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OXIDATIVE METABOLISM OF MITOXANTRONE BY THE HUMAN NEUTROPHIL ENZYME MYELOPEROXIDASE

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Abstract—The anti-cancer drug mitoxantrone is readily oxidized by the human heme enzyme myeloperoxidase (MPO) and H_2O_2. Direct oxidation yielded up to three products, which depended on the ratio of H_2O_2 to mitoxantrone. At an H_2O_2:mitoxantrone ratio of 1.0, one major product was obtained, with a spectrum and HPLC retention time identical to that resulting from oxidation by horseradish peroxidase. This metabolite is a substituted hexahydronaphtho[2,3-*f*]quinoxaline-7,12-dione and has been discovered in the urine of patients treated with mitoxantrone, hence implicating MPO in the *in vivo* metabolism of mitoxantrone. At higher concentrations of H_2O_2, the oxidation of mitoxantrone was more complex, with two further metabolites being identified. When mitoxantrone was incubated with neutrophils that had been stimulated with phorbol myristate acetate, it was oxidized by an MPO-dependent mechanism. Therefore, it appears that MPO may play a significant role in the clinical activity displayed by mitoxantrone against acute myelogenous leukemias, as neutrophils, monocytes and their bone marrow precursors contain high levels of the enzyme.

Key words: myeloperoxidase; mitoxantrone; oxidized metabolites; HPLC; neutrophil; azide inhibition

The anthracenedione-based anti-cancer drug mitoxantrone (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]-ethyl]amino]-9,10-anthracenedione dihydrochloride), a synthetic anti-cancer analogue of the anthracycline antibiotics (Fig. 1), has shown significant clinical effectiveness in the treatment of a range of human malignancies [1, 2]. In contrast to the anthracyclines, mitoxantrone produces less side-effects, such as nausea, vomiting, alopecia and cardiac toxicity [3, 4].

Mitoxantrone exhibits a redox potential significantly more negative than those found for the anthracyclines [5]. This more negative redox potential is believed to be responsible for the lack of significant reductive metabolism of mitoxantrone and poor redox cycling compared with the anthracyclines [5, 6]. However, mitoxantrone has been shown recently to be oxidized by horseradish peroxidase to yield metabolites that bind covalently and non-covalently to DNA [7–9]. Other anti-cancer agents have also been shown to form reactive metabolites after enzymic activation [10, 11], and the role of oxidative activation of mitoxantrone may also have significant implications regarding its mode of action.

The heme enzyme MPO§ has oxidative activity similar to that of horseradish peroxidase and, therefore, is potentially capable of metabolizing mitoxantrone in humans. MPO is the most abundant protein in neutrophils, constituting 5% of their dry weight, and is also found in monocytes [12]. MPO uses H_2O_2 to catalyse the direct oxidation of many xenobiotics including paracetamol and hydroquinone, and has been shown recently to oxidatively metabolize the anti-cancer drug amsacrine [13–15]. It also uses H_2O_2 to convert chloride ions to HOCl, the major strong oxidant produced by neutrophils [12]. Therefore, we have undertaken this study to investigate the direct oxidation of mitoxantrone by MPO.

MATERIALS AND METHODS

Materials. Mitoxantrone was supplied by Lederle Laboratories (Pearl River, NY). The drug was prepared daily by dissolving it in distilled water to a final concentration of 2 mM. Horseradish peroxidase (EC 1.11.1.7) and glutathione were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade.

MPO was purified to a purity index (A_{430}/A_{280}) of 0.75 as described previously [16], and its concentration was determined using $\varepsilon_{430} = 91{,}000\ M^{-1}\ cm^{-1}$ [17]. The concentration of HOCl (Reckitt & Colman Ltd., Avondale, New Zealand) was determined by reacting with monochlorodimedon ($\varepsilon_{290} = 19{,}000\ M^{-1}\ cm^{-1}$) and measuring the change in absorbance at 290 nm due to the production of dichlorodimedon [16].

