Supporting Information

Thioxo-dihydroquinazolin-one Compounds as Novel Inhibitors of Myeloperoxidase

Yang Li, [1] Thota Ganesh,[2] Becky A. Diebold,[1] Yerun Zhu,[1] James McCoy,[1] Susan M.E. Smith,[3] Aiming Sun, [1,*] J. David Lambeth[1,*]

[1]Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322

[2]Department of Pharmacology, Emory University, Atlanta, Georgia 30322

[3]Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, Georgia 30144

List of Contents

METHODS, page 1-2

GENERAL PROCEDURE AND CHARACTERIZATION OF COMPOUNDS, page 3-6

Table 2, page 7-8

Figure 1 S.I, page 8

Figure 2 S.I, page 8

METHODS

Measurement of oxidant generation

In initial experiments reported in Supplemental Information, L-012 chemiluminescence was used to detect reactive oxygen species in a cell-free "semi-recombinant" system consisting of isolated plasma membrane plus recombinant regulatory subunits.[1,2] To generate a strong signal, the assay requires the presence of a peroxidase (*e.g.*, added horseradish peroxidase or endogenous MPO), as well as a source of H_2O_2 that is produced by the neutrophil NADPH-oxidase (NOX) [3] The NOX2 catalytic subunit is well known to be present in these membrane preparations, and MPO was also evident by Western blots at 65 ng/μg protein, based on MPO activity (assayed as described below), using a standard curve generated with pure human MPO. Hence, compounds that inhibit *either* the NADPH-oxidase or the MPO are detected as inhibitors of the L-012 signal,[3]–Plasma membranes (0.3 μg) were prepared from human neutrophils as described previously,[4] and were mixed with 0.25 μg p67Np47N fusion protein (human p67phox residues 1-210 fused to human p47phox residues 1-286)[5], 0.2 μg Rac1Q61L, 500 nM flavin adenine dinucleotide (FAD), 50 μM L-012 (Wako Chemicals, Richmond, VA), NADPH (10 μM), and 1 μL inhibitor (dissolved in dimethyl sulfoxide; DMSO) in 100 μL of assay buffer (100 mM PIPES, 30 mM NaCl, 35 mM $MgCl_2$, 1M KCl, pH 7.4). The final DMSO concentration in all assays was 1%. Luminescence was recorded in a Synergy 2 microplate reader (Biotek Instruments, Winooski,VT). After two minutes, 90 μM sodium dodecyl sulfate (SDS) was added and luminescence was recorded for an additional 20 minutes. Maximum luminescence was achieved by 5 minutes, and is reported as the mean from 3–5 consecutive readings. For control xanthine oxidase assays, each well of a 96-well plate contained 5.0 mU/mL final xanthine oxidase (Roche Diagnostics, Indianapolis, IN) in 100 μL of PBS. Xanthine (50 μM) was added to initiate the reaction and luminescence was recorded for 20 minutes using a Synergy 2 microplate reader (Biotek).[6] Superoxide generation was quantified using NAPDH-dependent, superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction, as described previously.[4]

MPO Activity Assays

MPO activity was assayed at 25 °C by monitoring H_2O_2-dependent Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine; Life Technologies, Grand Island, NY) oxidation using purified human MPO (Planta Natural Products, Vienna, Austria). Each well of a 96 well microplate (Corning Special Optics microplate, Cat. No. 3720 Corning, NY) received 1 µL of test compound in DMSO or vehicle alone. DMSO was 1% v/v final concentration. Each compound was tested in triplicate at final concentrations ranging from 0.1 µM to 100 µM. All wells contained the following: 0.17 nM MPO, 10 µM H_2O_2, and 50 µM Amplex Red in a total volume of 100 µL of 20 mM potassium phosphate, 0.15 M NaCl, pH 7.4. The Amplex Red generates the highly fluorescent resorufin, which was detected at 590 nm using an excitation wavelength of 540 nm using a Synergy 2 microplate reader. Fluorescence was monitored over the course of 20 min, and the mean of the maximum slopes obtained from the linear portion of the rate curves were calculated (Vmean) using the Gen5 software (Biotek). IC_{50} values +/- standard deviation were calculated from a 4-parameter fit of logarithmic dose response curves generated by the Gen5 software. Under our conditions, approximately 60% of the Amplex Red-generated signal was dependent on chloride, suggesting that it detects both chlorination-dependent and -independent reactions catalyzed by MPO. MPO-dependent chlorination of taurine was measured as described previously.[7] As a control assay for scavenging of H_2O_2, 20 nM horseradish peroxidase (HRP) (Type II, Sigma-Aldrich, St. Louis, MO) was used in place of MPO in the Amplex Red assay. In the absence of inhibitors, this amount of HRP gave the same rate as 0.17 nM MPO under the same assay conditions.

U.V.-visible Absorbance Spectroscopy

Absorbance spectra were recorded in triplicate wells using a BMG Omega plate reader and 96 well UV/VIS half-area plates (Grenier). Spectra were averaged and smoothed, and corrected for path length (MARS software, BMG). Each well contained 45 µL of 1.1 µM MPO (2.2 µM heme) (Cell Sciences, Canton, MA) in 50 mM potassium phosphate buffer, pH 7.4 containing 150 mM NaCl plus 5 µL inhibitor solution in 10% DMSO or 5 µL of 10% DMSO (1% final concentration). After 15 mins at 25°C, spectra were recorded in the absence of H_2O_2, and recorded again 1 min after the addition of 2 µL of H_2O_2 (250 µM final). No absorption changes due to inhibitor were seen in the absence of H_2O_2. The concentration of ferric MPO in the absence of H_2O_2 was calculated using A_{430nm}, ε = 89 mM^{-1} cm^{-1} per heme.[8,9]. Difference spectra were obtained by subtracting the spectra obtained in the absence of H_2O_2 from spectra obtained in the presence of H_2O_2. The concentration of H_2O_2 was determined using A_{240nm} and using ε = 43.6 mM^{-1} cm^{-1} [8]

