

## REVIEW

## Myeloperoxidase: a target for new drug development?

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Myeloperoxidase (MPO), a member of the haem peroxidase-cyclooxygenase superfamily, is abundantly expressed in neutrophils and to a lesser extent in monocytes and certain type of macrophages. MPO participates in innate immune defence mechanism through formation of microbicidal reactive oxidants and diffusible radical species. A unique activity of MPO is its ability to use chloride as a cosubstrate with hydrogen peroxide to generate chlorinating oxidants such as hypochlorous acid, a potent antimicrobial agent. However, evidence has emerged that MPO-derived oxidants contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory disease. The fact that circulating levels of MPO have been shown to predict risks for major adverse cardiac events and that levels of MPO-derived chlorinated compounds are specific biomarkers for disease progression, has attracted considerable interest in the development of therapeutically useful MPO inhibitors. Today, detailed information on the structure of ferric MPO and its complexes with low- and high-spin ligands is available. This, together with a thorough understanding of reaction mechanisms including redox properties of intermediates, enables a rationale attempt in developing specific MPO inhibitors that still maintain MPO activity during host defence and bacterial killing but interfere with pathophysiologically persistent activation of MPO. The various approaches to inhibit enzyme activity of MPO and to ameliorate adverse effects of MPO-derived oxidants will be discussed. Emphasis will be put on mechanism-based inhibitors and high-throughput screening of compounds as well as the discussion of physiologically useful HOCl scavengers.

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**Abbreviations:** 2-ClHDA, 2-chlorohexadecanal; ABAH, 4-aminobenzoic acid hydrazide; BHA, benzylhydroxamic acid; *E*<sup>o</sup>, standard reduction potential; HCN/CN<sup>-</sup>, hydrocyanic acid (hydrogen cyanide)/cyanide; HOCl/OCl<sup>-</sup>, hypochlorous acid/hypochlorite; HOSCN/OSCN<sup>-</sup>, hypothiocyanous acid/hypothiocyanate; HSCN/SCN<sup>-</sup>, thiocyanous acid/thiocyanate; HOX/X<sup>-</sup>, hypohalous acid/halide; MCD, monochlorodimedon; MPO, myeloperoxidase; PDB, Protein Data Bank; ROOH, lipid-hydroperoxide; SHA, salicylhydroxamic acid (2-hydroxybenzohydroxamic acid); TauNH<sub>2</sub>, taurine; TauNHCl, taurine chloramine; TMB, 3,3',5,5'-tetramethylbenzidine; TNB, 5,5'-dithiobis(2-nitrobenzoic acid)

## Introduction

An intensely green protein containing iron that had peroxidase activity was originally isolated from canine pus and purulent fluid of patients with tuberculous empyema (Agner, 1941). Observations that expression of this enzyme is restricted to the myeloid series of haematopoietic cells where

it is entirely located in azurophil (primary) granules, resulted in renaming of this protein, verdoperoxidase, to myeloperoxidase (MPO) (Yamada and Kurahashi, 1984; Koeffler *et al.*, 1985). Promyelocytes and promyelomonocytes actively synthesize MPO during granulocyte differentiation in the bone marrow. MPO levels in neutrophilic polymorphonuclear leucocytes (neutrophils) make up in between 2 and 5% of the total cellular protein or 2 to 4 μg per 10<sup>6</sup> cells (Schultz and Kaminker, 1962; Bos *et al.*, 1978). Human monocytes also contain MPO-positive granules although they are fewer in number than in neutrophils and lost during differentiation into tissue macrophages (for a review see: Klebanoff, 2005).

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Observations that destruction of microorganisms occurs in the phagosome of neutrophils and that MPO is among the enzymes discharged into these phagocytic vacuoles from cytoplasmic granules suggest an important role of MPO in bacterial killing (Klebanoff, 2005). Among the antimicrobial systems present in the phagosome, a major proportion consists of MPO, hydrogen peroxide ( $H_2O_2$ , formed during the respiratory burst), and a halide ( $X^-$ ), particularly chloride ( $Cl^-$ ) (Hampton *et al.*, 1998). The initial product of the MPO– $H_2O_2$ – $Cl^-$  system is the potent antimicrobial oxidant hypochlorous acid/hypochlorite ( $HOCl/OCl^-$ ,  $pK_a$  7.53) (Figure 1). However, under pathological conditions, persistent activation of the MPO– $H_2O_2$  system of activated phagocytes may adversely affect tissues.  $HOCl$  is able to initiate modification reactions targeting lipids, DNA and (lipo)proteins, including halogenation, nitration and oxidative cross-linking (Figure 1).

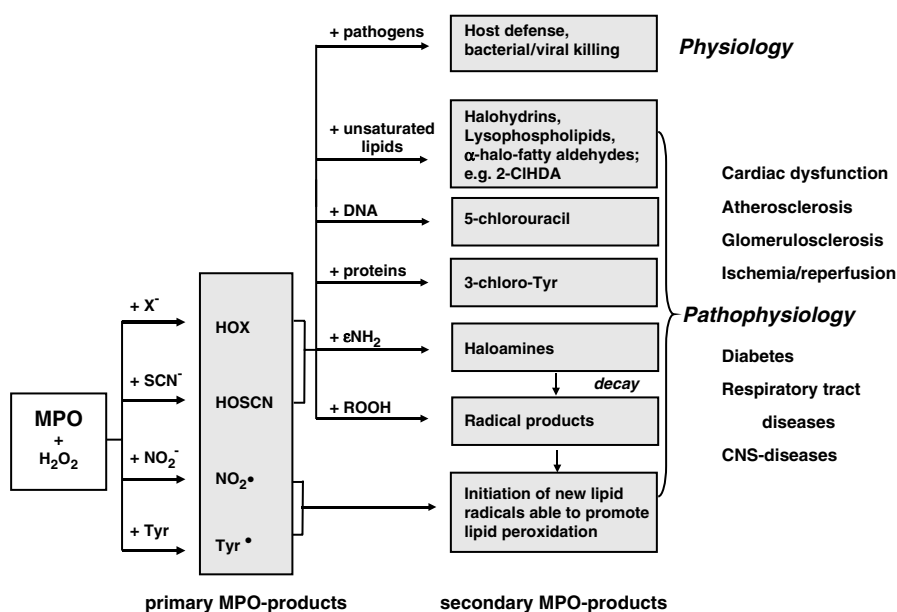
Chlorohydrins are formed by addition of  $HOCl$  to double bonds present either on cholesterol or various unsaturated ester- and ether-phospholipid species (Malle *et al.*, 2006a; Leßig *et al.*, 2007); however, the usefulness of chlorohydrins to be used as biomarkers is limited. Most importantly, the remaining lyso-phospholipid remnants formed by  $HOCl$ -mediated attack of ester-/ether-phospholipids (plasmalogens) may adversely affect membrane dynamics and cause cell lysis. In contrast to ester-phospholipids,  $HOCl$ -mediated attack of ether-phospholipids (present in biological membranes and lipoprotein particles) results in formation of 2-chlorohexadecanal (2-ClHDA), a chlorinated fatty aldehyde that is enriched in human atherosclerotic lesions (Thukkani *et al.*, 2003) and infarcted myocardium (Thukkani *et al.*, 2005). 2-ClHDA is a potent chemoattractant *in vitro* initiating recruitment of circulating neutrophils to areas of inflammation and promotes endothelial dysfunction (Marsche *et al.*, 2004). After release from activated phago-

cytes, MPO colocalizes with  $HOCl$ -modified epitopes on the endothelial layer lining the blood vessel (Malle *et al.*, 2006b) and contributes to endothelial dysfunction (Eiserich *et al.*, 2002).

$HOCl$  has also been reported to react with nucleobases resulting in the formation of 5-chlorouracil, a marker for DNA damage during inflammation, which is enriched in human atherosclerotic tissue (Takeshita *et al.*, 2006).

Another indicator for MPO-catalysed oxidation and specific protein-associated biomarker, formed either directly by  $HOCl$  attack or via intermediate formation of chlorine gas from  $HOCl$  (Hazen *et al.*, 1996), is 3-chlorotyrosine. 3-Chlorotyrosine was identified in human atherosclerotic lesion and lipoproteins extracted from lesions (Hazen and Heinecke, 1997; Zheng *et al.*, 2004), in respiratory tract disease (Buss *et al.*, 2003; Kettle *et al.*, 2004), and neutrophil-induced liver injury (Gujral *et al.*, 2004).

Immunocytochemistry and immunohistochemistry with specific monoclonal antibodies generated against  $HOCl$ -modified proteins/epitopes (Malle *et al.*, 1995) provides an immunological tool to identify chlorinated biomarkers in kidney disease (Malle *et al.*, 1997, 2003; Gröne *et al.*, 2002), ischaemia reperfusion (Hasegawa *et al.*, 2005), Parkinson's disease (Choi *et al.*, 2005), and atherosclerosis (Hazell *et al.*, 1996; Malle *et al.*, 2000). Fractionation of human plaque homogenate by density gradient centrifugation and subsequent immunoblot analysis of the low-density lipoprotein-bouyant fraction revealed that apoB-100, the major apolipoprotein of low-density lipoprotein is modified by the MPO– $H_2O_2$ – $Cl^-$  system of activated phagocytes in lesion material (Hazell *et al.*, 1996). *In vitro* studies have shown that lipoproteins, modified by  $HOCl$  (added as reagent or generated by the MPO– $H_2O_2$ – $Cl^-$  system), display a number of pathophysiological effects on phagocytes and vascular cells, contributing to initiation and maintenance of the



**Figure 1** Primary and secondary MPO-reaction products and (patho)physiology. Adapted and modified from Malle *et al.* (2006a). CNS, central nervous system.

inflammatory process during atherosclerotic lesion development (Daugherty *et al.*, 1994; Malle *et al.*, 2006a, b). In particular, the uptake and degradation of HOCl-modified lipoproteins by monocyte-derived macrophages via scavenger receptor-mediated pathways (Marsche *et al.*, 2001, 2003) is a leading event in the formation of cholesterol-enriched foam cells (Malle *et al.*, 2006a), representing the hallmark of fatty streaks and the earliest recognisable lesion of atherosclerosis. HOCl-modified (lipo)proteins are also high-affinity signalling transducing ligands for RAGE (Marsche *et al.*, 2007a, b), a multiligand receptor that, originally reported to bind advanced glycation end products, promotes an array of inflammatory complications.

