Biochemical Pharmacology 79 (2010) 1156–1164

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# Acetaminophen (paracetamol) inhibits myeloperoxidase-catalyzed oxidant production and biological damage at therapeutically achievable concentrations

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ARTICLE INFO

*Article history:*
Received 2 October 2009
Accepted 30 November 2009

*Keywords:*
Paracetamol
Acetaminophen
Myeloperoxidase
Oxidation
Neutrophil

ABSTRACT

The heme peroxidase enzyme myeloperoxidase (MPO) is released by activated neutrophils and monocytes, where it uses hydrogen peroxide ($H_2O_2$) to catalyze the production of the potent oxidants hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) from halide and pseudohalide ($SCN^-$) ions. These oxidants have been implicated as key mediators of tissue damage in many human inflammatory diseases including atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis and some cancers. It is shown here that acetaminophen (paracetamol), a phenol-based drug with analgesic and antipyretic actions, is an efficient inhibitor of HOCl and HOBr generation by isolated MPO–$H_2O_2$–halide systems. With physiological halide concentrations, acetaminophen concentrations required for 50% inhibition of oxidant formation ($IC_{50}$) were $77 \pm 6$ μM (100 mM $Cl^-$) and $92 \pm 2$ μM (100 mM $Cl^-$ plus 100 μM $Br^-$), as measured by trapping of oxidants with taurine. The $IC_{50}$ for inhibition of HOCl generation by human neutrophils was ca. 100 μM. These values are lower than the maximal therapeutic plasma concentrations of acetaminophen (≤150 μM) resulting from typical dosing regimes. Acetaminophen did not diminish superoxide generation by neutrophils, as measured by lucigenin-dependent chemiluminescence. Inhibition of HOCl production was associated with the generation of fluorescent acetaminophen oxidation products, consistent with acetaminophen acting as a competitive substrate of MPO. Inhibition by acetaminophen was maintained in the presence of heparan sulfate and extracellular matrix, materials implicated in the sequestration of MPO at sites of inflammation *in vivo*. Overall, these data indicate that acetaminophen may be an important modulator of MPO activity *in vivo*.


## 1. Introduction

Stimulated neutrophils, monocytes and some tissue macrophages release the heme peroxidase enzyme myeloperoxidase (MPO) extracellularly and in phagosomes, where it uses hydrogen peroxide ($H_2O_2$) as a co-substrate to catalyze the production of the potent oxidants hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) from halide ions ($Cl^-$/$Br^-$) and the pseudohalide ion thiocyanate ($SCN^-$), respectively (reviewed [1,2]). The $H_2O_2$ required for these reactions is generated by these cells, primarily via plasma membrane NADPH oxidase (NOX) complexes [3]. Vascular cells can also act as a source of $H_2O_2$ [4]. HOCl, HOBr and HOSCN play a key role in the immune response, and are believed to be responsible, in part, for the killing of invading pathogens (bacteria and yeast in the case of MPO, parasites and viruses in the case of the related heme protein eosinophil peroxidase) (reviewed [1,5]). Despite these positive effects, inappropriate generation of these oxidants may result in

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doi:10.1016/j.bcp.2009.11.024

host tissue damage, with this implicated in a wide range of human inflammatory diseases including atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis and some cancers (reviewed [1,2,5]. As a result, there is considerable interest in novel therapeutic strategies for these diseases, targeted at preventing biological damage by MPO-derived oxidants [1].

The potential of strategies directed at preventing HOCl- and HOBr-mediated damage by scavenging these oxidants, once formed, appears limited. Kinetic modelling studies have predicted that low molecular mass antioxidants (e.g. thiols, ascorbate, α-tocopherol and urate) do not prevent biological damage by HOCl and HOBr to any significant extent (e.g. in plasma, where proteins are the major initial target for oxidation) [6–8]. Removal of the $H_2O_2$ required for oxidation of $Cl^-$ and $Br^-$, or prevention of its production, is not an attractive therapeutic approach, as multiple processes generate this species [9]. As a consequence, attention has been focused on inhibition of MPO (reviewed [2]). A number of compounds that effectively inhibit MPO *in vitro* (e.g. heme poisons and some suicide substrates [10,11]) are impractical *in vivo* due their lack of specificity and toxicity [12]. An alternative approach has been the use of compounds that reversibly divert the enzyme from its halogenation cycle, which yields HOCl and HOBr (Fig. 1), by acting as peroxidase substrates for the enzyme. A number of such compounds have been identified that act in this manner, including some anti-inflammatory drugs (and related materials) [13], tryptophan and derivatives [14,15] and nitroxide radicals [16].

Acetaminophen (paracetamol) is a well-tolerated phenol-based drug with analgesic and antipyretic actions [17]. These actions have been proposed to reflect inhibition of cyclooxygenase-2 via metabolism of acetaminophen by the peroxidase activity of this enzyme [18,19] and recent studies have shown that acetaminophen is a selective inhibitor of this enzyme in humans [20,21]. Acetaminophen is also a peroxidase substrate for MPO and, like other phenol derivatives [22], undergoes rapid one-electron oxidation by both MPO compound I and MPO compound II [23]. These reactions may underlie an important additional biological action of acetaminophen as a modulator of oxidant production by MPO. The reaction of acetaminophen with MPO compound I can compete with $Cl^-$ oxidation by this redox intermediate [24] and

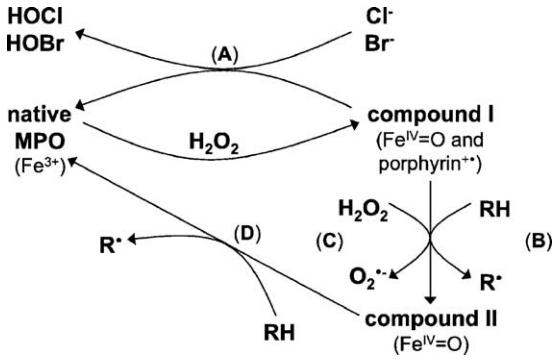


**Fig. 1.** HOCl and HOBr production by MPO and its proposed modulation by acetaminophen. Reaction of $H_2O_2$ with native MPO yields compound I. Two-electron oxidation of $Cl^-$ and $Br^-$ by compound I yields HOCl and HOBr, respectively, and regenerates native MPO (reaction A) (the halogenation cycle). Competing one-electron oxidation of acetaminophen (RH) by compound I, yields compound II and the acetaminophen phenoxyl radical ($R^•$) (reaction B) diverting the catalytic activity of MPO from the halogenation cycle and HOCl and HOBr formation. Reaction of $H_2O_2$ with compound I can also yield compound II (reaction C). Reduction of compound II by acetaminophen (reaction D) regenerates native MPO and can increase enzyme turnover and the initial rate of oxidant production. However, competitive inhibition of $Cl^-$ and $Br^-$ oxidation by MPO compound I (reaction B versus reaction A) limits the total yield of HOCl and HOBr. Biomolecular rate constant data are available for reactions A, C and D, but not reaction B (see [1,23]).

