HOSCN is a more potent inducer of apoptosis and protein thiol depletion in murine macrophage cells than HOCl or HOBr.

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Running Title: *Hypothiocyanous acid is a potent inducer of apoptosis*

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ABSTRACT

Hypohalous acids are generated by activated leukocytes, via the formation of H_2O_2 and the release of peroxidase enzymes (myeloperoxidase, eosinophil peroxidase). These species are important bactericidal agents, but hypochlorous acid (HOCl) and hypobromous acid (HOBr) have also been implicated in tissue damage in a number of inflammatory diseases. Hypothiocyanous acid (HOSCN) is a milder, more thiol-specific, oxidant than HOCl or HOBr, and as such may be a more potent inducer of cellular dysfunction due to selective targeting of critical thiol residues on proteins. In this study, HOCl and HOBr are shown to react rapidly with macrophage (J774A.1) cells resulting in a greater extent of cell lysis compared to HOSCN. However, HOSCN induces apoptosis and necrosis with greater efficacy, and at lower concentrations than HOCl or HOBr. Apoptosis occurs in conjunction with an increased release of cytochrome *c* into the cytosol, but no associated increase in caspase activity. Similarly, apoptosis is observed on treating the cells in the presence of a caspase inhibitor, suggesting that it is mediated by a caspase-independent pathway. HOSCN oxidised protein thiols more efficiently than either HOCl or HOBr. The greater efficacy of HOSCN in inducing apoptosis, is attributed to selective damage to critical mitochondrial membrane protein thiol groups, resulting in increased permeability and subsequent leakage of cytochrome *c* into the cytosol. This induction of damage by HOSCN may be of critical importance in people with elevated levels of SCN^- arising from cigarette smoking, and play a role in the pathologies associated with this biological insult.

Keywords : Hypohalous acid, apoptosis, eosinophil peroxidase, myeloperoxidase, thiocyanate

Abbreviations : DTT, dithiothreitol; DTNB, 5,5'-dithio-2-nitrobenzoic acid; DTPA, di-ethylene-triaminepentaacetic acid; EPO, eosinophil peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidised glutathione; GST, glutathione *S*-transferase; H_2O_2, hydrogen peroxide; HOBr, the physiological mixture of hypobromous acid and its anion; HOCl, the physiological mixture of hypochlorous acid and its anion; HOSCN, the physiological mixture of hypothiocyanous acid and its anion; IAF, 5-iodoacetamidofluorescein; LPO, lactoperoxidase; MPO, myeloperoxidase; PBS, phosphate buffered saline; SCN^-, thiocyanate ions; TCA, trichloroacetic acid; TNB, 5-thio-2-nitrobenzoic acid.

INTRODUCTION

Activation of leukocytes results in the release of a range of enzymes and lytic factors from intracellular granules, and concurrent generation of superoxide radicals ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) by a membrane-bound NADPH complex [1]. Neutrophils and monocytes release the haem enzyme myeloperoxidase (MPO) both into phagosomal compartments and extracellularly, whilst eosinophils release the related species eosinophil peroxidase (EPO) [1, 2]. These enzymes catalyse the reaction of H_2O_2 with physiological concentrations of halide ions, with MPO oxidising primarily Cl^- and SCN^-, and EPO preferentially utilising Br^- and SCN^-. The products of these reactions are hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) [3-5]. At physiological halide ion concentrations (100-140 mM Cl^-, 20-100 μM Br^-, < 1 μM I^-, ≤ 120 μM SCN^-), SCN^- is a major substrate for both peroxidases [3-5]. In the case of MPO, it has been suggested that equimolar amounts of HOCl and HOSCN are produced under physiological conditions [3]. Whilst these oxidants are important components of antibacterial defence mechanisms, misplaced or excessive generation of these species has been implicated in a wide range of inflammatory diseases, with higher concentrations of the specific biomarkers for HOCl- and HOBr-mediated damage, 3-chloro-Tyr and 3-bromo-Tyr, observed in diseased tissue (reviewed [6]). Recently, the importance of SCN^- -derived oxidants produced by MPO in atherosclerosis has been highlighted, by the detection of elevated levels of carbamylated proteins [7].

The toxic effects of activated leukocytes on cells has been attributed, at least in part, to reactions mediated by HOCl [8]. Exposure of a range of different cells, including erythrocytes, neutrophils, monocytes, macrophages, endothelial, epithelial and tumour cells to pathologically relevant concentrations of HOCl results in cell lysis [9-12]. In contrast, the reactions of HOBr and HOSCN with cells have not been studied widely. HOBr has been shown to induce more rapid red blood cell lysis than HOCl, but this effect was not observed with nucleated cells [9]. HOSCN has been reported to be bacteriostatic, but to have minimal effects on mammalian cell viability [13, 14].

The mechanisms involved in hypohalous acid induced cellular damage are not well defined. It has been demonstrated that exposure of many cell types to HOCl results in the depletion of reduced glutathione (GSH) and protein thiol groups, and inactivation of some intracellular enzymes [10, 11, 15]. HOSCN has also been reported to deplete cellular thiols, including GSH, in mammalian cells [16], and inactivate some thiol-dependent enzymes, (e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione *S*-transferases (GST) and membrane ATPases [4]). HOSCN has been shown to inactivate membrane ATPases more potently than HOCl or HOBr, with this attributed to an increased specificity of HOSCN for thiol groups [17].

HOCl has also been reported to affect cell function by modulating signal transduction pathways [18-21]. Exposure of cells to HOCl can initiate growth arrest and apoptosis via the activation of caspases [18, 19]. These effects occur long after (24 h) initial oxidant exposure despite the rapid consumption of HOCl, suggesting that HOCl initiates a time-dependent, downstream, response [18]. In HepG2 cells, the HOCl-mediated induction of apoptosis, has been shown to occur via activation of the mitochondrial permeability transition and subsequent release of cytochrome *c* from the inner mitochondrial membrane [20]. Mitochondrial damage resulting in cytochrome *c* release has also been observed with HOCl-treated endothelial cells [22].

The induction of apoptosis by HOBr and HOSCN has not been studied widely. H_2O_2 can induce apoptosis in HL-60 cells in the presence of Br^-, implicating HOBr as a causative agent [23]. In contrast, SCN^- inhibited H_2O_2-induced apoptosis in the presence of physiological levels of Cl^- in the same cell type, suggesting that SCN^- can suppress the pathways that result

in apoptosis [23]. Similarly, SCN^- has been reported to inhibit both spontaneous and agonist-induced apoptosis of eosinophils by EPO [24]. In this case, HOSCN was postulated to play a role in maintaining the viability of these cells [24].

In the study reported here, we have examined the effects of HOCl, HOBr and HOSCN on cellular integrity (lysis, necrosis and apoptosis), and thiol levels in a murine macrophage-like cell line (J774A.1 cells). These cells were chosen for study as macrophage dysfunction is a key event in the development and progression of atherosclerosis, a disease in which MPO and hypohalous acid-mediated damage has been implicated (reviewed [6, 25]). The ability of HOCl, HOBr and HOSCN to induce apoptosis and necrosis has been determined, and the level of cellular thiol oxidation observed with each oxidant quantified.

