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MECHANISM OF INHIBITION OF MYELOPEROXIDASE BY ANTI-INFLAMMATORY DRUGS

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Abstract—Hypochlorous acid (HOCl) is the most powerful oxidant produced by human neutrophils, and should therefore be expected to contribute to the damage caused by these inflammatory cells. It is produced from H_2O_2 and Cl^- by the heme enzyme myeloperoxidase (MPO). We used a H_2O_2-electrode to assess the ability of a variety of anti-inflammatory drugs to inhibit conversion of H_2O_2 to HOCl. Dapsone, mefenamic acid, sulfapyridine, quinacrine, primaquine and aminopyrine were potent inhibitors, giving 50% inhibition of the initial rate of H_2O_2 loss at concentrations of about 1 μM or less. Phenylbutazone, piroxicam, salicylate, olsalazine and sulfasalazine were also effective inhibitors. Spectral investigations showed that the inhibitors acted by promoting the formation of compound II, which is an inactive redox intermediate of MPO. Ascorbate reversed inhibition by reducing compound II back to the active enzyme. The characteristic properties that allowed the drugs to inhibit MPO reversibly were ascertained by determining the inhibitory capacity of related phenols and anilines. Inhibition increased as substituents on the aromatic ring became more electron withdrawing, until an optimum reduction potential was reached. Beyond this optimum, their inhibitory capacity declined. The best inhibitor was 4-bromoaniline which had an i_{50} of 45 nM. An optimum reduction potential enables inhibitors to reduce MPO to compound II, but prevents them from reducing compound II back to the active enzyme. Exploitation of this optimum reduction potential will help in targeting drugs against HOCl-dependent tissue damage.

Neutrophils are implicated in the tissue damage that occurs in a broad spectrum of inflammatory diseases including ulcerative colitis, pyelonephritis, glomerulonephritis, and rheumatoid arthritis [1]. The oxidants generated during the respiratory burst of neutrophils undoubtedly contribute to the damage that occurs in these pathologies. When stimulated, neutrophils discharge superoxide (O_2^-) which is converted into more reactive secondary oxidants, including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [2]. The heme enzyme myeloperoxidase (MPO) uses H_2O_2 and Cl^- to produce HOCl, which is the most powerful neutrophil oxidant. It is extremely cytotoxic and reacts readily with most biological molecules, degrading structural proteins and inactivating enzymes. It readily inactivates the major plasma protease inhibitor α_1-antitrypsin and can activate neutrophil collagenase [3]. Thus, HOCl may also promote tissue damage indirectly by facilitating proteolysis at inflammatory sites. HOCl should therefore be expected to contribute to inflammatory tissue damage caused by neutrophils.

Scavengers of HOCl have been suggested as potential inhibitors of inflammatory tissue damage [4]. However, they may be of limited use because the extreme and indiscriminate reactivity of HOCl would require high concentrations of scavengers to prevent oxidation of susceptible biological targets. A more feasible approach is to develop specific inhibitors of MPO. Several anti-inflammatory drugs inhibit MPO [5–8], however, with the exception of D-penicillamine [9], there has been no detailed account of how they act. This information is essential if specific inhibitors of MPO are to be developed.

The normal catalytic cycle of MPO involves the reaction of H_2O_2 with the ferric enzyme (MP^{3+}) to produce compound I, which then reacts with Cl^- to regenerate the native enzyme and produce HOCl (Fig. 1 reactions 1 and 2) [2]. We showed that monochlorodimedon, which is a structural analogue of phenylbutazone, inhibits chlorination because it is oxidized by MPO to promote the accumulation of inactive compound II (Fig. 1 reactions 3 and 4) [10].

Anti-inflammatory drugs with oxidizable functional groups may act similarly to monochlorodimedon, and inhibition of MPO may in part account for their anti-inflammatory effects. To investigate this possibility we chose a range of anti-inflammatory drugs that have the potential to be oxidized by peroxidases, and determined their ability to inhibit conversion of H_2O_2 to HOCl. We have also determined the mechanism of inhibition and the characteristics of the drugs that were essential for preventing production of HOCl.

MATERIALS AND METHODS

Materials. Sodium salicylate, anthranilic acid, phenolsulphonic acid and 4-bromoaniline were from BDH Ltd (Poole, U.K.). 4-Nitroaniline and 4-aminophenazone (aminopyrine) were from Fisons Scientific Ltd (Loughborough, U.K.). 3-Hydroxybenzoic acid was from Fluka and 2,3-dihydroxybenzoic acid was from Aldrich Chemical Co. (Dorset, U.K.) Sulfosalicylic acid was from J. T.