has the potential to compete with $Br^-$ oxidation. The former process has been observed to inhibit HOCl production [25], however inhibition of HOBr production has yet to be demonstrated. In contrast to its reaction with MPO compound I, the reaction of acetaminophen with MPO compound II has been reported to stimulate HOCl production by regenerating the native enzyme [23]; it has been proposed that this stimulation of HOCl production could predominate over inhibition at therapeutically relevant acetaminophen concentrations [23]. Leukocyte HOCl production depends upon cellular production of superoxide to provide the MPO co-substrate $H_2O_2$. With human neutrophils, acetaminophen has been reported to inhibit superoxide production (measured by the reduction of ferricytochrome *c*) [26], a finding contradicted in another study [27], and to inhibit luminol-dependent chemiluminescence [26], a non-specific measure of HOCl production [28]. Thus, despite the potentially important role that acetaminophen may play in modulating MPO activity *in vivo*, the extent to which acetaminophen stimulates or inhibits, production of MPO-derived oxidants at therapeutically relevant concentrations, and its effects on leukocyte superoxide and HOCl production are currently unclear.

The present study was undertaken to address these issues and quantify the ability of acetaminophen to inhibit or stimulate HOCl and HOBr production by isolated MPO, and superoxide and HOCl production by human neutrophils. Given that binding of MPO by macromolecular ligands has the potential to limit the action of inhibitors, studies were also undertaken to determine whether inhibition is efficient in the presence of heparan sulfate and extracellular matrix, materials implicated in sequestering the enzyme at sites of oxidant production *in vivo* [1,29,30].

## 2. Materials and methods

### 2.1. Materials

Phosphate buffered saline ($Ca^{2+}$ and $Mg^{2+}$ free; PBS), heat inactivated fetal calf serum were from Flow Laboratories (Sydney, Australia). Ficoll-Paque and Dextran T-500 were from Pharmacia AB (Uppsala, Sweden). Basement membrane extracellular matrix extract was from R&D Systems (Minneapolis, MN, USA). Heparan sulfate was from Celsus Laboratories (Cincinnati, OH, USA). MPO was from Planta Natural Products (Austria). $H_2O_2$ was from Merck (Darmstadt, Germany). 5,5′-Bis(2-nitro-5-thiobenzoic acid) (DTNB), catalase, dimethyl sulfoxide (DMSO), Krebs/Ringer phosphate buffer, luminol, lucigenin, phorbol myristate acetate (PMA), sodium chloride, sodium bromide, sodium hypochlorite, taurine, zymosan and general reagents were from Sigma–Aldrich (St. Louis, USA). Horseradish peroxidase was from Roche (Basel, Switzerland). Stock solutions of PMA (1 mg $mL^{-1}$) and luminol (0.1 mg $mL^{-1}$) were prepared in DMSO, stored at 4 °C and diluted with PBS before use. Acetaminophen (paracetamol) was from GlaxoSmithKline (Australia). Solutions were prepared using water filtered through a fourstage MilliQ system and chelex-treated buffers (0.05 or 0.1 M phosphate buffer, pH 5.5, 6.5, 7.4 or 8.0; PBS, pH 7.4). Stock solutions of HOCl and HOBr were prepared and diluted into phosphate buffer immediately before use as described previously [31,32].

### 2.2. Incubations with MPO

HOCl/HOBr formation was quantified in reaction mixtures containing MPO (100 nM), $H_2O_2$ (50 μM), $Cl^-$ (100 mM) and/or $Br^-$ (100 μM), taurine (10 mM) and acetaminophen (15 μM–3.84 mM). Reactions were performed at 37 °C and pH 5.5, 6.5, 7.4 or 8.0 and were initiated by addition of $H_2O_2$. After 30 min reaction, the taurine chloramine and/or taurine bromamine

(resulting from the rapid reactions of HOCl and HOBr with the free amine function of taurine: $RNH_2 + HOX \rightarrow RNHX + H_2O$, X = Cl or Br) were assayed with 2-nitro-5-thiobenzoic acid (TNB, ca. 45 μM) [33]; under these conditions, product formation was complete within 30 min.

Formation of heparan sulfate-derived chloramines and bromamines (arising from the rapid reaction of HOCl and HOBr with its free amine functions: $RNH_2 + HOX \rightarrow RNHX + H_2O$, X = Cl or Br [31,32]) was quantified in reaction mixtures equivalent to those described above, but containing heparan sulfate (1.6 mg $mL^{-1}$, 56 μM in amine groups, as determined previously [31]) instead of taurine. Reactions were performed at 37 °C and pH 5.5, 6.5, 7.4 or 8.0 and were initiated by addition of $H_2O_2$; choramine/bromamine formation was measured after 30 min reaction by assay with TNB. Chloramine/chloramide formation was quantified in a similar manner in equivalent experiments at pH 7.4 with basement membrane extracellular matrix (0.5 mg protein $mL^{-1}$), with and without added taurine (10 mM).

The initial rate of HOCl formation was quantified in reaction mixtures containing MPO (1 nM), $H_2O_2$ (50 μM), $Cl^-$ (100 mM), taurine (10 mM) and acetaminophen (10–500 μM). Reactions were performed at 37 °C and pH 7.4 and were initiated by addition of $H_2O_2$ and were stopped after 5 min by addition of catalase (50 μg $mL^{-1}$) before assaying the taurine chloramine with TNB; under these conditions, the rate of product formation remained constant for >5 min. These reactions were performed in 96-well microtitre plate (final reaction volume 200 μL, path length 0.5 cm) maintained at 37 °C in a plate reader (Spectramax 340, Molecular Devices, Sunnyvale CA).

Direct oxidation of TNB was examined in reaction mixtures containing MPO (20 nM), $H_2O_2$ (25 μM), $Cl^-$ (100 mM) and/or acetaminophen (100 μM). Reactions were performed at 37 °C and pH 7.4 and were initiated by addition of $H_2O_2$, with the decrease in absorbance of TNB at 412 nm monitored continuously using a Hitachi spectrophotometer.

### 2.3. Incubations with human neutrophils

Neutrophils were isolated from heparinized blood (10 mL) collected from healthy volunteers, who gave informed consent, as outlined previously [34] and were suspended in PBS ($5 \times 10^6$ cells $mL^{-1}$) and stored on ice for up to 5 h. In all treatments, cells ($2 \times 10^6$ cells $mL^{-1}$) were diluted into Krebs/Ringer phosphate buffer containing $Mg^{2+}$ (1 mM), $Ca^{2+}$ (1 mM) and glucose (1 mg $mL^{-1}$) and were pre-incubated for 10 min at 37 °C with acetaminophen (1–500 μM) and other agents before stimulation of the cells by addition of opsonized zymosan (1 mg $mL^{-1}$) or PMA (100 ng $mL^{-1}$).