EXPERIMENTAL

Reagents and preparation of hypohalous acids – All aqueous solutions and buffers were prepared using nanopure water filtered through a four-stage Milli Q system (Millipore-Water, Lane Cove, NSW, Australia). HOCl was prepared by dilution of a concentrated stock solution of NaOCl (BDH, Poole, Dorset, UK) into water to a final concentration of 20 mM. This stock solution was diluted in phosphate buffered saline (PBS – Amresco, Solon, Ohio, USA) prior to addition to cells. HOCl concentrations were standardised at pH 11, at 292 nm using an extinction coefficient of 350 $M^{-1}cm^{-1}$ [26]. HOBr was prepared by mixing equal volumes of 45 mM KBr (Ajax, Seven Hills, NSW, Australia) and 40 mM HOCl for 60 min to ensure complete conversion of HOCl to HOBr [9]. Conversion of HOCl to HOBr was monitored by the loss of UV absorption of $^{-}$OCl (292 nm) and the formation of $^{-}$OBr (329 nm) at pH 9.

HOSCN was prepared enzymatically using lactoperoxidase (LPO, from bovine milk, Calbiochem, Kilsyth, Victoria, Australia) essentially as described previously [27]. H_2O_2 (30 % v/v solution, Merck, Kilsyth, Victoria, Australia) was quantified by UV absorbance at 240 nm using an extinction coefficient of 39.4 M^{-1} cm^{-1} [28]. Briefly, LPO (1.5 – 2 μM) was incubated with NaSCN (7.5 mM; Sigma-Aldrich, Castle Hill, NSW, Australia) and H_2O_2 (3.75 mM) at pH 6.6 in potassium phosphate buffer (10 mM) for 15 min. The concentration of LPO was determined by absorbance measurement using an extinction coefficient of 112,000 M^{-1} cm^{-1} [29]. Catalase (from bovine liver, 140 U, Sigma-Aldrich) was added to remove unreacted H_2O_2 before filtration by centrifugation (5 min, 10,000 *g*, 4 °C) through nanosep devices (Pall Life Sciences, Ann Arbor, MI) with a 10,000 Da molecular-weight cut-off to remove catalase and LPO. The concentration of HOSCN was assessed immediately by quantifying the consumption of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm using an extinction coefficient of 14,150 M^{-1} cm^{-1} [30, 31].

Tissue culture – All experiments were carried out with the J774A.1 mouse macrophage-like cell line (ATCC: TIB-67). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM – JRH Biosciences, Lenexa, Kansas, USA) supplemented with 10 % (v/v) Fetal Bovine Serum (Invitrogen, Eugene, Oregon, USA), 2 mM L-glutamine (Thermotrace, Melbourne, Victoria, Australia), 100 U/ml penicillin (Invitrogen) and 0.1 mg/ml streptomycin (Invitrogen) in 175 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2. For experiments, cells were plated down in 6- or 12-well culture plates at a concentration of 0.5 x 10^6 cells/ml and allowed to adhere overnight. Prior to experimentation, cell media was washed from the cells with warm (37 °C) PBS to prevent confounding reactions of the hypohalous acids with cell media components.

Cell lysis assay – Cell lysis was assessed by measuring DNA release into the cell media, using ethidium bromide (Roche, Mannheim, Germany) [9]. Results are expressed as as a percentage of the total DNA present in the same number of non-treated, control cells after lysis by addition of Triton X-100 (0.1 % v/v, Sigma-Aldrich). The change in ethidium bromide fluorescence observed on DNA binding was measured using λ_{Ex} 360 nm and λ_{Em} 580 nm.

Measurement of apoptosis and necrosis – The extent of apoptosis and necrosis was quantified using the Annexin V-FITC Apoptosis Detection Kit (BD, North Ryde, NSW, Australia). Cells were harvested in binding buffer and labelled with Annexin V-FITC and propidium iodide (PI) prior to quantification of staining by flow cytometry using a Cytomics FC 500 flow cytometer (Beckman Coulter, Gladesville, NSW, Australia).

Quantification of caspase activity and cytochrome c release – Cells (0.5 x 10^6) were lysed in

H_2O and added to buffer containing 100 mM HEPES (Sigma-Aldrich), 10 % sucrose (Sigma-Aldrich), 0.1 % CHAPS (Sigma-Aldrich), 0.1 % NP-40 (Calbiochem), 5 mM DTT (Sigma-Aldrich) and 50 µM Ac-DEVD-MCA (Peptide Institute Inc., Osaka, Japan) at pH 7.25 as previously [32]. The release of MCA was measured continuously in a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Foster City, California, USA) at λ_{ex} 390 nm and λ_{em} 460 nm at room temperature. The release of cytochrome *c* from the mitochondria into the cytosol of apoptotic cells was measured using a Cytochrome *C* ELISA kit (Millipore, Bedford, Massachusetts, USA). Caspase inhibition was achieved by incubating cells for 1 h in media containing 100 µM Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (Z-VAD-fmk) as previously [33, 34].

Quantification of total cell thiols **–** The concentration of cellular thiol groups was assessed by reaction with 5,5'-dithio-2-nitrobenzoic acid (DTNB, Sigma-Aldrich). Cell thiols were measured after lysing the cells with pH 7.0 phosphate buffer (50 mM) containing EDTA (10 mM) and the addition of DTNB (200 µM) before incubation for 1 h at 37 °C in the dark, and measuring the gain in absorbance at 412 nm. Thiol concentration was quantified using an extinction coefficient of 13,600 M^{-1} cm^{-1} [31].

Determination of reduced and oxidised glutathione – Loss of GSH and generation of oxidised glutathione (GSSG) was quantified by reaction with dansyl chloride and HPLC separation using a Supelco 5 µM LiChrospher Amino column with Pelliguard LC-NH_2 guard column maintained at 30 °C, with a flow rate of 1.3 ml min^{-1} [35]. A linear gradient from 0 – 100 % solvent B was applied for 20 min, where solvent A contained 80 % (v/v) methanol and solvent B contained 400 mM sodium acetate and 72 % v/v methanol [35]. The dansyl chloride derivatives of GSH, GSSG and the γ-glutamylglutamine internal standard were observed by fluorescence detection (RF10A-XL, Shimadzu, Rydalmere, NSW, Australia) at λ_{ex} 328 nm and λ_{em} 542 nm.

Oxidation of thiol-containing proteins – Cell lysate samples were labelled with 5-iodoacetamidofluorescein (IAF- Invitrogen, 200 µM from a stock solution of 10 mM in DMSO) after centrifugation to remove cell debris. The protein concentration was determined for each sample by BCA protein assay (Pierce, Rockford, IL, NSW) and samples were adjusted to ensure that equal amounts of protein were loaded onto each lane of the gels. Samples were reduced by addition of an equal volume of loading buffer (2 % (w/v) SDS (Sigma-Aldrich), 10 % (v/v) glycerol (Chem-Supply, Gillman, South Australia), saturated bromophenol blue (ICN Biomedicals, Aurora, Ohio, USA), 0.7 M 2-mercaptoethanol (Sigma-Aldrich) in 0.3 M Tris (Amresco) pH 6.8) and heating at 95 °C for 5 minutes, before separation by SDS-PAGE 1D gel electrophoresis. Gels were run in the dark for 15 hours at a constant current of 17 mA per gel. Following electrophoresis, gels were transferred into H_2O and scanned immediately using a Bio-Rad Molecular Imager® PharosFX™ system with λ_{Ex} 488 nm and λ_{Em} 530 nm. Gels were then silver-stained to visualise protein loading, following the protocol of Shevchenko *et al.* [36]. The density of gel bands were analysed using the Bio-Rad Quantity One software.

Statistical analyses **–** All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA, www.graphpad.com), with $p < 0.05$ taken as significant. Details of specific tests performed in each case are given in the Figure legends.