Methods. Absorption spectra were measured in 1 cm quartz cuvettes using a Cary 1E spectrophotometer. The oxidation of mitoxantrone was initiated by the addition of H_2O_2 to the other reactants, unless stated otherwise. Spectral studies

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§ Abbreviations: MPO, myeloperoxidase; MXH_2, mitoxantrone; HOCl, hypochlorous acid; PMA, phorbol 12-myristate 13-acetate; AML, acute myelogenous leukemia.

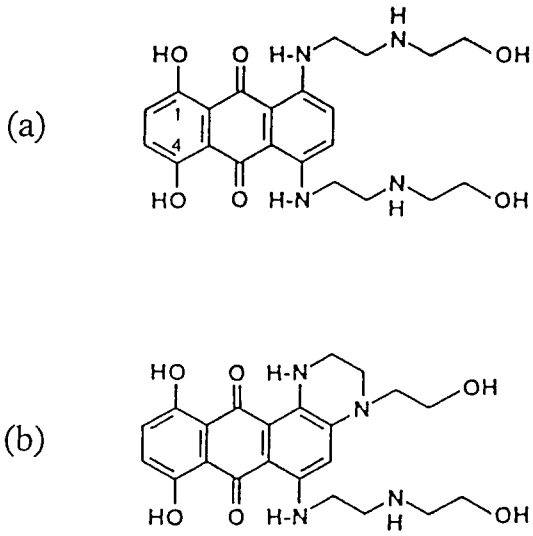

Fig. 1. Structures of mitoxantrone (a) and the initial oxidized metabolite, product B (b).

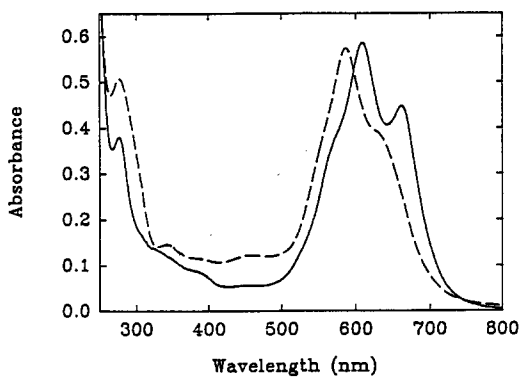

Fig. 2. Absorption spectra resulting from the MPO-catalysed oxidation of mitoxantrone at equimolar concentrations of mitoxantrone and H_2O_2. Addition of 40 μM H_2O_2 and 100 nM MPO to 40 μM mitoxantrone (——) for 10 min, resulted in the formation of product B (– – –).

were conducted at room temperature in 50 mM phosphate buffer, pH 7.4.

HPLC studies were conducted using a Waters 600E instrument equipped with a Waters 990$^+$ photodiode array detector. A Nova-Pak® C8 HPLC cartridge column was utilized for all HPLC experiments and was purchased from Waters Millipore. The isocratic mobile phase consisted of H_2O, acetonitrile, 2-methoxyethanol and 90% formic acid, at a ratio of 1550:250:100:100 with a flow rate of 1 mL/min.

Human neutrophils were isolated from healthy donors using a procedure described previously [18]. For studies of the metabolites arising from the oxidation of mitoxantrone by PMA-stimulated human neutrophils, freshly isolated human neutrophils were resuspended in PBS to a final concentration of 1×10^6 cells/mL. PMA was added to a final concentration of 0.1 μg/mL, and the solution was incubated at 37° for 5 min to initiate a respiratory burst. Mitoxantrone was then added to a final concentration of 25 μM, and the solution was incubated for a further 75 min. At the end of the incubation the solution was centrifuged (1000 *g* for 10 min), and 100 μL of the resulting supernatant was analysed for metabolites by HPLC. Conditions for the HPLC studies were as described above.

For studies of the MPO dependence of mitoxantrone oxidation by human neutrophils, freshly isolated human neutrophils (2×10^6 cells/mL) were stimulated with PMA in the presence of various inhibitors of MPO. Mitoxantrone (20 μM) was then added and the cells were incubated at 37° for 30 min. Following incubation the cells were pelleted, and the loss in absorbance at 608 nm of the supernatant was compared with that for unstimulated cells.