To measure HOCl production, neutrophils were stimulated by opsonized zymosan in the presence of taurine (10 mM) and taurine chloramine formation was measured, after stopping the reaction at 30 min by the addition of catalase (50 μg $mL^{-1}$), by assay with TNB.

Luminol-dependent chemiluminescence (a less specific measure of HOCl production [28]) and lucigenin-dependent chemiluminescence (a measure of superoxide production) were measured before and after stimulation of the cells with PMA in the presence of luminol (0.6 μg $mL^{-1}$) or lucigenin (0.3 mg $mL^{-1}$) using a Packard Picolite 6500 luminometer; in some incubations, 50% autologous serum (prepared from blood by allowing it to clot in plastic tubes) was included. Photon counts were taken over 5 s intervals every 2 min, for 5 min before and 30 min after stimulation. The cumulative counts over the latter 30 min period are termed the total chemiluminescence.

To measure formation of fluorescent acetaminophen metabolites, neutrophils were stimulated with opsonized zymosan (0.5 mg $mL^{-1}$) in the presence of acetaminophen (100 μM), with and without taurine (10 mM), and after 30 min the cells were removed by centrifugation and the fluorescence of supernatants measured.

### 2.4. HPLC analysis of acetaminophen oxidation products

A Shimadzu HPLC system equipped with a RF-10A XL Shimadzu fluorescence detector ($\lambda_{ex}$ 366 nm, $\lambda_{em}$ 420 nm) and a SPD-M10A VP Shimadzu diode array detector (set at 254 nm) was used to analyse acetaminophen and its oxidation products. Samples (50 μL) were separated using a Beckman Ultrasphere ODS column (4.6 mm × 250 mm) by gradient elution. Samples were eluted at a flow rate of 1 mL $min^{-1}$ with the following gradient: 100% buffer A for 10 min, a linear gradient to 81% buffer A over the next 9 min which was then held for 11 min, followed by a linear gradient to 100% buffer B over the next 10 min, which was held for 5 min, before returning to 100% buffer A over the next 10 min and re-equilibrating with 100% buffer A for 5 min prior to the next injection. Buffer A was composed of 10% methanol, 2% glacial acetic acid and 0.1% ethyl acetate in water; buffer B was 100% methanol. The column was maintained at 30 °C. Typical elution times were 7.7 min for parent paracetamol and 19.5 min for the dimer oxidation product. The assignment of these peaks was confirmed by both spiking with authentic materials and mass spectroscopic analysis (see below) of both unfractionated reaction mixtures, and collected materials eluted from the HPLC column. Authentic paracetamol dimer was synthesized using a previously reported procedure using horseradish peroxidase [35].

### 2.5. Mass spectroscopic analysis of acetaminophen oxidation products

Samples eluted from the HPLC column were evaporated to dryness using a SpeediVac concentration system, reconstituted in a minimum volume of water, and diluted 1:1 with a solution of methanol and 2% formic acid (FA). Samples were infused into a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer, operating in the positive ion mode, at a rate of 10 μL $min^{-1}$. The electrospray needle was held at 4.5 kV. A nitrogen sheath gas (11 arbitrary units) and a helium collision gas were utilized. The capillary was held at 250 °C and the normalized collision energy was 35%. Full mass spectra and $MS^n$ data were collected.

### 2.6. Statistics

Data are presented as mean ± SEM unless stated otherwise. The effects of acetaminophen were determined by either one-way ANOVA with Newman–Keuls post-testing, or two-way ANOVA with Bonferroni post-hoc tests; $p \leq 0.05$ was taken as significant. The concentrations of drug that induced 50% inhibition ($IC_{50}$ values) were calculated by fitting a rectangular hyperbola to the dose response curve using nonlinear regression.

## 3. Results

### 3.1. Effect of acetaminophen on the production of HOCl and superoxide by neutrophils

The yield of HOCl produced by opsonized zymosan-stimulated neutrophils, measured by trapping the oxidant with taurine (10 mM) and assaying the resulting taurine chloramine with TNB, was decreased in a dose-dependent manner by acetaminophen (Fig. 2; 48 ± 9% inhibition at 100 μM). Luminol-dependent chemiluminescence generated by the cells was also decreased ($IC_{50}$ ca. 10 μM; Fig. 2). In the presence of serum, acetaminophen maintained its ability to inhibit luminol-dependent chemilumines-

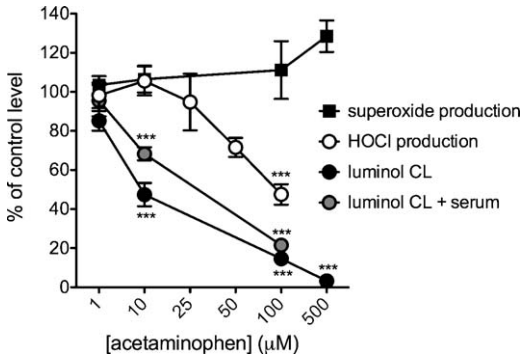


**Fig. 2.** Effects of acetaminophen on HOCl production and superoxide production by human neutrophils. Human neutrophils ($2 \times 10^6$ cells $mL^{-1}$) were stimulated with PMA (100 ng $mL^{-1}$) or opsonized zymosan (1 mg $mL^{-1}$) in the presence of acetaminophen (1–500 μM) and superoxide production was measured by lucigenin-dependent chemiluminescence ('superoxide production'; PMA-stimulated cells), HOCl production measured by taurine chloramine formation using the TNB assay ('HOCl production'; opsonized zymosan-stimulated cells), luminol-dependent chemiluminescence in the absence ('luminol CL'; PMA-stimulated cells) and presence of 50% serum ('luminol CL + serum'; PMA-stimulated cells). Data are mean ± SEM of $n = 4$ experiments and are expressed as % of control level obtained in the absence of acetaminophen. $^{***}p < 0.001$ relative to control level.

cence (Fig. 2). Acetaminophen did not diminish and even slightly increased, at high concentrations, superoxide production by neutrophils (Fig. 2) establishing that the decrease in HOCl production did not involve inhibition of NADPH oxidase, which has the potential to inhibit HOCl production by decreasing the availability of the MPO co-substrate $H_2O_2$.

### 3.2. Effect of acetaminophen on the yields of HOCl and HOBr generated by isolated MPO, and modulation of damage to biological targets

The effect of acetaminophen on the formation of HOCl and HOBr by MPO (100 nM) and $H_2O_2$ (50 μM) in the presence of typical physiological levels of $Cl^-$ (100 mM) and/or $Br^-$ (100 μM) was determined by trapping the oxidants with taurine (10 mM) to form the taurine chloramine and bromamine, respectively, and assaying these species with TNB. To assess the effect of acetaminophen on the total yields of HOCl and HOBr, reactions were allowed to proceed for 30 min, at which point oxidant production was complete (reflecting complete consumption of $H_2O_2$) under all experimental conditions examined. Taurine chloramine and bromamine yields obtained by reaction of taurine (10 mM) with reagent HOCl or HOBr (50 μM) were not significantly decreased by acetaminophen (100 μM; data not shown), establishing that it is a poor scavenger of these oxidants.