RESULTS

Hypohalous acid-induced loss in cellular integrity – The extent of cell lysis induced by treating washed J774A.1 cells (0.5 x 10^6 cells) with HOCl, HOBr and HOSCN (0 – 200 μM, in PBS), was investigated by quantifying DNA release from the treated cells using a fluorescent dye-binding assay. DNA release was observed on reaction of HOCl with J774A.1 cells in a dose- and time-dependent manner (Fig. 1a). Significant levels of DNA release were observed with ≥ 100 μM HOCl when 0.5 x 10^6 cells were exposed to the oxidant for 15 min (Fig. 1a). Similar data were obtained with HOBr (Fig. 1b), and no significant differences were observed between these two oxidants, in accord with previous studies [9]. In contrast, no significant DNA release was observed with HOSCN (50 – 200 μM) on reaction for ≤ 2 h (Fig. 1c). However, significant DNA release was observed at ≥ 50 μM HOSCN after 4 h incubation (Fig. 1c). Under these conditions, with ≥ 100 μM oxidant, HOSCN induced a greater extent of DNA release than either HOCl or HOBr (Fig. 1).

To determine whether these differences were due to varying rates of reaction of the oxidant with the cells, the consumption of the hypohalous acids was investigated by treating the J774A.1 cells (0.5 x 10^6) with HOCl, HOBr and HOSCN (200 μM) for 2 – 240 min before removing the cells and quantifying the remaining oxidant by TNB assay. HOCl and HOBr were consumed rapidly by the cells, with > 80 % of the oxidant removed after 2 min incubation, and no detectable levels of oxidant remaining after 240 min treatment (Fig. 2). In contrast, in experiments with HOSCN, significantly less oxidant was consumed, with 50 % of the HOSCN still detected after 240 min incubation (Fig. 2). It should be noted that the TNB assay will also quantify potential reactive secondary species generated from the added oxidant, including chloramines, bromamines, and amino thiocyanate (RN-SCN) derivatives, resulting in an underestimation of the consumption of the oxidants by the cells.

Effect of hypohalous acid exposure on cell apoptosis and necrosis – J774A.1 cells (0.5 x 10^6) were treated with 0, 25, 50 or 100 μM of each oxidant for 1 or 2 h, before washing to remove residual oxidant and addition of Annexin V-FITC and propidium iodide analysis of the cell populations by flow cytometry. Exposure of the cells to each of the oxidants resulted in an increase in the proportions of both non-viable and apoptotic cells in the total population (Fig. 3). No significant increase in the level of apoptotic cells, compared to controls was observed with either HOCl or HOBr at < 100 μM at either time point (Fig. 3a and c). In contrast, treatment with ≥ 25 μM HOSCN increased the number of apoptotic cells at both 1 and 2 h, relative to both control cells, and those treated with either HOCl or HOBr (Fig. 3). No significant necrosis was detected with HOCl or HOBr compared to controls with 25 – 100 μM oxidant for either 1 or 2 h (Fig. 3b and d), whereas HOSCN increased the extent of necrosis in a significant manner, compared to both control and HOCl- and HOBr-treated cells, with this being both dose and time-dependent.

The mechanism of hypohalous acid-induced apoptosis was investigated further by quantifying the release of cytochrome *c* from the mitochondria into the cell cytosol by ELISA, after exposing the cells (0.5 x 10^6) to HOCl, HOBr and HOSCN (25 or 50 μM) for 2 h, before subcellular fractionation. A significant increase in cytosolic cytochrome *c* concentration was observed on treating the cells with HOSCN (25 μM), but not HOCl or HOBr (Fig. 4). However, in all cases, a decrease in cytosolic cytochrome *c* was observed on treating the cells with a higher concentration (50 μM) of oxidant; this may be due to the loss of cellular material by lysis (cf. Fig. 1) or the loss of the epitope as a result of cytochrome *c* oxidation by the hypohalous acids. However, the release of cytochrome *c* was not associated with a corresponding increase in caspase-3 activity. Thus, no significant change in the activity of caspase-3 was observed in cells exposed to HOCl, HOBr and HOSCN (25 – 100 μM) for 1 h before the addition of the caspase-specific substrate Ac-DEVD-MCA and continual

monitoring of the caspase-3 activity (Fig. 5a). Incubation of the cells with HOCl and HOSCN (both 100 μM) for 2 h resulted in a decrease in caspase activity, which was significant with HOSCN (Fig. 5b). Moreover, no significant difference in the extent of apoptosis induced by HOCl, HOBr or HOSCN (100 μM) after 2 h treatment time was observed in experiments with cells pre-treated with the caspase inhibitor Z-VAD-fmk (100 μM, 1 h) compared to non-inhibitor treated cells (Fig. 6a). Pre-treatment of the cells with Z-VAD-fmk significantly reduced cellular caspase 3 activity by > 50 % (Fig. 6b), confirming that under these conditions, Z-VAD-fmk is an effective caspase inhibitor.

Hypohalous acid-induced oxidation of cellular thiols – The effects of HOCl, HOBr and HOSCN on total cell thiol levels (i.e. sum of low molecular-mass and protein-bound) was quantified after incubating cells with oxidant (0 – 100 μM) for 15 min to minimise loss of thiols resulting from cell lysis using DTNB. In each case, oxidant treatment resulted in a decrease in the concentration of cell thiols (Fig. 7). There was no significant difference between the extent of thiol oxidation observed with either HOCl, HOBr or HOSCN. Similarly, reaction of the cells with the hypohalous acids resulted in a decrease in the concentration of GSH, and an increase in GSSG concentration compared to the non-treated controls (Fig. 8a and b), with no significant differences in GSH loss observed between the three oxidants. However, the extent of GSSG formation was greater in cells treated with 25 μM HOSCN compared to 25 μM HOCl or HOBr (Fig. 8b).

The effect of HOCl, HOBr and HOSCN on the sum of GSH and GSSG levels (calculated as GSH + 2 x GSSG concentration) was also determined (Fig. 8c). With HOCl and HOBr these totals remained constant whereas there was a significant ($p < 0.05$) decrease with HOSCN indicating that the mechanism of GSH oxidation by HOSCN is likely to be different to that for HOCl and HOBr. Furthermore, quantification of GSH loss versus GSSG formation indicated that with each oxidant 15 – 25 % of the GSH lost could not be accounted for by GSSG formation. Treating the samples with sodium borohydride ($NaBH_4$) prior to analysis had no effect on the results obtained (data not shown), suggesting that the formation of mixed disulphides between GSH and protein thiols is not occurring to a significant extent under these conditions.

Oxidation of specific thiol-containing proteins in cells by hypohalous acids – J774A.1 cells (0.5×10^6 cells) were treated with HOCl, HOBr and HOSCN (≤ 50 μM) for 15 min before removal of any residual oxidant, lysis, and thiol-labelling with IAF. A decrease in the fluorescence intensity of the gel bands from proteins containing free thiols was observed upon oxidant treatment compared to controls on separation of the cellular proteins by SDS-PAGE (Fig. 9a). Under the conditions employed, no significant association of the IAF with non-thiol residues was observed, as evidenced by a lack of fluorescent protein bands on pre-treatment of the proteins with the thiol-specific blocking agent *N*-ethylmaleimide (NEM), prior to IAF treatment (data not shown). The extent of thiol oxidation, as evidenced by the loss of fluorescence of particular protein bands, was dependent on the oxidant employed, with HOSCN inducing a greater extent of loss compared to identical concentrations of HOCl or HOBr (Fig. 9a, Table 1). The relative fluorescence intensity of 18 clearly-defined and reproducible bands was quantified using the Quantity One software (Fig. 9c). Any differences in protein loading between lanes was corrected by expressing the relative fluorescence intensity values as a ratio of the relative density of at least two other specific protein bands visualised by silver staining (Table 1). For all the 18 proteins examined, HOSCN-treatment of the cells caused a significant decrease in fluorescent band density, and hence protein thiol concentration, compared to control proteins from untreated cells. In contrast, with HOCl and HOBr, a significant decrease in fluorescent band density was observed with some, but not all

of the proteins examined (Table 1).

