Biochemical Pharmacology, Vol. 42, No. 3, pp. 599–608, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

MECHANISMS BY WHICH CLOFAZIMINE AND DAPSONE INHIBIT THE MYELOPEROXIDASE SYSTEM

A POSSIBLE CORRELATION WITH THEIR ANTI-INFLAMMATORY PROPERTIES

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(*Received* 19 *November* 1990; *accepted* 25 *March* 1991)

Abstract—The mechanisms by which two anti-leprotic drugs (clofazimine and dapsone), both with anti-inflammatory properties, inhibit myeloperoxidase (MPO)-catalysed reactions, were investigated. The disappearance of NADH fluorescence was used as an assay for its oxidation. Chloride stimulated the oxidation of NADH in the MPO–H_2O_2 system in a concentration-dependent manner (50-fold at 150 mM NaCl). Under these conditions Cl^- is oxidized and the oxidant formed, presumably hypochlorous acid (HOCl), oxidizes NADH. Observations demonstrating the effect of the drugs on the MPO system, are: (1) Inhibition of Cl^--stimulated oxidation of NADH. (2) Inhibition of polypeptide modification in a model protein, thyroglobulin (TG). (3) Protection of MPO against loss of catalytic activity caused by chlorinating oxidants generated by the system. (4) Inhibition of haemoglobin oxidation. Only dapsone was active here. HPLC analyses suggested that the drugs were not significantly metabolized in the MPO–H_2O_2 system in the absence of Cl^-. Bleaching of clofazimine was stimulated by Cl^- in the MPO system, suggesting the involvement of HOCl. Clofazimine was found to be a more potent scavenger of HOCl than dapsone when the inhibition of NADH oxidation by reagent HOCl was used as an assay. This finding is also supported by HPLC analyses which indicated a greater sensitivity of HOCl for clofazimine than for dapsone. Relatively low concentrations of dapsone inhibited the oxidation of oxygenated haemoglobin (HbO_2), suggesting that the drug was not metabolized to its N-hydroxylated derivative which is thought to be responsible for methaemoglobin (metHb) formation *in vivo*. It is proposed that the inhibitory mechanism of action of clofazimine is to scavenge chlorinating oxidants generated by the MPO–Cl^-–H_2O_2 system, while dapsone converts MPO into its inactive compound II (ferryl) form. The different inhibitory mechanisms of clofazimine and dapsone towards the MPO system may contribute to the anti-inflammatory actions of the drugs.

In addition to their anti-leprotic properties, clofazimine and dapsone (Fig. 1) are also effective anti-inflammatory agents [1–4]. Both drugs have been shown to influence various aspects of neutrophil function [4–6]. Neutrophils contain high concentrations of MPO†; levels up to 5% of the dry weight of the cell have been reported [7]. MPO catalyses a two-electron oxidation of Cl^- by H_2O_2 to hypochlorite, which exists in equilibrium with its protonated form (HOCl) at physiological pH. HOCl and OCl^- are powerful chlorinating oxidants which can attack a wide range of biomolecules. One major extracellular target of attack by HOCl is α_1-antiproteinase, the major circulating inhibitor of serine proteases such as elastase in body fluids (reviewed in [8]). HOCl (and OCl^-) rapidly inactivates α_1-antiproteinase which should facilitate the uncontrolled action of proteinases such as elastase and collagenase. These hydrolytic enzymes, together with MPO, are released from activated neutrophils. This, as well as other actions of HOCl, may contribute to inflammatory conditions.

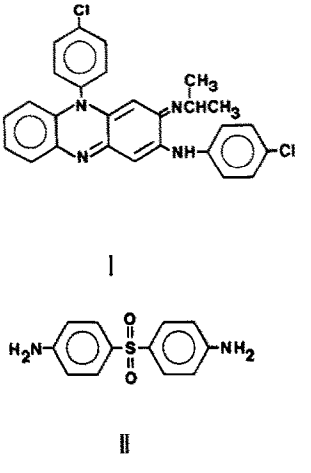

Fig. 1. Structural formulae of clofazimine (I) and dapsone (II).

Conflicting results regarding the effect of the drugs on neutrophil functions have been reported. Luminol-enhanced chemiluminescence studies showed that clofazimine enhanced the production

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† Abbreviations: MPO, myeloperoxidase; TG, thryoglobulin; HOCl, hypochlorous acid; HbO_2, oxygenated haemoglobin; metHb, methaemoglobin; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

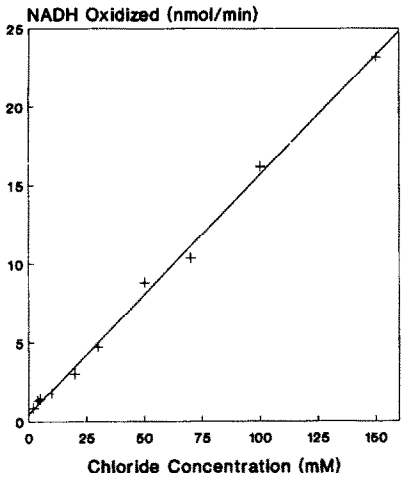

Fig. 2. Stimulation of NADH oxidation by Cl^- in the MPO system. Increasing concentrations of Cl^- were added to incubation mixtures containing 150 nM MPO and 5 μM NADH in 50 mM phosphate buffer (pH 7.4). Reactions were initiated with the addition of 50 μM H_2O_2 and the NADH fluorescence were immediately recorded. The amount of NADH oxidized per min was calculated from the initial slopes of the decrease of NADH fluorescence (excitation/emission wavelengths; 340 nm/450 nm) with time.