Determination of the reversibility of inhibitor binding

A custom ELISA plate to which MPO was bound to the surface of each well was prepared as follows: wells of a 96-well plate (Corning Special Optics plate #3720) were incubated with 0.3 µg (2.1 pmol) of purified human MPO (Cell Sciences, City, Cat#CS19692B, isoform C) in 0.1 mL of 0.1M potassium phosphate buffer pH 7.4 (PPB). The covered, parafilm-sealed plate was incubated at 4°C for 48 hr to allow MPO to bind. Plates were then gently washed once with 100 µL per well of 100 mM PPB to remove unbound MPO. Inhibitors or vehicle control (1% DMSO) were added to wells along with H_2O_2 (50 µM final), all in 100 µL of 100 mM PPB containing 100 µM DTPA, and the incubation was continued for 1 hr at 25°C. To determine reversibility of inhibition, after incubation, half of the wells were gently washed 3 times with 100 µL per well of 100 mM PPB containing 0.1% Tween-20, incubating for 1 min per wash. Activity in all wells was measured.[10,11] Each well received 1.5 mM (final concentration) of TMB (Sigma #T2885) in 100 µL of 100 mM PPB containing 100 µM DTPA. The absorbance at 652 nm was measured for 3 min to obtain a baseline, 200 µM H_2O_2 (final) was added to all the wells and the absorbance change at 652 nm was recorded for an additional 15 min using a BMG FLUOstar Omega plate reader at 25°C. Reversibility of inhibition was indicated by recovery of significant activity following washing.

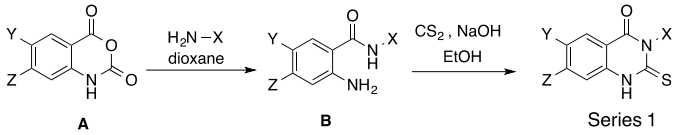
Solubility

Solubility of select compounds was evaluated by nephelometry using a nephelometer (BMG LABTECH Nephelostar, Cary, North Carolina) according to manufacturer instructions.

General procedures for chemistry

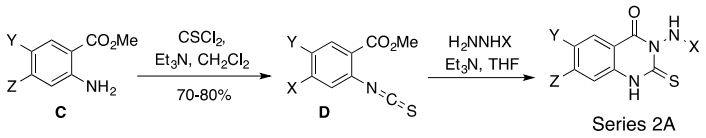
Unless otherwise noted, all materials were obtained from commercial suppliers and were used without purification. Dry organic solvents (DriSolv) were packaged under nitrogen in Sure Seal bottles. Reactions were monitored using thin-layer chromatography (TLC) on 250 µm plates or using LC/MS with UV detection at 254 nm and low resonance electrospray mode (ESI). After synthesis, individual chemical compounds dissolved in hexane, dichloromethane or a mixtue of the two, were loaded onto a silica gel 60 column (particle size 0.04-0.063 mm, 230-400 mesh) and chromatographed using a mobile phase of the same solvent,[12] using a CombiFlash RF instrument. Eluting compounds were monitored by absorbance at 254 nm, and purified fractions were collected using the built-in fraction collector. In some cases, compounds were purified by crystallization. ^{1}H NMR spectra were recorded on a Varian spectrometer (400 MHz) at ambient temperature. Chemical shifts are reported in ppm relative to DMSO or $CDCl_3$ and coupling constants (J) are reported in hertz (Hz). Solvents for NMR were $CDCl_3$ or DMSO-d_6. The residual shifts were taken as internal references and reported in parts per million (ppm). Purity of final compounds was ≥95% based on analytical HPLC or NMR analysis.

General procedure for synthesis of series 1 compounds.



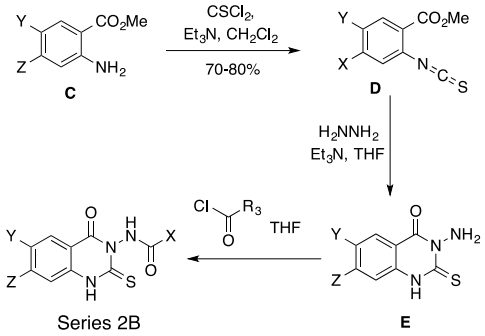
Alkyl amine (2 mmol) was added dropwise to a solution of isatoic anhydride (**A**) (2 mmol) in dioxane (4 mL), and the resulting solution was stirred at room temperature for 45 min. The solvent was removed under vacuum. The crude product **B** (2 mmol) was dissolved in ethanol (8 ml) and was treated with NaOH (3 mmol) followed by carbon disulfide (3 mmol 1.5eq) and refluxed for 12h. The reaction mixture was cooled and quenched with 5% HCl to furnish **1a-p** (Series 1) upon crystallization with methanol.

General procedure for synthesis of series 2A compounds



To a solution of methyl aminobenzoate **C** (10 mmol) and Et_3N (4.0 mL, 30 mmol) in CH_2Cl_2 (30 mL) was added thiophosgene (1.2 ml, 15 mmol) at 0 °C. The mixture was stirred for 3 hours at room temperature and water was added dropwise. The mixture was extracted with CH_2Cl_2 (3 x 20 mL), washed with brine and the combined extracts were dried with Na_2SO_4 and filtered. The filtrate was concentrated under vacuum to afford a brown oil which was purified by chromatography on silica gel (hexanes: EtOAc 10:1) to give thiocyanate **D**. To a suspension of **D** (1 mmol) in THF was added Et_3N (3 mmol) and amine or hydrazine (1 mmol). The mixture was refluxed for 12 hours and concentrated in vacuo to afford crude **2k** and **2l**. The crude product was purified by chromatography on silica gel (hexanes: EtOAc) to give pure products **2k**, **2l** (series 2A).

General procedure for synthesis of series 2B compounds.