RESULTS

Direct oxidation of mitoxantrone by MPO. The addition of H_2O_2 to a solution containing mitoxantrone and MPO caused a change in the spectral characteristics of the drug. This oxidation of mitoxantrone by MPO was dependent on the ratio of H_2O_2 to mitoxantrone, and did not occur in the absence of either MPO or H_2O_2.

Spectral studies conducted at equimolar concentrations of mitoxantrone and H_2O_2 revealed that mitoxantrone was oxidized to a new metabolite(s) (Fig. 2). HPLC studies were conducted on the product(s) and revealed one dominant compound (product B) (Fig. 3B), with a substantially altered retention time (50 min) compared with that of mitoxantrone (6 min). The spectral and HPLC characteristics of this compound were identical to those of the metabolite obtained by oxidation of mitoxantrone with horseradish peroxidase under identical conditions (data not shown). Also apparent from Fig. 3B is an additional minor component, designated as product C.

When the oxidation of mitoxantrone was conducted at an H_2O_2:mitoxantrone ratio of 2.0, there was a substantial increase in the amount of product C (Fig. 3C). This product arose from the further MPO-catalysed oxidation of product B, and was quite distinct from product B, with a relatively short retention time of 22 min.

At an H_2O_2:mitoxantrone ratio of 3.0, spectral studies of the oxidation of mitoxantrone by MPO revealed a complex behaviour (Fig. 4). Initially, the visible absorbance peaks of mitoxantrone at 608 and 656 nm decreased; however, after approximately 6 min, there was a progressive increase in the absorbance at 590 and 630 nm. The conversion of these products (at 6 min) to the final products was independent of MPO, as the addition of azide (MPO inhibitor) or catalase at this time did not inhibit the reaction (data not shown). To interpret these spectral results, the products were resolved by HPLC. These studies revealed that after a 10-min incubation at an H_2O_2:mitoxantrone ratio of 3.0, a further metabolite