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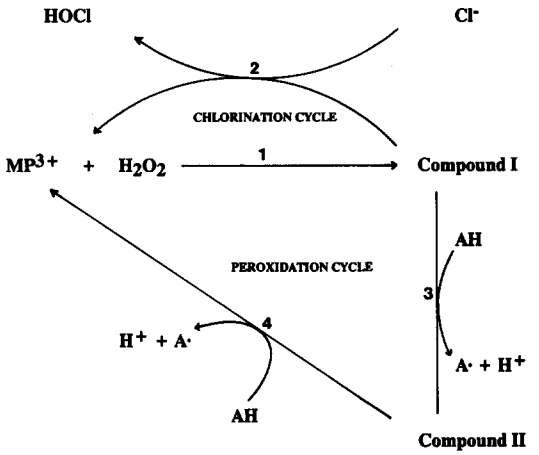

Fig. 1. The chlorination and peroxidation pathways of myeloperoxidase. AH is a peroxidase substrate or a reducing compound substrate such as monochlorodimedon.

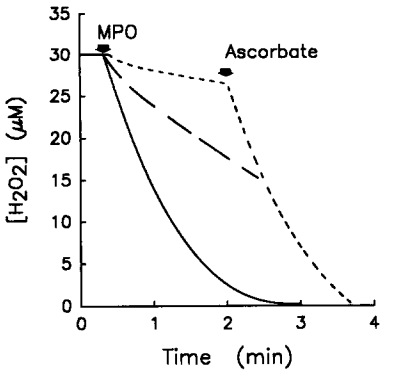

Fig. 2. The effect of dapsone on the loss of H_2O_2 catalysed by MPO. Reactions were started by adding 15 nM MPO to 30 μM H_2O_2 in 50 mM phosphate buffer (pH 7.4) containing 150 mM Cl^-, 30 μM H_2O_2 and either 0 μM (——), 0.5 μM (——) or 5 μM (-----) dapsone. Thirty μM ascorbate was added as indicated by the arrow.

Baker Chemical Co. (NJ, U.S.A.) 5-aminosalicylate, sulfasalazine, olsalazine and sulfapyridine were a gift from Mr Peter Chapman, Pharmacia (North Ryde, Australia). All other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Ten mM stock solutions of chemicals were prepared daily in water, dilute HCl or dilute NaOH. MPO was purified to a purity index of greater than 0.75 as described previously [11] and its concentration was determined using ε_{430} 91,000 $M^{-1}\,cm^{-1}$ [12]. Horseradish peroxidase (HRP) (type 1) and bovine liver catalase were purchased from the Sigma Chemical Co. Hydrogen peroxide solutions were prepared daily by diluting a stock solution and concentrations were determined by using ε_{240} 43.6 $M^{-1}\,cm^{-1}$ [13].

Methods. Myeloperoxidase activity was determined by measuring the loss of H_2O_2 continuously with a YSI model 25 oxidase meter fitted with a YSI 2510 oxidase probe (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The electrode was covered with an inner membrane of cellulose acetate to prevent interference from low molecular weight compounds, and an outer membrane of collagen film. Any compound that interfered with the H_2O_2 signal was excluded from this investigation. The electrode gave a linear response between 0 and 100 μM H_2O_2. Reactions were carried out at 25 ± 2° in 50 mM phosphate buffer (pH 7.4) with or without 150 mM Cl^-, plus 300 μM methionine to scavenge HOCl. Reactions were started by adding MPO. Initial rates were determined by drawing a tangent to the initial linear part of the curve for H_2O_2 loss (see Fig. 2). The effect of each compound on the loss of H_2O_2 was determined at a minimum of six concentrations which gave inhibition between 0% and the maximum possible for that compound. The maximum initial rate for the loss of H_2O_2 was determined in the absence of potential inhibitors, and replicate values for these controls had a standard deviation of less than ± 5% (N = 4). I_{50} values were calculated by fitting a rectangular hyperbola to the dose response curve using nonlinear regression. The correlation coefficient was always greater than 0.96.

Conversion of H_2O_2 to HOCl by MPO was determined by stopping the reaction after one minute with 100 μg/mL of catalase, and comparing H_2O_2 loss with the formation of taurine chloramine, which was assayed with 2-nitro-5-thiobenzoate (TNB) (ε_{412} 13,600 $M^{-1}\,cm^{-1}$) [14]. In these assays methionine was replaced with 10 mM taurine. TNB was formed by raising the pH of a 0.5 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to 10 and then readjusting it to 7.4 [15]. In agreement with our previous work [16], there was 100% conversion of H_2O_2 to HOCl by MPO with concentrations of H_2O_2 between 5 and 30 μM (data not shown). With 100 μM 5-aminosalicylate or 4-aminoantipyrine only 85% of HOCl reacted with taurine, which was accounted for when determining inhibitory effects of these drugs. In the presence of anti-inflammatory drugs taurine chloramine was assayed immediately and was found to be stable for at least 30 min.