The extent of thiol protein oxidation, shown by fluorescent band density loss, was greater with increasing concentrations of HOSCN (5 – 50 μM; Fig. 9b, Table 1). The loss in band density was not significant in experiments with 5 or 10 μM HOSCN (Fig. 9b, Table 1), however with 25 μM HOSCN, significant loss of fluorescent band density was observed compared to controls (Fig. 9b, Table 1). The extent of fluorescent band density loss was not the same for each protein, suggesting that there may be some selectivity of damage between protein thiols. The specific identity of the proteins modified by HOSCN was not investigated further in this study.

DISCUSSION

In this study, the ability of HOCl, HOBr and HOSCN to induce cellular damage has been directly compared for the first time. HOSCN has been reported to be a mild, cell permeable, oxidant that reacts preferentially with free thiol groups, without inducing significant damage to mammalian cells [37]. This is clearly not the case with murine macrophage cells, where we have shown that HOSCN is able to induce cellular dysfunction more potently than either HOCl or HOBr under pathologically relevant oxidant concentrations (≤ 200 μM). Estimates of the physiological / pathological levels of HOCl can be made from calculating the concentration of oxidant produced on activation of normal circulating concentrations of neutrophils (5 x 10^6 cells/ml), which is 150 – 425 μM of HOCl per hour (e.g. [38, 39]). As approximately 50 % of the H_2O_2 used by MPO is believed to converted to HOSCN [3], it can be estimated that the concentration of HOSCN produced by neutrophils may be in the same range assuming that the levels of SCN^- are not depleted. Eosinophils are also likely to contribute to HOSCN generation [4, 17], and it has been reported that HOCl and HOBr formed by both MPO and EPO can react rapidly and directly with SCN^- ions to generate HOSCN [40].

Reaction of HOCl and HOBr with J774A.1 cells results in the rapid release of cellular DNA, as assessed by ethidium bromide binding, due to cell lysis, in a time- and concentration-dependant manner, with significant release of DNA only observed with HOSCN at long time points (4 h). However, after 4 h treatment, the extent of lysis induced by HOSCN is greater than that observed with either HOCl or HOBr. The fact that 50 % of the HOSCN (or oxidising species derived from HOSCN) remains after 4 h treatment suggests that HOSCN is a far more potent cell damaging agent than HOCl and HOBr with this cell type. The increased extent of DNA release observed with HOCl and HOBr compared to HOSCN at short time points (≤ 2 h) may reflect the increased reactivity of HOCl and HOBr with cell membrane components (either protein or lipid). Indeed, these oxidants were consumed rapidly by the cells. HOCl and HOBr have been shown to react rapidly with red blood cell membrane proteins, resulting in disruption of membrane structure and cell lysis [12, 41]. The significant time lag between HOSCN exposure and DNA release observed in the current study is consistent with alternative mechanisms of damage, rather than direct membrane perturbation, with the induction of apoptosis a potential pathway.

HOSCN has been shown to induce significant apoptosis, as evidenced by a significant increase in the population of cells expressing phosphatidylserine on the outer plasma membrane that stains positively with Annexin V-FITC compared to cells incubated in the absence of the oxidant. Analogous increases were not detected with < 100 μM HOCl or HOBr for 1 or 2 h. Even at high oxidant concentrations (100 μM), the extent of apoptosis induced by HOSCN was still significantly higher than that induced by HOCl or HOBr. Similarly, the proportion of necrotic cells (propidium iodide binding) was significantly higher with HOSCN compared to both control cells and those exposed to identical concentrations of HOCl or HOBr. It is possible that a greater extent of apoptosis may have been observed on incubation of the cells with oxidant for longer time periods (≥ 6 h). However, the cells were treated in the absence of cell media, and hence the extent of apoptosis in the non-treated control cells also increases.

The HOSCN-induced apoptosis at low oxidant concentrations (25 μM) occurred in conjunction with the release of mitochondrial cytochrome *c* into the cytosol, suggesting that caspases may play a role in this apoptosis, as cytochrome *c* release can lead to downstream caspase activation. However, HOSCN treatment of cells resulted in a decrease in the activity of caspase-3, and no significant changes in the extent of apoptosis was observed in the presence of a potent caspase inhibitor, Z-VAD-fmk. A number of caspase-independent cell death pathways have been suggested, including those involving apoptosis inducing factor

(AIF) and endonuclease G (endoG) which contribute to nuclear DNA degradation [42]. Other proteins released from the mitochondria such as second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI (Smac/DIABLO) and high-temperature requirement protein A2 (HtrA2/OMI) may also exert caspase-independent cytotoxic activity; however the exact mechanisms by which they mediate this remain unknown [42]. Release of these mitochondrial proteins may be playing a role in the observed hypohalous acid-induced caspase-independent apoptosis, however this is beyond the scope of the present study.

The results from our study on the mechanism of apoptosis in the J774A.1 cells are comparable to those obtained with mesenchymal progenitor cells by Whiteman and colleagues [21], where mitochondrial cytochrome *c* release was detected with an associated decrease in caspase activity. In the mesenchymal cells, it was suggested that HOCl treatment induced cytochrome *c* release from the mitochondria of apoptotic cells which leads to activation of the caspase cascade, followed by rapid inactivation of the caspase enzymes by HOCl and/or its reactive intermediates [21]. In these cells, other pro-apoptotic proteins released from the mitochondria were postulated to play an integral role in the apoptotic pathway, such as AIF and EndoG; and this may also be the case in the J774A.1 cells.

The ability of HOSCN to induce a greater extent of apoptosis than HOCl or HOBr in J774A.1 macrophage cells was unexpected, as production of HOSCN by a MPO/H_2O_2/SCN^- system has previously been reported to cause no appreciable apoptosis in HL-60 cells (a human leukaemia cell line) [23]. Indeed, physiological levels of SCN^- ions were reported to protect these cells from apoptosis induced by H_2O_2 in the presence of Cl^- [23]. This previous study also supports that hypothesis that MPO can catalyse the oxidation of SCN^- even in the presence of high concentrations of Cl^-, in accord with other data [3, 5, 43]. The ability of SCN^- to protect eosinophils from both spontaneous and agonist-induced apoptosis has also been reported [24]. A number of major differences between the current and these previous studies, including the cell types examined, and the method of oxidant generation and exposure may explain this apparent discrepancy.

HOSCN is known to target thiol (Cys) residues with a high degree of selectively, while HOCl and (particularly) HOBr do so to a much lesser extent [17, 44]; this may explain the differential effects of these oxidants in terms of the induction of apoptosis and necrosis. No significant differences were observed between the three oxidants in terms of the extent of total (low-molecular-mass and protein-bound) thiol oxidation under conditions where DNA release was low ($\leq$ 100 μM oxidant, 15 min incubation). Similarly, there was no difference in the extent of GSH loss or GSSG formation. Not all of the GSH lost could be accounted for by GSSG formation, suggesting that either additional product(s) are being generated together with GSSG, or that further oxidation of GSSG is occurring. It has been shown previously that treatment of cells with HOCl generates further oxidation products, including glutathione sulfonamide and oxy-acids [45]. These products cannot be quantified by the method employed in this study. Further oxidation of GSSG by the hypohalous acids and / or reactive species derived from them is also possible (cf. high rate constants for reaction of cystine with HOCl and HOBr [44]). No loss of GSSG was observed on treatment of this isolated material with HOSCN (data not shown). The fate of the GSH and potential oxidation of GSSG by HOCl and HOBr was not investigated further in this study.