In experiments with $Cl^-$ alone, the effect of acetaminophen on the yield of taurine chloramine was examined over the pH range 5.5–8.0. Maximum oxidant yields ($E_{max}$ values) for oxidant production by all MPO–$H_2O_2$–halide systems, summarized in Table 1, show that the taurine chloramine yields detected in the absence of acetaminophen were approximately equal to the concentration of $H_2O_2$ added (50 μM) at all pH values. Inclusion of acetaminophen resulted in a dose-dependent decrease in the chloramine yields (Fig. 3A); from these data, $IC_{50}$ values for acetaminophen inhibition of taurine chloramine generation were calculated (Table 1), with inhibition being greatest at pH 8.0 and decreasing at lower pH values. In control reactions containing high concentrations of acetaminophen (≥480 μM), but no $Cl^-$, low levels of TNB-reactive species were generated (Fig. 4 shows data

**Table 1**
$IC_{50}$ values for inhibition of HOCl and HOBr production, as assayed by chloramine and bromamine formation, respectively, on taurine and heparan sulfate, by isolated MPO in the presence of $H_2O_2$ (50 μM) and $Cl^-$ (100 mM) and/or $Br^-$ (100 μM) over the pH range 5.5–8.0. $E_{max}$ (MPO maximum activity recorded as the concentration of *N*-halo species generated in the absence of acetaminophen) and $IC_{50}$ (acetaminophen concentration leading to 50% inhibition of *N*-halo derivative formation) are means ± SD of $n = 3$ experiments. $R^2$ for plots were ≥0.98.

| | Halide/s | pH | $E_{max}$ (μM) | $IC_{50}$ (μM) |
|---|---|---|---|---|
| Taurine | $Cl^-$ | 8.0 | 47 ± 1 | 52 ± 2 |
| | | 7.4 | 56 ± 1 | 77 ± 6 |
| | | 6.5 | 57 ± 1 | 336 ± 107 |
| | | 5.5 | 51 ± 1 | 695 ± 81 |
| | $Br^-$ | 7.4 | 20 ± 3 | 19 ± 4 |
| | $Cl^- + Br^-$ | 7.4 | 57 ± 7 | 92 ± 2 |
| Heparan sulfate | $Cl^-$ | 8.0 | 40 ± 3 | 49 ± 6 |
| | | 7.4 | 36 ± 1 | 98 ± 2 |
| | | 6.5 | 35 ± 1 | 320 ± 142 |
| | | 5.5 | 35 ± 1 | 509 ± 39 |
| | $Br^-$ | 7.4 | 7 ± 1 | 67 ± 2 |
| | $Cl^- + Br^-$ | 7.4 | 34 ± 2 | 115 ± 7 |

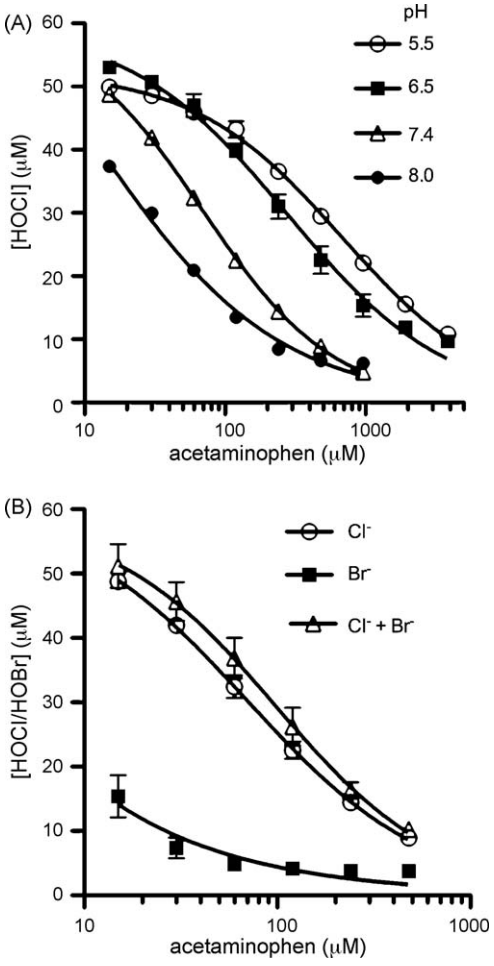


**Fig. 3.** Effects of acetaminophen on the total yields of HOCl and HOBr produced by isolated MPO. HOCl and HOBr production by MPO–$H_2O_2$–halide systems in the presence of acetaminophen (15 μM–3.84 mM) was measured by taurine chloramine and bromamine formation using the TNB assay. Reactions were performed at 37 °C and contained taurine (10 mM), MPO (100 nM) and $Cl^-$ (100 mM) and/or $Br^-$ (100 μM) and were initiated by the addition of $H_2O_2$ (50 μM). Chloramine and bromamine formation was quantified after 30 min. Data are mean ± SEM of $n = 3$ experiments. (A) Effect of pH on inhibition of HOCl production by acetaminophen. (B) Inhibition of HOCl and/or HOBr production by acetaminophen at pH 7.4. At pH 7.4, the effect of acetaminophen on oxidant production by the MPO–$H_2O_2$–$Cl^-$ and MPO–$H_2O_2$–$Cl^-$/$Br^-$ systems was significant ($p < 0.01$) at 15 μM and greater.