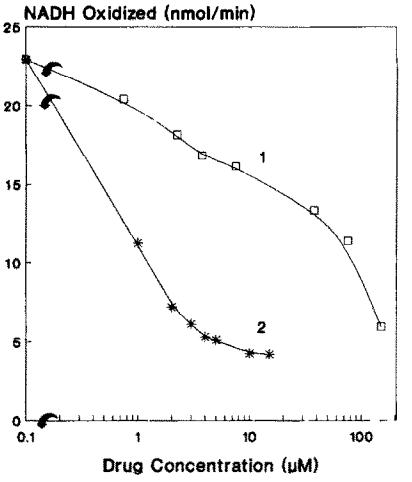

Fig. 3. Inhibition of MPO-catalysed reactions with clofazimine and dapsone. Increasing concentrations of the drugs were added to incubation mixtures containing 150 nM MPO, 5 μM NADH and 150 mM NaCl in 50 mM phosphate buffer (pH 7.4). Reactions were initiated with the addition of H_2O_2 (50 μM in reaction) and the NADH fluorescence immediately recorded. The amount of NADH oxidized per min was calculated from the initial slopes of the decrease of NADH fluorescence with time. The horizontal axis is calibrated in log scale. Curves 1: clofazimine; 2: dapsone.

of reactive oxidants by human phagocytes and potentiated the reactivity of the MPO–H_2O_2–halide system [6]. In view of this it seems paradoxical that clofazimine has anti-inflammatory activity, but it was suggested that the anti-inflammatory activity of the drug might outweigh its pro-inflammatory potential by deactivating leukocyte migration and potentiating oxidative inactivation of leukoattractants [9]. Studies by other workers, however, failed to demonstrate an effect of clofazimine on the phagocytic function of neutrophils *in vitro* [10].

Dapsone was shown to interfere with the MPO–H_2O_2–halide-mediated cytotoxic system of the neutrophil [5]. More recently, it has been reported that the drug is metabolized to a hydroxylamine by activated human neutrophils (or MPO in a cell-free system) and it was proposed that this oxidation reaction was the mechanism for the anti-inflammatory action of the drug [11].

In the present study, evidence will be presented that both clofazimine and dapsone are inhibitors of the MPO–H_2O_2–Cl^- system, but by different mechanisms. Clofazimine was found to be an effective scavenger of chlorinating oxidants generated during the MPO-catalysed oxidation of Cl^-. Dapsone was found to be a less potent scavenger of HOCl, but it stimulated accumulation of the compound II (ferryl) form of MPO, which is catalytically inactive in the oxidation of Cl^-.

MATERIALS AND METHODS

Reagents. Pure substance of clofazimine, 3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine, was kindly donated by Prof. R. Anderson, Institute of Pathology, Pretoria, R.S.A. Dapsone, 4-aminophenyl sulfone, was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Stock solutions of the drugs were made up in 95% ethanol and control experiments were performed to evaluate the effect of the alcohol in the experimental systems. NADH (disodium salt) was from Boehringer Mannheim (Hamburg, F.R.G.). Sodium hypochlorite (approx. 1 N in 0.1 NaOH) was from BDH. HOCl was obtained by adjusting NaOCl to pH 6.2 with dilute H_2SO_4 and its concentration determined iodometrically [12].

Preparation of proteins. Detergent-solubilized MPO from human neutrophils was isolated as previously described [13] and only enzyme with purity indexes ($A_{423\,nm}/A_{280\,nm}$) above 0.75 was used in this study. Its concentration was calculated using an absorption coefficient of $89 \times 10^3\,M^{-1}\,cm^{-1}$ at 428 nm [14]. Haemoglobin was isolated from red blood cell lysates and its concentration determined using an absorption coefficient of $3 \times 10^4\,M^{-1}\,cm^{-1}$ at 522 nm for the tetrameric molecule [15]. Bovine TG was prepared by linear gradient density centrifugation [16].

Oxidation of NADH. NADH fluorescence was measured on a Perkin Elmer MPF-44A fluorescence spectrophotometer. The disappearance of NADH fluorescence (excitation at 340 nm and emission at 450 nm) was used as a measure of oxidation in the following assays:

(1) stimulation by Cl^- of the oxidation of NADH (5 μM) by MPO (150 nM) and H_2O_2 (50 μM). Increasing concentrations of NaCl (up to 150 mM) were cooxidized in the MPO system.

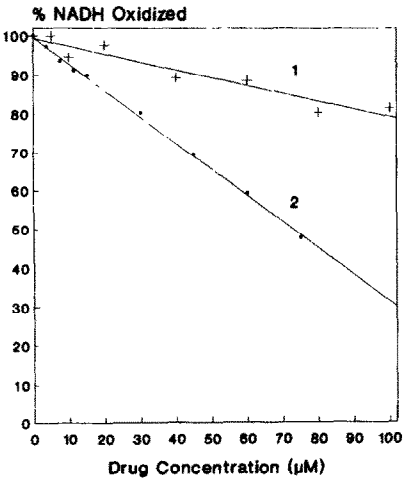

Fig. 4. Relative abilities of clofazimine and dapsone to scavenge HOCl. Reagent HOCl (30 μM) was added to solutions of 5 μM NADH and increasing concentrations of the drugs in 50 mM phosphate buffer (pH 7.4). The relative fluorescence of NADH was determined and expressed as a percentage of NADH oxidized relative to the amount of NADH oxidized in the absence of any drug. Curves 1, dapsone; 2, clofazimine.

(2) Inhibition of the MPO system by clofazimine and dapsone. Increasing concentrations of the drugs were included in a system containing 150 nM MPO, 150 mM NaCl, 5 μM NADH and 50 μM H_2O_2.
(3) Relative abilities of clofazimine and dapsone to scavenge HOCl. Volumes of reagent HOCl (30 μM in mixture) were added to solutions containing 5 μM NADH and increasing concentrations of the drugs.

For further experimental details, see the legends of Figs 2, 3 and 4, respectively.

Effect of drugs on MPO suicide inhibition. Aliquots of clofazimine and dapsone in 95% ethanol were included in the MPO–H_2O_2 system in the absence and presence of 150 mM NaCl. Reactions were terminated by passing each mixture through a Sephadex G-10 column (2 × 1.3 cm) to separate the MPO from reactants and products. Peroxidase activities, determined by the guaiacol assay, were expressed as a percentage of the activity of the same concentration of unreacted MPO. See the legend of Fig. 5 for more experimental detail.