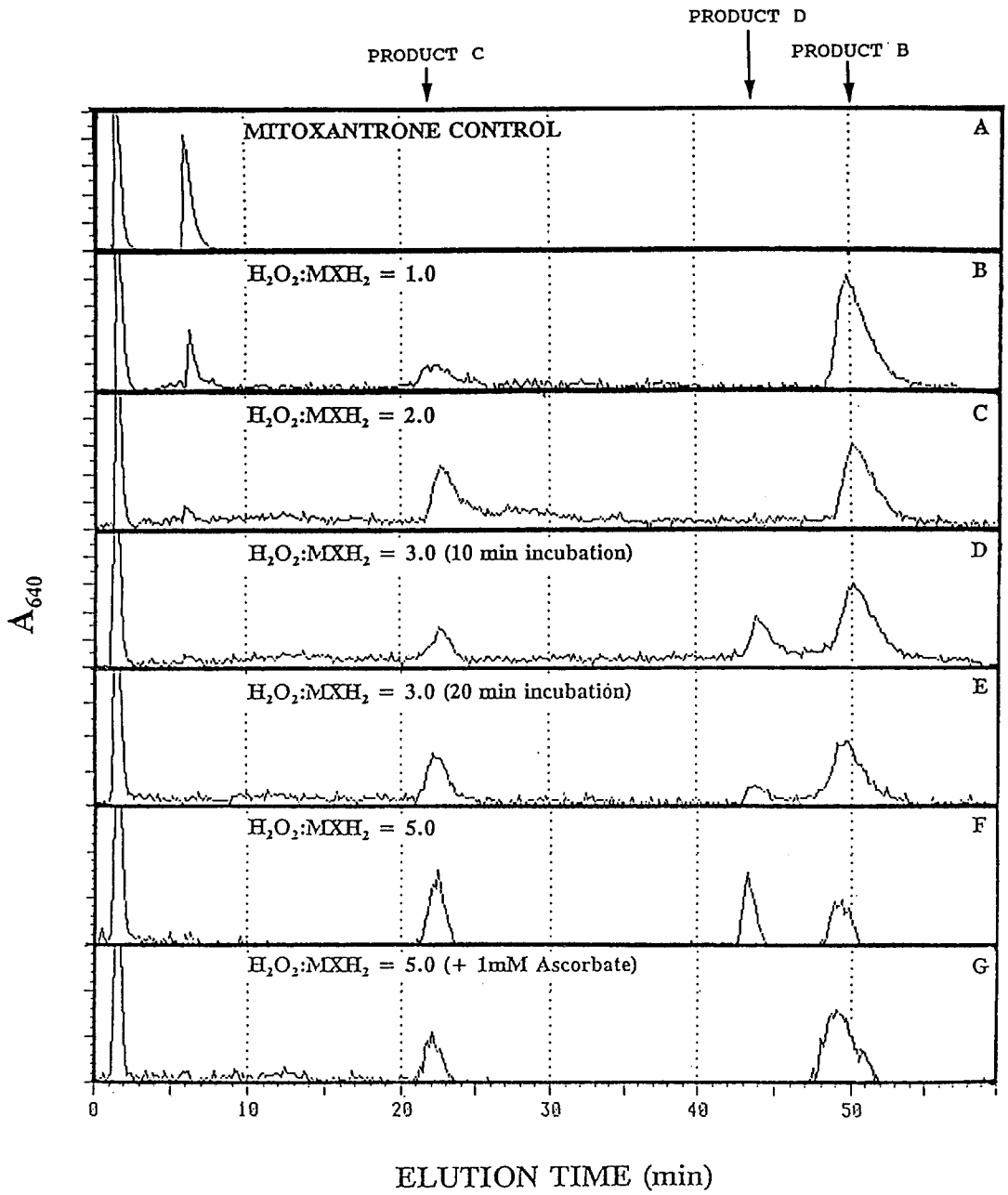

Fig. 3. HPLC resolution of the products resulting from the MPO-catalysed oxidation of mitoxantrone (MXH_2) at various ratios of H_2O_2:mitoxantrone concentrations. (A) mitoxantrone, (B) 10-min incubation at an H_2O_2:mitoxantrone ratio of 1.0, (C) 10-min incubation at an H_2O_2:mitoxantrone ratio of 2.0, (D) 10-min incubation at an H_2O_2:mitoxantrone ratio of 3.0, (E) 20-min incubation at an H_2O_2:mitoxantrone ratio of 3.0, (F) H_2O_2:mitoxantrone ratio of 5.0, and (G) addition of 1 mM ascorbate to the products resulting from the MPO-catalysed oxidation of mitoxantrone at an H_2O_2:mitoxantrone ratio of 5.0 (panel F). As can be seen in all panels (A–G), a peak eluting at 1.5 min is apparent. This is due to the solvent front and does not represent any products resulting from the MPO-catalysed oxidation of mitoxantrone. Complete details of the HPLC conditions utilized are described in Materials and Methods.

was produced and eluted at 44 min (Fig. 3D). This metabolite has been designated as product D. When the incubation time was extended to 20 min, the amount of product D diminished (Fig. 3E).

When the oxidation of mitoxantrone by MPO was conducted at an H_2O_2:mitoxantrone ratio of 5.0 (Fig. 3F), products C and D were obtained at a higher level than at a ratio of 3.0. At

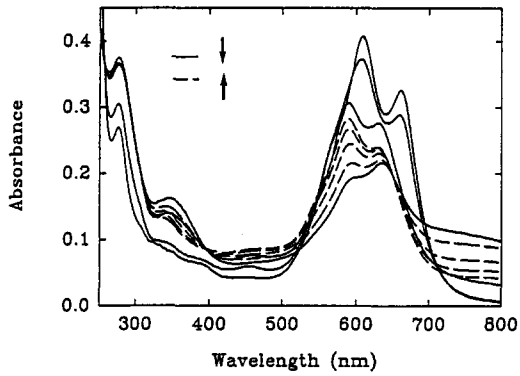

Fig. 4. Serial scans of 25 μM mitoxantrone after the addition of 75 μM H_2O_2 and 100 nM MPO. Solid lines indicate progressive loss in the major peaks of mitoxantrone at 608 and 656 nm. Broken lines show subsequent increase in absorbance at 590 nm. Scans were recorded every 2 min.