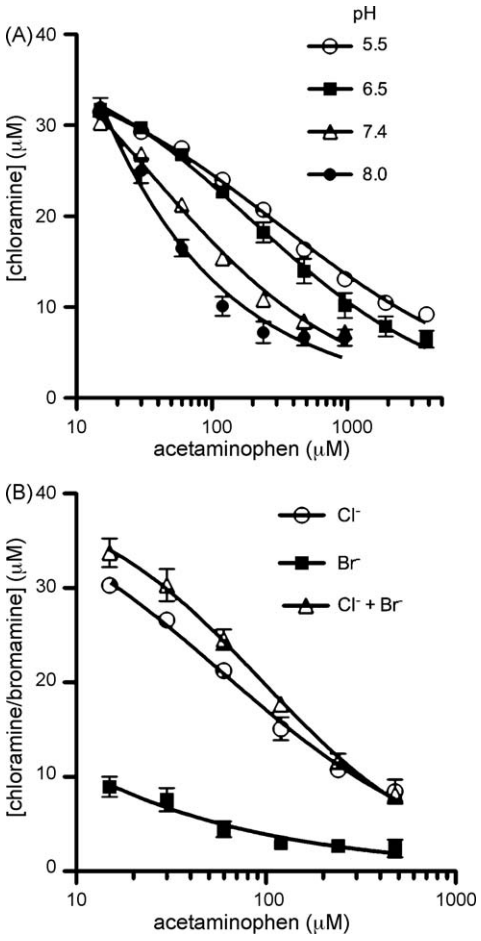


**Fig. 4.** Effects of acetaminophen on the generation of chloramines and bromamines on heparan sulfate by isolated MPO. Heparan sulfate chloramine and bromamine production by MPO–$H_2O_2$–$Cl^-$ system in the presence of acetaminophen (15 μM–3.84 mM) was measured using the TNB assay. Reactions were performed at 37 °C and contained heparan sulfate (1.6 mg $mL^{-1}$, 56 μM in amine groups), MPO (100 nM) and $Cl^-$ (100 mM) and/or $Br^-$ (100 μM) and were initiated by the addition of $H_2O_2$ (50 μM). Chloramine and bromamine formation was quantified after 30 min. Data are mean ± SEM of $n = 3$ experiments. (A) Effect of pH on inhibition of heparan sulfate chloramine production by acetaminophen. (B) Inhibition of heparan sulfate chloramine and/or bromamine production by acetaminophen at pH 7.4. At pH 7.4, the effect of acetaminophen on heparan sulfate oxidation by the MPO–$H_2O_2$–$Cl^-$ and MPO–$H_2O_2$–$Cl^-$/$Br^-$ systems was significant ($p < 0.001$) at 15 μM and greater.

obtained at pH 7.4; similar values were obtained at other pH values). This is consistent with conversion of acetaminophen by MPO to low concentrations of thiol-reactive products in a $Cl^-$-independent manner.

In the presence of both 100 mM $Cl^-$ and 100 μM $Br^-$ at pH 7.4, conversion of $H_2O_2$ to the taurine chloramine and taurine bromamine was essentially quantitative (Table 1) and inclusion of acetaminophen resulted in a dose-dependent decrease in choramine/bromamine yields (Fig. 3B). The $IC_{50}$ value for the combined inhibition of HOCl and HOBr production in this system (92 ± 2 μM; Table 1) was slightly higher than that for inhibition of HOCl production alone (77 ± 6 μM; Table 1). Inhibition of HOBr production was confirmed in experiments with $Br^-$ alone (Fig. 3B).

As MPO-binding materials have the potential to alter the efficacy of MPO inhibitors, the ability of acetaminophen to inhibit MPO in the presence of heparan sulfate and extracellular matrix was examined. The ability of acetaminophen to inhibit formation of heparan sulfate chloramines and bromamines was examined in an identical manner to that for taurine at pH 5.5–8.0 in the presence of $Cl^-$ (Fig. 4A) and also at pH 7.4 in the presence of $Cl^-$ and/or $Br^-$ (Fig. 4B). The chloramine/bromamine yields obtained in the absence of acetaminophen were slightly lower than those obtained with taurine (Table 1), however the $IC_{50}$ values for inhibition of chloramine/bromamine production from heparan sulfate and taurine were essentially identical (Table 1). These data indicate that the association of MPO with heparan sulfate does not prevent, or perturb the inhibition of MPO by acetaminophen.

These studies with isolated MPO were extended to examine the ability of acetaminophen to inhibit MPO-mediated HOCl formation in the presence of an extracellular matrix preparation. The yield of chloramines generated on this material by the MPO–$H_2O_2$–$Cl^-$ system in the absence of acetaminophen (ca. 25% based on $H_2O_2$ added; Fig. 5) was substantially lower than those obtained with other targets (cf. data in Fig. 5 for taurine). Corresponding experiments with the MPO–$H_2O_2$–$Br^-$ system were not carried out due to the greater instability of the bromamines that are formed on matrix materials, which precluded accurate quantification. With the MPO–$H_2O_2$–$Cl^-$ system, inclusion of taurine resulted in higher yields of chloramines (ca. 60% based on $H_2O_2$ added; Fig. 5), which is consistent with taurine chloramine formation and confirmed that the activity of MPO is maintained whilst bound to extracellular matrix components (e.g. heparan sulfate). Inclusion of acetaminophen resulted in a dose-dependent decrease in the yield of chloramines/chloramides, with maximal (but not complete) inhibition in the presence of extracellular matrix observed

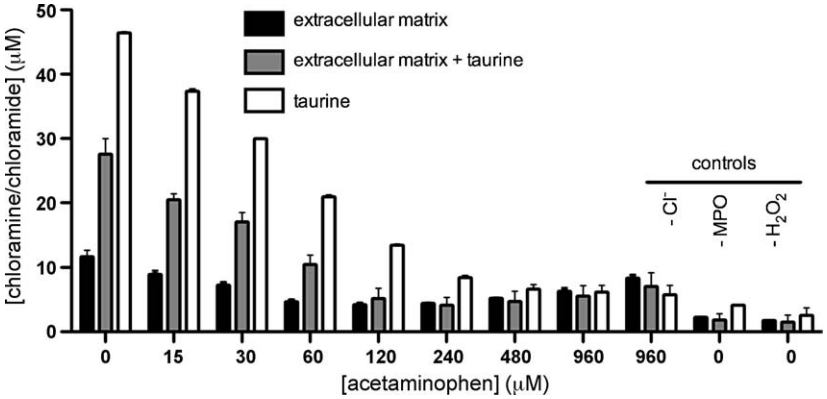


**Fig. 5.** Effect of acetaminophen on the generation of chloramines/chloramides on extracellular matrix by isolated MPO. Production of extracellular matrix-chloramines/chloramides (and/or the taurine chloramine) by the MPO–$H_2O_2$–$Cl^-$ system in the presence of acetaminophen (15 μM–960 μM) was measured using the TNB assay. Reactions were performed at 37 °C and contained extracellular matrix (0.5 mg protein $mL^{-1}$) and/or taurine (10 mM), MPO (100 nM) and $Cl^-$ (100 mM) and were initiated by the addition of $H_2O_2$ (50 μM). Chloramine/chloramide formation was quantified after 30 min. Control reactions omitting $Cl^-$, MPO and $H_2O_2$ were also performed. Data are mean ± SEM of $n = 3$ experiments. The effect of acetaminophen on extracellular matrix oxidation by the MPO–$H_2O_2$–$Cl^-$ system was significant ($p < 0.01$) at 15 μM and greater.

with 120 μM acetaminophen, both in the absence and presence of taurine (Fig. 5). The TNB-reactive species detected in reactions containing high concentrations of acetaminophen (480 μM) were generated at equivalent levels in control reactions where $Cl^-$ was omitted, indicating that the incomplete inhibition reflected generation of thiol-reactive acetaminophen products. Despite the generation of these products, these data establish that acetaminophen is an effective inhibitor of HOCl-mediated damage in complex systems.