SDS–PAGE of TG polypeptides. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of TG polypeptides was performed on 4% slab gels (3 mm thick) according to Laemmli [17], but without a stacking gel. A constant current of 50 mA was applied until satisfactory separation of polypeptides was achieved. The gel was stained with Coomassie Brilliant Blue R-250.

Spectrophotometric analyses. Spectral recordings were made on a Cary 219 spectrophotometer. Fractional contents of metHb were determined using an approach described by Szebeni *et al.* [18]. The percentage of methaemoglobin (metHb) was calculated as follows:

$$\% \text{ metHb} = \frac{100 \times (7.0\,A_{577\,nm} + 76.8\,A_{630\,nm} - 13.8\,A_{560\,nm})}{(3.6\,A_{577\,nm} + 31.0\,A_{630\,nm} + 22.2\,A_{560\,nm})}.$$

High performance liquid chromatography. HPLC was performed on a Hewlett Packard HP 1090 liquid chromatograph equipped with a HP 79994A analytical workstation and a C8 Whatman (25 cm length) column. The liquid phase consisted of two components; solvent A (50 mM KH_2PO_4 in water) and solvent B (acetonitrile : isopropanol; 8:20, v/v). For dapsone, a linear gradient starting with a mixture of 90% and 10% and reaching a final composition of 30% and 70% of A and B respectively, within 8 min at a flow rate of 1.5 mL/min, was used. Isocratic elution was then continued under the conditions which existed at the end of the gradient. Absorbance was monitored at 254 nm. In this system, the retention time of dapsone was 6.6 min.

For clofazimine, isocratic elution with a liquid phase consisting of 30% and 70% of A and B, respectively, was used. The flow rate was also 1.5 mL/min and absorbance was monitored at 290 nm. Under these conditions, the retention time of clofazimine was 6.5 min.

RESULTS

Effect of chloride on MPO-induced NADH oxidation

Figure 2 shows that the oxidation of NADH by MPO and H_2O_2 is stimulated by Cl^-. In the absence of Cl^-, the oxidation of NADH was very low (<1 nmol/min). The plot of NADH oxidation as a function of Cl^- concentration shows a linear relationship up to 150 mM, where about 23 nmol NADH were oxidized per min.

Inhibition of NADH oxidation by clofazimine and dapsone

Figure 3 shows the rate of NADH oxidation in the MPO–Cl^-–H_2O_2 system as a function of drug concentration. Dapsone (curve 2) was at least 10-fold more potent than clofazimine (curve 1) in inhibiting NADH oxidation.

Effect of drugs on MPO-induced polypeptide modification

Figure 6 shows SDS–PAGE patterns of TG under non-reducing conditions. Thyroglobulin was incubated in different reaction systems for 10 min at 37° before electrophoresis. The first three lanes are patterns of control TG samples (2 mg/mL) incubated with 133 nM MPO + 500 μM H_2O_2 (lane 1); MPO + 100 μM dapsone + H_2O_2 (lane 2) and MPO + 100 μM clofazimine + H_2O_2 (lane 3). The TG half-molecule (M_r = 330 kDa) as well as the undissociated (mostly disulphide-linked) TG are clearly visible. In lane 4, the oxidizing mixture contained 150 mM NaCl (without drug). It is evident that chlorinating oxidants generated by the MPO system caused covalent polymerization of the half-molecules, while the Coomassie Blue stains of the half-molecules are clearly less intense than the corresponding stains in lanes 1–3. The presence of 200 mM ethanol in any of the reaction mixtures of the first four lanes did not appear to change their

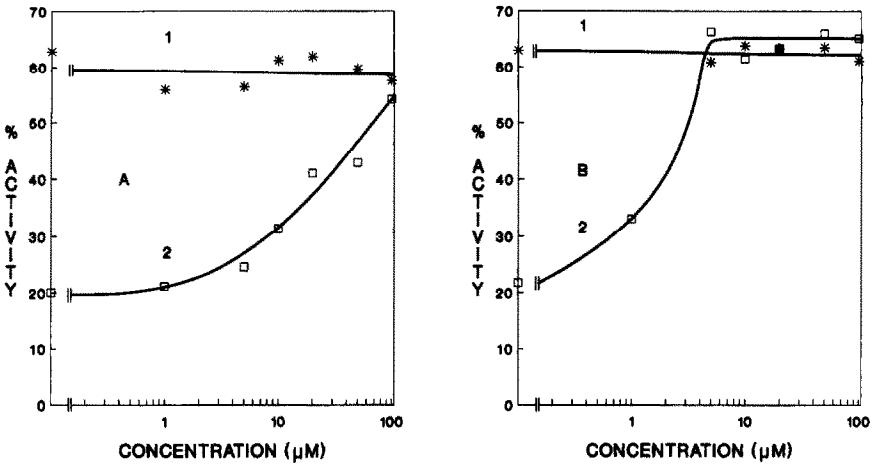

Fig. 5. Effect of clofazimine and dapsone on MPO suicide inhibition. To each of a series of solutions of 350 nM MPO in 50 mM phosphate buffer (pH 7.4) in the absence and presence of 150 mM NaCl, were added increasing concentrations of drug. Reactions were initiated with H_2O_2 (300 μM in mixture) and proceeded for 15 min at 37°. Aliquots (100 μL) were then passed through Sephadex G-10 columns. The MPO-containing fractions were analysed for peroxidase activity (guaiacol assay) and expressed as a percentage of activity of the same concentration of unreacted MPO. (A) Clofazimine; curve 1, without NaCl; curve 2, in the presence of 150 mM NaCl. (B) Dapsone; curve 1, without NaCl; curve 2, in the presence of 150 mM NaCl. Horizontal axes are calibrated in log scale. The data points on the left are per cent activities at zero drug concentrations. Each data point represents the average from three experiments done in duplicate.