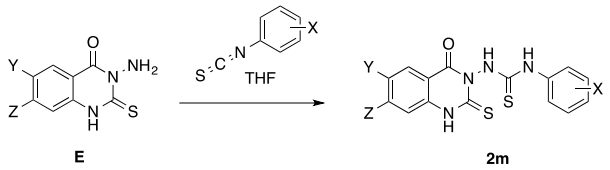


To a solution of methyl aminobenzoate **C** (10 mmol) and Et_3N (4.0 mL, 30 mmol) in CH_2Cl_2 (30 mL) was added thiophosgene (1.2 ml, 15 mmol) at 0 °C. The mixture was stirred for 3 hours at room temperature and water was added dropwise. The mixture was extracted with CH_2Cl_2 (3 x 20 mL), washed with brine and the combined extracts were dried with Na_2SO_4 and filtered. The filtrate was concentrated under vacuum to afford a brown oil which was purified by chromatography on silica gel (hexanes: EtOAc 10:1) to give thiocyanate **D**.

To a solution of thiocyanate **D** (1.0 mmol) in THF was added Et_3N (0.28 mL, 2.0 mmol) and hydrazine (32 μL, 1.0 mmol) at room temperature. The mixture was stirred for 2 hours and the resulting white solid was filtered out to provide **E**.

To a suspension of **E** (1 mmol) in THF was added carbonyl chloride (1 mmol). The mixture was stirred for 12 hours and concentrated in vacuo to afford crude products. The crude product was purified by chromatography on silica gel (hexanes: EtOAc) to give pure **2a-i** (series 2B).

Synthesis of thiourea derivative 2m



E 2m

To a suspension of **E** (1 mmol) in THF was added thiocyanate (1 mmol). The mixture was refluxed for 12 hours and concentrated in vacuo to afford crude **2m**. The crude product was purified by chromatography on silica gel (hexanes: EtOAc) to give pure **2m** as a white solid.

CHARACTERIZATION OF COMPOUNDS

1a: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.98 (m, 2H), 2.61 (s, 6H), 3.11 (dd, *J* = 9.2 Hz, 1.8 Hz 2H), 4.43 (t, *J* = 7.2 Hz, 2H), 7.31 (t, *J* = 7.2 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.69 (dt, *J* = 7.8, 1.6 Hz, 1H), 7.91 (dd, *J* = 7.8, 1.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ22.5, 42.1, 43.4, 55.3, 110.5, 113.8, 117.2, 118.6, 125.8, 128.3, 136.2, 158.1, 175.9; LCMS: m/z (M+H) 264; found 264. Anal Calcd for $C_{13}H_{17}N_3OS$; C, 59.29; H, 6.51; N, 15.96; O, 6.08; S, 12.18; found C, 59.24; H, 6.41; N, 15.94; S, 11.97. Physical form: white powder.

1b: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.95 (m, 2H), 2.58 (s, 6H), 3.16 (dd, *J* = 8.9 Hz, 2.0 Hz 2H), 4.54 (t, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 7.0 Hz, 1H), 7.42 (d, *J* = 8.2 Hz, 1H), 7.72 (dt, *J* = 8.2, 1.6 Hz, 1H), 7.88 (dd, *J* = 7.8, 1.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ22.6, 41.9, 43.1, 55.4, 109.1, 113.1, 116.9, 118.3, 125.6, 127.3, 133.2, 149.8, 158.5; HRMS calcd for $C_{13}H_{18}N_3O_2$ *m/z* +1 248.1399, found 248.1385. Physical form: white powder.

1c: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.08 (m, 2H), 2.69 (s, 6H), 3.09 (dd, *J* = 9.6, 5.6 Hz, 2H), 4.42 (t, *J* = 6.9 Hz, 2H), 7.40 (d, *J* = 8.8 Hz, 1H), 7.78 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.85 (d, *J* = 2.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ22.4, 42.4, 43.6, 54.6, 110.0, 117.5, 118.4, 125.6, 128.7, 135.8, 138.4, 159.2, 175.4; LCMS: m/z (M+H) 298; found 298. Anal Calcd for $C_{13}H_{17}Cl_2N_3OS$; C, 46.71; H, 5.13; N, 12.57; S, 9.59, found C, 45.19; H, 5.14; N, 12.19; S, 10.94. Physical form: pale yellow powder.

1d: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.78 (m, 2H), 2.14 (s, 6H), 2.33 (m, 2H), 4.34 (dd, *J* = 9, 5.2 Hz, 2H), 7.31 (dd, *J* = 8.4, 2 Hz, 1H), 7.34 (d, *J* = 2 Hz, 1H), 7.90 (d, *J* = 8.4 Hz, 1H); LCMS: m/z (M+H) 298; found 298. Physical form: white powder.

1e: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.01 (m, 2H), 2.69 (s, 3H), 2.71 (s, 3H), 3.11 (m, 2H), 4.28 (t, *J* = 6.8 Hz, 2H), 7.44 (dd, *J* = 9.8, 4.4 Hz, 1H), 7.66 (m, 2H), 10.0 (bs, 1H), 13.0 (bs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ22.3, 42.8, 44.0, 57.0, 112.9, 113.5, 118.1, 119.2, 125.8 (d, J=16.1 Hz), 136.2, 157.2, 159.8, 175.9; LCMS: m/z (M+H) 282; found 282. Anal Calcd for $C_{13}H_{17}ClFN_3OS$; C, 49.13; H, 5.39; N, 13.22; S,10.09, found C, 48.86; H, 5.44; N, 13.19; S, 9.88. Physical form: white powder.

1e-L: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.28 (m, 4H), 1.35 (m, 2H), 1.62 (m, 2H), 2.09 (s, 6H), 2.18 (t, *J* = 6.8 Hz, 2H), 4.33 (t, *J* = 6.8 Hz, 2H), 7.38 (m, 1H), 7.59 (m, 2H), 13.0 (bs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.5, 26.6, 26.8, 27.1, 45.4, 46.2, 59.3, 112.4 (d, *J* = 24 Hz), 117.2, 119.1, 123.9 (d, *J* = 15 Hz), 136.9, 157.4, 159.1, 159.8, 174.9; HRMS calcd for $C_{16}H_{23}FN_3OS$ *m/z* +1 324.1540, found 324.1455. Physical form: white powder.