In general, oxidation of amino-acid side chain appears to play a specific role during HOCl-mediated protein backbone fragmentation. The first step involves chloramine formation (probably from lysine side chains), giving rise to nitrogen-centred radicals in a time- and HOCl-concentration-dependent manner. These radicals may further form carbon-centred radicals at peptide bonds thereby promoting protein fragmentation and/or promote lipid peroxidation (Hawkins and Davies, 1998).

Although several groups have detected accumulation of lipid peroxidation products in HOCl-modified liposomes and lipoproteins, the mechanisms of lipid-hydroperoxide (ROOH) formation is not entirely clear (Malle *et al.*, 2006a). Panasenko *et al.* (1997) have suggested that this requires the presence of preformed ROOH able to induce further lipid peroxidation. During reaction with hydroperoxide, HOCl induces the formation of singlet molecular oxygen and comparable observations were reported for fatty acid and phospholipid hydroperoxides (Miyamoto *et al.*, 2006). The reaction of HOCl with preformed ROOH may be of particular importance at sites of inflammation where both, MPO/HOCl and ROOH concentrations are significantly elevated leading to the formation of secondary lipid/protein oxidation products.

In the absence of physiological  $\text{Cl}^-$  concentrations, formation of tyrosyl radicals promotes protein crosslinks via dityrosine formation, whereas nitrogen dioxide radical generates nitrated lipids. In addition, both radical species are able to induce lipid peroxidation (Figure 1).

As MPO levels of leucocyte- and blood-MPO are associated with the presence of coronary artery disease (Baldus *et al.*, 2003; Brennan *et al.*, 2003) and MPO considerably contributes to plaque vulnerability (for a review see: Nicholls and Hazen, 2005), quantitation of MPO mass and activity, as well as analysis of MPO-derived biomarkers (secondary MPO products, Figure 1) is of prognostic value to follow disease progression.

Development of therapeutic intervention strategies aiming at efficient MPO inhibition can be envisaged at different levels: (i) active site blockade of MPO, (ii) diversion of MPO from the chlorination cycle, (iii) irreversible suicide inhibition by use of oxidized inhibitors, and (iv) application of HOCl scavengers to prevent initiation and propagation of diseases with an inflammatory component. All these strategies have already been followed, but in a limited and non-systematic way. The provided information is distributed in journals of varying disciplines and often fragmentary or even

contradictory. It is the aim of this review to provide a systematic overview about potential inhibitors and their mode of action that is based on our present knowledge about structure-function relationship and (patho)physiological relevance of MPO.

#### *MPO transcription, translation, processing and release*

MPO is encoded by a single gene (approximately 14 kb in size), composed of 11 introns and 12 exons, and located on the long arm of chromosome 17 in segment q12-24. The primary 80 kDa translation product preproMPO is processed in the endoplasmic reticulum undergoing cotranslational N-glycosylation (resulting in an 90 kDa apoproMPO), transient interaction with molecular chaperones, and haem incorporation to generate enzymatically active proMPO that is exported into the Golgi compartment (for a review see: Hansson *et al.*, 2006). After exiting the Golgi, the propeptide is removed before final proteolytic processing in azurophilic granules. The processed MPO protein is a glycosylated, predominantly  $\alpha$ -helical cationic 146 kDa dimer with a single disulphide bridge between symmetry-related halves (73 kDa), each containing two polypeptides of 108 (14.5 kDa, light chain) and 466 amino acids (58.5 kDa, heavy chain). Dimeric MPO is found in neutrophils and monocytes. All available structural data refer to this mature dimeric MPO (Zeng and Fenna, 1992; Fiedler *et al.*, 2000; Blair-Johnson *et al.*, 2001) and the majority of studies on MPO inhibitors have been performed with this protein.

However, some proMPO escapes granule targeting and becomes constitutively secreted to the extracellular environment as a monomer (Hansson *et al.*, 2006). There is also evidence of *de novo* MPO synthesis in certain subtypes of macrophages and it has been suggested that foam cells newly engaged in MPO gene transcription lack the cellular machinery to proteolytically process proMPO into subunits of mature MPO. As a result, only proMPO enters the secretory pathway and is released in the extracellular environment. It is important to note that recombinant MPO overexpressed in Chinese hamster ovary cell lines (Moguilevsky *et al.*, 1991) resembles monomeric and partially unprocessed proMPO. Nevertheless, several spectral and kinetic investigations clearly demonstrated that mature MPO and recombinant proMPO exhibit identical enzymatic features (Jacquet *et al.*, 1991; Moguilevsky *et al.*, 1991; Furtmüller *et al.*, 2001), indicating identical molecular architecture of the haem cavity and substrate channel. Thus, recombinant proMPO represents a valid model for inhibitor studies and it is reasonable to assume that the action of inhibitors on mature dimeric MPO and monomeric (recombinant) proMPO is identical.

#### *Structural information about MPO*

The overall protein fold of MPO was first revealed by a 3 Å resolution crystal structure of the canine enzyme (Protein Data Bank (PDB) code: 1MYP) (Zeng and Fenna, 1992). By now, the structure of human MPO at 2.3 Å resolution is solved and refined to 1.8 Å using X-ray data recorded at  $-180^\circ\text{C}$  (1CXP) (Fiedler *et al.*, 2000). The structure of MPO

and its interaction with bromide ( $\text{Br}^-$ ) (1D2V) and thiocyanate ( $\text{SCN}^-$ ) (1DNU) (Fiedler *et al.*, 2000) as well as of the MPO-cyanide (MPO- $\text{CN}^-$ ) complex (1D5L) and its interaction with  $\text{Br}^-$  (1D7W) and  $\text{SCN}^-$  (1DNW) was published (Blair-Johnson *et al.*, 2001). In addition, there is one report on the crystal structure of salicylhydroxamic acid (SHA) bound to human MPO (Davey and Fenna, 1996), but unfortunately no entry in the PDB exists. Additionally, computational analysis of indole derivative binding to MPO by computational docking is reported (Hallingbäck *et al.*, 2006).

## Enzymatic properties and activity assays

### Chlorination activity

The principal reaction catalysed by MPO under physiological conditions is the oxidation of  $\text{X}^-$ , for example  $\text{Cl}^-$  and  $\text{Br}^-$  as well as  $\text{SCN}^-$  to the corresponding hypohalous acids (HOX) and hypothiocyanous acid (HOSCN) according to equation 1 (halogenation activity). HOX/HOSCN can participate in subsequent nonenzymatic reactions such as oxidation and halogenation of compounds present in the immediate environment (Figure 1). There is no well-defined pH optimum for MPO-catalysed chlorination because it depends on the relative concentrations of  $\text{Cl}^-$  and  $\text{H}_2\text{O}_2$ . However, at constant concentrations of these reaction partners, the rate of HOCl release increases with decreasing pH (Andrews and Krinsky, 1982). It is important to realize that at high concentrations,  $\text{H}_2\text{O}_2$  inactivates MPO. To minimize inactivation, the concentration should be kept at  $\leq 100 \mu\text{M}$ . Because amine-containing buffer systems are potent scavengers of HOCl activity, measurements must be performed in phosphate buffers.



Two assays are routinely used to measure the chlorination activity of MPO. The first assay involves chlorination of monochlorodimedon (MCD) by HOCl, a process that is accompanied by a decrease in absorbance ( $\epsilon_{290} = 1.99 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ ) (Kettle and Winterbourn, 1988). However, MCD cannot be regarded as an inert detector of HOCl production by MPO because it has been shown to function as competitive electron donor (Kettle and Winterbourn, 1988). Thus, the MCD assay underestimates the chlorinating activity (Kettle and Winterbourn, 1994). Nevertheless, this method is useful for detecting HOCl, as shown by its complete inhibition by methionine, and thus is applicable in inhibitor studies.

The second assay involves chlorination of nitrogen compounds, particularly primary amines. The reaction of HOCl with these compounds yields chloramines containing covalent nitrogen-chlorine bonds. The taurine chloramine assay is based on the reaction of HOCl with taurine ( $\text{TauNH}_2$ ) to produce taurine chloramine ( $\text{TauNHCl}$ ). Usually, chloramines are unstable and decompose to the corresponding aldehydes and nitriles. By contrast,  $\text{TauNHCl}$  is relatively stable and its formation can be either followed spectrophotometrically [ $\epsilon_{252} = 429 \text{M}^{-1} \text{cm}^{-1}$ ] (Thomas *et al.*, 1986)

or by measuring the oxidation of yellow 5-thio-2-nitrobenzoic acid ( $\epsilon_{412} = 14000 \text{M}^{-1} \text{cm}^{-1}$ ) to colourless 5,5'-dithio-bis(2-nitrobenzoic acid) (TNB) by  $\text{TauNHCl}$  (Weiss *et al.*, 1982). This is an extremely sensitive assay and can accurately measure concentrations of HOCl as low as  $5 \mu\text{M}$ .

Alternatively,  $\text{I}^-$  can be used to detect  $\text{TauNHCl}$  produced by MPO (Dyppbukt *et al.*, 2005).  $\text{TauNHCl}$  oxidizes  $\text{I}^-$  to HOI, which finally oxidizes 3,3',5,5'-tetramethylbenzidine (TMB) to a strongly absorbing blue product or dihydrorhodamine to highly fluorescent rhodamine, respectively (Dyppbukt *et al.*, 2005). The advantage over the TNB assay is that in this assay a signal is produced rather than quenched, which enhances its sensitivity and accuracy and offers its application to microtitre plate format (Dyppbukt *et al.*, 2005).