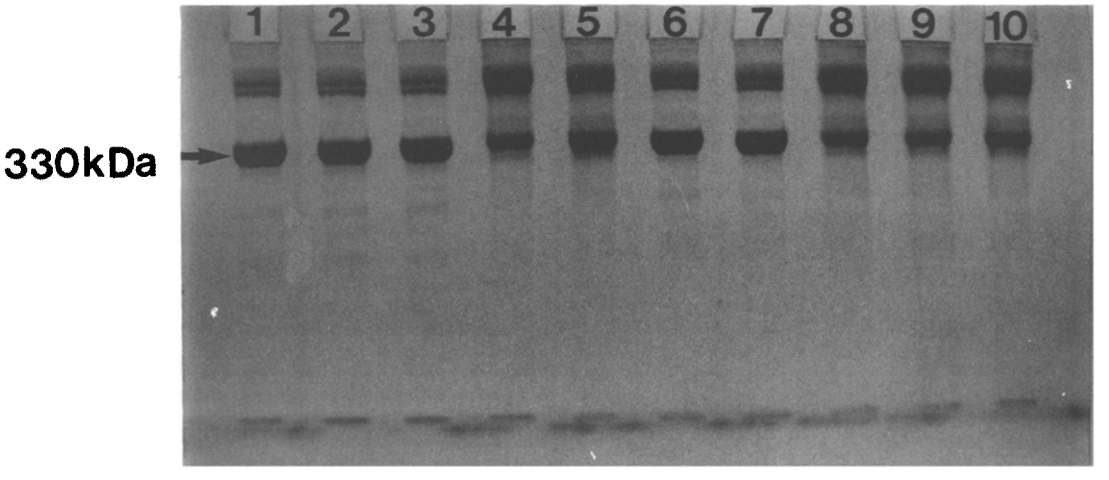

Fig. 6. SDS–PAGE (4% gel) demonstrating the effect of clofazimine, dapsone and Cl^- on TG polypeptide composition in the MPO-H_2O_2 system. TG (2 mg/mL) was incubated with 133 nM MPO in 50 mM phosphate buffer (pH 6). The reactions were initiated with the addition of H_2O_2 (500 μM in solution) and proceeded for 10 min at 37°. Before electrophoresis, the samples were treated with SDS (1% in solution) and 50 μL aliquots applied to the gel. A constant current of 50 mA was applied until satisfactory separation of polypeptides was achieved. The gel was stained with 0.02% Coomassie Brilliant Blue R-250 and destained with acetic acid/methanol/water (1:2:7). The following mixtures were analysed: Lanes 1, TG + MPO + H_2O_2; 2, TG + MPO + 100 μM dapsone + H_2O_2; 3, TG + MPO + 100 μM clofazimine + H_2O_2; 4, TG + MPO + 150 mM NaCl + H_2O_2; 5, TG + MPO + NaCl + 1 μM dapsone + H_2O_2; 6, same as lane 5, but with 5 μM dapsone; 7, with 50 μM dapsone; 8–10, same as lanes 5–7, but with clofazimine instead of dapsone.

electrophoresis patterns. Furthermore, omission of MPO or H_2O_2, or both, did not alter the patterns of the first three lanes, which look identical. The incubation mixtures for lanes 5, 6 and 7 were the same as for lane 4, but contained additionally 1, 5 and 50 μM dapsone, respectively. Even 1 μM dapsone had a significant protective effect on TG (lane 5), while 5 and 50 μM dapsone rendered full protection to TG polypeptides (lanes 6 and 7). The incubation mixtures for lanes 8, 9 and 10 contained 1, 5 and 50 μM clofazimine instead of dapsone. Clofazimine was clearly less potent than dapsone in protecting TG polypeptides against polymerization. Only at 50 μM clofazimine, some effect is visible (lane 10). The Coomassie Blue stain of the TG half-molecule in lane 10 is somewhat more intense than the corresponding stains in lanes 8 and 9 or in lane 4.

Protection against self-induced loss of MPO activity by the drugs

Figure 5 demonstrates the effects of clofazimine (A) and dapsone (B) on suicide inhibition of MPO. Increasing concentrations of the drugs were included in the MPO–H_2O_2 reaction mixtures in the absence (curves 1) or the presence of 150 mM NaCl (curves 2). Neither of the drugs had any appreciable effect on MPO activity in the absence of Cl^- and curves 1 of A and B were nearly parallel to the concentration axes at about 60% activity, i.e. the activity remaining after incubation of 350 nM MPO with 300 μM H_2O_2 only. In the presence of 150 mM NaCl, without any drug, about 80% of the MPO activity was lost after the reaction had taken place. A concentration-dependent protection of MPO catalytic activity by the drugs is evident in the Cl^--containing media. The effect of dapsone (B; curve 2) was more pronounced than that of clofazimine (A; curve 2). At a concentration of 5 μM and higher, dapsone gave its maximum response, while maximum protection was reached only at about 100 μM in the case of clofazimine.

Relative abilities of clofazimine and dapsone to scavenge HOCl

Figure 4 shows the relative amount of NADH oxidized as a function of dapsone (curve 1) and clofazimine (curve 2) concentration when reagent HOCl (30 μM in mixture) was added to mixtures of 5 μM NADH and increasing concentrations of the drugs. The extent of NADH oxidation caused by HOCl in the absence of any drug was taken as 100%. It is obvious that clofazimine is a better scavenger of HOCl than dapsone. Ethanol concentrations up to 200 mM did not scavenge HOCl in this system.