1e-OH: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.30 (m, 2H), 1.40 (m, 2H), 1.50 (m, 2H), 3.21 (m, 2H), 3.35 (t, *J* = 7.8 Hz, 2H), 7.23 (t, *J* = 7.2 Hz, 1H), 7.32 (d, *J* = 7.6 Hz, 1H), 7.46 (t, *J* = 6.8 Hz, 2H), 7.76 (d, *J* = 6.8 Hz, 1H), 8.82 (bs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 23.5, 29.2, 32.6, 61.0, 123.0, 124.8, 129.0, 132.3, 167.1; HRMS calcd for $C_{13}H_{18}N_2O_2S$ *m/z* +1 265.3505, found 265.3512. Physical form: white powder.

1f: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.02 (m, 2H), 3.11 (dd, *J* = 9.2, 6.1 Hz, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 4.50 (t, *J* = 6.4 Hz, 2H), 6.96 (s, 1H), 7.30 (s, 1H), 13.07 (s, 1H); HRMS calcd for $C_{15}H_{22}N_3O_3S$ *m/z*+1 324.1382, found 324.1375. Physical form: white powder.

1g: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.0 (m, 2H), 2.7 (s, 6H), 3.10 (t, *J* = 8 Hz, 2H), 4.4 (t, *J* = 6.8 Hz, 2H), 7.33 (dd, *J* = 11, 6.8 Hz, 1H), 7.73 (dd, *J* = 10, 8.4 Hz, 1H), 9.9 (bs, 1H), 13.0 (bs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ22.2, 42.9, 44.3, 57.9, 113.9, 114.7, 119.1 (dd, *J*=16.2, 24.6 Hz), 125.6 (dd, *J*=16.3, 15.1 Hz), 136.4, 139.8, 157.2, 159.8, 175.9; LCMS: m/z (M+H) 300; found 300. Anal Calcd for $C_{13}H_{16}ClF_2N_3OS$; C, 46.50; H, 4.80; N, 12.51; S, 9.55, found C, 46.29; H, 4.85; N, 12.26; S, 9.27. Physical form: white powder.

1h: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.83 (m, 2H), 2.02 (s, 6H), 2.19 (t, *J* = 7.2 Hz, 2H), 3.99 (m, 2H), 7.06 (d, *J* = 2.4 Hz, 1H), 7.95 (d, *J* = 2.4 Hz, 1H); HRMS calcd for $C_{13}H_{26}N_3OS$ *m/z* +1 332.0386, found 332.0365. Physical form: white powder.

1i: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.62 (m, 2H), 1.78 (m, 2H), 2.22 (s, 6H), 2.33 (t, *J* = 7.6 Hz, 2H), 4.51 (t, *J* = 7.6 Hz, 2H), 7.13 (d, *J* = 8 Hz, 1H), 7.28 (t, *J* = 7.6 Hz, 1H), 7.62 (t x d, *J* = 7.6, 1.2 Hz, 1H), 8.08 (dd, *J* = 8, 1.6 Hz, 1H); LCMS: m/z (M+H) 278; found 278. Physical form: pale yellow powder.

1j: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.38 (m, 2H), 1.48 (m, 2H), 2.01 (s, 6H), 2.10 (m, 2H), 4.64 (t, *J* = 7.6 Hz, 2H), 6.51 (t, *J* = 8 Hz, 1H), 6.70 (t, *J* = 1.2 Hz, 1H), 6.95 (t, *J* = 1.2 Hz, 1H), 8.0 (d, *J* = 8 Hz, 1H); LCMS: m/z (M+H) 292; found 292. Physical form: pale yellow powder.

1k: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (t, *J* = 7.6 Hz, 6H), 3.33 (q, *J* = 7.2 Hz, 4H), 4.45 (q, *J* = 7.2 Hz, 2H), 4.89 (m, 2H), 7.15 (d, *J* = 8.4 Hz, 1H), 7.32 (t, *J* = 1.8 Hz, 1H), 7.68 (t, *J* = 1.6 Hz, 1H), 7.68 (d, *J* = 1.6 Hz, 1H), 8.09 (d, *J* = 1.2 Hz, 1H); LCMS: m/z (M+H) 278; found 278. Physical form: pale yellow powder.

1l: ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.93 (m, 6H), 2.46 (m, 4H), 2.54 (m, 2H), 3.90 (t, *J* = 7.2 Hz, 2H), 7.15 (d, *J* = 8.8 Hz, 1H), 7.66 (dd, *J* = 8.6, 2.8 Hz, 1H), 7.82 (d, *J* = 2.4 Hz, 1H); LCMS: m/z (M+H) 312; found 312. Physical form: white powder.

1m: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.18 (s, 6H), 2.52 (t, *J* = 7.2 Hz, 2H), 4.47 (t, *J* = 7.2 Hz, 2H), 7.29 (t, *J* = 7.6 Hz, 1H), 7.34 (d, *J* = 8 Hz, 1H), 7.70 (td, *J* = 7.2, 1.6 Hz, 1H), 7.91 (dd, *J* = 8, 1.2 Hz, 1H), 12.99 (bs, <1H); LCMS: m/z (M+H) 250; found 250. Physical form: white powder.

1n: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (m, 2H), 1.49 (m, 4H), 2.46 (bs, 4H), 2.77 (t, *J* = 7.6 Hz, 2H), 4.76 (t, *J* = 7.2 Hz, 2H), 6.78 (d, *J* = 8.8 Hz, 1H), 7.07 (dd, *J* = 8.6, 2.8 Hz, 1H), 8.08 (d, *J* = 2.4 Hz, 1H); LCMS: m/z (M+H) 324; found 324. Physical form: white powder.