Besides MCD and  $\text{TauNH}_2$ , ascorbate (Chesney *et al.*, 1991) and TMB (Suzuki *et al.*, 1983) have been used as the basis for assaying the chlorination activity of MPO. The loss of ascorbate ( $\epsilon_{267} = 15000 \text{M}^{-1} \text{cm}^{-1}$ ) can be related to its oxidation by HOCl because the reaction is inhibited by methionine. However, results must be interpreted with caution because ascorbate can act directly as peroxidase substrate (equation 2). Similarly, oxidation of TMB ( $\epsilon_{285} = 2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ ) by HOCl or via the peroxidation cycle of MPO gives a blue product with an absorbance maximum ( $\epsilon_{652} = 3.9 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ ) (Josephy *et al.*, 1982). Generally, the TMB assay is extremely sensitive and has been used to quantitate the total utilization of  $\text{H}_2\text{O}_2$  by MPO. However, under usual assay conditions, the lack of inhibition by methionine indicates that the peroxidation activity completely overrides the chlorination activity.

### Peroxidase activity

MPO is also able to catalyse typical peroxidation reactions (equation 2) with  $\text{AH}_2$  representing the peroxidase substrate oxidized to the corresponding radical ( $\bullet\text{AH}$ ). Principally, each peroxidase assay described in the literature can also be performed with MPO.



Oxidation of tyrosine to dityrosine can be followed spectrofluorimetrically using an excitation wavelength of 325 nm and an emission wavelength at 405 nm (Marquez and Dunford, 1995). As already mentioned, TMB functions both as scavenger of HOCl and as peroxidase substrate. In the absence of  $\text{Cl}^-$ , the TMB assay can be used to monitor the peroxidatic activity of MPO (Marquez and Dunford, 1997). Another assay, following the oxidation of guaiacol to tetraguaiacol ( $\epsilon_{470} = 2.6 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ ) (Maehly and Chance, 1954) is also often used to follow MPO activity. Furthermore, a luciferin derivative (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazol[1,2- $\alpha$ ]pyrazin-3-one) has also been used for the determination of singlet oxygen produced from the reaction of HOBr with  $\text{H}_2\text{O}_2$  (Nakano and Koga, 1994).

### Hydrogen peroxide electrode assay

The loss of  $\text{H}_2\text{O}_2$  catalysed by MPO during halogenation and peroxidatic cycles (equations 1 and 2) can be continuously

monitored using a  $\text{H}_2\text{O}_2$  electrode (Kettle and Winterbourn, 1994). In the presence of  $\text{Cl}^-$  (up to 100 mM) as the only electron donor, this method allows direct assessment of the chlorination activity without the need for a detector compound that might potentially interfere with MPO. The formation of HOCl accounts completely for the loss of  $\text{H}_2\text{O}_2$ . It is recommended to include  $\text{TauNH}_2$  (10 mM) or methionine (500  $\mu\text{M}$ ) in the reaction buffer to scavenge HOCl and prevent it from inactivating MPO (Kettle and Winterbourn, 1994).

#### Assays for inhibitor studies

In our opinion screening for putative MPO inhibitors should include both, the use of the  $\text{H}_2\text{O}_2$  electrode and the  $\text{TauNHCl}$  assay, respectively. Polarographic monitoring of the loss of  $\text{H}_2\text{O}_2$  should be followed either in the presence of  $\text{Cl}^-$  or a peroxidase substrate (for example, tyrosine). This approach allows a first conclusion of (i) whether the inhibitor specifically interferes with the chlorination and/or peroxidatic cycle, (ii) whether the inhibitory mode is reversible or irreversible, and (iii) whether the putative inhibitor simply acts as a scavenger for HOCl or  $\text{TauNH}_2$ . In any case, measuring inhibition of only peroxidative activity is an inappropriate strategy in searching for inhibitors of HOCl production because many reversible inhibitors act by diverting MPO from the chlorinating cycle to the peroxidase cycle where they act as competitive electron donors for compounds I and II (see below).

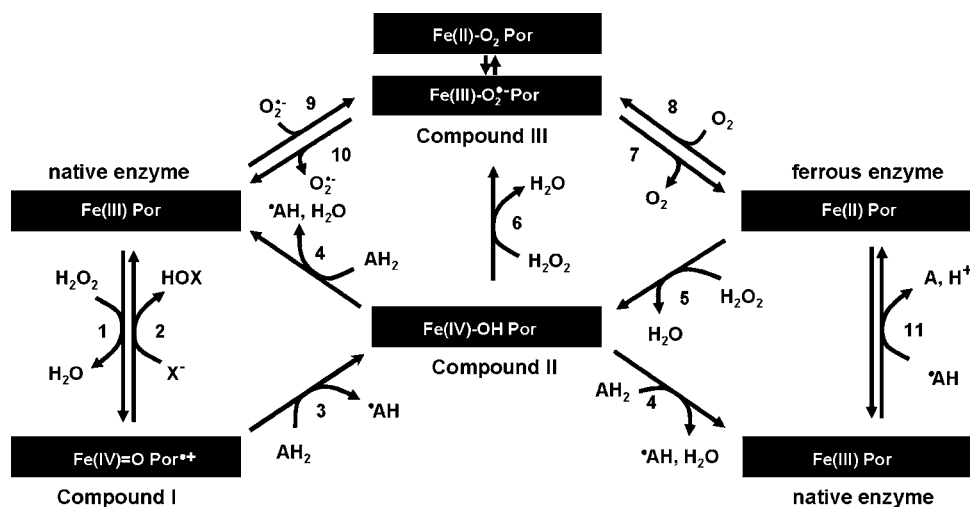
In high-throughput screenings, the polarographic method is inapplicable. Here, remaining  $\text{H}_2\text{O}_2$ , which has not been consumed either in the chlorination (equation 1) or peroxidase activity (equation 2) due to inhibitory effects, can be monitored photometrically or fluorimetrically

(Tarpey *et al.*, 2004). Caution is advised when horseradish peroxidase and a peroxidase substrate is used for  $\text{H}_2\text{O}_2$  visualisation, because the inhibitor of MPO could also impair the activity of horseradish peroxidase. Alternatively,  $\text{I}^-$ -mediated oxidation of TMB or dihydrorhodamine by  $\text{TauNHCl}$  can be used to elucidate the effect of putative inhibitors.

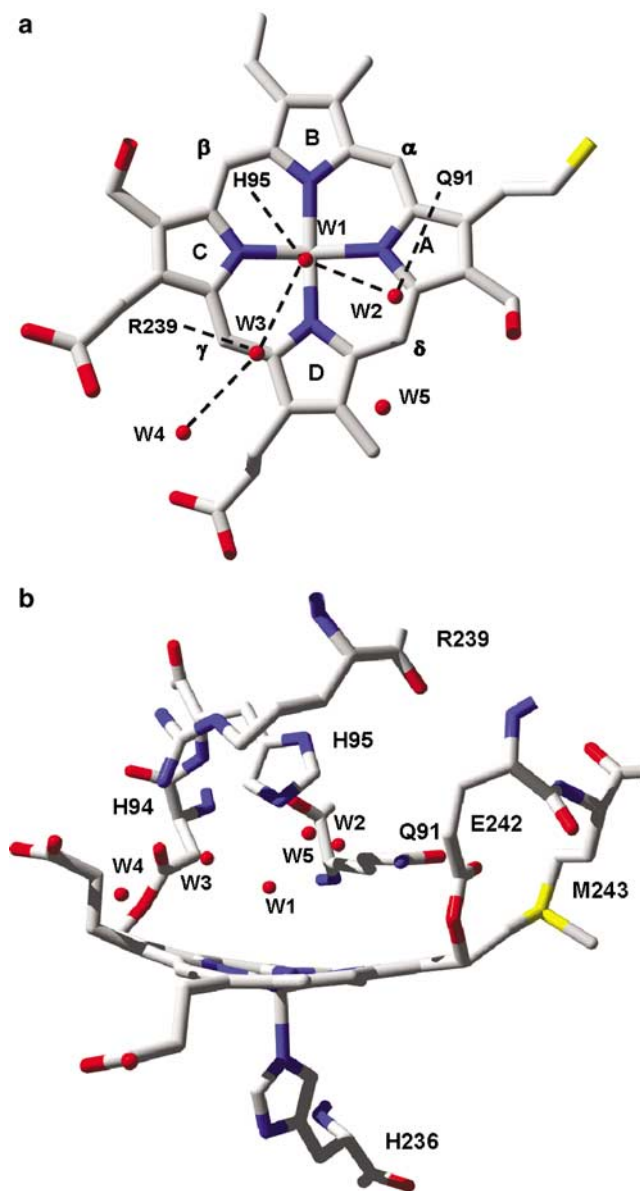
#### Redox intermediates of MPO

To understand the underlying mechanism of MPO inhibition it is necessary to investigate the interaction of potential inhibitors with the individual reaction steps both in the halogenation (equation 1) and peroxidation (equation 2) reactions. Firstly, the inhibitor could block the access of  $\text{H}_2\text{O}_2$  or its binding and reduction. As a consequence it would inhibit both cycles because both start by reaction of the Fe(III) form of MPO with  $\text{H}_2\text{O}_2$  to form compound I, which contains two oxidizing equivalents more than the resting enzyme (Figure 2, reaction 1). The reaction occurs at the distal haem cavity and amino acids His95 and Arg239 (Figure 3b) plays critical roles in the binding/orientation/activation and finally heterolytic cleavage of  $\text{H}_2\text{O}_2$ . Compound I is an oxoiron(IV) intermediate  $[\text{Fe}(\text{IV})=\text{O}]$  containing a porphyrin  $\pi$ -cation radical ( $\text{Por}^{\bullet+}$ ) (Dolphin *et al.*, 1971).