Peroxidation of aminobenzoates was determined by measuring the formation of a brown product that absorbed at 440 nm. Visible absorption spectra of MPO were recorded on a Pye-Unicam PU 8800 spectrophotometer.

RESULTS

Effects of anti-inflammatory drugs on the activity of MPO

Production of HOCl by MPO, H_2O_2 and Cl^- was measured by continuously monitoring the loss of H_2O_2 in the presence of methionine. We have shown that all the H_2O_2 is converted to HOCl which reacts with methionine to form the sulphoxide [16]. The concentration of each anti-inflammatory drug that gave 50% inhibition of H_2O_2 loss (I_{50}) was determined by measuring initial rates over a range of drug concentrations. For example, the effect of dapsone on the loss of H_2O_2 is shown in Fig. 2. The I_{50} value

Table 1. The concentrations of anti-inflammatory drugs that gave 50% inhibition of the initial rate of H_2O_2 loss catalysed by MPO

Anti-inflammatory	I_{50} ± SE (μM)	(N)
Acetylsalicylate	>200	(4)
Salicylate	9.8 ± 1.8	(7)
Mefenamic acid	0.90 ± 0.08	(10)
Indomethacin	61.6 ± 5.8	(7)
Piroxicam	12.8 ± 2.2	(6)
5-Aminosalicylate	≈175	(9)
Olsalazine	14.7 ± 2.3	(8)
Sulfasalazine	10.4 ± 0.9	(7)
Sulfapyridine	1.0 ± 0.2	(11)
Dapsone	0.42 ± 0.05	(9)
Chloroquine	>200	(2)
Quinacrine	1.1 ± 0.1	(10)
Primaquine	0.23 ± 0.05	(10)
Phenylbutazone	30.5 ± 6.1	(6)
Aminopyrine	0.72 ± 0.09	(9)
Antipyrine	>200	(3)
4-Aminoantipyrine	P.S.	(3)

I_{50} values with their associated standard errors (SE) were calculated using N concentrations of drug as described in the methods section. P.S. indicates that the drug was a peroxidase substrate that promoted the loss of H_2O_2 in the absence of Cl^-. The inhibitors prevented the loss of H_2O_2 to a maximum of at least 90%. Conditions were as described in Fig. 1.

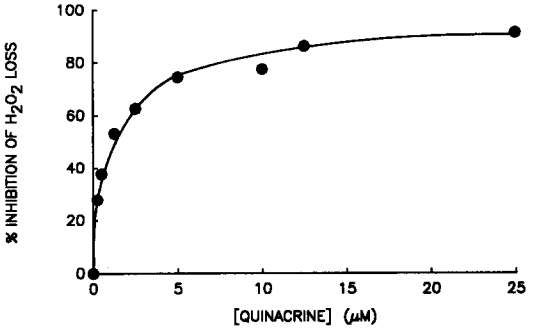

Fig. 3. The effect of quinacrine on H_2O_2 loss catalysed by MPO. The % inhibition of the initial rate of H_2O_2 loss was determined by adding 15 nM MPO to 30 μM H_2O_2 in 50 mM phosphate buffer (pH 7.4) containing 150 mM Cl^-, 30 μM H_2O_2 and varying concentrations of quinacrine.