1o: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.83 (m, 2H), 2.34 (t, *J* = 6 Hz, 2H), 2.46 (m, 4H), 3.43 (m, 4H), 4.43 (t, *J* = 7.6 Hz, 2H), 4.43 (t, *J* = 7.6 Hz, 2H), 7.30 (t, *J* = 7.2 Hz, 1H), 7.35 (d, *J* = 8.4 Hz, 1H), 7.70 (t x d, *J* = 8, 1.2 Hz, 1H), 7.92 (d, *J* = 8 Hz, 1H), 12.95 (bs, <1H); LCMS: m/z (M+H) 306; found 306. Anal Calcd for $C_{15}H_{19}N_3O_2S$; C, 58.99; H, 6.27; N, 13.76; S, 10.50, found C, 58.86; H, 6.24; N, 13.70; S, 10.58. Physical form: white powder.

1p: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.9 (s, 3H), 2.0 (m, 2H), 2.18 (bs, 4H), 2.34 (m, 4H), 4.62 (m, 4H), 6.85 (t, *J* = 7.2 Hz, 1H), 7.05 (d, *J* = 8 Hz, 1H), 7.22 (d, *J* = 8 Hz, 1H), 8.04 (dd, *J* = 8, 1.2 Hz, 1H); LCMS: m/z (M+H) 319; found 319. Physical form: white powder.

2a-E: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.11 (t, *J*= 7.2 Hz, 3H), 2.14 (q, *J*= 7.2 Hz, 2H), 7.41 (m, 1H), 7.68 (m, 2H), 10.52 (s, 1H), 13.09 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 11.2, 36.1, 113.0 (d, *J*=16.0 Hz), 117.6, 119.3, 125.2 (d, *J*=17.7 Hz), 136.4, 157.3, 158.0, 159.6, 175.6 (d, *J*= 48 Hz); HRMS calcd for $C_{11}H_{11}FN_3O_2S$ *m/z* +1 268.0478, found 268.0466. Physical form: white powder.

2a: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.21 (s, 9H), 7.41 (m, 1H), 7.68 (m, 2H), 10.52 (s, 1H), 13.09 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.4, 38.1, 113.0 (d, *J*=16.0 Hz), 117.1, 119.1, 124.7 (d, *J*=17.7 Hz), 136.4, 157.3, 158.0, 159.6, 175.6 (d, *J*= 48 Hz); HRMS calcd for $C_{13}H_{15}FN_3O_2S$ *m/z* +1 296.0791, found 296.0866. Physical form: white powder.

2b: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (m, 2H), 7.61 (m, 1H), 7.73 (m, 3H), 7.81 (s, 1H), 11.66 (s, 1H), 13.34 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 113.2 (d, *J*=24.5 Hz), 114.9 (d, *J*=22.9 Hz), 117.0, 119.3 (d, *J*=7.6 Hz), 119.8 (d, *J*=20.6 Hz), 124.5, 124.9 (d, *J*=24.4 Hz), 131.5, 134.3, 136.5, 157.5 (d, *J*=24.4 Hz), 160.1, 161.1, 163.7, 175.5; HRMS calcd for $C_{15}H_9F_2N_3O_2SNa$ *m/z* +Na 356.0281, found 356.0280; Elementary analysis calcd C 54.05, H 2.72, N 12.61, found C 54.23, H 2.85, N 12.51. Physical form: white powder.

2c: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (m, 1H), 7.73 (m, 2H), 8.18 (m, 2H), 8.40 (m, 2H) 11.94 (s, 1H), 13.37 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ113.2 (d, J=23.6 Hz), 117.1, 119.3, 124.2, 124.9 (d, J=14.6 Hz), 129.5 (d, *J*=27.4 Hz), 136.5, 137.7, 150.1, 157.5 (d, *J*=38.1 Hz), 160.2, 163.5, 175.3; HRMS calcd for $C_{15}H_9FN_4O_4SNa$ *m/z* +Na 383.0226, found 383.0228; Elementary analysis calcd C 50.00, H 2.52, N 15.55, found C 50.17, H 2.67, N 15.47. Physical form: white powder.

2d: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.46 (m, 2H), 7.54 (t, *J* = 6 Hz, 1H), 7.62 (t, *J* = 6 Hz, 1H), 7.71 (m, 2H), 7.95 (d, *J* = 6 Hz, 2H), 11.52 (s, 1H), 13.31 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 113.1 (d, *J*=16.8 Hz), 117.1, 119.2, 124.8 (d, J=16.1 Hz), 128.2, 129.1, 132.2, 132.8, 136.5, 157.4, 158.1, 159.7, 164.8, 175. HRMS calcd for $C_{15}H_{11}FN_3O_2S$ *m/z* +1 316.0551, found 316.0554. Physical form: white powder.

2e: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.44 (m, 1H), 7.73 (m, 2H), 7.95 (m, 2H), 8.14 (m, 2H) 11.82 (s, 1H), 13.35 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 110.0, 113.2 (d, *J*=16.1 Hz), 117.1, 119.3, 125.0 (d, *J*=16.1 Hz), 126.2, 129.1, 135.9, 136.5, 157.4, 158.1, 163.9, 175.4; HRMS calcd for $C_{16}H_{10}F_4N_3O_2S$ *m/z* +1 384.0430, found 384.0428. Physical form: white powder.

2f: ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.82 (s, 3H), 3.86 (s, 3H), 6.96 (s, 1H), 7.30 (s, 1H), 7.49 (m, 1H), 7.61 (m, 1H), 7.71 (m, 1H), 7.81 (m, 1H), 11.54 (s, 1H), 13.07 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.3, 56.5, 98.6, 107.4, 108.3, 114.9 (d, J=15.3 Hz), 119.7, 124.4, 131.5, 134.6, 135.6, 147.3, 156.1, 157.6, 161.6, 163.8 (d, *J*=40.0 Hz), 175.1; HRMS calcd for $C_{17}H_{15}FN_3O_4S$ *m/z* +1 376.0762, found 376.0763. Physical form: white powder.