In contrast, the reactivity of HOSCN with thiol-containing proteins was significantly greater than HOCl or HOBr as evidenced by the dramatic decrease in the binding of a fluorescent, thiol-specific tag. Whilst all the proteins quantified (18 in total) displayed a significant decrease in the fluorescent staining intensity with HOSCN, there appears to be some proteins within this cohort that are more susceptible to oxidation by HOCl, with relatively few more sensitive to HOBr. The difference in reactivity of some protein thiols compared to GSH may be associated with the lower pK_a values of the former. It has been

shown previously that chloramines react preferentially with proteins with low pK_a thiols compared to GSH [46]. The identity of the 18 thiol-containing proteins that have been shown to be affected by HOSCN, was not investigated further.

The greater extent of apoptosis and necrosis observed in cells treated with HOSCN, compared to HOCl and HOBr, may be associated with the greater selectivity of this oxidant for cellular thiol-containing proteins, and hence an increased extent of damage to these species. This hypothesis is supported by data from studies on other mammalian cell types, where it has been demonstrated that oxidation of critical protein thiols results in the induction of apoptosis [47-49]. In particular, modification of mitochondrial thiols results in the activation of the mitochondrial permeability transition, which is a critical pathway in cell death [49, 50] as it results in release of cytochrome *c* and caspase activation, or necrosis via a significant reduction in ATP production [50]. This pathway has been shown to be critical in HepG2 cells treated with HOCl [20]. The redox state of cellular thiols is also known to be important in the regulation of apoptosis via the modulation of protein kinase activities [48]. Thus, thiol oxidation has been shown to lead to selective phosphorylation of p38 mitogen activated protein kinase, which induces apoptosis in fibroblast cells [48].

In summary, the data reported here indicate that the hypohalous acids react with cells via different mechanisms. HOCl and HOBr appear to target membrane components (proteins or lipids) in addition to intracellular species, with these reactions resulting in cell lysis and leakage of DNA from the cell. In contrast, HOSCN appears to induce apoptosis and necrosis via alternative pathways potentially involving the specific targeting of intracellular thiol-containing proteins. Depletion of GSH does not appear to contribute directly to these different mechanisms, as the extent of GSH oxidation induced by all three oxidants is similar in extent, at least in this cell type under the conditions employed. This unexpected greater propensity for HOSCN to induce apoptosis and necrosis, possibly via the selective targeting of specific proteins, may have important implications in various inflammatory diseases, particularly in people who smoke who can have markedly elevated levels of SCN^-, and hence might be expected to be exposed to greater concentrations of HOSCN.

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Table 1 : Effect of the hypohalous acids on thiol-containing proteins in J774A.1 cells.

Band	Standardised Average Density Ratio of Fluorescent Band					
	50 µM HOCl	**50 µM HOBr**	**5 µM HOSCN**	**10 µM HOSCN**	**25 µM HOSCN**	**50 µM HOSCN**
1	83.1	* 76.9	134.9	86.7	83.7	** 57.8
2	* 77.8	** 72.3	117.8	83.3	72.0	** 46.1
3	* 79.2	84.8	106.4	90.4	70.8	** 47.2
4	* 80.3	* 79.8	114.7	96.5	76.1	** 43.7
5	* 77.9	* 75.4	115.0	95.0	* 66.9	** 36.9
6	79.8	75.6	118.7	102.6	66.5	** 42.5
7	* 80.9	86.4	117.3	92.9	70.8	** 51.6
8	* 84.9	90.7	114.0	98.2	79.5	** 60.7
9	87.2	98.3	122.7	111.8	85.8	* 56.9
10	88.3	90.1	115.7	101.2	77.4	** 46.3
11	* 83.9	* 84.8	112.3	110.7	77.3	** 49.5
12	90.8	90.0	129.2	119.7	87.6	** 45.0
13	88.2	93.9	115.8	114.7	78.9	** 33.7
14	* 69.6	85.6	120.5	103.2	79.3	** 38.8
15	* 74.2	79.9	114.2	100.0	81.5	** 49.6
16	83.6	90.7	107.3	109.6	87.0	* 62.3
17	89.2	97.7	116.7	109.7	88.4	** 56.6
18	99.7	90.1	112.5	101.5	77.5	** 54.0

The relative average density ratios were standardised by dividing the average density for each band obtained from the fluorescent scans by the average density for each lane obtained by analysing the selected band on the corresponding total gel stained (by silver-staining) for protein. Results are expressed as percentage of control (n=5, duplicate measurements). * and ** indicate a significant difference ($p<0.05$ and $p<0.01$ respectively) in relative average band density when compared to control by repeated measures ANOVA with Dunnett's post-hoc testing.

FIGURE LEGENDS

Figure 1: Hypohalous acid-induced cell lysis.
The rate and extent of J774A.1 cell lysis induced on incubation of the cells (0.5 x 10^6 cells) with (a) HOCl, (b) HOBr and (c) HOSCN was determined by quantification of the change in ethidium bromide fluorescence on DNA release. In each case, 0 (●), 50 μM (■), 100 μM (◆), 200 μM (▲) oxidant. Data are means ± S.E.M. (n = 12). In (a) and (b) cell lysis is significant at concentrations > 50 μM after exposure to HOCl or HOBr for 15 min, and ≥ 50 μM after exposure for 60 min and in (c) at concentrations ≥ 50 μM after exposure to HOSCN for 240 min according to ANOVA with Dunnett's post-hoc testing.

Figure 2: Consumption of the hypohalous acids by J774A.1 cells
Cells (0.5 x 10^6) were exposed to 200 μM HOCl (■), HOBr (▲) or HOSCN (●) for 2 – 240 min before removing an aliquot of the supernatant and quantifying the oxidant concentration by TNB assay. Data are expressed as a percentage of the initial oxidant added to the cells as mean ± S.E.M. (n = 6)

Figure 3: Hypohalous acid-induced apoptosis and necrosis in J774A.1 cells.
Cells (0.5 x 10^6) were exposed to HOCl (black bars), HOBr (white bars), HOSCN (hatched bars) or incubated in PBS as a control (chequered bars), for 1 or 2 hours, and the percentage of healthy, apoptotic and necrotic cells in the population assessed using the Annexin V-FITC Apoptosis Detection Kit with flow cytometric analysis. Results are expressed as the percentage of apoptotic or necrotic cells in the total cell population. Data are presented as mean ± S.E.M. (n = 6). (a) Assessment of apoptosis following 1 h incubation of cells with oxidant; (b) assessment of necrosis following 1 h incubation of cells with oxidant; (c) assessment of apoptosis following 2 h incubation of cells with oxidant; (d) assessment of necrosis following 2 h incubation of cells with oxidant. * indicates a significant increase in the percentage of apoptotic or necrotic cells compared to control, according to repeated measures ANOVA with Dunnett's post-hoc testing. "a" and "b" indicate a significant increase in the percentage of apoptotic or necrotic cells compared to HOCl, or HOBr treatment, respectively, by 2-way ANOVA with Bonferroni post-hoc testing.