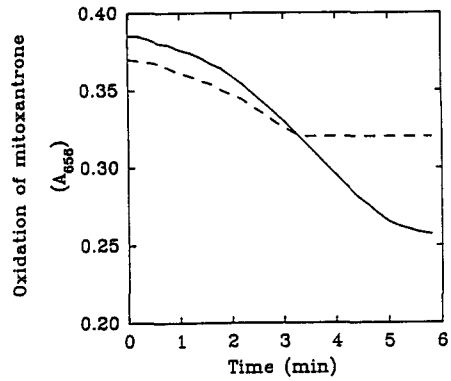

Fig. 6. Effect of chloride on the oxidation of mitoxantrone by MPO. H_2O_2 (50 μM) was added to 20 μM mitoxantrone and 100 nM MPO in 50 mM phosphate buffer, pH 7.4, containing either 100 mM sodium chloride and 1 mM methionine (– – –) or 33 mM sodium sulphate (——). Oxidation of mitoxantrone was followed by monitoring the loss in absorbance at 656 nm.

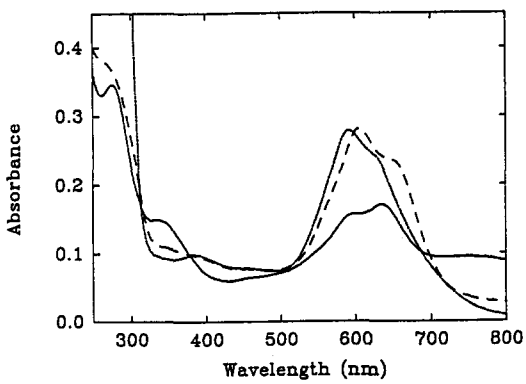

Fig. 5. Effect of reducing agents on the MPO-derived oxidation products. To the products resulting from the MPO-catalysed oxidation of mitoxantrone at an H_2O_2:mitoxantrone ratio of 5.0 (——) was added 1 mM ascorbate (—··—) or 100 μM glutathione (— — —).

H_2O_2:mitoxantrone ratios greater than 5.0, the oxidation process was more complex, and a range of products was observed (data not shown).

Figure 5 shows the effect of reducing agents on the products arising from the oxidation of mitoxantrone by MPO at an H_2O_2:mitoxantrone ratio of 5.0. The addition of 1 mM ascorbate to these products caused a substantial increase in the absorbance of the solution, producing a spectrum with an absorbance maximum at 590 nm. HPLC analysis revealed that the addition of ascorbate to the products (from the oxidation of mitoxantrone at an H_2O_2:mitoxantrone ratio of 5.0) caused the complete loss of product D and a reduction in the peak area of product C (Fig. 3G). An analysis of the peak areas revealed that the reduction of products C and D by ascorbate did not yield new metabolites; rather, they had been reduced back to product B. From these results it is apparent that product D shows the greatest propensity to undergo chemical reduction. Product C is also reduced, but to a lesser extent. Product B does not undergo chemical reduction, indicating that the oxidation of mitoxantrone to product B is irreversible under these conditions, as has been reported previously for HRP-derived product B [7]. The use of reduced glutathione as a reducing substrate yielded a similar absorbance spectrum to that induced by ascorbate, except that the absorbance maximum was shifted to 602 nm. The small shift in absorbance maximum may reflect the formation of a thiol adduct between glutathione and product D, in a manner similar to that suggested for the glutathione reduction of horseradish peroxidase-derived mitoxantrone metabolites [7].

Effect of HOCl and chloride. In the presence of choride ions, MPO converts H_2O_2 to HOCl. It was therefore of interest to determine if HOCl reacts with mitoxantrone, and to determine whether chloride affects the ability of MPO to oxidize mitoxantrone. Direct addition of equimolar reagent HOCl to a solution of mitoxantrone resulted in the formation of a blue precipitate. The precipitate was dissolved in dimethyl formate, and subsequent HPLC analysis revealed only mitoxantrone (data not shown). This result supports previous studies that showed that HOCl forms complexes with amine groups, yielding unstable chloramines [19].