2g: ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.82 (s, 3H), 3.86 (s, 3H), 6.97 (s, 1H), 7.30 (s, 1H), 7.94 (d, *J*=1.5 Hz, 2H), 8.13 (d, *J*=1.5 Hz, 2H), 11.70 (s, 1H), 13.02 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.3, 56.5, 98.6, 107.4, 108.3, 126.2, 129.1, 135.6, 136.2, 147.3, 156.1, 157.6, 163.9, 175.0; HRMS calcd for $C_{18}H_{15}F_3N_3O_4S$ *m/z* +1 426.0730, found 426.0741. Physical form: white powder.

2h: ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29 (m, 2H), 4.38 (m, 2H), 6.89 (s, 1H), 7.36 (s, 1H), 7.94 (d, *J*=1.5 Hz, 1H), 8.12 (d, *J*=1.5Hz, 1H), 11.68 (s, 1H), 13.01 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 64.3, 65.4, 103.7, 109.6, 114.5, 126.2, 129.1, 130.6, 132.6 (d,

J=21.3 Hz), 134.9, 136.1, 142.1, 151.0, 157.3, 163.9, 175.1; HRMS calcd for $C_{18}H_{13}F_3N_3O_4S$ *m/z*+1 424.0501, found 424.0599. Physical form: white powder.

2i: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.75 (bs, 6H), 2.92 (bs, 2H), 3.23 (bs, 2H), 4.31 (s, 2H), 7.52 (m, 1H), 7.72 (m, 2H), 7.80 (m, 2H), 8.00 (m, 2H), 11.65 (s, 1H), 13.38 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.8, 22.6, 52.1, 58.7, 113.1 (d, *J*=16.0 Hz), 117.1, 119.3, 124.8, 128.5, 132.2, 132.8, 136.5, 157.4, 158.1, 159.7, 164.3, 175.5; HRMS calcd for $C_{21}H_{22}FN_4O_2S$ *m/z*+1 413.1442, found 413.1436. Physical form: white powder.

2j: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.37 (s, 3H), 7.33 (m, 2H), 7.41 (m, 1H), 7.61 (m, 2H), 7.70 (m, 2H), 10.82 (s, 1H), 13.02 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.5, 113.2 (d, *J*=17.3 Hz), 117.0, 119.0, 124.8 (d, J= 16.0 Hz), 127.8, 129.6, 129.8, 136.3, 138.9, 143.6, 158.1 (d, J=22.1 Hz), 175.9; HRMS calcd for $C_{15}H_{11}FN_3O_3S_2$ *m/z* 364.0231, found 364.0224. Physical form: white powder.

2k: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (s, 1H), 7.70 (m, 2H), 8.22 (s, 1H), 8.34 (s, 1H), 10.27 (s, 1H), 13.30 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 113.1 (d, *J*=24.5 Hz), 114.4, 117.1, 117.8 (dd, *J*=32.8, 32.2 Hz), 119.2, 122.6, 124.9 (ddd, *J*=23.4, 22.1, 24.5 Hz), 135.1, 136.5, 144.1, 154.5, 157.8 (d, J=19.0 Hz), 160.1, 175.9; HRMS calcd for $C_{14}H_7ClF_4N_4OS$ *m/z* +1 391.0074, found 391.0074; Elementary analysis calcd C 43.03, H 1.81, N 14.34, F 19.45, found C 43.16, H 1.89, N 14.33, F 19.81. Physical form: white powder.

2l: ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.68 (m, 2H), 6.95 (m, 2H), 7.45 (m, 1H), 7.65 (m, 2H), 8.71 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ112.9 (d, *J*=16.0 Hz), 114.6 (d, *J*=5.4 Hz), 115.6 (d, *J*=13.5 Hz), 117.6, 119.0, 124.3 (d, J=16.8 Hz), 136.6, 143.1, 156.1, 157.7, 157.9, 158.6, 159.6, 176.0; HRMS calcd for $C_{14}H_{10}F_2N_3OS$ *m/z*+1 306.0434, found 306.0508. Physical form: white powder.

2m: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (m, 2H), 7.61 (m, 1H), 7.73 (m, 3H), 7.81 (s, 1H), 11.66 (s, 1H), 13.34 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 104.8 (d, *J*=17.6 Hz), 108.2 (d, *J*=12.5 Hz), 114.1, 117.2 (dd, *J*=13.1, 16.0 Hz), 123.7 (d, J=16.0 Hz), 125.9, 130.6 (d, *J*=7.0 Hz), 131.4, 134.3, 135.6, 142.2, 148.1, 163.4 (d, *J*=16.8 Hz), 165.7, 169.4; HRMS calcd for $C_{15}H_{10}F_2N_4OS_2$ *m/z* +1 365.0264, found 365.0280. Physical form: pale yellow powder.

3a: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.34 (s, 3H), 2.37 (s, 3H), 3.82 (s, 3H), 3.86 (s, 3H), 6.96 (s, 1H), 7.30 (s, 1H), 13.07 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 46.1 54.2 108.2, 121.7, 133.1 136.9, 154.1, 161.8, 180.3; HRMS calcd for $C_{12}H_{16}N_3O_3S$ *m/z*+1 282.0834, found 282.0822. Physical form: white powder.

3b: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.19 (t, *J* = 6.8 Hz, 3 H), 4.40 (q, *J* = 6.8 Hz, 2 H), 7.40 (s, 1H), 7.63 (dd, *J* = 8.4 Hz, 2H), 13.07 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.4, 43.1, 113.06(d, *J*=16.0 Hz), 117.5, 120.1, 125.1 (d, *J*=17.7 Hz), 136.4, 157.3, 158.0, 159.6, 175.6 (d, *J*= 48 Hz); HRMS calcd for $C_{10}H_{10}FN_2OS$ *m/z*+1 225.0498, found 225.0457. Physical form: white powder.