### 3.3. Effect of acetaminophen on the kinetics of HOCl production by isolated MPO

The preceding studies establish that acetaminophen decreases the total yield of HOCl generated by isolated MPO. The ability of acetaminophen to stimulate the initial rate of HOCl production was examined by quantifying HOCl production by MPO (1 nM) and $H_2O_2$ (50 μM) in the presence of $Cl^-$ (100 mM) 5 min after initiating reactions, using catalase (50 μg $mL^{-1}$) to stop reactions by quenching residual $H_2O_2$. At this time point, HOCl production was incomplete and displayed steady-state (linear) kinetics. Low levels of acetaminophen stimulated the initial rate of HOCl formation (Fig. 6), even though they suppressed the total yield of the oxidant (cf. Fig. 3). Both these phenomena were also apparent when oxidant production was monitored continuously by including TNB (instead of taurine) and quantifying its direct oxidation by HOCl spectrophotometrically (Fig. 7). Thus, although the initial rate of TNB oxidation was more rapid in the presence of acetaminophen than in its absence, the total extent of TNB oxidation was smaller (Fig. 7). Notably, acetaminophen stimulated some TNB oxidation in the absence of $Cl^-$ (Fig. 7); this is attributed to metabolism of acetaminophen by MPO compound I and MPO compound II to thiol-reactive species.

### 3.4. Generation and characterization of fluorescent acetaminophen oxidation products

Acetaminophen is metabolized to radicals by myeloperoxidase and other peroxidase enzymes [35,36] and termination reactions of these radicals yield a fluorescent dimer and higher polymers [35]. The formation of fluorescent acetaminophen oxidation products by a reaction system consisting of MPO (50 nM), $H_2O_2$ (50 μM) and acetaminophen (100 μM) was examined by HPLC. In the absence of $H_2O_2$ a single peak, eluting at ca. 7.5 min, from parent acetaminophen was detected on the basis of its UV absorbance at 254 nm. The identity of this species was confirmed by spiking with authentic material, and MS analysis (see below). With the complete oxidation system, the area of the peak from acetaminophen decreased and an additional peak eluting at ca. 19 min was detected (Fig. 8A); additional peaks of low intensity were detected at longer elution times. Unlike the parent compound, these products were also detected using in-line fluorescence detection ($\lambda_{ex}$ 366 nm, $\lambda_{em}$ 420 nm; data not shown). The major product eluting at ca. 19 min had an identical retention time to authentic acetaminophen dimer (generated using horseradish peroxidase/$H_2O_2$/acetaminophen [35]); the minor additional products are assigned to higher polymers (e.g. trimer and tetramer) on the basis of previous data [35]. MS analysis of the collected HPLC fractions from the two major UV-active peaks provided further support for assignment of these materials as parent acetaminophen and its dimer. The peak that eluted at ca. 7.5 min gave rise to a major ion at $m/z$ 152 consistent with the molecular ion from parent acetaminophen, and a major fragment ion at $m/z$ 110 consistent with the expected loss of the acetyl function (data not shown). The species assigned to the dimer gave a major ion with $m/z$ 301 and fragment ions in $MS^n$ experiments at $m/z$ 259 and 217 consistent with the successive loss of two acetyl functions (Fig. 8B–D). These data are consistent with the fluorescent acetaminophen dimer being the major metabolite generated by MPO.

To further establish whether acetaminophen inhibits HOCl production by human neutrophils by acting as a peroxidase substrate for MPO, the generation of fluorescent acetaminophen oxidation products by opsonized zymosan-stimulated neutrophils was examined. In the presence of acetaminophen (100 μM), which effectively inhibited HOCl production (Fig. 2), the stimulated human neutrophils generated products that exhibited fluorescence spectra with excitation $\lambda_{max}$ 322 nm and emission $\lambda_{max}$ 426 nm (Supplementary Fig. 1). Products with identical fluorescence spectra are generated by isolated MPO [25]. Direct reaction of HOCl with acetaminophen also generated fluorescent products,

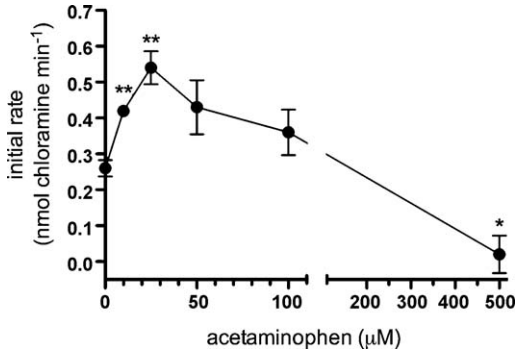


**Fig. 6.** Effects of acetaminophen on the initial rate of HOCl production by isolated MPO. The initial rate of HOCl production by the MPO–$H_2O_2$–$Cl^-$ system in the presence of acetaminophen (10–500 μM) was measured by taurine chloramine formation using the TNB assay. Reactions were performed at 37 °C and contained taurine (10 mM), MPO (1 nM) and $Cl^-$ (100 mM) and were initiated by the addition of $H_2O_2$ (50 μM) and were stopped with catalase (50 μg $mL^{-1}$) after 5 min before quantifying the taurine chloramine by the TNB assay and calculating the rate of chloramine generation over this period. Data are means ± SEM of $n = 3$ experiments. $^*p < 0.05$, $^{**}p < 0.01$ relative to the initial rate of HOCl production in the absence of acetaminophen as assessed by unpaired $t$-tests.

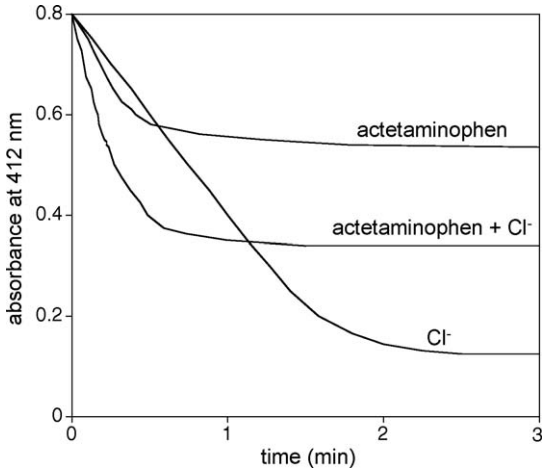


**Fig. 7.** Direct oxidation of TNB by isolated MPO in the presence of $Cl^-$ and/or acetaminophen. The effect of acetaminophen on the direct oxidation of TNB by isolated MPO was examined by continuously monitoring the bleaching of the absorbance of the thiolate form of TNB at 412 nm. Reactions were performed at 37 °C and pH 7.4 and contained TNB (adjusted such that initial absorbance was 0.8; ca. 75 μM), MPO (20 nM) and $Cl^-$ (100 mM) and/or acetaminophen (100 μM), and were initiated by the addition of $H_2O_2$ (25 μM). Data are representative of three independent experiments.