Once produced, compound I of MPO is a strong oxidant that mediates both one- and two-electron oxidation reactions. Depending on the apparent bimolecular rate constant and donor concentration, the enzyme follows either the halogenation (Figure 2, reactions 1 and 2) or the peroxidase cycle (Figure 2, reactions 1, 3 and 4). In the halogenation cycle  $\text{X}^-$  and  $\text{SCN}^-$  directly reduce compound I to the Fe(III)



**Figure 2** General reaction scheme of MPO. In the first step  $\text{H}_2\text{O}_2$  is used for compound I formation (reaction 1). Compound I is two oxidizing equivalents above the native enzyme with a porphyrin  $\pi$ -cation radical in combination with an oxoiron(IV) centre. Compound I can react with halides ( $\text{X}^-$ ) reducing the enzyme back to the ferric state (reaction 2, halogenation activity). In the peroxidase reaction, compound I is transformed in the first one-electron reduction to compound II, which contains an oxoiron(IV) centre (reaction 3). Compound II is finally reduced back to ferric peroxidase in a second one-electron reduction (reaction 4). Compound III (oxyperoxidase) is formed either from ferric peroxidase with superoxide anion (reaction 9), from ferrous MPO with  $\text{O}_2$  (reaction 8) or from compound II with  $\text{H}_2\text{O}_2$  (reaction 6). Compound III is a complex of ferrous-dioxygen in resonance with ferric superoxide. Ferrous  $[\text{Fe}(\text{II})]\text{MPO}$  can be formed from ferric  $[\text{Fe}(\text{III})]\text{MPO}$  by reducing radicals produced in the peroxidase cycle (reaction 11).



**Figure 3** (a) The hydrogen bonding network and locations of five water molecules (W1–W5) at the distal haem cavity of ferric high-spin MPO. (b) The non-planar porphyrin ring in MPO and its covalent attachments to the protein via two ester bonds (Glu242 and Asp94) and one sulfonium ion linkage (Met243). In addition, the proximal His336 and the distal catalytic residues His95, Arg239, and Gln91 are shown, the latter being important in halide binding. The figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1CXP).

form of MPO (Figure 2, reaction 2) releasing the corresponding HOX/HOSCN. The standard reduction potential of the couple compound I/native MPO is quite high (+1.16 V) (Arnhold *et al.*, 2001, 2006) enabling the enzyme to oxidize  $\text{Cl}^-$ . The putative binding site(s) of these small anionic inorganic two-electron donors and the impact of inhibiting substances on compound I reduction by  $\text{X}^-/\text{SCN}^-$  are described below.

In the peroxidase cycle, compound I is reduced by two successive one-electron steps via compound II, a protonated

oxoiron(IV) species (Figure 2, reactions 3 and 4). Many inorganic and organic substrates were found to act as electron donors for both compounds I and II (for a review see: Dunford, 1999; Furtmüller *et al.*, 2006). MPO compound I is an extremely strong one-electron oxidant, because the standard reduction potential of the couple compound I/compound II is 1.35 V; the standard reduction potential for the couple compound II/native enzyme is only 0.97 V (Furtmüller *et al.*, 2003). This marked difference in the oxidation capacity of MPO compounds I and II is strongly reflected by the actual reaction rate constants of these intermediates with substrates of one-electron reduction potentials  $>0.95$  V, for example indole and tryptamine derivatives (Jantschko *et al.*, 2005). The latter molecules are good electron donors for compound I but do not react with compound II thus diverting MPO from the chlorination cycle.

Owing to its strong oxidation capacity, MPO compound I is responsible for drug bioactivation (Yang *et al.*, 2006) including oxidation of phenytoin (Utrecht and Zahid, 1988; Kubow and Wells, 1989; Mays *et al.*, 1995), carbamazepine (Furst and Utrecht, 1993), L-dopa (Nappi and Vass, 2001), clozapine (Utrecht *et al.*, 1997; Williams *et al.*, 1997), trimethoprim (Lai *et al.*, 1999), fluperlapine (Lai *et al.*, 2000) and procainamide (Utrecht and Zahid, 1991) to reactive metabolites/intermediates (for example, nitroso, nitrogen free radical and iminium species). Diclofenac (Miyamoto *et al.*, 1997) undergoes *p*-hydroxylation by MPO, resulting in *p*-aminophenols, which can be further oxidized to reactive quinoneimines. However, MPO itself also oxidizes many potential inhibitors. The present knowledge concerning their binding and electron transfer site(s) is summarized below.

With respect to inhibitory mechanisms further redox intermediates have to be discussed, which neither participate in the halogenation nor in the peroxidase cycle, namely the Fe(II) form of MPO and compound III, the latter being the ferrous-dioxy/ferric-superoxide anion complex of MPO (Figure 2). Similar to higher oxidation states the reduction potential of Fe(III)/Fe(II) of MPO is significantly higher (5 mV) (Battistuzzi *et al.*, 2006) than that of other haem peroxidases. Thus, MPO is very susceptible to reduction to its Fe(II) form, that is by radical intermediates formed during the peroxidase cycle (Figure 2, reaction 11). In the presence of oxygen Fe(II), MPO is readily converted into compound III (Figure 2, reaction 8). Alternatively, compound III can be formed by reaction of ferric MPO with superoxide anion (Figure 2, reaction 9) or of compound II with  $\text{H}_2\text{O}_2$  (Figure 2, reaction 6). It is important to note that compound III should be regarded as a complex that can decay to ferric MPO and superoxide anion (Figure 2, reaction 10) or ferrous MPO and oxygen (Figure 2, reaction 7).

### Substrate and inhibitor binding sites

To be able to rationally design therapeutically useful MPO inhibitors, a detailed knowledge of the structural features of the enzyme is essential. Each monomer or catalytic domain of dimeric MPO or monomeric proMPO contains one iron

atom (present as covalently bound ferri-protoporphyrin IX derivative) and one  $\text{Ca}^{2+}$  ion. The  $\text{Ca}^{2+}$ -binding site has typical pentagonal bipyramidal coordination (Zeng and Fenna, 1992; Fiedler *et al.*, 2000). Because one of its ligand, Asp96, is in close vicinity to the distal His95 (Figure 3b),  $\text{Ca}^{2+}$  is important in maintaining the distal architecture but does not participate immediately in substrate and/or inhibitor binding. A unique structural feature of MPO concerns the modification of the 1- and 5-methyl groups on pyrrole ring A and C of the haem group (Figures 3a and b) enabling formation of ester linkages with the carboxyl groups of an Asp94 and Glu242. In addition the  $\beta$ -carbon of the vinyl group on pyrrole ring A forms a covalent bond with the sulphur atom of methionine 243, giving rise to a sulphonium ion linkage (Figures 3a and b) (Zeng and Fenna, 1992; Fiedler *et al.*, 2000), which is responsible for the peculiar redox properties of MPO (Arnhold *et al.*, 2006; Zederbauer *et al.*, 2007b). As a consequence, the haem porphyrine ring is considerably distorted from planarity (Figure 3b). Although pyrrole rings B and D are nearly coplanar, ring A and, to a lesser extent ring C are tilted toward the distal side, resulting in a bow-shaped haem structure. The proximal haem ligand is a histidine, which is hydrogen bound with an asparagine.

The haem of MPO is located in a crevice, about 15 Å in depth, with access to the solvent via an open channel, approximately 10 Å in diameter (Figure 5). It discharges into the distal cavity that accommodates the amino-acid triade histidine, arginine and glutamine (Figure 3b). The distal His95 is hydrogen bonded to a water molecule (W1), which is positioned approximately mid-way between the  $\text{N}_\epsilon$  atom of His95 and the haem iron (Figures 3a and b). Its distance from both the histidine nitrogen and the iron atom suggests that it is hydrogen bonded to the histidine and not strongly coordinated to the haem iron. Four additional water molecules (W2–W5) form hydrogen bonds with His95, Arg239, Gln91, the haem pyrrole ring C propionate and between themselves (Figure 3a). Water W2 is hydrogen bonded to the  $\text{N}_\epsilon$  atom of Gln91, W3 to  $\text{NH}_2$  of Arg239, W4 to haem propionate of pyrrole ring C and W5 having no additional hydrogen bonding (Figure 3a) (Fiedler *et al.*, 2000). Generally, the covalent haem attachment in MPO causes small solvent reorganisation effects in the reduction reaction of Fe(III) MPO (Battistuzzi *et al.*, 2006), suggesting a high rigidity of the hydrogen bond network involving water molecules in the substrate channel leading to the distal haem site.

#### Halide binding site

In the MPO– $\text{Br}^-$  complex (data not shown),  $\text{Br}^-$  replaces water molecule W2, which is hydrogen bonded to the amide of Gln91 in the proximity of the  $\text{N}_\epsilon$  atom of His95. Unfortunately, there is no structure of a  $\text{Cl}^-$  bound to the distal haem cavity. Figures 4a and b allow a comparison of the distal cavities of the MPO– $\text{CN}^-$  complex with the MPO– $\text{CN}^-$ – $\text{Br}^-$  double complex. In the MPO– $\text{CN}^-$ – $\text{Br}^-$  double complex, which could be regarded as a model for compound I with bound  $\text{Br}^-$ , W2 is present and  $\text{Br}^-$  binds in place of W5 thereby preventing direct hydrogen bonding to protonated His95. When  $\text{CN}^-$  is bound to the iron, the W2 site appears