3c: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.57 (d, *J* = 6.8 Hz, 6 H), 4.23 (m, 2H), 4.26 (m, 2H), 6.02 (m, 1 H), 6.59 (s, 1H), 7.24 (s, 1H), 10.30 (bs, 1H); HRMS calcd for $C_{13}H_{15}N_2O_3S$ *m/z*+1 279.0803, found 279.0807. Physical form: white powder.

3d: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 4.26 (m, 2H), 4.33 (m, 2H), 6.26 (s, 2H), 6.82 (s, 1H) 7.33 (s, 1H), 13.01 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ64.2, 64.4, 103.7, 109.6, 114.5, 122.2, 126.1, 142.1, 161.3, 175.1; HRMS calcd for $C_{10}H_9N_3O_3S$ *m/z* +1 251.0365, found 251.0678. Physical form: white powder.

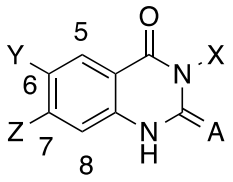
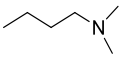
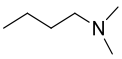
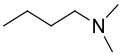
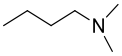
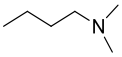
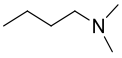
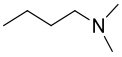
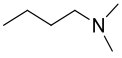
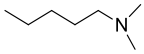
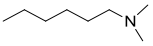
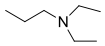
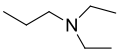
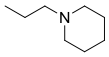
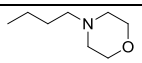
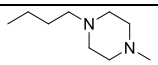
3e: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 6H), 6.30 (s, 2H), 6.88 (s, 1H) 7.32 (s, 1H), 13.01 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.3, 56.5, 104.0, 110.1, 114.6, 122.7, 126.1, 142.2, 161.4, 177.1; HRMS calcd for $C_{10}H_{12}N_3O_3S$ *m/z*+1 254.0599, found 254.0557. Physical form: white powder.

3f: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.32 (s, 2H), 7.44 (m, 1H) 7.67 (m, 2H), 13.21 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 113.0 (d, *J*=16.0 Hz), 117.1, 119.1, 124.7 (d, *J*=17.7 Hz), 136.4, 157.3, 158.0, 175.6 (d, *J*= 48 Hz); HRMS calcd for $C_8H_7FN_3OS$ *m/z*+1 212.0294, found 212.0288. Physical form: white powder.

3g: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.81 (s, 6H), 6.86 (s, 1H) 7.25 (s, 1H), 12.91 (s, 1H); HRMS calcd for $C_{10}H_{11}N_2O_4S$ *m/z*+1 255.0440, found 255.0445. Physical form: white powder.

3h: ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.11 (s, 2H), 7.64 (m, 1H) 7.77 (m, 1H), 13.11 (s, 1H); HRMS calcd for $C_8H_6F_2N_3OS$ *m/z*+1 230.0200, found 230.0228. Physical form: white powder.

Table 2. Initial synthesized compounds. ROS-generation was measured in a neutrophil membrane cell-free system using a NADPH-dependent L-012 chemiluminescence (see METHODS). IC_{50} values and maximum % inhibition were calculated as described in METHODS. Values represent the average +/- S.E.M. of 2 to 6 determinations. N.I., no inhibition.

Entry	 X	Y/Z	A	Cell-Free L-012 Oxidation	
				IC_{50} (μM)	Maximum Inhibition (%)
1a		H	S	1.9 ± 0.4	80%
1b		H	O	N.I.	N.I.
1c		6-Cl	S	0.8 ± 0.1	85%
1d		7-Cl	S	2.5 ± 0.5	63%
1e		6-F	S	0.8 ± 0.04	60%
1f		6,7-OMe	S	1.5 ± 0.5	60%
1g		6,7-F	S	0.6 ± 0.2	60%
1h		6,8-Cl	S	N.I.	N.I.
1i		H	S	3.0 ± 1	95%
1j		H	S	4.0 ± 1	65%
1k		H	S	3.0 ± 1	95%
1l		6-Cl	S	2.5 ± 0.5	83%
1m		H	S	3.0 ± 1	63%
1n		H	S	1.0 ± 0	88%
1o		H	S	1.5 ± 0.5	75%
1p		H	S	2.5 ± 0.5	65%
3a	NMe_2	6,7-OMe	S	2.7 ± 0.2	80%
3b	CH_2CH_3	6-F	S	0.7 ± 0.1	60%
3c	$CH(CH_3)_2$	6,7-OCH_2-OCH_2	S	0.7 ± 0.1	60%
3d	NH_2	6,7-OCH_2-OCH_2	S	1.3 ± 0.7	98%
3e	NH_2	6,7-OMe	S	0.2 ± 0.04	63%
3f	NH_2	6-F	S	0.4 ± 0.2	95%
3g	OH	6,7-OMe	S	2.0 ± 0	85%