To establish whether chloride affects the oxidation of mitoxantrone, reactions were carried out in the absence or presence of physiological concentrations of chloride. Methionine was included to scavenge HOCl and prevent it from reacting with mitoxantrone, so that only the effects of chloride on the enzyme were observed. Sodium sulphate was included in the chlorine-free buffer to keep the ionic strength constant. As shown in Fig. 6, the rate of mitoxantrone oxidation was unaffected by chloride, but the extent of oxidation was approximately

halved. The reduction in the extent of oxidation would occur because in the presence of chloride some of the H_2O_2 would be diverted to the formation of HOCl.

Oxidation of mitoxantrone by PMA-stimulated human neutrophils. Although it is clear from the above results that mitoxantrone is oxidized by MPO *in vitro*, it was not known if neutrophils (which contain high levels of MPO) were capable of metabolizing mitoxantrone. As shown in Fig. 7, human neutrophils stimulated with PMA oxidized mitoxantrone to product B. To determine if oxidation of mitoxantrone by these cells was dependent on MPO, spectral studies were performed in the presence of various inhibitors of the enzyme (Fig. 8). Catalase and the MPO inhibitors azide and 4-aminobenzoic acid hydrazide (Kettle *et al.*, unpublished results) almost completely abolished oxidation of the drug. From these results we conclude that MPO is involved in the oxidative metabolism of mitoxantrone by neutrophils. The addition of superoxide dismutase enhanced the metabolism of mitoxantrone by neutrophils.

DISCUSSION

We have shown that in the presence of H_2O_2, MPO readily oxidizes mitoxantrone. Furthermore, we have found that neutrophils stimulated with PMA metabolize mitoxantrone by an MPO-dependent mechanism. Even in the presence of physiological concentrations of chloride, mitoxantrone reacts directly with MPO. From these results we propose that oxidative metabolism of mitoxantrone by MPO should be considered as a possible mechanism in the cytotoxicity of mitoxantrone. This proposal is strengthened by the recent finding that product B, the initial metabolite of MPO-dependent oxidation of mitoxantrone, is present in the urine of patients undergoing chemotherapy with this drug [20].

In the cellular environment, HOCl will also be formed, and this could therefore complex with mitoxantrone or directly oxidize the drug. Both of these processes are likely to be minimal since HOCl is likely to be scavenged by a multitude of biological molecules *in vivo*.

The mechanism of oxidation of mitoxantrone by MPO is complex. However, it is known that MPO oxidizes phenols and anilines in two successive one-electron steps to give free radical intermediates. These free radicals then disproportionate or dimerize to form stable end products [21]. The substituents on mitoxantrone that are susceptible to oxidation by MPO are the amino and hydroxyl groups on the aromatic rings. We found that MPO (MP^{3+}) and horseradish peroxidase oxidize mitoxantrone (MXH_2) to the same initial product, i.e. a hexahydronapthol[2,3-*f*]quinoxaline-7,12-dione (Fig. 1). Thus, the amino group must be the primary site of attack as proposed by Lown and co-workers [7, 8] for the plant enzyme. This proposal is supported by the finding that ametantrone, which does not contain the 1,4-hydroxyl groups on the chromophore, was oxidized by MPO to give spectral changes similar to those observed with mitoxantrone (data not shown). In this scheme, the initial radical ($MXH_2^{+\cdot}$) is proposed to cyclize to a secondary radical ($MH_2^{+\cdot}$), which then leads to the formation of MH_2 (product B), either by disproportionation or reduction by mitoxantrone [7, 8]. Thus, the mechanism of formation of product B can be written as:

$$MP^{3+} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + MXH_2 \rightarrow \text{Compound II} + MXH_2^{+\cdot} \quad (2)$$

$$\text{Compound II} + MXH_2 \rightarrow MP^{3+} + MXH_2^{+\cdot} \quad (3)$$

$$MXH_2^{+\cdot} \rightarrow MH_2^{+\cdot} \quad (4)$$

$$MH_2^{+\cdot} \xrightarrow{1e^-} MH_2 \quad (5)$$

Subsequent oxidation of product B by MPO differs from that for horseradish peroxidase. Without detailed knowledge of the further oxidation products, we are unable to propose a likely mechanism for their formation. However, since the redox intermediates of MPO have higher reduction potentials than those for horseradish peroxidase [22], they may be able to oxidize the 1,4-hydroxyl groups on the aromatic ring of product B to form distinctly different products to those produced by the plant enzyme.