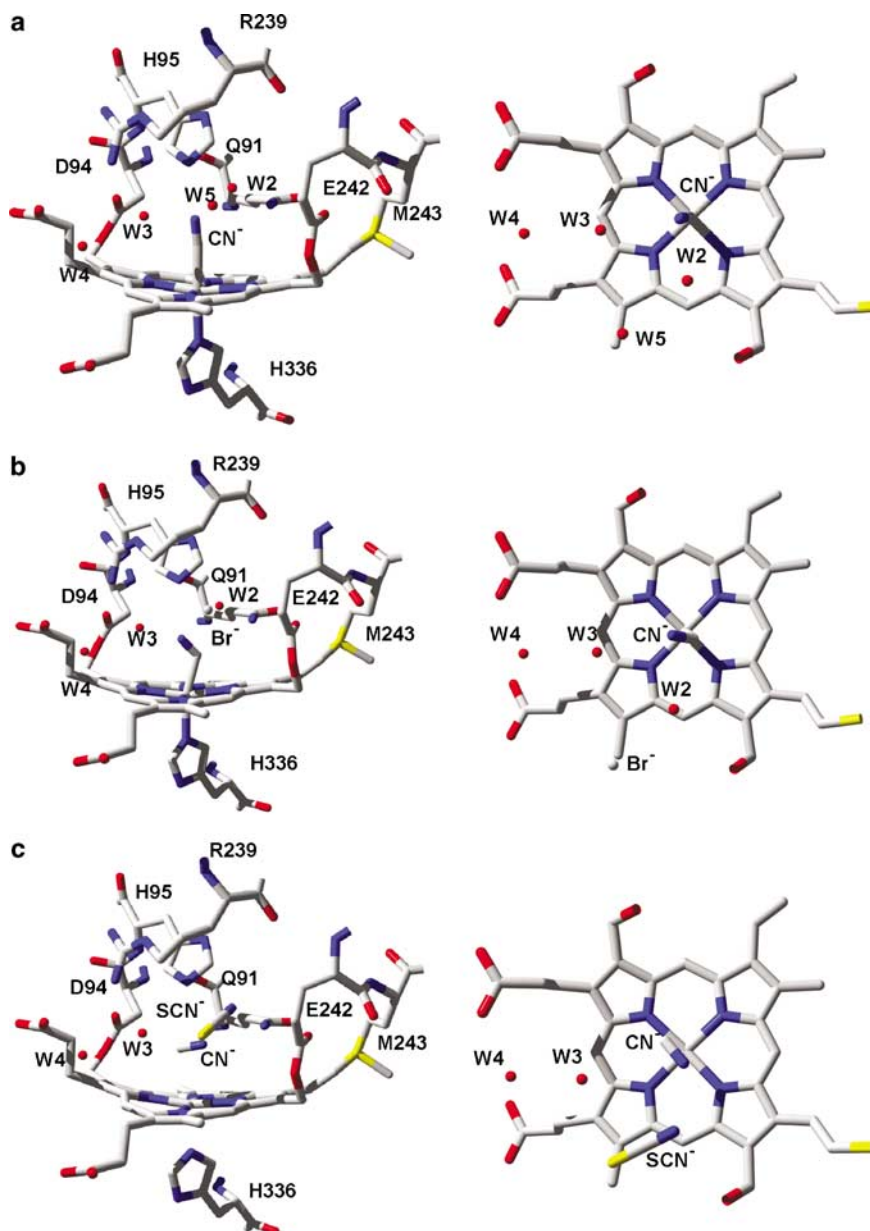
to be inaccessible for larger anions like  $\text{Br}^-$  or  $\text{I}^-$ . However, it is possible that the W2 position could accommodate  $\text{Cl}^-$  equally well in both native MPO and its  $\text{CN}^-$  complex, because  $\text{Cl}^-$  (1.81 Å) is significantly smaller in radius than  $\text{Br}^-$  (1.96 Å) or  $\text{I}^-$  (2.2 Å). The sterical hindrance of  $\text{Br}^-$  and  $\text{I}^-$  to bind at the W2 position in compound I might be reflected by the fact that with increasing radius of the anionic substrates, the increase in the rate of compound I reduction by these donors at acidic pH is less pronounced compared to  $\text{Cl}^-$  oxidation (Furtmüller *et al.*, 1998). Binding of  $\text{Cl}^-$  at position W2 together with the MPO typical distal water network of low mobility could be crucial in fixing the position of  $\text{Cl}^-$  and in favouring the transfer and incorporation of the oxyferryl oxygen into HOCl. In any case at neutral pH,  $\text{Cl}^-$  is bound weakly ( $K_D > 100$  mM) and needs a rigid hydrogen-bonding network, which is easily destabilized by peroxidase substrates or inhibitor binding at the haem edge (see below).

There is evidence from kinetic studies that the  $\text{Cl}^-$  level (2 mM) used to obtain the X-ray structure of the MPO– $\text{Cl}^-$  complex could be insufficient to occupy the distal binding site (Proteasa *et al.*, 2007). Moreover, based on the biphasic behaviour of nitric oxide binding to MPO in the presence of increasing  $\text{Cl}^-$  concentrations, two distal  $\text{Cl}^-$  binding sites of different affinities have been proposed (Proteasa *et al.*, 2007).

In the X-ray structure, the closest haem atom of  $\text{X}^-$  bound at the position of W2 is the  $\delta$ -methine bridge between pyrrole rings A and D (Figures 4a and b) at a distance of 3.8 Å, and the haem iron is at a distance of 5.0 Å. A  $\text{X}^-$  bound at the position of W2 is also in vicinity to the side chain of Glu242, which is only 3.5 Å away from W2 in the native enzyme. Although Met243 is relatively distant and not involved in positioning of W2 in native MPO or  $\text{Br}^-$  in the MPO– $\text{Br}^-$  complex (data not shown), its exchange has a tremendous effect on the  $\text{Cl}^-$  affinity. The affinity is decreased by 100-fold, compared to recombinant proMPO (Kooter *et al.*, 1997). In contrast, the exchange of Glu242 and Asp94 by 242Gln and 94Val in MPO mutants is relatively small (Zederbauer *et al.*, 2005, 2007a). A specific role of Met243 in  $\text{X}^-$  binding cannot be inferred by inspection of the structures but might be again related to its role in maintaining a rigid structural environment at the distal site that enables the binding of small anionic ligands (Battistuzzi *et al.*, 2006).

In the MPO– $\text{CN}^-$ – $\text{Br}^-$  double complex (Figure 4b) (Blair-Johnson *et al.*, 2001) the  $\text{Br}^-$  binding site is close to the  $\delta$ -methine bridge carbon and does not appear to form any readily identifiable electrostatic interactions with the enzyme, the haem, or the bound  $\text{CN}^-$ . The closest amino acid is Glu242 and the nearest neighbouring haem atom is the pyrrole ring D methyl carbon (Blair-Johnson *et al.*, 2001). The close proximity of Glu242 to positions of both W2 and W5 is underlined by the fact that compound I reduction by  $\text{X}^-$  is generally decreased in Glu242Gln mutants irrespective of the nature of the  $\text{X}^-$  (Zederbauer *et al.*, 2005). By contrast, disruption of the haem to Asp94 ester linkage affected the oxidation of  $\text{Cl}^-$  and  $\text{Br}^-$  but not of  $\text{I}^-$  and  $\text{SCN}^-$  (Zederbauer *et al.*, 2007b).

In the MPO– $\text{SCN}^-$  complex (data not shown),  $\text{SCN}^-$  is arranged almost parallel with the plane of the haem replacing water molecules W2 and W5 in the native enzyme.



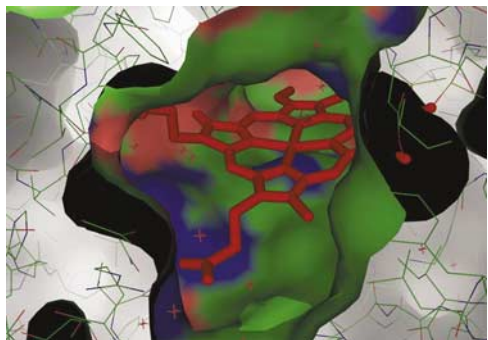
**Figure 4** (a) Structure of the myeloperoxidase-cyanide (MPO-CN<sup>-</sup>) complex (accession code 1D5L), which can be regarded as a model of compound I. In the CN<sup>-</sup>-complex water molecule W1 is displaced by CN<sup>-</sup>. (b) Structure of the MPO-CN<sup>-</sup>-bromide (Br<sup>-</sup>) double complex (accession code 1D7W). CN<sup>-</sup> and Br<sup>-</sup> are located at positions of W1 and W5. Note that in the MPO-Br<sup>-</sup> complex (data not shown), the halide is binding at W2. (c) Structure of the MPO-CN<sup>-</sup>-thiocyanate (SCN<sup>-</sup>) double complex (accession code 1DNW). In this complex CN<sup>-</sup> displaces W1, whereas SCN<sup>-</sup> displaces both W2 and W5.

The nitrogen of SCN<sup>-</sup>, at the W2 position, forms hydrogen bonds with the amide NH<sub>2</sub>-group of Gln91 and water molecule W2 as well as a weak interaction with the N<sub>ε</sub> atom of distal His95, whereas the sulphur makes van der Waals contacts with C<sub>γ</sub> of Glu242, C<sub>δ</sub> of Arg239, and the haem pyrrole ring D methyl carbon. SCN<sup>-</sup> in the distal cavity of the MPO-CN<sup>-</sup> complex (Figure 4c) (Blair-Johnson *et al.*, 2001) occupies the same position as in the native enzyme, except that it is inclined ≈ 15° from its former orientation parallel with the haem. In the presence of CN<sup>-</sup>, the nitrogen atom in SCN<sup>-</sup> can form only a single hydrogen bond with Gln91.

#### Aromatic substrate/inhibitor binding site

Binding studies have indicated that aromatic molecules bind near to the distal haem pocket in MPO (Hori *et al.*, 1994). On the basis of electron paramagnetic spectroscopy and molecular modelling it was proposed that the hydroxamic acid side chains of benzylhydroxamic acid (BHA) and SHA interact with the haem iron in MPO (Ikeda-Saito *et al.*, 1991). Finally, the 2.3 Å resolution X-ray structure of the MPO-SHA complex was published (Davey and Fenna, 1996) showing the aromatic ring of SHA binding to a hydrophobic region at the entrance to the distal haem pocket between pyrrole ring D and the side chain of Arg239 (Figure 6a). The