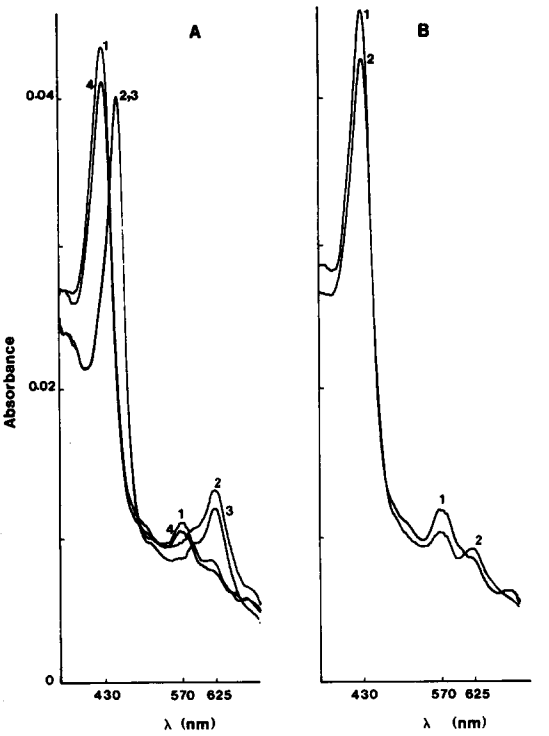

Fig. 4. The effects of dapsone and acetylsalicylate on the absorption spectrum of MPO. Spectrum (1): 0.5 μM MPO, 300 μM methionine and (A) 20 μM dapsone or (B) 20 μM acetylsalicylate; Spectrum (2): (1) plus 90 μM H_2O_2; Spectrum (3): two minutes after recording (2); Spectrum (4): (3) plus 50 μM ascorbate. Other conditions were as described in Fig. 1.

for each drug (Table 1) was then calculated from its dose–response curve, such as that shown for quinacrine (Fig. 3).

The majority of the anti-inflammatory drugs tested affected the activity of MPO. Mefenamic acid was the most effective inhibitor of the aspirin-like non-steroidal anti-inflammatory drugs, having an I_{50} of 0.9 μM. Salicylate and piroxicam were moderately effective, while indomethacin was a comparatively weak inhibitor. Acetylsalicylic acid gave minimal inhibition at concentrations less than 200 μM. The 5-aminosalicylic acid based drugs, sulfasalazine and olsalazine, inhibited MPO to approximately the same extent as did salicylate. However, 5-aminosalicylate was a relatively poor inhibitor and at low concentrations actually enhanced the loss of H_2O_2 (see below). In contrast, the related drugs sulfapyridine and dapsone were potent inhibitors of MPO. The best inhibitor was the antimalarial primaquine. Of the other antimalarials, quinacrine was a good inhibitor, while chloroquine failed to inhibit MPO. The two pyrazalones aminopyrine and phenylbutazone were good and moderate inhibitors respectively. However, 4-aminoantipyrine was a peroxidase substrate that hardly prevented the loss of H_2O_2 (see below). Antipyrine did not inhibit MPO.

To confirm that inhibition by the anti-inflammatory drugs accurately represented inhibition of HOCl production, we related the amount of HOCl produced to the H_2O_2 lost after one minute of reaction (Table 2). HOCl was scavenged by taurine to give its chloramine, which was detected with TNB. Within the sensitivity of our assays, all the H_2O_2 lost in the presence of dapsone, salicylate, 5-aminosalicylate or 4-aminoantipyrine was converted to HOCl. Thus, inhibition of H_2O_2 loss accurately represented inhibition of HOCl production.

As illustrated for dapsone (Fig. 2), inhibition of MPO by all the drugs listed in Table 1 was reversed by ascorbate. Ascorbate reduces compound II to

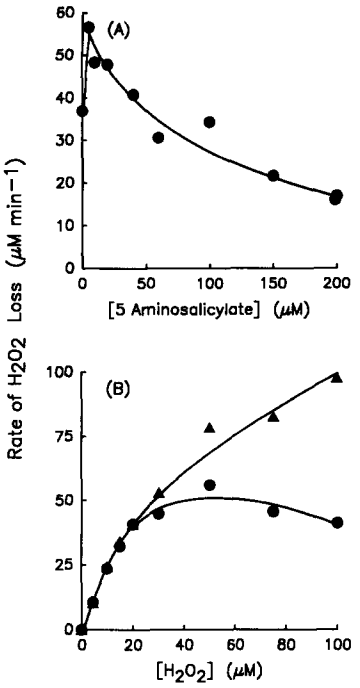

Fig. 5. The effect of 5-aminosalicylate on the initial rate of H_2O_2 loss catalysed by MPO. (A) The effect of the concentration of 5-aminosalicylate on H_2O_2 loss. (B) The effect of 5-aminosalicylate on H_2O_2 loss at varying concentrations of H_2O_2. (●) No 5-aminosalicylate and (▲) 10 μM 5-aminosalicylate. Other conditions were as in Fig. 1.

ferric MPO [17]. Therefore, this result strongly suggested that these drugs were acting by promoting the accumulation of compound II. We confirmed this hypothesis by showing that in the presence of H_2O_2 and Cl^-, MPO was converted to compound II by dapsone (Fig. 4A), sulfapyridine and aminopyrine. Compound II was converted back to ferric MPO by ascorbate. Spectral changes with primaquine, quinacrine and mefenamic acid were also indicative of compound II formation, although they were complicated by interference from oxidation products of these drugs. In contrast, there was little formation of compound II when H_2O_2 was added to MPO, Cl^- and acetylsalicyclic acid (Fig. 4B).