Oxidative changes in the absorption spectra of the drugs

Figure 7 shows the change in absorbance of clofazimine when oxidized by MPO and H_2O_2 in the presence (scan 3) or absence (scan 2) of Cl^- or by reagent HOCl (scan 4). Both sample and reference cuvettes contained 700 nM MPO and 75 μM clofazimine in 50 mM phosphate buffer (pH 7.4). Reactions were initiated by the addition of H_2O_2 (150 μM in solution) or reagent HOCl (20 μM in solution). Clofazimine has an absorbance peak at

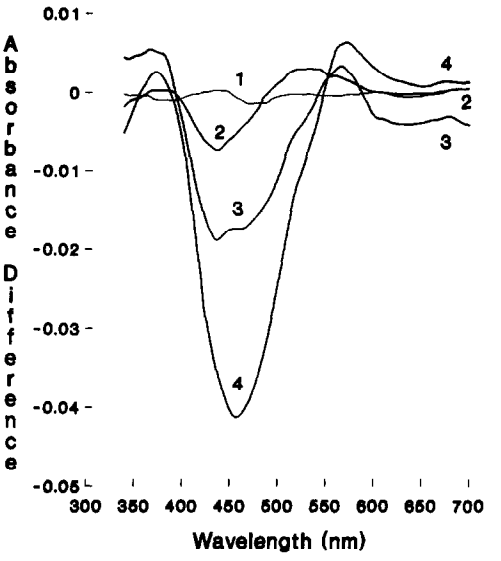

Fig. 7. Difference scans demonstrating oxidative changes in the absorbance spectrum of clofazimine. Reactions were performed 50 mM phosphate buffer (pH 7.4). Scan 1; both sample and reference cuvettes contained 700 nM MPO. Recording was made 15 min after adding H_2O_2 (150 μM in solution) to the sample cuvette. Scan 2; both cuvettes contained 700 nM MPO and 75 μM clofazimine. Recording was made 15 min after adding H_2O_2 to the sample cuvette. Scan 3; same as scan 2, but both cuvettes contained 150 mM NaCl additionally. Scan 4; cuvettes contained 75 μM clofazimine. Recording was made 15 min after adding HOCl (20 μM in solution) to the sample cuvette.

455 nm which decreased somewhat during the MPO reaction in the absence of Cl^-. This is shown as a trough at about 440 nm in the difference scan (scan 2). The decrease in absorbance of clofazimine was obviously stimulated when the MPO reaction mixture contained Cl^- (scan 3). Reaction with 20 μM HOCl shows a still greater decrease in clofazimine absorbance (scan 4). Scan 1 shows that the absorbance change due to the reaction of MPO with H_2O_2 in the absence of drug was negligible under our experimental conditions.

Dapsone has absorbance maxima at 255 and 290 nm. Addition of 20 μM HOCl had little effect on the dapsone spectrum. Only a slight rise in the baseline around 350 nm was observed (results not shown). MPO-catalysed reaction conditions similar to those described above for clofazimine had little (if any) effect on the dapsone spectrum.

HPLC analyses

Various experimental conditions in the presence or absence of Cl^- (i.e. changing the MPO/H_2O_2 ratio or increasing the time of incubation), failed to demonstrate any significant metabolism of dapsone in the MPO–H_2O_2 system. The dapsone used in our study had minor impurities with retention times of 7.7 and 8 min. The amount of these impurities, however, never exceeded 1% of the dapsone peak which had a retention time of 6.6 min. Only in some cases, a trace amount of product with retention time

of 8.7 min was formed in the MPO system. This peak had an area less than 0.5% of that under the dapsone peak, but had a UV absorption spectrum related to that of dapsone. In all cases, the absorption spectra at different positions on the dapsone peak were identical. It is thus reasonable to assume that the dapsone peaks were not contaminated with possible metabolites having similar retention times. Various peaks with retention times greater than 8 min eluted when dapsone was treated with reagent HOCl. When 75 μM dapsone was treated with 100 μM HOCl, these peaks comprised in total about 7% of that of the dapsone peak.

When clofazimine (75 μM) was incubated in the MPO–H_2O_2 system (MPO, 700 nM; H_2O_2, 150 μM) in the absence of Cl^-, HPLC under the conditions described in the "Methods" section did not reveal significant metabolism of the drug. When the drug was co-oxidized with 150 mM NaCl, the area under the clofazimine peak was 84% of the control. When the drug solution was incubated with 30 μM reagent HOCl only, the area under the clofazimine peak was only 38% of that of the control. Spectral comparisons at different positions on the clofazimine peaks suggested homogeneity.

Inhibition of MPO-induced metHb formation

The formation of metHb in the MPO system and its inhibition by dapsone is shown in Fig. 8. Scan 1 of A shows the spectrophotometric scan of 1.7 μM HbO_2 between 450 and 700 nm in 50 mM phosphate buffer containing 150 mM NaCl. Both sample and reference cuvettes contained 1 μM MPO. When 100 μM dapsone was present in the sample cuvette, the HbO_2 spectrum remained unchanged, suggesting that the drug alone does not induce metHb formation. On addition of 500 μM H_2O_2 (without drug), oxidation of HbO_2 can be noted as a progressive decrease in absorbance at 540 and 576 nm in scans 2–4 which were recorded at 2 min intervals. Scan 2 was recorded 15 sec after adding the H_2O_2. From scan 4, a metHb content of 95% was calculated. In the presence of 10 μM dapsone, formation of metHb was significantly slower (Fig. 8B). Scan 2 was also recorded 15 sec after adding 500 μM H_2O_2 to the MPO/HbO_2/dapsone mixture and then at 2 min intervals. Scan 4, which nearly coincided with scan 3, shows a metHb content of 49%. Figure 8C demonstrates the effect of 500 μM H_2O_2 alone on the HbO_2 preparation. The somewhat lower rate of metHb formation in comparison to Fig. 8B is also evident. A metHb content of 31% was calculated from scan 4.

