OXIDATION OF A METABOLITE OF INDOMETHACIN
(DES METHYL DE SCHLORO BENZOYLINDOMETHACIN) TO REACTIVE INTERMEDIATES
BY ACTIVATED NEUTROPHILS, HYPOCHLOROUS ACID, AND THE
MYELOPEROXIDASE SYSTEM

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ABSTRACT:
The use of indomethacin is associated with a relatively high incidence of adverse reactions such as agranulocytosis. Many other drugs associated with agranulocytosis are metabolized to reactive metabolites by activated neutrophils. Therefore, we studied the oxidation of indomethacin and its metabolites by activated neutrophils, myeloperoxidase (MPO) (the major oxidizing enzyme in neutrophils), and HOCl (the major oxidant produced by activated neutrophils). No oxidation of indomethacin by activated neutrophils was observed. However, desmethyldeschlorobenzoylindomethacin (DMBI), a major metabolite of indomethacin, was oxidized to a reactive iminoquinone that could be trapped with glutathione (GSH) or N-acetylcysteine (NAC) to form conjugates, with MH ions at m/z 511 and 367, respectively. No metabolism was detected in neutrophils that had not been activated, and the oxidation was inhibited by azide (which inhibits MPO) and by catalase (which catalyzes the breakdown of H2O2). In reactions with HOCl, the same reactive intermediate was formed; its mass spectrum, with a MH+ ion at m/z 204, was obtained by using a flow system in which the reactants were fed into a mixing chamber and the products flowed directly into the mass spectrometer. The same GSH and NAC conjugates were also observed when DMBI was oxidized by HOCl or by the MPO system, followed by addition of GSH or NAC. NMR data for the NAC conjugate indicated that the sulfur was substituted in the 4-position on the aromatic ring. The reactive intermediate generated from DMBI by activated neutrophils may be responsible for indomethacin-induced agranulocytosis.

Indomethacin is a very effective nonsteroidal anti-inflammatory drug, but its use is limited by a high incidence of adverse reactions, including blood disorders. Of a total of 1261 cases of adverse reactions to indomethacin that were reported to the United Kingdom Committee on Safety of Medicines between June 1964 and January 1973, blood disorders were recorded in 157 cases (with 25 fatalities), including thrombocytopenia (35 cases, with 5 fatalities), aplastic anemia (17 cases, with no fatalities), and agranulocytosis or leukopenia (21 cases, with 3 fatalities) (Cuthbert, 1974). Subsequently, the First Report from the International Agranulocytosis and Aplastic Anemia Study confirmed a significant relationship between the use of indomethacin and agranulocytosis and aplastic anemia. In this population-based case-control study conducted in Europe and Israel, the excess risk estimated for agranulocytosis and aplastic anemia was 0.6/10^6 for indomethacin exposure in a 1-week period (Shapiro, et al. 2001). A preliminary report of this work was presented at the Society of Toxicology 36th Annual Meeting, Cincinnati, OH.

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SYNTHESIS OF DMI. DMI was synthesized by demethylation of indomethacin using a modification of the method of McOmie et al. (McOmie et al. 1968). Indomethacin (0.7 g; Sigma Chemical Co., St. Louis, MO) was dissolved in 200 ml of dichloromethane. To this solution, 10 ml of 1 M Br2 was added dropwise. The reaction was protected from moisture by bubbling N2 through the reaction mixture. The reaction was carried out at room temperature for 3 hr. The reaction mixture was then shaken with water to hydrolyze excess reagent and boron complexes. DMI was obtained by extraction into ether. The mass

1 Abbreviations used are: MPO, myeloperoxidase; GSH, glutathione; NAC, N-acetylcysteine; DMBI, desmethyldeschlorobenzoylindomethacin; DMI, O-desmethylindomethacin.
spectrum of the synthesized DMI included a MH\(^+\) ion at \(m/z\) 344. The synthesized DMI demonstrated the same HPLC retention time as did an authentic sample provided by Merck Research Laboratories, and it was used to synthesize DMBI without further purification.