At a concentration of 200 μM, 4-aminoantipyrine inhibited MPO by only 25%. However, it changed the kinetics of H_2O_2 loss from exponential to linear, indicating that it was acting as a peroxidase substrate. This was confirmed by showing that with 200 μM 4-aminoantipyrine H_2O_2 was still consumed in the absence of Cl^-, although at only 25% of the rate that occurred in the presence of Cl^- (data not shown). Even though 4-aminoantipyrine is a peroxidase substrate, 10 or 100 μM scarcely affected the production of HOCl (Table 2).

5-Aminosalicylate behaved differently to the other drugs. At 5 μM it enhanced the initial rate of H_2O_2 loss but it became inhibitory as its concentration was increased (Fig. 5A). Virtually all the H_2O_2 lost at high or low concentrations of 5-aminosalicylate was accounted for by the HOCl produced (Table 2). 5-Aminosalicylate enhanced activity only when the concentration of H_2O_2 was greater than 20 μM (Fig. 5B). With concentrations of 5-aminosalicylate greater than 50 μM the rate of H_2O_2 loss declined appreciably after 30 sec. This inhibition may be irreversible since it was unaffected by ascorbate (results not shown).

Electronic effects as determinants of inhibition by phenols and anilines

All the anti-inflammatory drugs that inhibited MPO contained oxidizable substituents. It became apparent that oxidation of these groups contributed to inhibition when we compared salicylate (I_{50} 10 μM) with its substituted derivatives. Electron donating and withdrawing groups had differing effects, since 10 μM of 5-amino or 6-hydroxysalicylate increased the initial rate of H_2O_2 loss by 31 or 47% respectively, while 5-sulphosalicylic acid did not inhibit MPO.

To clarify the role electronic effects play in the inhibition of MPO, we determined I_{50} values for 3 and 4-substituted phenols and anilines (Tables 3 and 4). 4-Hydroxybenzoate (compound I), 4-phenolsulphonate (compound 4) and 4-aminobenzoate (compound 9) did not inhibit the loss of H_2O_2 by more than 50%, but they changed the kinetics of loss to first order, which indicates that they were acting as peroxidase substrates. Except for trinitrophenol (compound 8), the remaining compounds within each series inhibited MPO. 4-Bromoaniline was by far the best inhibitor. However there were several other excellent inhibitors with I_{50} values less than 1 μM. We verified that these inhibitors acted in the same way as the anti-inflammatory drugs, by showing that inhibition was reversed by ascorbate. In addition, in the presence of 100 mM Cl^- and 100 μM H_2O_2, 100 μM of 4-aminobenzoic acid ethyl ester (compound 13) converted 400 nM MPO to compound II, which was reduced back to ferric MPO by ascorbate (data not shown). However, this compound was not peroxidized by MPO (Fig. 6). Thus, the phenols and anilines inhibited MPO reversibly by promoting the accumulation of compound II.

Reactivity of substituted aromatic compounds is often determined by the electron donating or withdrawing capacity of their substituents. The magnitude of these effects is related to Hammett's substituent constant σ. A positive value of σ indicates that the substituent is electron withdrawing, while a negative value indicates that it is electron donating. These constants are not applied to 2-substituted compounds because of complicating steric effects. When direct resonance interaction is available between the substituent and an electron deficient reaction site, the modified Hammett constant σ^+ is required for successful correlation with reactivity. Resonance effects are possible for electron donating groups only when they are in the 2 and 4 positions. For the majority of electron withdrawing groups σ is essentially the same as σ^+. Their values are given in Tables 3 and 4 [18].

We expected σ to be related to I_{50} because inhibition was due to the accumulation of compound II, and the rate of reduction of horseradish peroxidase

Table 2. The effect of anti-inflammatory drugs on the conversion of H_2O_2 to HOCl by MPO

Addition	H_2O_2 utilized (μM ± SD)	HOCl produced (μM ± SD)
None	14.2 ± 2.0	13.3 ± 1.4
Dapsone 10 μM	1.8 ± 0.5	0.2 ± 0.5
Salicylate 10 μM	6.0 ± 0.8	4.7 ± 0.6
5-Aminosalicylate		
10 μM	21.8 ± 1.3	19.2 ± 0.3
100 μM	6.5 ± 0.3	4.3 ± 0.8
4-aminoantipyrine		
10 μM	13.7 ± 0.6	11.3 ± 1.2
100 μM	13.2 ± 1.2	10.3 ± 0.9

Reactions were started by adding 8 nM MPO to 10 mM taurine in 50 mM phosphate buffer (pH 7.4) containing 30 μM H_2O_2, and 150 mM Cl^-. Reactions were stopped at 1 min by adding 100 μg/mL of catalase, and the H_2O_2 utilized, measured with the H_2O_2 electrode, was compared to the HOCl produced, which was assayed by the oxidation of TNB by taurine chloramine. Values are means and standard deviations (SD) of triplicate experiments.