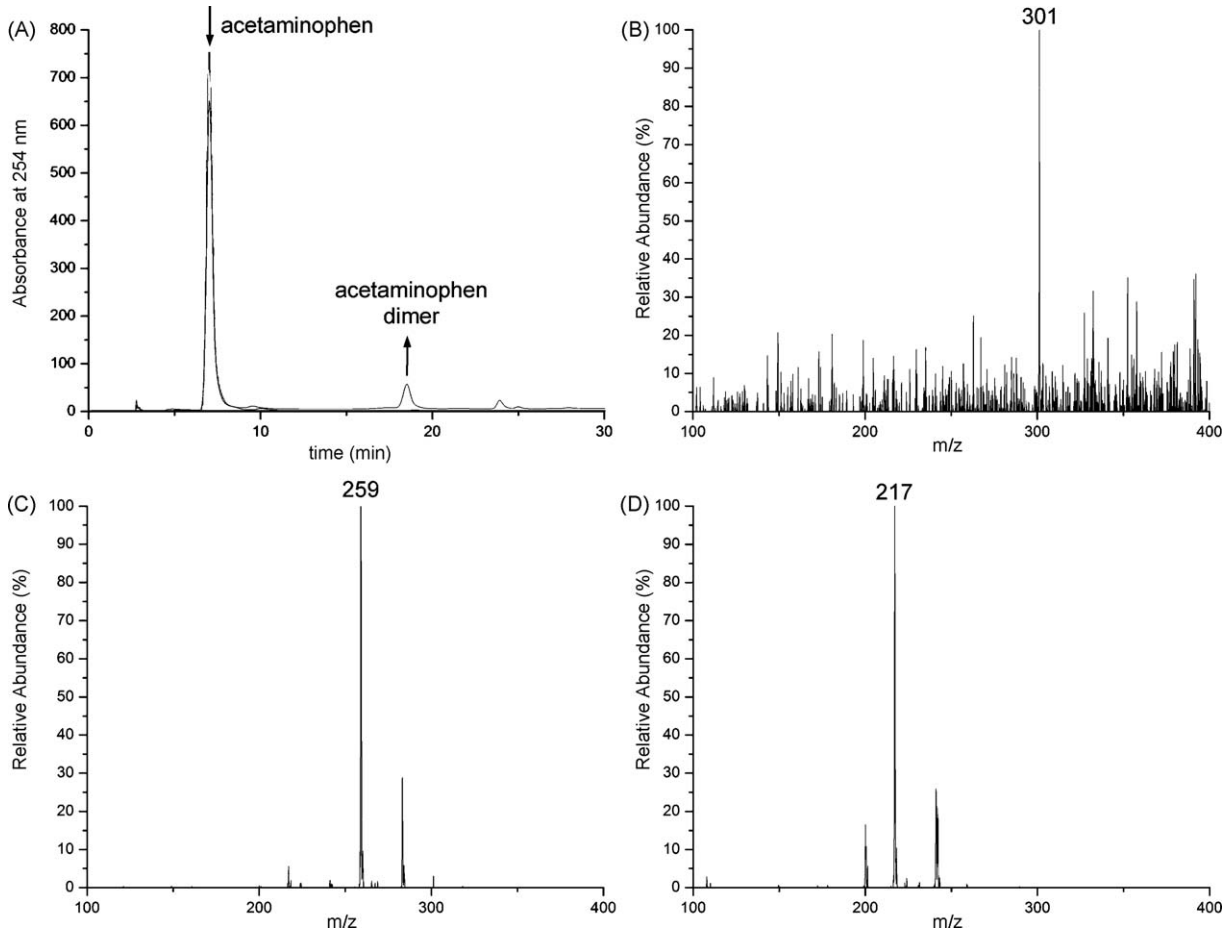


**Fig. 8.** Characterization of acetaminophen oxidation products. MPO (50 nM) was reacted without or with $H_2O_2$ (50 μM), $Cl^-$ (100 mM), taurine (10 mM) and acetaminophen (960 μM) in 0.1 M phosphate buffer, pH 7.4 at 37 °C for 30 min. Samples were subsequently injected on to a HPLC column with the column eluent monitored by UV absorbance at 254 nm (panel A). For further details see Section 2. Panel (A): complete reaction system with and without added $H_2O_2$; arrows indicate loss and gain in peak intensities observed upon addition of $H_2O_2$. The peak eluting at ca. 7.5 min is assigned to parent acetaminophen on the basis of co-elution with authentic standards and mass spectroscopic (MS) analysis of collected fraction (see text). Peak at ca. 19 min assigned to the acetaminophen dimer on the basis of co-elution with authentic material, its fluorescent properties and $MS^n$ analysis. Panel (B): MS, panel (C): MS/MS, and panel (D): MS/MS/MS analysis of the collected fraction that elutes at ca. 19 min. See text for further details.

but this was completely blocked by taurine (10 mM) (data not shown), which rapidly scavenges HOCl [37]. With the stimulated neutrophils, formation of the fluorescent acetaminophen products was unaffected by taurine (10 mM) (Supplementary Fig. 1), indicating the major source of these materials was oxidation of acetaminophen by MPO compound I and MPO compound II, and not direct reaction with HOCl.

## 4. Discussion

Oxidants generated by the heme peroxidase enzyme MPO have been reported to play a role in a wide range of human inflammatory diseases including atherosclerosis, asthma, rheumatoid arthritis, cystic fibrosis and some cancers [1]. HOCl and HOSCN are generated in high yields by MPO in the presence of typical physiological halide/pseudohalide ion plasma concentrations, with these oxidants accounting for about 90% of the $H_2O_2$ consumed by the enzyme; HOBr formation accounts for most of the remainder [38–40]. Kinetic data for the reaction of HOCl and HOBr with biological targets (reviewed [37]) indicate that scavenging of these species once formed is probably not a realistic strategy to prevent biological damage [6], so attention has been focused on the development of therapeutically applicable enzyme inhibitors [10,11,13–16]. Previous studies have indicated that acetaminophen can inhibit MPO-mediated HOCl formation [24,25], but information as to whether this results in the protection of biological targets (and particularly materials to which MPO is bound [29,41–43]) and the effects of acetaminophen on HOBr-mediated damage have not been explored previously.

The reactions of acetaminophen with MPO compound I and MPO compound II have opposing effects on HOCl and HOBr production by the enzyme, with the former reaction decreasing oxidant production, and the latter stimulating oxidant production by preventing the accumulation of MPO compound II. It has previously been observed that acetaminophen increases the initial rate of HOCl production by isolated MPO in reaction systems containing the poor peroxidase substrate monochlorodimedon [44], which inhibits MPO activity and promotes the accumulation of MPO compound II [23]. It has been shown here that acetaminophen similarly increases the initial rate of production of HOCl production by isolated MPO in the absence of a poor peroxidase substrate. This is consistent with substrate-independent accumulation of MPO compound II during catalysis due to reaction of $H_2O_2$ with MPO compound I (Fig. 1, reaction C), and with acetaminophen acting to stimulate the rate of HOCl production by reacting with MPO compound II and regenerating the native enzyme (Fig. 1, reaction D). However, the data obtained here demonstrates that the overall effect of acetaminophen is to inhibit the total yield of oxidants.