Clofazimine was inactive in this system. In a set of four experiments, H_2O_2 (250 μM) was included in a mixture containing 150 mM NaCl and 1 μM each of HbO_2 and MPO in 50 mM phosphate buffer (pH 7.4). In the absence of drug, the percentage metHb formed was 32.9 ± 3.4 ($P < 0.01$) within 15 sec and

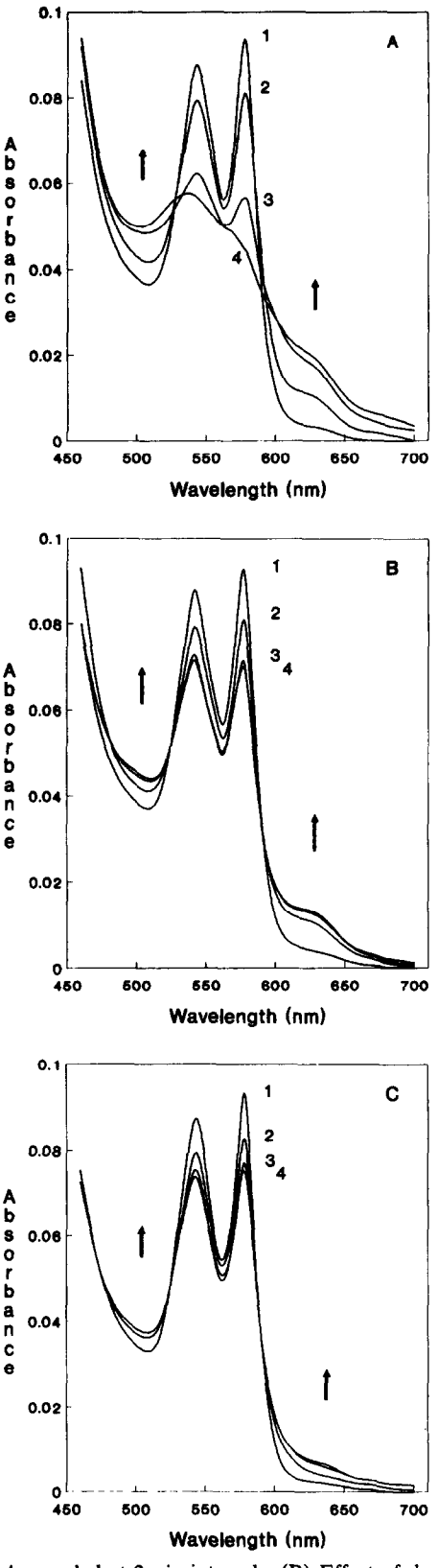

Fig. 8. Inhibition of MPO-induced metHb formation. Reactions were performed in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. (A) Absorbance spectrum of 1.7 μM HbO_2. Both sample and reference cuvettes contained 1 μM MPO. Scan 1, before H_2O_2 addition; scan 2, 15 sec after adding H_2O_2 (500 μM in solution); scans 3 and 4 recorded at 2 min intervals. (B) Effect of dapsone on HbO_2 (1.7 μM) spectrum. Reference and sample cuvettes contained 1 μM MPO and 10 μM dapsone. Recordings were made as in A. (C) Effect of H_2O_2 alone on HbO_2 spectrum. Recordings were made as in A.

in the presence of 50 μM clofazimine, it was 33.1 ± 3.5 ($P < 0.01$) within 15 sec. In both cases, full conversion to metHb was achieved within 10 min. These results also serve as a control to evaluate the effect of ethanol in the MPO system. An aliquot of 10 μL clofazimine in 95% ethanol was included in the 2 mL reaction mixture, giving a concentration of about 80 mM ethanol. It is evident that this amount of ethanol did not influence the MPO–HbO_2 system. As in the case of dapsone, clofazimine alone had no effect on the oxidation of HbO_2. When both Cl^- and drug was omitted from the MPO system, the proportion of HbO_2 oxidized was 18%, 10 min after initiating the reaction with H_2O_2. In the absence of MPO, H_2O_2 alone (250 μM) oxidized about 20% of the 1 μM HbO_2 within the same period. Thus, the proportion of metHb formed by MPO and H_2O_2 in the absence of Cl^- was close to that formed by H_2O_2 alone. Reagent HOCl (100 μM) also did not oxidize HbO_2. The conversion of only 3% within 10 min cannot be considered as significant.

Accumulation of compound II

The effect of dapsone on the MPO spectrum in the presence and absence of 150 mM NaCl, is shown in the difference scans of Fig. 9. Scan 1 (Fig. 9A) was recorded 15 sec after adding 100 μM H_2O_2 to the sample cuvette containing 5 μM dapsone and 800 nM MPO in 50 mM phosphate buffer (pH 7.4; without Cl^-). The reference cuvette also contained the same concentrations MPO and dapsone. Scan 2 was recorded 30 min later and shows a conversion of about only 12% to the native ferric form. In the absence of dapsone and under the same conditions as above, conversion to the native ferric form of MPO was about 80% (scan 3) as judged from the decrease in the difference peak at 455 nm. Spontaneous decay of compound II in the presence of 200 mM ethanol was about 90% after 30 min, which implies that ethanol had some electron-donatory properties in the MPO system in the absence of Cl^- and dapsone. The same experiment was repeated in the presence of 150 mM NaCl (Fig. 9B). Six min after adding 100 μM H_2O_2 to the reaction mixture containing dapsone and Cl^-, relatively little change in the difference scan was observed (scan 2). At 8 min, however, a rapid change in the spectrum was observed (scan 3) which shows a conversion of more than 70% to the native enzyme. In the absence of dapsone, complete conversion to the native enzyme was achieved within 2 min after adding the H_2O_2 (scan 4). The trough at 430 nm in scan 4 indicates haem loss induced by the MPO–Cl^-–H_2O_2 system. An effect of ethanol was only observed in the absence of Cl^-.

The absorbance of clofazimine interfered with the Compound II peak at 455 nm. The minor Compound II peak at 630 nm [19], however, was free from this interference. Our results show that there was no build-up of Compound II in the presence of clofazimine. In the absence of Cl^-, the decay of Compound II was not influenced by the presence of clofazimine. When Cl^- was present, the decay of Compound II was complete within 2 min, irrespective of the presence of clofazimine (see also Fig. 9B; scan 4).