Table 3. The relationship between Hammett's substituent constant for substituted phenols and the concentration that gave 50% inhibition of the initial rate of H_2O_2 loss catalysed by MPO

Substituted phenol	I_{50} ± SE (μM)	(N)	σ	σ^+	σ_c
1 4 COO^-	P.S.	(2)	0.13	−0.02	−0.02
2 3 COO^-	52.0 ± 11.8	(6)	0.10	−0.03	0.10
3 3 CHO	0.22 ± 0.01	(6)	0.36	—	0.36
4 p SO_3^{2-}	P.S.	(6)	0.38	—	—
5 4 $COOCH_3$	0.22 ± 0.01	(10)	0.413	—	0.413
6 4 CHO	0.16 ± 0.01	(8)	0.45	—	0.45
7 4 NO_2	0.42 ± 0.03	(9)	0.78	0.79	0.79
8 2,4,5 NO_2	>100	(3)	>2	>2	>2

I_{50} values were calculated from N drug concentrations and are given with their associated standard errors (SE). σ_c values for Hammett's substituent constant are σ^+ values for four substituted compounds and σ for three substituted compounds [18]. Unknown values are designated by —. P.S. indicates that the compound was a peroxidase substrate because H_2O_2 loss was linear, and it was not inhibited by more than 50%. The inhibitors prevented the loss of H_2O_2 to a maximum of at least 90%. Reaction conditions were described in Fig. 1.

Table 4. The relationship between Hammett's substituent constant for substituted anilines and the concentration that gave 50% inhibition of the initial rate of H_2O_2 loss catalysed by MPO

Substituted aniline	I_{50} ± SE (μM)	(N)	σ	σ^+	σ_c
9 4 COO^-	P.S.	(3)	0.13	−0.02	−0.02
10 3 COO^-	7.6 ± 1.0	(7)	0.10	−0.03	0.10
11 4 SO_3^{2-}	8.9 ± 1.7	(6)	0.38	—	—
12 4 Br	0.045 ± 0.003	(10)	0.23	0.15	0.15
13 4 $COOCH_2CH_3$	0.13 ± 0.01	(6)	0.52	0.48	0.48
14 4 NO_2	0.93 ± 0.06	(9)	0.78	0.79	0.79

I_{50} values were calculated from N drug concentrations and are given with their associated standard errors (SE). σ_c values for Hammett's substituent constant are σ^+ values for four substituted compounds and σ for three substituted compounds [18]. Unknown values are designated by —. P.S. indicates that the compound was a peroxidase substrate because H_2O_2 loss was linear, and it was not inhibited by more than 50%. The inhibitors prevented the loss of H_2O_2 to a maximum of at least 90%. Reaction conditions were described in Fig. 1.

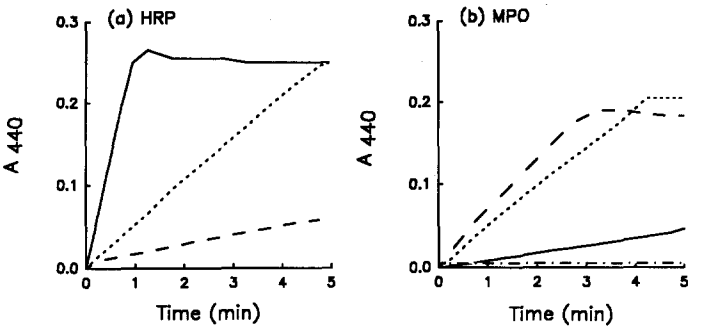

Fig. 6. Peroxidation of aminobenzoates by HRP and MPO. (a) HRP (24 μg/mL) or (b) MPO (120 nM) were used to peroxidize 2 mM (----) 2-aminobenzoic acid, (——) 3-aminobenzoic acid, (--------) 4-aminobenzoic acid or (—·—·—) 4-aminobenzoic acid ethyl ester in 50 mM phosphate buffer (pH 7.4) containing 200 μM H_2O_2. Peroxidation was determined at 25° by measuring the formation of a brown product at 440 nm.