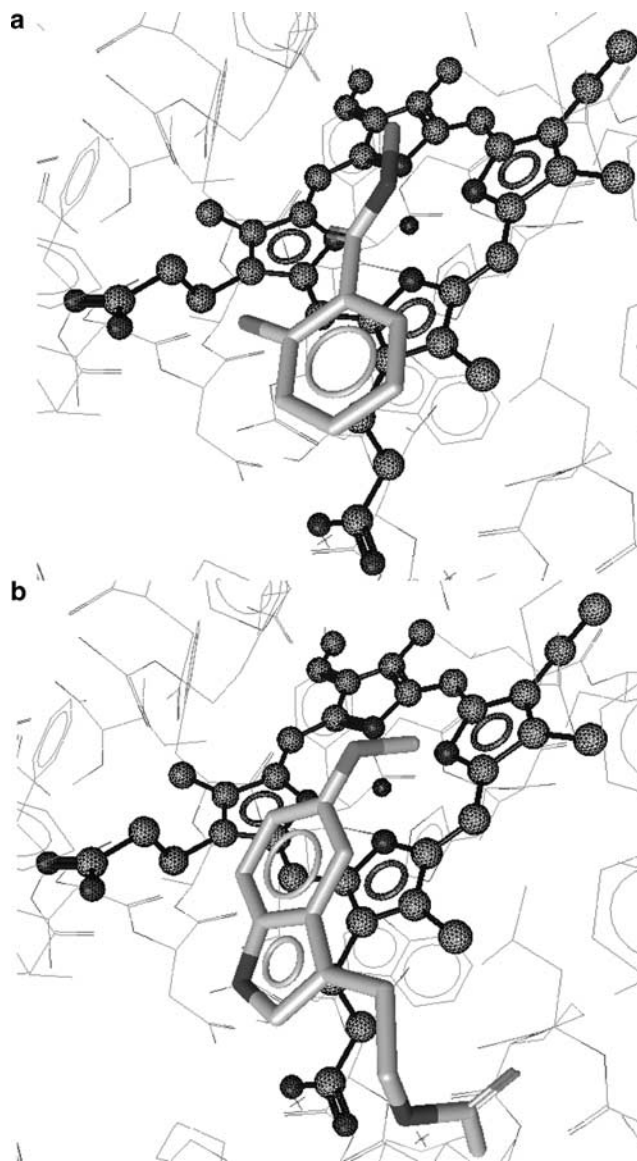
**Figure 5** View through the access channel to the active site (complexed with cyanide) showing exposure of the haem pyrrole ring D with its 8-methyl and of the  $\delta$ -methine bridge (that is, electron transfer site). The figure was constructed using PyMol Viewer (version 0.99).

hydroxamic acid moiety is hydrogen bonded to both the distal His95 and Gln91 amide group but is not coordinated to the haem iron. Although SHA binding displaces three water molecules from the distal cavity (W1, W2 and W3), no significant conformational differences between the active site regions of the complex and the native enzyme became apparent.

The hydrophobic region at the entrance to the distal cavity is conserved among mammalian peroxidases (Figure 5) (Furtmüller *et al.*, 2006). The aromatic ring of SHA (Figure 6a) is tilted about  $20^\circ$  with respect to that of the haem pyrrole ring D which forms the lower surface of the hydrophobic cavity, whereas the  $\beta$ ,  $\gamma$  and  $\delta$  carbons of Arg239 form the upper surface (Davey and Fenna, 1996). In the MPO–SHA complex, the aromatic ring of SHA is almost centred above the pyrrole ring D 8-methyl group. Additional van der Waals interactions occur with adjacent phenylalanines, that is Phe99, Phe366 and Phe407, each of which has an aromatic ring within 4.2–4.6 Å of the aromatic ring of the SHA molecule.

It is reasonable to assume that substrate binding to compounds I and II (Figure 2, reactions 3 and 4) is identical. The proposed electron transfer most probably occurs near the  $\delta$ -methine bridge carbon (Davey and Fenna, 1996). Mutation of neighbored Glu242Gln decreases the overall rate of guaiacol and tyrosine oxidation compared to non-mutated recombinant proMPO (Zederbauer *et al.*, 2005). Similar to the negative effect on  $X^-$  oxidation, disruption of the neighbored Glu242 ester bond seems to decrease the electron transfer between the donor molecule and the haem moiety. Interestingly, oxidation of the hydrophilic donor ascorbate by both compounds I and II was enhanced in Glu242Gln mutated compared to non-mutated recombinant proMPO (Zederbauer *et al.*, 2005), suggesting that the described binding scenario fits to aromatic but not to hydrophilic one-electron donors.

Recently, the binding of melatonin (*N*-acetyl-5-methoxytryptamine) and serotonin (5-hydroxytryptamine) to MPO in its Fe(III) compounds I and II state was studied by computational docking (Hallingbäck *et al.*, 2006). Both indole derivatives could be docked into all three forms with their indole rings oriented parallel to the haem plane and



**Figure 6** Docking models for SHA (a) and melatonin (b) bound to MPO. The positions of the three oxygen atoms of SHA are close to the positions occupied by the three water molecules W1–W3 in the native enzyme (see Figure 3a). Both models were calculated using LigandScout 1.03 (Wolber and Langer, 2005) from Inte:Ligand GmbH ([www.inteligand.com](http://www.inteligand.com)). SHA, salicylhydroxamic acid.

close enough to the D pyrrole ring to achieve stacking (Figure 6b). The distances of the closest indole atom to the centre of the D ring were about 3.4 Å for both substrates. The ligand side chain was never directed towards the distal cavity, whereas the indole substituent at position 5 was close to the haem centre (Hallingbäck *et al.*, 2006). In detail, the distance between the oxygen of the 5-methoxy or 5-hydroxyl substituent on the indole ring and the haem iron ion was calculated to be around 3.0 Å.

Computational docking of compounds I and II states of MPO (data not shown) demonstrated, that both, melatonin and serotonin were pushed about 1 Å away from the ferryl oxygen, which abolished the 5-substituent to point to the haem centre thus favouring an alternative binding mode

with a rotated indole still parallel to pyrrole ring D (Hallingbäck *et al.*, 2006).

## MPO inhibitors

### Hydroxamic acids

Hydroxamic acids [RCNOHOH or RC(O)NHOH] are known to act as reducing substrates for compounds I and II of haem peroxidases (Figure 2, reactions 3 and 4) and can also substitute for H<sub>2</sub>O<sub>2</sub> in compound I formation (reaction 3) (Schonbaum and Lo, 1972; Schonbaum, 1973). SHA has been reported to inhibit the luminol-dependent chemiluminescence of human neutrophils stimulated by phorbol 12-myristate 13-acetate or the chemotactic peptide *N*-formyl-methionyl-leucyl-phenyl-alanine (Davies and Edwards, 1989). Furthermore, SHA had no inhibitory effect on the kinetics of superoxide anion generation or O<sub>2</sub> uptake during the respiratory burst of phagocytes, but inhibited the peroxidase activity of purified MPO (IC<sub>50</sub> value of 3–5 μM). SHA also prevented the formation of compounds I and II but only at low H<sub>2</sub>O<sub>2</sub> concentrations, suggesting that it may compete for the H<sub>2</sub>O<sub>2</sub> binding site on the enzyme (Davies and Edwards, 1989).

Binding of SHA and BHA to dimeric leucocytic MPO, as studied by optical absorption (Ikeda-Saito *et al.*, 1991) and electron paramagnetic spectroscopy (Hori *et al.*, 1994), showed dissociation constants of 2 μM (SHA) and 5 mM (BHA) as well as the involvement of an ionizable group on the protein with a pK<sub>a</sub> of approximately 4. In the SHA–MPO inhibitory complex determined at 2.3 Å resolution (Davey and Fenna, 1996), it is clearly shown that this residue is the distal histidine, because the hydroxamic acid moiety is hydrogen bonded to His95 (Figure 6b). The ability of the three SHA oxygen atoms to closely duplicate the hydrogen-bonding pattern of W1–W3 in the native state (Figure 3a) could account for the strong binding of SHA to MPO. The salicyl-ring hydroxyl oxygen participates in hydrogen bonding to the guanidinium group of Arg239 and to water molecule W2 that remains hydrogen bonded to the pyrrole ring C propionate carboxyl group. As described above, a separate hydrophobic interaction occurs between the aromatic ring and a hydrophobic region at the entrance to the distal haem cavity suggesting that oxidation occurs at the haem edge, in the vicinity of the δ-meso carbon and the 8-methyl group on pyrrole ring D (Davey and Fenna, 1996).

In any case, SHA plays dual inhibitory roles in peroxidase catalysis by inhibiting hydroperoxide binding to the iron and by competing with other donors in reactions with higher oxidation states. However, this inhibition is not irreversible and strongly depends both on H<sub>2</sub>O<sub>2</sub> and competing electron donor concentration.

### Benzoic acid hydrazides

Hydrazines (RNHNH<sub>2</sub>) and hydrazides (RCONHNH<sub>2</sub>) have been reported to be suicide substrates of other haem peroxidases than MPO (Ator *et al.*, 1987). The antituberculous agent isonicotinic acid hydrazide (isoniazid) inhibits

both the chlorination and peroxidation activity of MPO irreversibly. Oxidation of isoniazid or hydrazine sulphate between pH values 6.5 and 7.8 by the MPO–H<sub>2</sub>O<sub>2</sub> system caused irreversible loss of haem absorbance associated with compound III formation (van Zyl *et al.*, 1989a). Finally, during a study using a series of benzoic acid hydrazides, it was demonstrated that these are general inhibitors of the peroxidation and chlorination activity of MPO (Kettle *et al.*, 1995). Unsubstituted benzoic acid hydrazide and its 4-chloroderivative were poor inhibitors, whereas derivatives with substituents containing oxygen or nitrogen were much better inhibitors. Benzoic acid hydrazides are readily oxidized by compound I (Figure 2, reaction 3) and the rate constants for these reactions are relatively insensitive to the substituents on the aromatic ring (Burner *et al.*, 1999). By contrast, reduction of compound II (Figure 2, reaction 4) by benzoic acid hydrazides strongly correlated with the substitution patterns and to the Hammett rule, and there were also significant correlations with Brown–Okamoto substituent constants (Hansch and Leo, 1979; Burner *et al.*, 1999). This indicates that the rates of these reactions were simply determined by the redox potential of the substrates and that the incipient free radical carried a positive charge, which is delocalized via resonance between the substituent on the aromatic ring and the hydrazide group.

4-Aminobenzoic acid hydrazide (ABAH) was the best electron donor of compound II and also the most potent inhibitor of peroxidation. ABAH irreversibly inhibited HOCl production of MPO isolated from leucocytes with an IC<sub>50</sub> value of 0.3 μM. The kinetic analysis of the inactivation conformed to that for a mechanism-based inhibitor (Kettle *et al.*, 1997). ABAH is oxidized in the peroxidase cycle by compounds I and II and the produced radicals readily convert the enzyme into ferrous MPO (Figure 2, reaction 11) and compound III (Figure 2, reaction 8). Compound III is neither part of the peroxidase nor of the chlorination cycle thus impeding oxidation of reducing substrates reversibly. Its turnover is slow and ABAH or an ABAH radical may reduce it in analogy to the reduction of oxyhaemoglobin by phenylhydrazine (Misra and Fridovich, 1976). In the absence of oxygen, ferrous MPO accumulates and irreversible inactivation is exacerbated by destruction of the haem prosthetic group (Kettle *et al.*, 1997). Protection by dioxygen suggests that ferrous MPO, permanently formed by reactions 11 and 7 (Figure 2) (that is, dissociation of the dioxygen–Fe(II) complex), is the actual target of irreversible inactivation but the detailed mechanism is still unknown (Burner *et al.*, 1999).