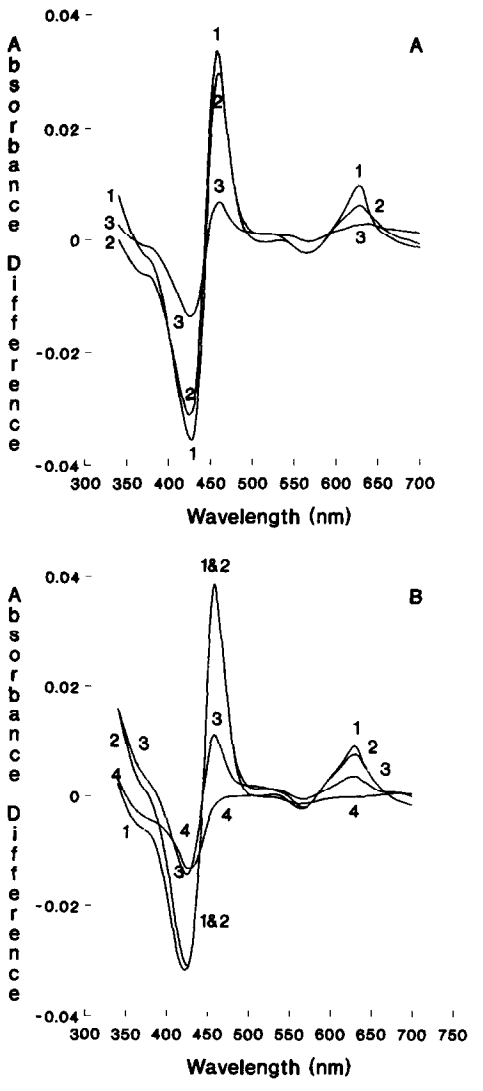

Fig. 9. Accumulation of Compound II induced by dapsone. (A) Accumulation of Compound II in the absence of Cl^-. Both sample and reference cuvettes contained 800 nM MPO in 50 mM phosphate buffer (pH 7.4). For scans 1 and 2 the cuvettes contained 5 μM dapsone, additionally. Reactions were initiated with the addition of H_2O_2 (100 μM in solution) to the sample cuvette. Scan 1, 15 sec after adding H_2O_2; scan 2, 30 min after adding the H_2O_2; scan 3, 30 min after adding the H_2O_2 to the MPO system without dapsone. (B) Accumulation of Compound II in the presence of Cl^-. Both sample and reference cuvettes contained 800 nM MPO and 150 mM NaCl in phosphate buffer and additionally 5 μM dapsone for scans 1–3 only. Reactions were initiated with the addition of H_2O_2 (100 μM in solution). Scan 1, 15 sec after adding H_2O_2; scan 2, 6 min after H_2O_2 addition; scan 3, 2 min later; scan 4, 2 min after adding the H_2O_2 to the MPO system without dapsone.

DISCUSSION

The results of the studies reported here indicate an inhibitory effect of clofazimine and dapsone on MPO-catalysed reactions. One of the assays used was the disappearance of NADH fluorescence during

oxidation. Oxidation of NADH by MPO and H_2O_2 is stimulated by Cl^- (Fig. 2). Under these conditions Cl^- is oxidized by MPO and H_2O_2 and the oxidant, presumably HOCl, oxidizes NADH. Although NADH itself can serve as a MPO substrate, the extent of stimulation of NADH oxidation by Cl^- (50-fold at 150 mM NaCl relative to zero Cl^- concentration) suggests that the chlorinating oxidants generated in the MPO system play a predominant role in the oxidation of NADH. Both drugs inhibit the oxidation of NADH in the MPO–Cl^-–H_2O_2 system (Fig. 3), but dapsone was considerably more potent in this respect. The difference in potencies of the drugs is also demonstrated in their relative abilities to inhibit MPO-induced polypeptide polymerization (SDS–PAGE patterns of Fig. 6). Relatively low concentrations of dapsone (5 μM) completely protected the model polypeptide, TG, against polymerization. Clofazimine, on the other hand, showed some effect only at a 10-fold higher concentration. Furthermore, possible chemically reacting metabolites derived from dapsone or clofazimine did not seem to affect the polypeptide composition of TG.

In the absence of the drugs, inhibition of MPO was likely to be caused by chlorinating oxidants generated in the MPO–Cl^-–H_2O_2 system. In the absence of Cl^-, the drugs had no effect on the loss of MPO activity (curve 1 of Fig. 5) above that caused by H_2O_2. This implies that 300 μM H_2O_2 alone destroyed about 40% of the activity of the 350 nM MPO solution. It is also evident that the inhibition of MPO-catalysed reactions caused by the drugs were fully reversible. Thus possible chemically reacting metabolites were not responsible for MPO inactivation. Dapsone was particularly effective in protecting MPO activity. At a concentration of 5 μM, MPO activity was maximally protected; i.e. 60% of the activity could be recovered, which is the percentage of activity remaining after reaction with 300 μM H_2O_2 alone. It is well known that either reagent HOCl or a similar species (if not identical) generated enzymatically by MPO causes rapid inactivation of MPO. Loss of activity is accompanied by haem loss [20, 21]. This suggests an inhibition of the generation of oxidants, such as HOCl, or scavenging of HOCl, or both. Clofazimine is a better scavenger of HOCl than dapsone when their relative potencies to inhibit NADH oxidation by reagent HOCl are compared (Fig. 4). The HPLC data also demonstrate the stronger ability of clofazimine to scavenge HOCl. When 30 μM HOCl reacted with 75 μM clofazimine, the area under the drug peak was 62% less than that of the control sample. When 100 μM HOCl reacted with 75 μM dapsone, the area under the drug peak decreased by less than 10% relative to the control. In contrast, dapsone was a considerably better inhibitor of the MPO system than clofazimine. These observations suggest that the inhibitory mechanisms of the two drugs must be different.

In order to elucidate the inhibitory action of the drugs, we further investigated alterations in their absorption spectra during cooxidation in the MPO system in the absence and presence of Cl^-. Clofazimine lost some absorbance in the visible region during the reaction (Fig. 7). The bleaching effect was enhanced when the reaction took place in the presence of Cl^-. This suggests that generated chlorinating oxidants are responsible for the increase in bleaching of clofazimine. Such a suggestion is supported by the fact that the reagent HOCl also caused bleaching of clofazimine. HPLC, however, did not reveal significant product formation when clofazimine was included in the MPO system in the absence of Cl^-. Metabolism was detected only in the presence of Cl^- in the MPO system or on reaction with reagent HOCl. In contrast, the spectrum of dapsone, which does not have absorbance in the visible region, did not show any significant changes on reaction with MPO and H_2O_2, irrespective of the presence of Cl^-. Attempts with HPLC to show significant metabolism of dapsone in the MPO system, also failed. The only significant product formation was observed after reaction with reagent HOCl, in which several products were formed.

