voluminous conformations of the ligands docked. On the other hand, ligand ring flexibility was explored, leading to the consideration of boat and twisted boat configuration of the six-membered piperidine ring and the puckered forms of the pyrrolidine ring system. Up to 20 poses were saved for each of the ligands based on the ‘total docking score’ values. The top-ranked docked poses for each of the ten ligands were analyzed for intermolecular (protein–ligand) hydrogen bonds using docking tool and Discovery Studio Visualizer.

In order to further validate on our approach to select the top two best molecules is based on Lipinski’s rule of five wherein compounds **2a** and **3** fulfilled the rule without any violation. On the aspects of MPO inhibition evaluation, compounds **2a** and **3** both exert inhibitory effects at submicromolar concentrations. However, these compounds sometimes had divergent effects on multiple measures such as thiobarbituric acid reactive substances and conjugated dienes. These effects are sometimes not congruent with the effects on *in vitro* RCT suggesting that the effects on measures of HDL functionality can sometimes diverge, necessitating comprehensive evaluation of a number of parameters. The molecules are nontoxic even at high concentrations (data not reported) using viability assays. We employed MTT cell viability assay and performed as per the literature method by using RAW macrophage cells (RAW 264.7 from the ATCC) [45]. *Ex vivo* RCT assays demonstrate an improvement in cholesterol efflux with both **2a** and **3**. Since MPO-oxidized apolipoprotein A-1 impairs the cellular cholesterol efflux by ABCA1 [34], it may be hypothesized that these novel MPO inhibitors may retard atherosclerosis development via inhibition of LDL oxidation and improvement of RCT. Generic antioxidant experiment showed that compounds **2a** and **3** are observed to be good antioxidant even though the antioxidant capacity was less when it is compared with known antioxidant, ascorbic acid. Based on our other *in vitro* results, we strongly believe that the MPOI **2a** and **3** are not only generic antioxidants, but are a specific inhibitor of MPO, which can further developed for further preclinical study useful in cardiovascular platform.

Conclusion

The docking results as well as biochemical assays of the proposed inhibitors may seem to point out that MPO inactivation could occur either via active site blockade or rendering MPO inactive by Compound II. This is corroborated by lead compounds fulfilling the Lipinski rule of drug likeness. This hypothesis is supported by literature reports that have shown the phenols and anilines prevent MPO from producing hypochlorous acid by trapping the enzyme in its inactive Compound II form [46]. This type of inhibition is reversible because superoxide reduces Compound II back to its native form. Therefore, at this point of time, we reasonably believe that compounds **2a** and **3** may act as reducing substrate and may promote Compound II formation and it could act as a substitute for hydrogen peroxide in Compound I formation. Results based on *in vitro* and cell-based MPO inhibition assay and effect of compounds **2a** and **3** on LDL and HDL revealed that compounds **2a** and **3** are potent MPO inhibitors and can offer favorable therapeutic properties against CVD and its risk factors. Further investigations are underway toward lead optimization and evaluate their potential as antiatherosclerotic agents and will be reported elsewhere.

Future perspective

The understanding of MPO-mediated pathways has improved dramatically and it is therefore essential to suitably earmark interventions and resources for the purposes of bringing out a drug with a minimal risk–benefits ratio. In order to develop MPO inhibitors as important therapeutic agents, it is important to consider the multiple intricate parameters involved impacting the outcome of the study. This is not only for cardiovascular disease but other diseases with which MPO is associated. The FrA analogs as MPO inhibitors are expected to exert their effect on disease target, but at the same time offer little effect on immune suppression – addressing the concern on the development of infections due to lack of MPO. This study undoubtedly would pave the way for developing cardioprotective agents that are biocompatible and nontoxic due to the natural origin of the chemical entities.

Summary points

- New chemical entities based on ferulic acid were designed, synthesized and screening as myeloperoxidase inhibitors for effective cardioprotective agents.
- Based on *in silico* docking and enzyme inhibition assays to filter out lead compounds from hit analogs.
- Two lead compounds **2a** and **3** exhibited favorable properties in critical bioassays like high-density lipoprotein oxidation, enhanced cholesterol efflux of high-density lipoprotein in presence of these compounds efflux assay, which seem to indicate that these two compounds can be useful as cardioprotective agents.
- Compounds **2a** and **3** can be further developed as potent antiatherosclerotic agents.
- Outcome of this study points to a fact that hydroxycinnamic acid family of phytochemical can be used as a potential building block against cardiovascular disease.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fmc-2019-0080

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