(HRP) compound I to compound II by substituted phenols and anilines decreases with increasing σ [19]. However, I_{50} for inhibition of MPO is not related to σ. This is evident from comparing 3-hydroxybenzaldehyde and 4-phenolsulphonic acid (compounds 3 and 4). They have similar σ values, but the former was an excellent inhibitor while the latter was a peroxidase substrate and could not inhibit H_2O_2 loss by more than 50%. Furthermore, the σ values for the carboxy groups in 3 and 4-hydroxybenzoic acid (compounds 2 and 1) and 3 and 4-aminobenzoic acid (compounds 10 and 9) are also similar. However the 3-substituted analogues inhibited MPO, while their 4-substituted derivatives were peroxidases substrate. σ^+ values for 3 and 4-carboxy groups are also practically identical. Thus, I_{50} is not related to σ^+ either.

Since the rate of reduction of HRP compound I is inversely proportional to σ [19], it would be predicted that 3-hydroxy and 3-aminobenzoic acid should have been better peroxidase substrates than their 4-substituted analogues. To understand why the HRP data was not valid for MPO we compared the ability of these enzymes to peroxidize 2, 3 or 4-aminobenzoic acid (Fig. 6). The relative rates of peroxidation by HRP were 1:24:5 respectively, which is consistent with the earlier results for HRP [19]. However, for MPO the relative rates of peroxidation were 1:0.2:0.8. These results imply that there are major differences between the mechanisms of peroxidation for MPO and HRP. It is apparent that resonance effects must have a dominant impact on the relative ease of oxidation of these compounds by MPO, because electron donation is available only when the carboxyl group is in the 2 and 4 positions.

These resonance effects will undoubtedly influence the I_{50} values for inhibition of HOCl production by MPO. We accounted for them by relating I_{50} to σ^+ for 4-substituted compounds, but used σ for 3-substituted compounds because of the unavailability of resonance stabilization in this position. Using these composite Hammett constant (σ_c) a trend was apparent for both phenols (Table 3) and anilines (Table 4). I_{50} decreased with increasing σ_c until a minimum value was obtained. Further increases in σ_c caused I_{50} to increase until the substituted compounds could no longer inhibit, e.g. trinitrophenol. The effective inhibitors had values of σ_c that ranged from 0.36 to 0.79 for phenols, and from 0.15 to 0.79 for anilines.

DISCUSSION

We have demonstrated that a variety of anti-inflammatory drugs interact with the neutrophil enzyme MPO. Most of the drugs inhibited the production of HOCl by MPO at concentrations well below those achieved *in vivo*. In particular, mefenamic acid, sulfapyridine, dapsone, quinacrine, primaquine and aminopyrine were potent inhibitors, having I_{50} values of about 1 μM or less. It is therefore conceivable that these and related drugs may inhibit MPO *in vivo*. This proposal is strongly supported by the effectiveness of sulfapyridine and dapsone in suppressing inflammation in conditions that are characterized by a predominance of neutrophils in the inflammatory infiltrate [20]. Recently it was found that treatment of rats with dapsone caused a 65% reduction in the kidney damage associated with *Escherichia coli* pyelonephritis [21]. The only plausible explanation for the anti-inflammatory effect of dapsone was that it acted by inhibiting MPO. Inhibition of MPO has also been suggested as an important mechanism by which dapsone and 5-aminosalicylate attenuate FMLP-induced mucosal injury in rats [22]. Lack of inhibition by chloroquine argues against our proposal, since it has similar efficacy to quinacrine in rheumatoid arthritis, yet it did not inhibit MPO. However, it is possible that metabolic hydroxylation would allow chloroquine to inhibit MPO. It is now considered that inhibition of prostaglandin production is unlikely to be the only mode of action of anti-inflammatory drugs [23]. We suggest that in some cases inhibition of MPO-dependent production of HOCl should be considered as an alternative mechanism for their action.

The anti-inflammatory drugs inhibited HOCl production by trapping MPO as inactive compound II. In effect they were poor peroxidase substrates.