Figure 4: The effect of hypohalous acid treatment on cytosolic cytochrome *c* concentration.
Cells (0.5 x 10^6) were exposed to HOCl (black bars), HOBr (white bars) or HOSCN (hatched bars) for 2 hours, and the cytosolic cytochrome *c* concentration assessed by ELISA following subcellular fractionation. Data represent mean ± S.E.M. (n=5), results expressed as a percentage of untreated control cell cytosolic cytochrome *c* concentration. * indicates a significant increase in cytochrome *c* concentration when compared to untreated control cells (normalised to 100 %), according to repeated measures ANOVA with Dunnett's post-hoc testing.

Figure 5: The effect of hypohalous acids on caspase activity.
Cells (0.5 x 10^6) were exposed to HOCl (black bars), HOBr (white bars) or HOSCN (hatched bars) for (a) 1 or (b) 2 hours, and the release of the fluorescent MCA subunit from the specific caspase substrate Ac-DEVD-MCA was monitored continuously in a fluorescence plate reader. Data represent mean ± S.E.M. (n=9), results expressed as a percentage of untreated control cell caspase activity. The dotted line indicates the caspase activity in untreated control cells. * indicates a significant decrease in caspase activity when compared to untreated control cells, according to repeated measures ANOVA with Dunnett's post-hoc testing.

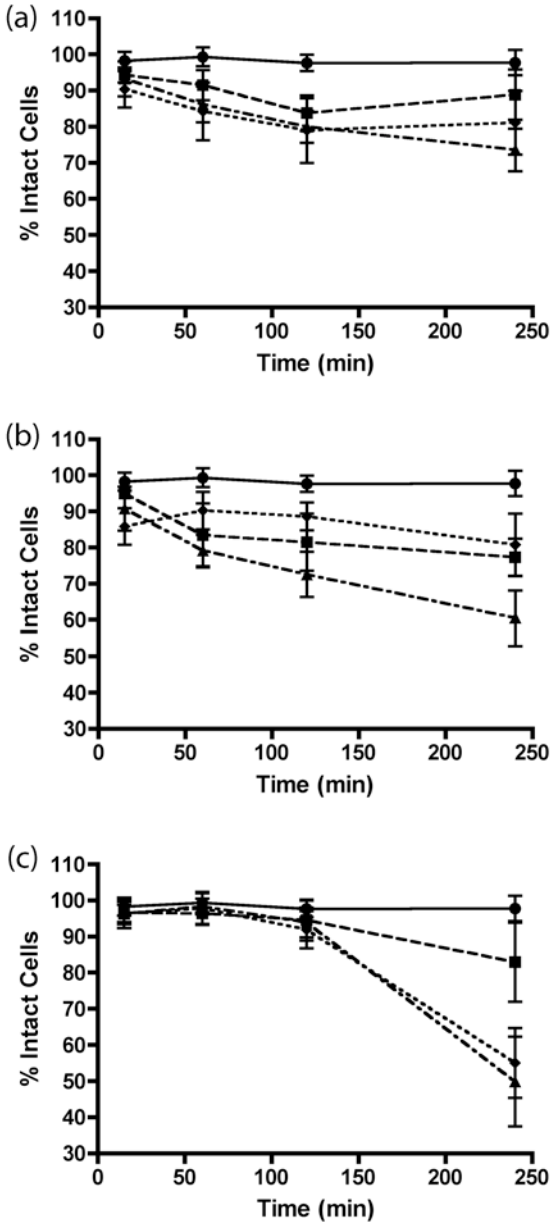
Figure 6: Caspase inhibition by Z-VAD-fmk
Cells (0.5 x 10^6) were incubated in normal media or media containing 100 μM Z-VAD-fmk for 1 hour, before - (a) treating the cells with HOCl (black bars), HOBr (white bars) or HOSCN (hatched bars) (100 μM) for 2 hours prior to assessing apoptosis using the Annexin V-FITC Apoptosis Detection Kit with flow cytometric analysis, chequered bars indicate untreated control cells. Results are expressed as the percentage of apoptotic cells in the cell population. Data are mean ± S.E.M. (n = 4). There is no significant difference in the percentage of apoptotic cells observed with and without pre-treatment with Z-VAD-fmk in each case; (b) measuring caspase activity by quantifying the release of the fluorescent MCA subunit from the caspase substrate Ac-DEVD-MCA in a fluorescence plate reader. The black bar indicates cells incubated in normal media, the white bar represents cells incubated in media containing Z-VAD-fmk. Data represent mean ± S.E.M. (n=9). * indicates a significant decrease in caspase activity according to a paired *t*-test.

Figure 7: The effect of hypohalous acids on total cell thiol levels.
Cells (0.5 x 10^6) were exposed to HOCl (black bars), HOBr (white bars) or HOSCN (hatched bars) for 15 minutes before the total cell thiol concentration was determined using the DTNB assay. Each data point represents the mean ± S.E.M. (n = 12). * indicates a significant decrease in total thiol concentration when compared to control cells. Paired *t*-tests were used for the statistical analysis. Experiments were performed in 6-well plates, each treatment well was paired with an untreated control well.

Figure 8: The effect of hypohalous acid treatment on cellular GSH and GSSG levels.
Cells (0.5 x 10^6) were exposed to HOCl (black bars), HOBr (white bars) or HOSCN (hatched bars) for 15 minutes before intracellular GSH and GSSG levels were determined after modification with dansyl chloride and separation by HPLC. (a) Effect of hypohalous acids on cellular GSH concentration; (b) effect of hypohalous acids on cellular GSSG concentration; (c) effect of hypohalous acids on total GSH + GSSG levels (calculated as GSH + 2 x GSSG concentration). Results expressed as a percentage GSH / GSSG in the non-treated control. Data represent mean ± S.E.M. (n=8). * indicates a significant difference when compared to control values. Paired t-tests were used for the statistical analyses, as each treatment well was paired with an untreated control well. There was no significant difference between the loss of GSH observed with each of the hypohalous acids. “a” indicates that HOSCN is significantly higher than HOCl and HOBr; “b” indicates that HOSCN is significant lower than HOCl, by 2-way ANOVA with Bonferroni post-hoc testing.

Figure 9: 1D-PAGE separation of IAF-labelled proteins in hypohalous acid-treated cells.
Comparison of the effects of (a) HOCl, HOBr and HOSCN (all 50 μM); and (b) varied concentrations (5 – 50 μM) of HOSCN on thiol-containing proteins. In each case, the cells were exposed to the oxidants for 15 minutes. In (a) lane 1 represents non-treated control cells, lane 2 represents HOCl-treated cells, lane 3 represents HOBr-treated cells, lane 4 represents HOSCN-treated cells. In (b) lane 1 represents non-treated control cells, lanes 2-5 represent cells treated with 5, 10, 25 and 50 μM HOSCN respectively. Cell thiol-containing proteins were labelled with IAF and scanned by a fluorescent scanner with λ_{Ex} 488 nm and λ_{Em} 530 nm. (c) Section of gel represents the 18 IAF-labelled, thiol-containing proteins, from non-treated control cells from the fluorescent scan (excitation wavelength of 488 nm and emission filter wavelength of 530 nm) selected for average density calculations and data comparison (see Table 1).