There are three lines of evidence that the further oxidation of product B involves the chromophore of the drug: (1) there were significant changes to the absorbance spectrum of product B with further oxidation (compare Fig. 2 and the 10-min spectrum of Fig. 4); (2) treatment of the highly oxidized products (H_2O_2: mitoxantrone = 5.0) with ascorbate resulted in restoration of the absorbance spectrum to that of product B; (3) the MPO-catalysed products of ametantrone (which lacks the 1,4-dihydroxy chromophore substituents of mitoxantrone) were not reduced by ascorbate (data not shown).

While investigating the MPO-dependent oxidation of mitoxantrone in stimulated human neutrophils, it was seen that superoxide dismutase enhanced the oxidation of mitoxantrone. One explanation for this effect is that the superoxide anion inhibits the peroxidation of mitoxantrone by MPO. This inhibition is likely to occur because when neutrophils are stimulated, co-released superoxide and MPO react to form oxymyeloperoxidase [23], which is inactive in the chlorination and peroxidation activities of the enzyme [22, 23]. It has also been shown that superoxide limits HOCl production by neutrophils [24]. Alternatively, superoxide may inhibit oxidation of mitoxantrone by reducing mitoxantrone radicals back to the parent compound. This proposal is supported by the recent finding that superoxide reduces tyrosine radicals back to tyrosine and limits the MPO-dependent formation of dityrosine [25]. Regardless of how superoxide affects the oxidation of mitoxantrone, it is apparent from the present results that neutrophils metabolize this drug by an MPO-dependent mechanism.

In recent times there has been an increasing interest in the use of mitoxantrone against AML [26, 27]. AML is a malignant clonal disease originating in a single deranged multipotent stem cell, leading to lethal overgrowth of myeloid progeny