At pH 7.4 and in the presence of physiological levels of $Cl^-$ and $Br^-$, the $IC_{50}$ values for inhibition of HOCl and HOBr production by

isolated MPO are below the maximal concentrations of this drug achieved in human plasma using typical dosing regimes (oral administration of 1 g four times daily, which achieves peak and trough plasma concentrations of ca. 150 μM and 15 μM, respectively, and a plasma half-life of 1.5–2.5 h [45,46]). Acetaminophen was also effective in inhibiting neutrophil HOCl production at pH 7.4; it did not diminish neutrophil superoxide production, indicating that inhibition of HOCl production reflected inhibition of MPO and not NADPH oxidase, the source of the MPO co-substrate $H_2O_2$.

Inhibition of HOCl formation by isolated MPO by acetaminophen was observed over the pH range 5.5–8.0, which reflects pH values at sites of inflammation and within the neutrophil phagosome [47–49]. The less effective inhibition of HOCl production observed at acidic pH can be rationalized in terms of a relative increase in the rate of $Cl^-$ oxidation by MPO compound I compared to that for acetaminophen due to protonation of the distal histidine ligand of the active site and a consequent increase in the affinity of the enzyme for $Cl^-$ [50,51]. Inhibition of HOBr production was observed in experiments with $Br^-$ alone; in this system, the low bromamine yields in the absence of acetaminophen are likely to reflect inhibition of $Br^-$ oxidation by reaction of $H_2O_2$ with MPO compound I [52].

Whilst acetaminophen can increase the turnover of the enzyme by preventing accumulation of MPO compound II during catalysis, the fraction of $H_2O_2$ used to generate HOCl and HOBr is decreased under all conditions by diverting the enzyme to the production of acetaminophen products. The generation of fluorescent acetaminophen oxidation products by isolated MPO, as reported previously with related systems [36,53,54], has been confirmed here using HPLC separation with UV and fluorescence detection, and MS analysis of the isolated species. The fluorescent acetaminophen dimer, the termination product of acetaminophen phenoxyl (*N*-acetyl-p-benzosemiquinone imine) radicals generated by one-electron oxidation of acetaminophen by MPO compound I and MPO compound II (Fig. 1, reactions B and D, respectively), was identified as the major metabolite. Previous studies on the metabolism of acetaminophen horseradish peroxidase, which also mediates one-electron oxidation of acetaminophen, have also identified the acetaminophen dimer as the major metabolite [35]. Products yielding identical fluorescence spectra to those generated by isolated MPO [25] were detected with stimulated human neutrophils in the presence of acetaminophen and $Cl^-$ and the yield of these species was unaffected by taurine, indicating that these arise via direct reaction of the acetaminophen with the redox intermediates of MPO and not *via* its reaction with HOCl; equivalent data have been obtained with isolated MPO [25]. As well as undergoing termination reactions to yield the acetaminophen dimer, *N*-acetyl-p-benzosemiquinone imine radicals can undergo disproportionation to yield low concentrations of NAPQI (*N*-acetyl-p-benzoquinone imine) [55]. Both the *N*-acetyl-p-benzosemiquinone imine radicals and NAPQI react with thiols [56], with these species potentially accounting for the generation of TNB-reactive species in the absence of $Cl^-$. Whilst generation of these thiol-reactive species has the potential to exert detrimental effects in biological systems (cf. the hepatoxic effects of NAPQI generated via cytochrome P450-dependent metabolism of acetaminophen [54,57]), it is noteworthy that acetaminophen was protective overall against direct oxidation of the thiol compound TNB by the MPO–$H_2O_2$–$Cl^-$ system. The significance of thiol modification by MPO-derived acetaminophen metabolites in complex biological systems is being examined in current studies.

Importantly, the inhibitory activity of acetaminophen was maintained in the presence of heparan sulfate and extracellular matrix, which sequester MPO *in vivo*. The former possesses free amino groups that, like taurine, react with rapidly with HOCl and HOBr ($k$ $3 \times 10^5$ $M^{-1}$ $s^{-1}$ for HOCl [31]) to give chloramines and bromamines, respectively [31,32]. The extracellular matrix material used, which is derived from EHS tumour cells, has a composition similar to that of the vascular sub-endothelial extracellular matrix, a known site of MPO deposition *in vivo* [1,29,30]. The yield of *N*-chloro species generated on this material by isolated MPO, in the absence of acetaminophen, were substantially lower than those obtained with other targets consistent with reaction of HOCl at alternative sites (e.g. protein Cys, Met and cystine residues [37]), that do not yield TNB-reactive products. Acetaminophen prevented chloramine/chloramide formation on heparan sulfate and extracellular matrix, an action that is of considerable significance, as these *N*-chloro species are key intermediates in biological damage mediated by HOCl and are involved in the fragmentation of heparan sulfate and extracellular matrix by this oxidant [31,58–61]. In addition, acetaminophen inhibited luminol-dependent chemiluminescence of neutrophils (a relatively non-specific measure of HOCl production) to similar extents in the presence and absence of serum. The maintenance of the inhibitory activity of acetaminophen in the presence of serum is likely to reflect the low binding of this drug to plasma proteins [25] and also indicates that binding of MPO to serum proteins (e.g. ceruloplasmin [41]) does not substantially perturb inhibition. Serum albumin is an excellent scavenger of HOCl [6,62] and the continued inhibition of acetaminophen in the presence of albumin is consistent with the direct metabolism of this compound by MPO. It is therefore concluded that reduced production of HOCl is likely to occur under physiological conditions.

Overall, the current data indicate that acetaminophen is a significant and efficacious inhibitor of both HOCl and HOBr formation by MPO, and subsequent damage induced by these oxidants, by competitively inhibiting $Cl^-$ and $Br^-$ oxidation by MPO compound I. Importantly, inhibition of MPO occurs with therapeutically achievable concentrations of acetaminophen, with both the isolated enzyme and stimulated human neutrophils, and is maintained in the presence of complex biological materials, including those implicated in sequestering the enzyme at sites of MPO-mediated damage *in vivo* (heparan sulfate, extracellular matrix). A number of compounds have been reported to inhibit HOCl production by purified MPO at pharmacologically achievable concentrations (e.g. some anti-inflammatory drugs and related materials [10,11,13], tryptophan and derivatives [14,15] and nitroxide radicals [16]), however some cannot efficiently inhibit HOCl production by human neutrophils and/or have unknown efficacy in the presence of complex biological materials. Acetaminophen not only satisfies these criteria, but is unique in that it is currently approved for use in humans and is known to have excellent tolerability at doses that yield high plasma concentrations of the drug [17]. Overall, our data indicate that paracetamol has considerable potential as a therapeutic inhibitor of MPO-mediated tissue damage and, as it is immediately applicable to human testing, studies into its effectiveness in human disease may be expedited.

## Acknowledgments

The authors would like to thank Manyiel Awet and Christopher Young for preliminary experiments. This work was funded by the Australian Research Council, through the Discovery Projects and Centres of Excellence programs. KFS is supported by a grant from the Cancer Council of New South Wales.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.11.024.

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