The ability of anti-inflammatory drugs to scavenge HOCl has been examined [12]. Most drugs tested were capable of reacting with HOCl, but the reactions seemed insufficiently rapid under physiological conditions to protect α_1-anti-proteinase against inactivation by HOCl. However, rapid scavenging of HOCl by penicillamine, gold sodium thiomalate, phenylbutazone and primaquine might contribute to their anti-inflammatory effects. Clofazimine is effective in various chronic inflammatory diseases of the skin including the management of leprosy reactions such as erythema nodosum leprosum [6]. The most prominent adverse effect of clofazimine is a dose-related skin pigmentation caused by the accumulation of the drug itself [1]. This red to brown discolouration of the skin tends to be more pronounced at the leprotic lesions [22]. Thus, relatively high concentrations of clofazimine are present at the site of the leprotic lesion which can scavenge extracellular HOCl generated by activated neutrophils *in situ*.

The mechanism of the inhibition of MPO-catalysed reactions by dapsone was different. Firstly, dapsone was a less potent scavenger of HOCl than clofazimine (Fig. 4). Dapsone is metabolized in man through N-hydroxylation by hepatic microsomal enzymes [23]. Since a similar metabolism of dapsone to a hydroxylamine has been reported to occur in neutrophils and mononuclear cells, it was proposed that the anti-inflammatory properties of dapsone are due to its oxidation by MPO, i.e. that dapsone is a competitive electron donor which depletes H_2O_2 and consequently decreases the generation of other pro-inflammatory compounds [11]. Furthermore, dapsone has been reported to inhibit MPO [5] and since Uetrecht *et al.* [11] reported that dapsone is also metabolized by isolated MPO and H_2O_2, the mechanism for the anti-inflammatory action of dapsone described above seems plausible.

Although aromatic amines have long been recognized as one of the groups of specific peroxidase substrates [24], we could find no significant metabolism of dapsone in our MPO system, irrespective of the presence of Cl^-. Evidence for such a statement, are as follows:

(1) lack of significant product information during the MPO-induced reaction. The only significant metabolism of dapsone occurred after reaction with reagent HOCl. Our HPLC experiments are described in detail in the Materials and Methods and the Results sections.
(2) Treatment of patients with dapsone can result in methaemoglobinaemia, which is thought to be caused by the *N*-hydroxyl derivatives of the drug [25]. In contrast, we found dapsone to be capable of suppressing metHb induced by the MPO–Cl^-–H_2O_2 system (Fig. 8). This suggests that dapsone was not N-hydroxylated in our system. Clofazimine at a concentration of 50 μM did not inhibit metHb formation in this system. Since reagent HOCl (up to 100 μM) did not oxidize HbO_2, dapsone must have blocked a MPO-catalysed reaction responsible for metHb formation, other than HOCl generation. Chloride, nevertheless, seems to be involved in this metHb formation, since MPO and H_2O_2 in the absence of the halide could not oxidize HbO_2 above that caused by H_2O_2 alone. As a matter of fact, metHb formation was even lower, since some H_2O_2, capable of forming metHb, would have been consumed by the MPO.

The reason for the discrepancy between our results and those of Uetrecht *et al.* [11] is unclear at this stage, but it is worth mentioning that they could not oxidize dapsone in a horseradish peroxidase system under conditions similar to those used in their MPO reaction system. We nevertheless do not rule out the possibility that dapsone can be metabolized to some extent in the MPO system; HPLC studies showed traces of product formation. Our results suggest that the inhibition of MPO activity by dapsone outweighs the ability of MPO to metabolize the drug. We thus want to propose an alternative mechanism by which dapsone can reversibly inhibit MPO activity.

MPO reacts with H_2O_2 to form a highly unstable, catalytically active complex (Compound I), which reacts with a variety of electron donors to regenerate the native ferric enzyme with oxidation of the electron donor. In the absence of a suitable electron donor or in the presence of an excess of H_2O_2, Compound I is converted to Compound II which is inactive as a catalyst of halide oxidation. Thus accumulation of Compound II leads to a progressive inhibition of MPO catalysis [26]. Conversely, it has been shown that vitamin C stimulates the chlorinating activity of MPO by reacting with Compound II and thereby preventing the build-up of this inactive species [27]. In Fig. 9 it is shown that dapsone, even at relatively low concentrations (5 μM), stimulated the build-up of Compound II (ferryl MPO) both in the absence and presence of Cl^-. Under similar conditions, clofazimine did not cause accumulation of Compound II. Recently, it has been shown that monochlorodimedon, a compound used to monitor chlorination reactions, also caused accumulation of compound II during its reaction in the MPO–Cl^-–H_2O_2 system [28].

In conclusion, both clofazimine and dapsone inhibit reactions catalysed by MPO. The reactions inhibited include the Cl^--stimulated oxidation of NADH, polymerization of a model polypeptide (TG) and suicide inhibition of MPO catalytic activity. Most of these inhibition reactions can be ascribed to a reduced availability (scavenging) or a reduced generation of chlorinating oxidants by the MPO system. Spectral evidence suggests that clofazimine is metabolized to some extent by the MPO system in the absence of Cl^-, but this could not be confirmed by HPLC. Metabolism of clofazimine was considerably enhanced in the presence of Cl^-, suggesting the involvement of chlorinating oxidants. Clofazimine was found to be a more potent scavenger than dapsone of HOCl. The fact that high concentrations of the lipophilic clofazimine accumulate in various body tissues give some *in vivo* relevancy to these findings. Dapsone was found to be a superior inhibitor of MPO and at relatively low concentrations completely inhibited various MPO-catalysed reactions. The effect of dapsone was to convert, or to promote accumulation of inactive Compound II. The different inhibitory effects of clofazimine and dapsone towards to MPO system may be mechanisms which contribute to their anti-inflammatory actions.

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