**Synthesis of DMBI.** DMI (15 mg) was hydrolyzed in 3 ml of 1 N sodium hydroxide. The reaction was carried out at room temperature for 5 min. The reaction mixture was made acidic with concentrated hydrochloric acid and then extracted with ethyl acetate. The ethyl acetate layer was dried with magnesium sulfate, and the solvent was evaporated under a stream of \(N_2\). The product was then purified by silica gel TLC, using a solvent of dichloromethane/methanol (9:1, v/v). The yield was approximately 10% [m.p. = 151–152°C; \(R_f = 0.35\); MS: m/z 206 (MH\(^+\)) and 160 ([MH−HCOOH]\(^+\))]\(^1\); \(1^H\) NMR (dimethylsulfoxide-\(d_6\)): \(\delta\) 10.64 ppm (1H, s); 6.85 ppm (1H, bs); H-7, \(\delta\) 7.00 ppm (1H, d, J = 8.54 Hz); H-4, \(\delta\) 6.72 ppm (1H, d, J = 2.20 Hz); H-6, \(\delta\) 6.48 ppm (1H, dd, J = 8.30 and 2.20 Hz); methylene protons, \(\delta\) 3.42 ppm (2H, s); methyl protons on the indole ring, \(\delta\) 2.28 ppm (3H, s)].

**Oxidation of DMBI by HOCl.** Sodium hypochlorite (50 \(\mu\)l of a 10 M phosphate buffer at room temperature. The pH of the phosphate buffer was varied from 4.0 to 7.0. Aliquots (20 \(\mu\)l) of the solution were analyzed by HPLC.

**Synthesis of Indomethacin and DMBI by HOCl.** Sodium hypochlorite (50 \(\mu\)l of a 4 M solution) was added to indomethacin or DMI (final concentration, 1 mM) in 150 \(\mu\)l of 0.1 M phosphate buffer (pH 6) at room temperature. Aliquots (20 \(\mu\)l) of the solution were analyzed by HPLC.

**Oxidation of DMBI by the MPO System.** DMBI (5 \(\mu\)l of a 10 M methanolic solution) was added to 50 \(\mu\)l of phosphate buffer (0.1 M, pH 6 or 7) to yield a final concentration of 0.1 mM. MPO (2.5 units/ml) was added, and then the reaction was initiated by the addition of hydrogen peroxide (final concentration, 0.4 mM). Incubations were carried out in the presence or in the absence of added chloride. Aliquots (20 \(\mu\)l) of the solution were analyzed by HPLC.

**Oxidation of DMBI, Indomethacin, and DMI by Activated Neutrophils.** Blood was drawn from normal volunteers, into heparinized syringes, and the neutrophils were isolated by differential centrifugation on Ficoll-paque (Pharmacia, Uppsala, Sweden) according to a standard procedure (Boyum, 1984).

**Results**

**Oxidation of DMBI by Activated Neutrophils.** DMBI was metabolized by neutrophils to a major metabolite with a MH\(^+\) ion at \(m/z\) 409 and a minor metabolite with a MH\(^+\) ion at \(m/z\) 407. The retention times of the products with MH\(^+\) ions at \(m/z\) 409 and 407 were 5.8 and 24.6 min, respectively. No significant metabolism occurred in the absence of activation of the cells. The metabolism increased with time, initial DMBI concentration, and cell number (fig. 2), and it was inhibited by azide and catalase. The formation of the product with a MH\(^+\) ion at \(m/z\) 409 was decreased by a factor of 2 or 10 when catalase (200 units/ml) or azide (0.2 mM), respectively, was included in the incubation mixture.

The MS/MS spectra of the metabolites with MH\(^+\) ions at \(m/z\) 409 and 407 are shown in fig. 3. To obtain enough material for UV and NMR analysis, manganese dioxide was used to oxidize DMBI; this procedure yielded the same products, except that the amount of the product with a MH\(^+\) ion at \(m/z\) 409 was greater than that of the product with a MH\(^+\) ion at \(m/z\) 407. The UV absorption spectrum of the product with a MH\(^+\) ion at \(m/z\) 409 demonstrated \(\lambda_{max}\) values of 215 and 300 nm. The UV absorption spectrum of the product with a MH\(^+\) ion at \(m/z\) 407 showed an additional broad peak with a \(\lambda_{max}\) value of approximately 480 nm (fig. 4). When the UV absorption spectrum was obtained at a higher concentration, this broad peak was more obvious. The \(^1^H\) NMR spectra of the two products were obtained. The \(\lambda_{max}\) values of the product with a MH\(^+\) ion at \(m/z\) 407 showed that the aromatic region consisted of five protons, as follows: H-7, \(\delta\) 7.29 ppm (1H, d, J = 8.55 Hz); H-4, \(\delta\) 6.71 ppm (1H, d, J = 1.71 Hz); H-6', \(\delta\) 6.65 ppm (1H, dd, J = 9.88 Hz, 1.58 Hz). The loss of the proton signal for the 4-position of the aromatic ring suggested the structure shown in fig. 5. A GSH conjugate with a MH\(^+\) ion at \(m/z\) 714 was formed when this product (MH\(^+\) ion at \(m/z\) 407) was reacted with GSH. This result is consistent with the structure of this product having an iminoquinone moiety (fig. 5). The simplicity of the NMR spectrum of the product with a MH\(^+\) ion at \(m/z\) 409 indicated a highly symmetrical molecule [H-7, \(\delta\) 6.72 ppm (1H, d, J = 8.55 Hz); H-6, \(\delta\) 6.78 ppm (1H, d, J = 8.55 Hz); H-4, \(\delta\) 6.70 ppm (1H, d, J = 1.71 Hz); H-6', \(\delta\) 6.65 ppm (1H, dd, J = 9.88 Hz, 1.58 Hz)]. The MS/MS spectra were recorded at 500 MHz with a Bruker AM 500 spectrometer (Bruker Canada, Milton, Ontario, Canada). Spectra were obtained in \(D_2\)O except for that for DMBI, which was obtained in dimethylsulfoxide-\(d_6\). The structure of the NAC conjugate of the iminoquinone intermediate was assigned by conventional \(^1^H\) NMR analysis as well as \(^1^H\-1^H\) and \(^1^H\-1^C\) correlation experiments.
formation of the two stable metabolites generated in the oxidation by activated neutrophils.