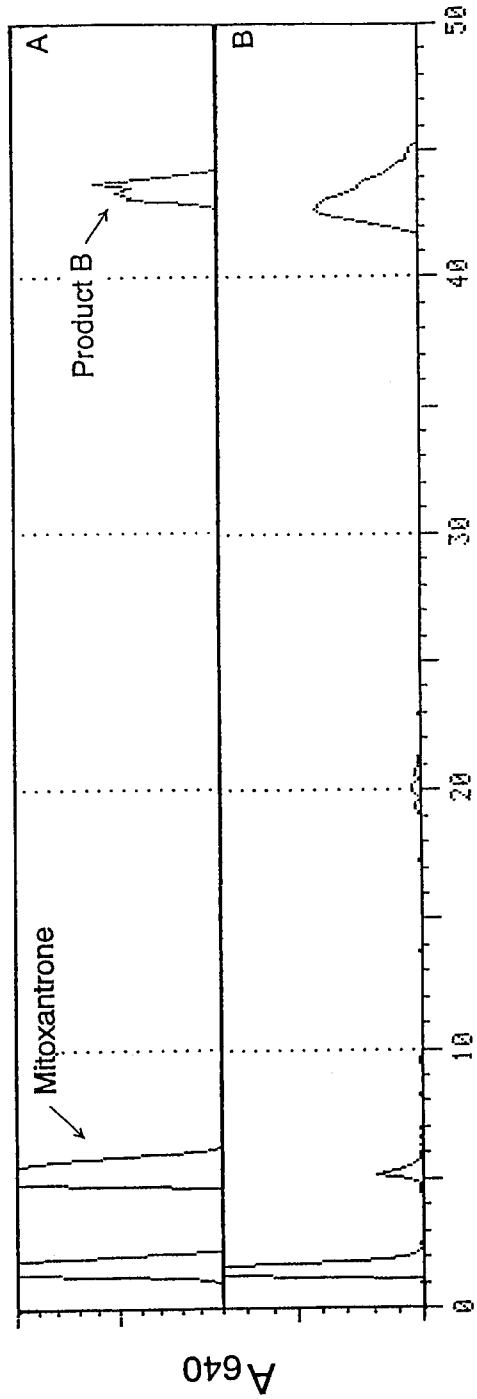

Fig. 7. Oxidation of mitoxantrone by human neutrophils. Mitoxantrone (25 μM) was added to a solution of PMA-stimulated human neutrophils (1×10^6 neutrophils/mL) and incubated for 75 min. After centrifugation, the resulting supernatant was analysed by HPLC, and the product detected is shown in panel A. Panel B shows the elution of *in vitro* derived product B. This was analysed by HPLC immediately following the panel A sample and allows for the identification of the product eluting in panel A. Additional experimental details are given in Materials and Methods.

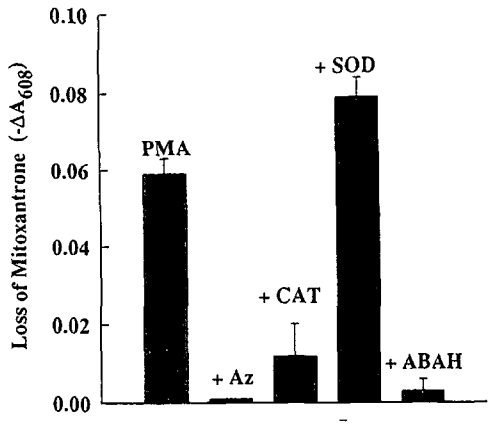

Fig. 8. Effect of MPO inhibitors on the oxidation of mitoxantrone by human neutrophils. Human neutrophils (2×10^6 cells/mL) were stimulated with PMA and then incubated with mitoxantrone (20 μM) in the presence of various MPO inhibitors [1 mM sodium azide (Az), 20 μg/mL catalase (CAT), 20 μg/mL superoxide dismutase (SOD) or 200 μM 4-aminobenzoic acid hydrazide, (ABAH)] for 20 min at 37°. Control cells were subjected to the same procedure but were not stimulated with PMA. After incubation, cells were pelleted, and the loss in absorbance (608 nm) of the supernatant was compared with that of the unstimulated cells. The results shown are the means of duplicate experiments, and the errors represent the maximum variation in these duplicates.

[28]. Since neutrophils and monocytes are myeloid cells and contain MPO, the significant clinical activity of mitoxantrone against AML may be due to the MPO-catalysed oxidation of mitoxantrone to form reactive metabolites of the drug in these cells, hence leading to cell death. Support for the involvement of MPO in the mode of action of mitoxantrone against AML can be found in a study that showed that MPO-containing granulocytes were significantly more sensitive to mitoxantrone than non-MPO-containing lymphocytes [29]. The finding that MPO is involved in the oxidation of mitoxantrone by human neutrophils (Fig. 8) is also consistent with this notion.

As well as apparently contributing to the mode of action of mitoxantrone against AML, MPO may also play a role in the clinical activity displayed by mitoxantrone against malignancies in general. In a recent study, it has been suggested that the close interaction of neutrophils with tumour cells may, in fact, target cytotoxic agents to malignant sites [30]. In this study, an *in vitro* neutrophil-mediated transfer of polar substances from liposomes to mammary tumour cells was described. Considering the significant clinical activity displayed by mitoxantrone against breast cancer [2, 3], it can be envisaged that once administered intravenously, mitoxantrone would be taken up and metabolically activated by neutrophils in the blood. The activated drug may then be transported to the malignancy, where it could exert its anti-tumour effect directly at the malignant site.

The MPO-catalysed oxidation of mitoxantrone described in this paper may be relevant to its biological activity. The discovery of product B in the urine of patients treated with mitoxantrone provides strong support for the involvement of MPO in the *in vivo* metabolism of mitoxantrone. It therefore appears that MPO may serve a critical role in the activation of mitoxantrone, and additional studies are currently in progress to gain further insight into this process.

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