With neutrophils stimulated with opsonized zymosan or phorbol myristate acetate, ABAH inhibited HOCl production by up to 90% and corresponding IC<sub>50</sub> values were 16 and 2.2 μM, respectively. However, in the presence of superoxide dismutase, these values decreased to 6.4 and 0.6 μM, indicating that superoxide anion similar to molecular oxygen protects MPO to some extent from irreversible inactivation. ABAH was shown to have no effect on superoxide anion production and degranulation of neutrophils, nor did it inhibit catalase or glutathione peroxidase (Kettle *et al.*, 1995).

### Indoles and tryptamines

Indole derivatives act as one-electron donors of compounds I and II of haem peroxidases (Figure 2, reactions 3 and 4) (Jantschko *et al.*, 2002). These molecules have been shown to act as reversible inhibitors of MPO when they exhibit a one-electron reduction potential  $E^{\circ}(A^{\bullet}, H^+/AH) > E^{\circ}$  (compound II/ferric MPO) = 0.97 V (Jantschko *et al.*, 2005). Generally, tryptamines were more effective in inhibition of chlorination than indoles and this strongly correlated with the determined ratios of rates of compound I to compound II reduction. 5-Fluorotryptamine and 5-chlorotryptamine were found to act as the most effective chlorination inhibitors during this study.

The putative binding mode of indole derivatives has been described above (Hallingbäck *et al.*, 2006). In ferric MPO, the indole ring binds parallel to the pyrrole ring D and the 5-OH group (serotonin, an excellent substrate) and 5-OCH<sub>3</sub> group (melatonin, a poor substrate) most probably points to the haem centre. In both cases, the indole ring seems to be in the same place. The orientation of the side chain constitutes the largest difference between melatonin and serotonin. In case of serotonin it points out into the access channel, whereas in case of melatonin it points into a pocket along the periphery of the distal cavity (Hallingbäck *et al.*, 2006). The ring substituent determines the actual rates of compounds I and II reduction with electron-donating groups by increasing the aromatic ring reactivity (Jantschko *et al.*, 2005). Electron-withdrawing substituents, for example -Cl and -F atoms, will increase the redox potential of the corresponding indole derivatives and, as a consequence, disqualify them to act as a peroxidase substrate. Owing to the extremely positive reduction potential of the redox couple compound I/compound II (Arnhold *et al.*, 2006), these substituents can still serve as electron donor for compound I but are unreactive towards compound II (Jantschko *et al.*, 2005). As a consequence, compound II accumulates and MPO is diverted from its chlorination cycle (Figure 2).

In effect these indole and tryptamine derivatives are poor peroxidase substrates and reversible inhibitors. Under *in vivo* conditions compound II can easily be reduced by alternative electron donors thus diminishing the efficacy of these reversible-inhibiting compounds. This has been demonstrated by the effect of tryptophan on formation of HOCl by human neutrophils (Kettle and Candaeis, 2000). Using neutrophil-derived MPO, IC<sub>50</sub> values increased from 5 to 80 μM, because physiological substrates such as ascorbate, tyrosine, urate or superoxide anion are able to reduce compound II enabling the enzyme to cycle.

### Drugs that promote compound II formation

As outlined above, hydroxamic acid, indole and tryptamine derivatives promote compound II formation of MPO thus diverting the enzyme from the chlorination cycle. Kettle and Winterbourn (1991) have demonstrated that many anti-inflammatory drugs that are already in clinical application, follow the same reaction pattern. By using the H<sub>2</sub>O<sub>2</sub> electrode to assess the ability of these compounds to inhibit the conversion of H<sub>2</sub>O<sub>2</sub> into HOCl, dapsone,

mefenamic acid, sulphapyridine, quinacrine, primaquine and aminopyrine were found to induce 50% inhibition of the initial rate of H<sub>2</sub>O<sub>2</sub> loss at concentrations of approximately 1 μM or less. Phenylbutazone, piroxicam, salicylate, olsalazine, benzocaine, sulfasalazine (Kettle and Winterbourn, 1991), diclofenac (Zuurbier *et al.*, 1990), chlorpromazine (van Zyl *et al.*, 1990), acetaminophen (van Zyl *et al.*, 1989b), deferoxamine (Klebanoff and Waltersdorph, 1988) were also effective inhibitors (IC<sub>50</sub> < 30 μM). However, inhibition of MPO by all these drugs was reversed by ascorbate, which is known to reduce compound II to ferric MPO (Figure 2, reaction 4) (Hsuanyu and Dunford, 1999). Additionally, the inhibitory effects of these molecules were significantly diminished in the presence of a superoxide anion generating system, because superoxide anion acts as electron donor for compound II (Kettle *et al.*, 1993).

The mechanism of inhibition of HOCl production, which relies on MPO being trapped as inactive compound II, was also supported by the findings that dapsone and indomethacin are competitive inhibitors with respect to I<sup>-</sup> and Cl<sup>-</sup> (Stendahl *et al.*, 1978; Shacter *et al.*, 1991). All these drugs are efficiently oxidized by compound I but have a limited capacity to reduce compound II. This indicates that they are unable to compete with high-affinity peroxidase substrates indicating that measurement of inhibited peroxidative activity is an inappropriate strategy to screen for potential inhibitors of HOCl production by MPO.

The IC<sub>50</sub> values are influenced by the electrochemical properties of these compounds. Their reduction potential determines their reactivity towards MPO compounds I and II and thus their effectiveness as inhibitors of HOCl production. Compounds with reduction potentials > 1.1 V could be relatively specific for MPO because they would react poorly with other haem enzymes. For all these reversible inhibitors it is unlikely that size or hydrophobicity have a major impact on inhibition properties. This is best demonstrated by salicylate analogues, sulphasalazine and olsalazine, which have similar IC<sub>50</sub> values as salicylate, even though they are much larger and more hydrophobic (Kettle and Winterbourn, 1991).

In a recent study, recombinant proMPO has been shown to be convenient for pharmacological purposes (Neve *et al.*, 2001). Fourteen drugs representative of a family of various nonsteroidal anti-inflammatory drugs were tested for their ability to inhibit the MPO-mediated chlorination of TauNH<sub>2</sub>. Most of them induced a dose-dependent inhibition of TauNH<sub>2</sub> and IC<sub>50</sub> values could be calculated. The most efficient inhibitors (IC<sub>50</sub> < 40 μM) were found to be flufenamic acid, diclofenac, niflumic acid, tenoxicam and piroxicam. In all cases, these drugs interacted with recombinant proMPO in the same way as already described for dimeric MPO from neutrophils (Kettle and Winterbourn, 1991).

In any case, within the phagosome inhibition of intracellular HOCl formation by reversible inhibitors will be limited by superoxide anion and other electron donors of compound II. To achieve more efficient inhibition, drugs that irreversibly inhibit MPO (like benzoic acid hydrazides, see above) are needed.

#### Drugs that promote compound III formation

Compound III is positioned outside both of the chlorination and peroxidation cycle of MPO (Figure 2). Thus, drugs that promote compound III formation divert MPO also from HOCl production. Many drugs and xenobiotics are oxidized by MPO via the peroxidase cycle (Figure 2, reactions 1, 3 and 4) but in some cases the kinetics of oxidation cannot adequately be explained by this pathway alone. Hydroquinones are known to be excellent substrates for MPO compounds I and II (Kettle and Winterbourn, 1992; Burner *et al.*, 2000). Semiquinone radicals are formed in a high flux, which efficiently reduce ferric MPO to ferrous MPO that rapidly binds oxygen to form compound III (Figure 2, reactions 11 and 8). The same reaction pathway seems to be followed in the oxidation of the anticancer drug amsacrine by the MPO-H<sub>2</sub>O<sub>2</sub> system (Kettle *et al.*, 1992); in both cases production of HOCl by MPO is inhibited. However, in contrast to hydrazides that follow the same mechanism but finally irreversibly modify the active site (see above), the hydroquinone derivatives and amsacrine are reversible inhibitors because compound III is in dynamic equilibrium with the native enzyme or ferrous MPO (Figure 2, reactions 10 and 7) or can even be reduced to the native enzyme by ascorbate (Marquez *et al.*, 1990).

#### Further studies on putative MPO inhibitors

In a comparative study of the effects of nonsteroidal anti-inflammatory cyclooxygenase 1 and 2 inhibitors on MPO activity, it has been shown that indomethacin and other representative tricyclic aromatics display only poor inhibition of the peroxidase activity of MPO (EC<sub>50</sub> > 100 µM) (Rider *et al.*, 1996).

More promising molecules with inhibitory effects on the TauNH<sub>2</sub> chlorination activity of MPO are 2(3*H*)-benzoxazolone derivatives. In a recent study 20 ω-[2-oxo-3*H*-benzoxazol-3-yl]-*N*-phenylacetamide and propionamide derivatives (carrying substituents of different lipophilic and electrophilic nature on the *N*-phenyl ring) have been synthesized and shown to exhibit inhibitory effects on the chlorinating capacity of MPO (IC<sub>50</sub> 2–95 µM) (Soyer *et al.*, 2005). In general, propionamide derivatives with chlorine and nitro-substituents and non-substituted ones were more active than the acetamide counterparts. The authors reported that these compounds were unable to react with HOCl suggesting that they interact directly with MPO. However, there are no detailed mechanistic studies available.