3h	NH_2	6,7-F	S	0.7 ± 0.1	83%

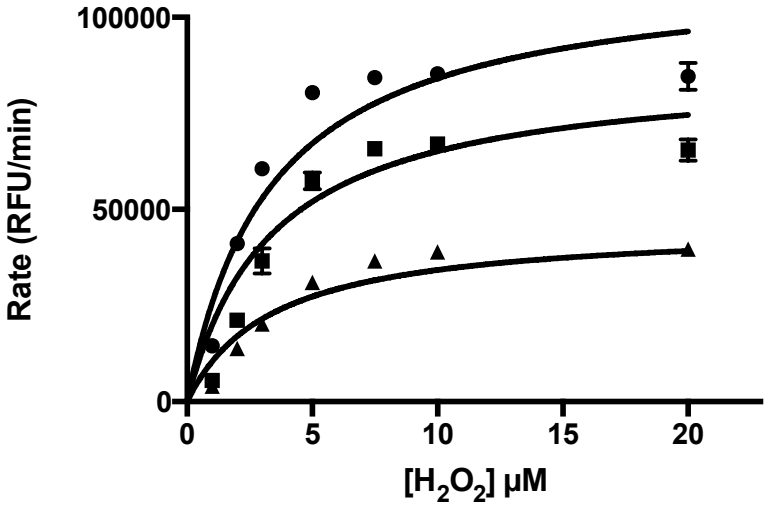


Figure 1 S.I. Inhibition of MPO by compound 1c is non-competitive with respect to H_2O_2. The rate of Amplex Red oxidation was measured at zero (filled circles), 1 µM (filled squares) or 5 µM compound 1c. Solid lines show a fit of the data to non-competitive kinetic inhibition model, which showed a Km for H_2O_2 of 3.4 µM and a Ki for compound 1c of 3.4 µM. Each point it the mean +/- S.E.M. of 3 determinations, and the experiment was repeated twice. Where error bars are not evident, the error was smaller than the size of the data point. Compound 2b also showed non-competitive inhibition with respect to H_2O_2 (data not shown).

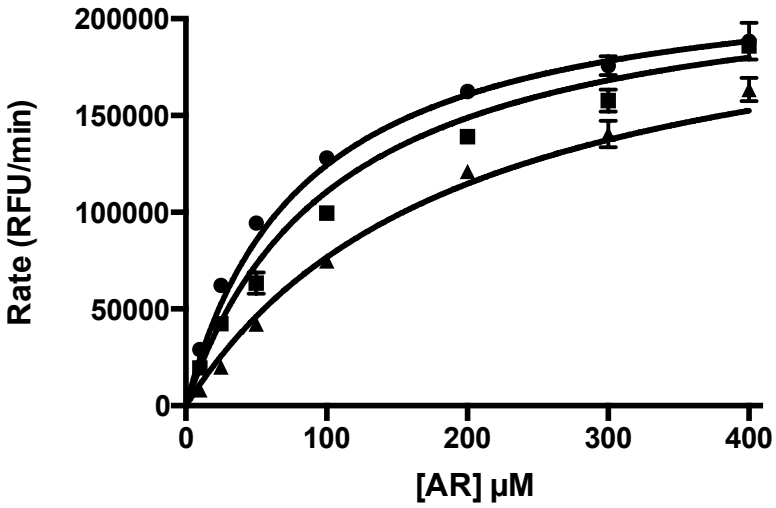


Figure 2 S.I. Inhibition of MPO by compound 2b is competitive with respect to Amplex Red. The rate of Amplex Red (AR) oxidation was measured at zero (filled circles), 0.1 µM (filled squares) and 0.5 µM inhibitor 2b. Solid lines show a fit of the data to kinetic inhibition model in which the inhibitor acts competitively with respect to Amplex Red. Data fit a model in which the K_m for Amplex Red is 83 µM and the Ki for inhibitor 2b is 0.05 µM. Each point it the mean +/- S.E.M. of 3 determinations, and the experiment was repeated twice. Where error bars are not

evident, the error was smaller than the size of the data point. The same experiment was also carried out for inhibitor 1c, and also showed that the inhibitor is competitive with respect to Amplex Red.

Bibliography

1. Bromberg, Y.; Pick, E., Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulfate. *J Biol Chem* **1985,** *260* (25), 13539-45.
2. Smith, S. M.; Min, J.; Ganesh, T.; Diebold, B.; Kawahara, T.; Zhu, Y.; McCoy, J.; Sun, A.; Snyder, J. P.; Fu, H.; Du, Y.; Lewis, I.; Lambeth, J. D., Ebselen and congeners inhibit NADPH oxidase 2-dependent superoxide generation by interrupting the binding of regulatory subunits. *Chem Biol* **2012,** *19* (6), 752-63.
3. Zielonka, J.; Lambeth, J. D.; Kalyanaraman, B., On the use of L-012, a luminol-based chemiluminescent probe, for detecting superoxide and identifying inhibitors of NADPH oxidase: a reevaluation. *Free Radic Biol Med* **2013,** *65*, 1310-4.
4. Curnutte, J. T.; Kuver, R.; Babior, B. M., Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J Biol Chem* **1987,** *262* (14), 6450-2.
5. Ebisu, K.; Nagasawa, T.; Watanabe, K.; Kakinuma, K.; Miyano, K.; Tamura, M., Fused p47phox and p67phox truncations efficiently reconstitute NADPH oxidase with higher activity and stability than the individual components. *J Biol Chem* **2001,** *276* (27), 24498-505.
6. Daiber, A.; Oelze, M.; August, M.; Wendt, M.; Sydow, K.; Wieboldt, H.; Kleschyov, A. L.; Munzel, T., Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. *Free Radic Res* **2004,** *38* (3), 259-69.
7. Dypbukt, J. M.; Bishop, C.; Brooks, W. M.; Thong, B.; Eriksson, H.; Kettle, A. J., A sensitive and selective assay for chloramine production by myeloperoxidase. *Free Radic Biol Med* **2005,** *39* (11), 1468-77.
8. Kettle, A. J.; Anderson, R. F.; Hampton, M. B.; Winterbourn, C. C., Reactions of superoxide with myeloperoxidase. *Biochemistry* **2007,** *46* (16), 4888-97.
9. Hoogland, H.; van Kuilenburg, A.; van Riel, C.; Muijsers, A. O.; Wever, R., Spectral properties of myeloperoxidase compounds II and III. *Biochim Biophys Acta* **1987,** *916* (1), 76-82.
10. Kettle, A. J.; Gedye, C. A.; Winterbourn, C. C., Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. *Biochem J* **1997,** *321 (Pt 2)*, 503-8.
11. Marquez, L. A.; Dunford, H. B., Mechanism of the oxidation of 3,5,3',5'-tetramethylbenzidine by myeloperoxidase determined by transient- and steady-state kinetics. *Biochemistry* **1997,** *36* (31), 9349-55.
12. Fair, J. D.; Kormos, C. M., Flash column chromatograms estimated from thin-layer chromatography data. *J Chromatogr A* **2008,** *1211* (1-2), 49-54.