They could divert MPO from its chlorination cycle by reducing compound I to compound II, but were unable to reduce compound II back to the active enzyme (see Fig. 1). Since we showed that ascorbate reverses inhibition by these drugs, it is conceivable that *in vivo* ascorbate would maintain the activity of MPO by reducing compound II. However, ascorbate is rapidly oxidized by HOCl [24], so that within the confines of a phagosome, its effect would be short-lived, and compound II would soon accumulate. The drugs that failed to inhibit HOCl production were either unable to reduce compound I, or they were good peroxidase substrates that continually turned over compound II. Thus, 4-aminoantipyrine and 5-aminosalicylate must have competed poorly with Cl^- for compound I, but converted any compound II formed to the active enzyme. This latter reaction occurs rapidly with 5-aminosalicylate [25]. Previously we have shown that H_2O_2 can inhibit MPO by reducing compound I to compound II [16]. Thus, 5-aminosalicylate would enhance production of HOCl at concentrations of H_2O_2 greater than 20 μM by increasing the rate of turnover of compound II, as proposed by Zuurbier *et al.* [25]. However at high concentrations it inhibits MPO by a different mechanism that may involve irreversible inactivation. Our results with 4-aminoantipyrine and 5-aminosalicylate indicate that drugs that are peroxidase substrates, such as acetaminophen [26] and methimazole [27], are likely to inhibit production of HOCl only when they are present at high concentrations that are probably not physiologically significant. Instead these compounds may exacerbate inflammatory conditions by being slowly peroxidized (reactions 3 and 4) to potentially damaging free radicals.

The mechanism of inhibition of HOCl production, which is reliant on MPO being trapped as inactive compound II, is supported by the findings that dapsone and indomethacin are competitive inhibitors with respect to I^- and Cl^- [7, 28]. It also explains why these two drugs were unable to inhibit the preoxidative activity of MPO (reactions 1, 3 and 4) [7, 21]. Peroxidative activity is dependent on the rate at which substrates reduce compound II to the ferric enzyme (reaction 4) [29]. However, since these drugs have a limited capacity to reduce compound II, they would be unable to compete with good peroxidase substrates and inhibit MPO. Thus, inhibition of peroxidative activity is an inappropriate measure of inhibition of HOCl production by MPO.

To reveal the characteristics that enabled anti-inflammatory drugs to be potent inhibitors of MPO, we related the I_{50} values of structurally similar phenols and anilines to Hammett's substituent constant σ. Although we found that I_{50} is influenced by electronic effects, it is not related to σ. However, we found that the mechanism by which MPO oxidizes anilines is quite different to that of the classical peroxidase mechanism typical of HRP. With MPO peroxidation is strongly influenced by resonance effects. These effects could be important in stabilizing either the transition state or the incipient free radical. In the latter case the incipient free radical may reduce compound II to complete the catalytic cycle. Thus MPO may be similar to thyroid peroxidase and oxidize different organic substrates by either a one or two electron mechanism [30].

To account for these resonance effects we used composite values for Hammett's substituent contant (σ_c), and found them to be non-linearly related to I_{50}, which had a minimum value. This type of relationship is usually characteristic of reactions that have two or more competing mechanisms [31]. For MPO, however, it is explained by a progressive failure of phenols and anilines to reduce first compound II and then compound I as σ_c increases. At the optimum value of σ_c inhibitors readily reduce compound I but are unable to reduce compound II. This explains, the action of 4-aminobenzoic acid ethyl ester, which was a potent inhibitor and promoted accumulation of compound II, but was not peroxidized by MPO. However, a decrease in σ_c allows inhibitors to reduce compound II and increase production of HOCl, whereas an increase impairs their ability to reduce compound I and divert MPO from its chlorination cycle.

Since I_{50} is strongly influenced by the electronic properties of phenols and anilines, the reduction potential of these compounds will determine their effectiveness as inhibitors of HOCl production. It is unlikely that size or hydrophobicity have a major impact on inhibition, because the salicylate analogues, sulfasalazine and olsalazine, had similar I_{50} values to salicylate, even though they are much larger and more hydrophobic. Their reduction potentials, however, should be similar. The reduction potentials of the majority of compounds we investigated are unknown. However, Lind *et al.* [32] have measured the reduction potentials of 4-substituted phenoxyl radicals (reaction 5), which have an excellent correlation with σ^+ ($R = 0.98$, equation 1).

$$R-\dot{O} + 1e + H^+ \rightarrow R-OH \qquad \text{(reaction 5)}$$

$$E_{7.0} = 0.36\sigma^+ + 0.92. \qquad (1)$$

Substitution of the σ_c's associated with the lowest I_{50} values for phenols into equation 1, gives an optimum range of reduction potentials of 1.05–1.20V. If drugs could be developed with reduction potentials in this range they should be excellent inhibitors of MPO. They should also be relatively specific since compounds with such high reduction potentials react poorly with other heme enzymes. In addition, the ability of MPO to oxidize these drugs to potentially toxic intermediates would be decreased. This may be especially important since MPO-dependent metabolism of phenylbutazone, dapsone and procainamide has been suggested as responsible for some of their toxic side effects [33].

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