Figure 1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% Intact Cells
Time (min)
(b)
% Intact Cells
Time (min)
(c)
% Intact Cells
Time (min)

Figure 2

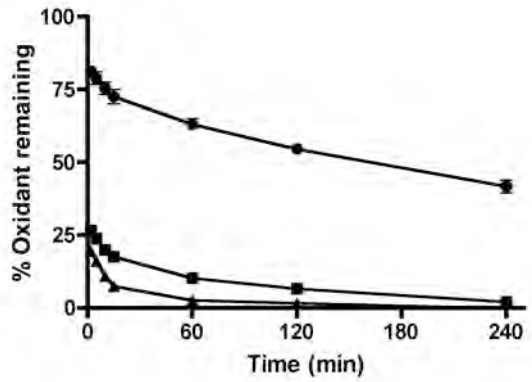


Figure 3

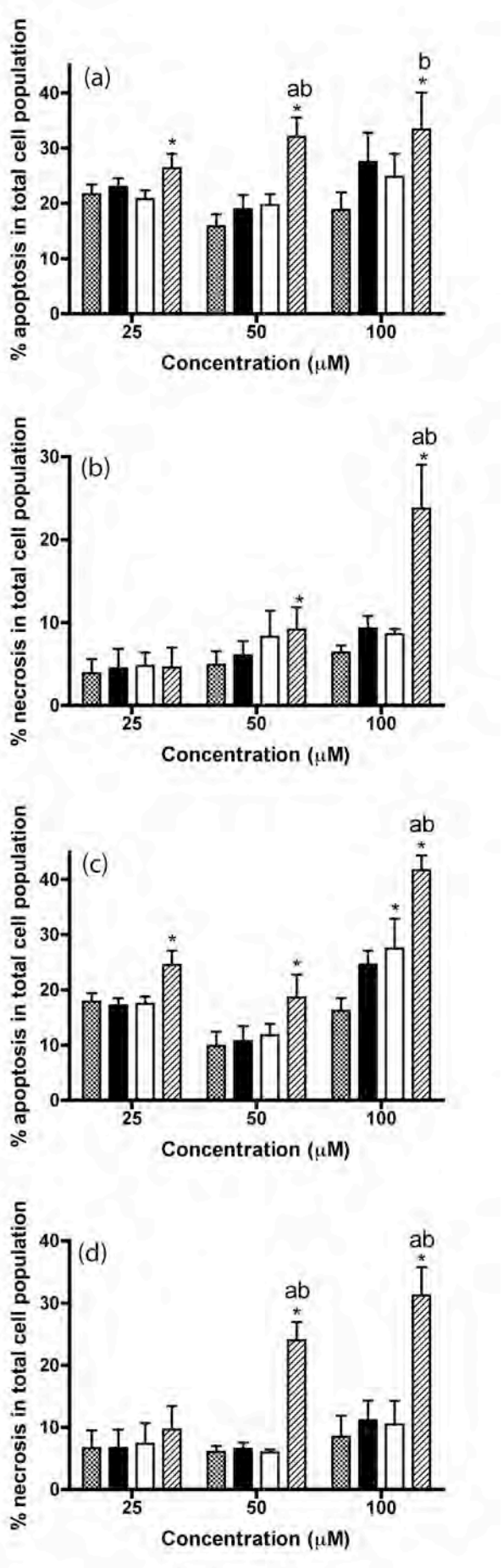

(a)
% apoptosis in total cell population
ab
b
*
0
10
20
30
40
25
50
100
Concentration (μM)
(b)
% necrosis in total cell population
ab
*
0
10
20
30
25
50
100
Concentration (μM)
(c)
% apoptosis in total cell population
ab
*
0
10
20
30
40
25
50
100
Concentration (μM)
(d)
% necrosis in total cell population
ab
*
0
10
20
30
40
25
50
100
Concentration (μM)

Figure 4

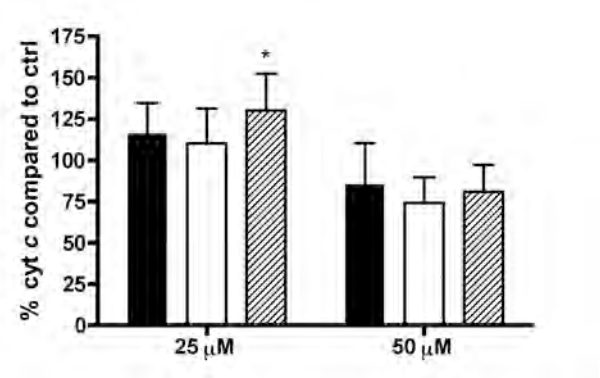


Figure 5

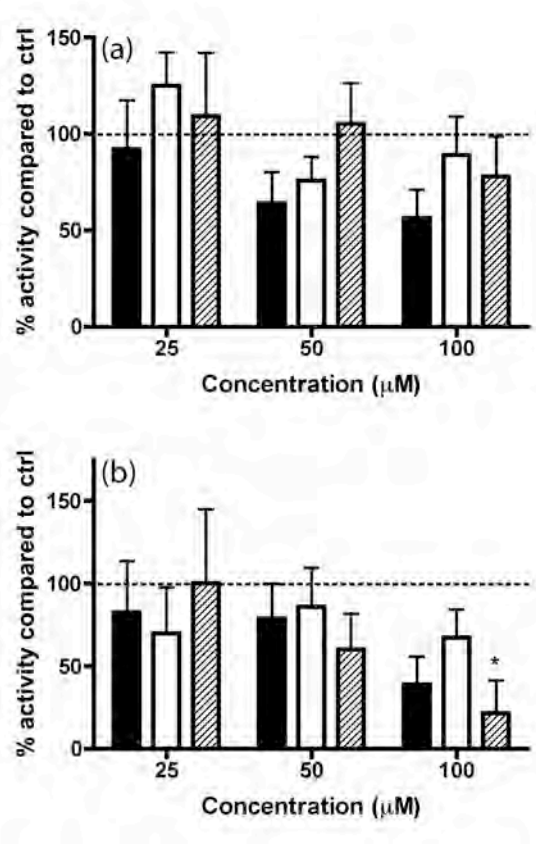

(a)
% activity compared to ctrl
150
100
50
0
25
50
100
Concentration (μM)
(b)
% activity compared to ctrl
150
100
50
0
*
25
50
100
Concentration (μM)

Figure 6

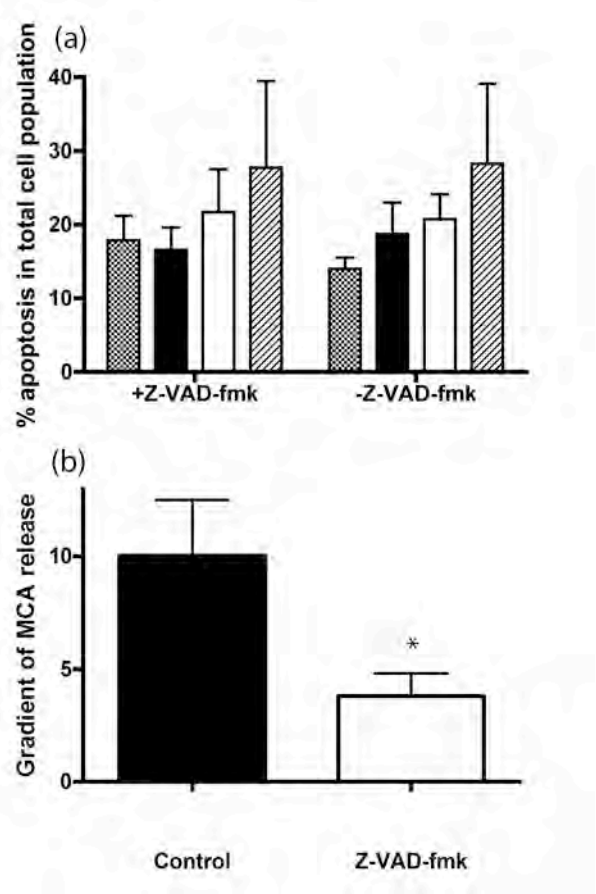

(a)
% apoptosis in total cell population
40
30
20
10
0
+Z-VAD-fmk
-Z-VAD-fmk
(b)
Gradient of MCA release
10
5
0
*
Control
Z-VAD-fmk