The antithyroid drug methimazole (2-mercapto-1-methylimidazole) (Bandyopadhyay *et al.*, 1993, 1995) and the selenium analogue (Roy and Mughesh, 2005) have been shown to be irreversible mechanism-based inhibitors of human peroxidases (Bandyopadhyay *et al.*, 1993, 1995). Methimazole is oxidized by thyroid peroxidase, lactoperoxidase and MPO, because no inactivation occurred in the absence of H<sub>2</sub>O<sub>2</sub>. With lactoperoxidase and a gastric peroxidase the kinetics of the inactivation process were described to be consistent with a mechanism-based mode. With I<sup>-</sup> (good electron donor for lactoperoxidase) and addition of the spin-trap 5,5'-dimethyl-1-pyrroline *N*-oxide, but not with Cl<sup>-</sup> (poor electron donor for lactoperoxidase),

lactoperoxidase could be protected against inactivation. This clearly demonstrates that methimazole is oxidized to thiy radicals, which finally could be responsible for irreversible modification of the haem cavity and inhibition of lactoperoxidase (Bandyopadhyay *et al.*, 1993, 1995). Inhibition of MPO-mediated chlorination by methimazole is also based on the oxidation of methimazole but in contrast to lactoperoxidase inhibition of MPO seems to be only reversible (McGirr *et al.*, 1990; Sayo and Saito, 1991).

Propylthiouracil (also used as antithyroid drug) was also reported to inhibit the peroxidase and chlorination activity of MPO (Lee *et al.*, 1990). The inactivation was dependent on H<sub>2</sub>O<sub>2</sub> and was still observed after propylthiouracil and H<sub>2</sub>O<sub>2</sub> were removed by gelfiltration, which suggests an irreversible inactivation mode. With a gastric peroxidase it has been shown that propylthiouracil is a mechanism-based inhibitor but with MPO detailed mechanistic studies are lacking.

The last group of sulphur-centred compounds with inhibitory effects on MPO are thiourea derivatives. Dimethylthiourea (1–10 mM) has been reported to completely block formation of HOCl by phorbol myristate acetate- and zymosan-stimulated neutrophils (Sagone *et al.*, 1989). The compound has been shown to act both as a substrate for MPO and a scavenger for HOCl. From these data there is no evidence that dimethylthiourea mediates an irreversible inactivation of MPO (Sagone *et al.*, 1989).

#### Alternative therapeutic options to influence the MPO-H<sub>2</sub>O<sub>2</sub>-chloride system

Subjects with profound deficiencies in MPO (approximately one out of 4000 subjects) appear to have a significant increase in risk for infection (Nauseef *et al.*, 1998; Klebanoff, 2005); observations that parallel severe impairment in early host defence in MPO<sup>-/-</sup> mice against *Candida albicans* (Aratani *et al.*, 1999) and enhanced inflammation after allogeneic marrow transplantation (Milla *et al.*, 2004). In contrast, cardiovascular problems appear to be very rare among MPO-deficient individuals in comparison to their high frequency among the reference population (Kutter *et al.*, 2000). Hoy *et al.* (2001) reported that subjects carrying the *A* allele of the G-463A MPO polymorphism display higher levels of triglycerides, total and low-density lipoprotein-cholesterol, and apoB-100 which are known risk factors for atherosclerosis. Recent reports demonstrated that lipid-lowering drugs downregulate MPO on gene (Kumar and Reynolds, 2005) and protein level (Stenvinkel *et al.*, 2006; Zhou *et al.*, 2006); statins block 3-hydroxy-3-methylglutaryl-CoA-reductase, the key enzyme of endogenous cholesterol biosynthesis pathway, thereby apparently blocking production of isoprenoid intermediates of the mevalonate pathway, which is required for human and mouse MPO expression. Observations that an Alu receptor response element in the human MPO promoter is regulated by T0901317 and GW9578, respectively, suggest that MPO regulation is under tight control of LXR and PPAR $\alpha$ , which normally regulate genes involved in cholesterol metabolism and inflammation (Reynolds *et al.*, 2006).

Neutrophils protect themselves from HOCl by maintaining an exceptionally high cytoplasmic concentration of TauNH<sub>2</sub> (approximately 50 mM). TauNH<sub>2</sub> reacts with HOCl to yield the relatively innocuous compound TauNHCl that may act as immunomodulator by compensating adverse effects of primary and secondary MPO-derived products (Schuller-Levis and Park, 2003). Therapeutic approaches using TauNH<sub>2</sub> have revealed that this sulphur-containing amino acid may blunt the contribution of phagocyte activation to acute coronary events (McCarty, 1999). Consequently, TauNH<sub>2</sub> was used as HOCl scavenger in a number of animal studies. Diminution of atherosclerotic lesions has been reported in control and WHHL rabbits (lacking functional low-density lipoprotein receptor) (Petty *et al.*, 1990; Murakami *et al.*, 2002) when dietary-induced hypercholesterolaemia was accompanied by TauNH<sub>2</sub> supplementation. In the absence of TauNH<sub>2</sub>, abundant staining for HOCl-modified (lipo)proteins is present in these animals (Malle *et al.*, 2001; Bräsen *et al.*, 2003). Although endogenous MPO levels in rodents are much lower than in humans, TauNH<sub>2</sub> supplementation leads to diminution and regression of lipid accumulation in murine lesions after cholesterol feeding (Murakami *et al.*, 1999a, b). Most importantly, overexpression of human MPO in murine models promoted formation of atherosclerosis (McMillen *et al.*, 2005; Castellani *et al.*, 2006). Hyperlipidemia-induced renal damage in mesangioproliferative glomerulonephritis is paralleled by occurrence of HOCl-modified (lipo)proteins in rats (Scheuer *et al.*, 2000). The protective effect of TauNH<sub>2</sub> in rat models of human kidney disease is paralleled by a decrease in total proteinuria and albuminuria, prevention of glomerular hypertrophy, and diminution of glomerulosclerosis and tubulointerstitial fibrosis (Malle *et al.*, 2003).

Another possibility is the application of antioxidants, for example, ambroxol (Cho *et al.*, 1999), dithiocarbamate (Zhu *et al.*, 2002) and vitamin C (Carr *et al.*, 2000), which protect against or even may reverse HOCl- and chloramine-dependent modification of proteins by pathways probably involving reduced glutathione and ATP. Even phenolic compounds, for example chlorogenic acid (Kono *et al.*, 1995) might be considered as HOCl scavengers. Chlorogenic acid is approved as food additive (Svetol) and used in coffee, chewing gum, and mints to promote weight reduction.

Very efficient HOCl scavengers are antiarthritic drugs containing thiol groups, such as *D*-penicillamine, tiopronin, sodium aurothiomalate and aurothioglucose; it has been suggested that the therapeutic effect of these drugs may be due to the protection of tissues against reactive HOCl released by activated granulocytes at inflamed sites (Cuperus *et al.*, 1985). *D*-penicillamine, tiopronin, aurothiomalate or aurothioglucose (10–50 µM) are relatively potential inhibitors of MCD chlorination by HOCl. It has to be mentioned that besides effectively scavenging HOCl, *D*-penicillamine and tiopronin inhibit the enzyme itself (Cuperus *et al.*, 1983, 1985). Extrapolation to *in vivo* conditions is possible because the concentration of *D*-penicillamine and tiopronin in serum from patients treated with these drugs is about 100 µM (van der Korst *et al.*, 1981). The concentration of

gold compounds in patients treated with aurothiomalate and aurothioglucose is about 50 µM (Lewis *et al.*, 1983). Interestingly, all four antiarthritic drugs develop their beneficial effects over a period of weeks of therapy. Although the therapeutic effect of these antirheumatics may be multifactorial, scavenging of HOCl and inhibition of MPO offers an attractive explanation for their mechanism of action.

Another drug already applied in practice is thiacetazone. It is used to treat tuberculosis and has been reported to be also an effective HOCl scavenger as well as to promote compound III formation of MPO (van Zyl and van der Walt, 1996).

## Future perspectives

MPO inhibitory properties of a variety of compounds were assessed with different methodologies. The information on promising MPO inhibiting compounds in the literature is sparse making a comparison and critical evaluation of their actual inhibitory potential difficult. Additionally, only a few compounds were investigated in detail to clarify the underlying molecular mechanism of inactivation. Nevertheless, the knowledge of (i) the three-dimensional structure of human MPO and (ii) binding sites of its endogenous substrates (that is, two-electron donating anionic halides) and of artificial aromatic one-electron donors/inhibitors (for example, SHA or indoles) as well as our knowledge of (iii) its reaction mechanisms and redox properties will accelerate the process for a rationale design of specific MPO inhibitors in the future.

Almost all known MPO inhibitors are oxidized at least by compound I, which is one of the strongest oxidizing enzyme intermediates found *in vivo*. Thus, these inhibitors divert the enzyme from the chlorination cycle and suppress HOCl production. In case the reaction products do not interact with the protein, compound II accumulates, which can be easily reduced by many electron donors found *in vivo*, for example superoxide anion, tyrosine or ascorbate. This inhibition mode is reversible and the *in vivo* inhibitory effect of these drugs on HOCl is questionable.

The interaction of MPO with some drugs results in the formation of radicals that either attack the haem prosthetic group or functionally important active site residues (mechanism-based irreversible inactivation). Alternatively, they can mediate the reduction of ferric MPO to ferrous MPO, which is readily converted into its dioxygen complex (compound III) under aerobic conditions. In case these radicals are unreactive toward the enzyme this inhibition mode is also reversible. With benzoic acid hydrazides the products generated in the peroxidase cycle mediate the irreversible destruction of the haem group of MPO in its ferrous form, with ABAH being the most efficient suicide substrate (irreversible inhibitor). However, we still do not fully understand the molecular basis of this suicide inactivation nor do we know the nature of the modification at the haem cavity. Understanding these features will facilitate the design of more specific and more potent irreversible MPO inhibitors.

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## Conflict of interest

E Malle has no conflict of interest. PG Furtmüller is coinvestigator of a project (protec-TRANS sponsored by the Austrian ERP Fonds) executed by Planta Naturstoffe Vertriebs GmbH that investigates natural anti-inflammatory substances (January 1, 2005–July 31, 2007). W Sattler has no conflict of interest. C Obinger is consultant to the Myeloperoxidase Programme Team of GlaxoSmithKline Research & Development Ltd (September 1, 2005–August 31, 2007).

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