Figure 7

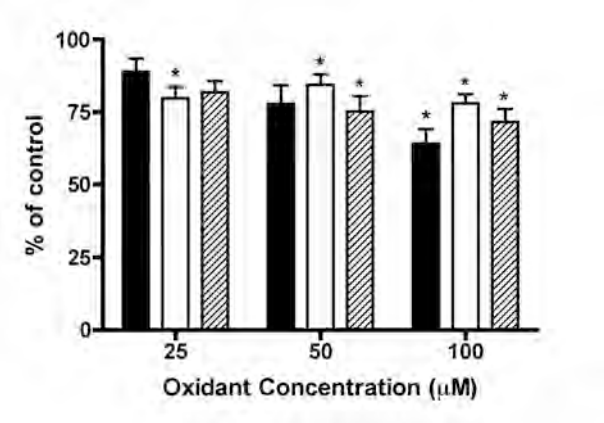


Figure 8

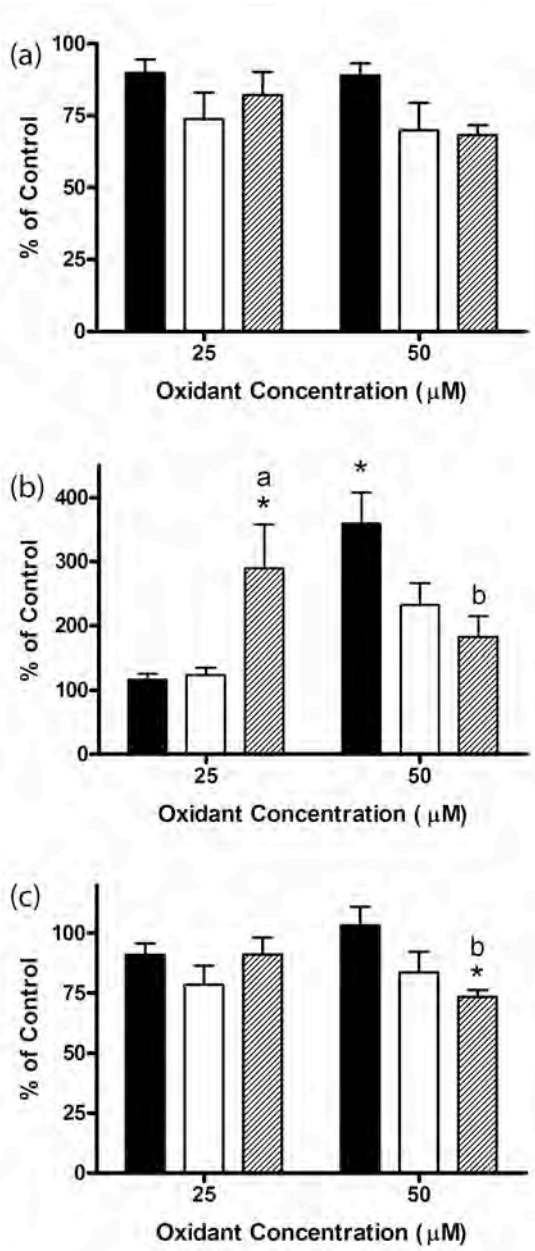
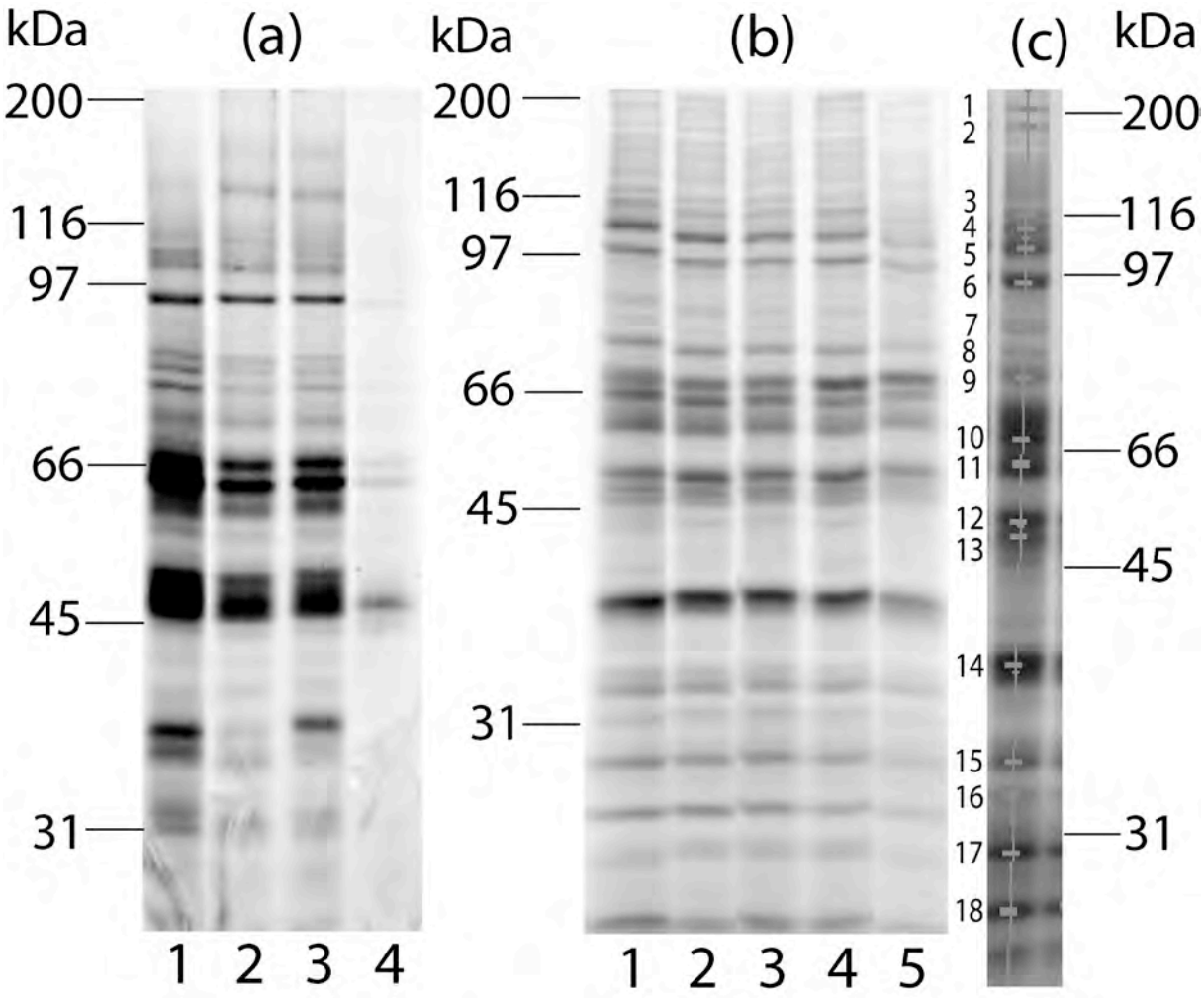

(a)
% of Control
100
75
50
25
0
25
50
Oxidant Concentration (μM)
(b)
% of Control
400
300
200
100
0
a
*
*
b
25
50
Oxidant Concentration (μM)
(c)
% of Control
100
75
50
25
0
b
*
25
50
Oxidant Concentration (μM)

Figure 9


kDa
(a)
200
116
97
66
45
31
1 2 3 4
kDa
(b)
200
116
97
66
45
31
1 2 3 4 5
(c)
kDa
200
116
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