When GSH or NAC was included in the incubation mixture, the GSH or NAC conjugates of a reactive intermediate were observed, with MH$^+$ ions at m/z 511 and 367, respectively. At the same time, the amount of the product with a MH$^+$ ion at m/z 409 was markedly decreased (data not shown).

Oxidation of DMBI by HOCl. DMBI was readily oxidized by HOCl. Scanning of the UV absorption spectrum of this mixture at 1-sec intervals resulted in the pattern shown in fig. 6. The absorbance of DMBI ($\lambda_{\text{max}} = 280$ nm) decreased rapidly and a new species was generated, with increased absorption at longer wavelengths.

When the products of the reaction between DMBI and HOCl were analyzed by MS in the flow system, so that the short-lived intermediate could be detected, an intermediate with a MH$^+$ ion at m/z 204 was observed. Using the MS/MS mode, the major fragment ions were at m/z 176 [MH$^+$CO] and 158 [MH$^+$HCOOH].

When GSH or NAC was added immediately to the reaction mixture of DMBI and HOCl, the GSH or NAC conjugate of the intermediate with a MH$^+$ ion at m/z 204 was observed. The GSH and NAC conjugates formed by trapping of the reactive intermediate generated during DMBI oxidation by neutrophils and HOCl were found to have the same retention times and molecular weights and identical MS/MS spectra. In addition, a n-butylamine conjugate of this intermediate with a MH$^+$ ion at m/z 277 was observed by LC/MS when n-butylamine, instead of NAC, was added to the mixture of DMBI and HOCl. The NAC conjugate was isolated and the NMR analysis was performed (fig. 7). The $^1$H NMR data, combined with the findings from the $^1$H-1H and $^1$H-13C correlation experiments, confirmed the structure of the conjugate where the sulfhydryl group of NAC was substituted in the 4-position on the aromatic ring (fig. 8).

When DMBI was oxidized by HOCl, another product was formed, with a MH$^+$ ion at m/z 238 and a chlorine isotope peak for the MH$^+$...
ion at m/z 240. The MS/MS spectrum of this product showed a major fragment ion at m/z 202, resulting from the loss of HCl, which suggested that this was a chlorinated species. This product was not observed when the HOCl concentration was lower than one fifth of the DMBI concentration. It was also not observed when GSH or NAC was added immediately to the reaction mixture of DMBI and HOCl. These results suggested that this chlorinated species was formed by further oxidation with excess HOCl.

Oxidation of DMBI by the MPO System. Compared with oxidation by neutrophils, a similar pattern of products was obtained when DMBI was oxidized by the MPO/H₂O₂ system without added Cl². The major product showed a MH⁺ ion at m/z 409. When GSH was included in the incubation mixture, the same GSH conjugate, with a MH⁺ ion at m/z 511, was observed. When 0.15 mM Cl² was added to the phosphate buffer, the reaction was more rapid and the major product observed had a MH⁺ ion at m/z 238. This is the same product as that observed when DMBI was oxidized by higher concentrations of HOCl.

Oxidation of Indomethacin by HOCl and Activated Neutrophils. Indomethacin was oxidized by HOCl to a major product with a molecular weight of 391, corresponding to indomethacin plus chlorine minus hydrogen. However, no GSH conjugates were observed when GSH was added to the reaction mixture of indomethacin and HOCl. When indomethacin was incubated with activated neutrophils under the same conditions as those used for DMBI, no oxidation was observed.

Oxidation of DMI by HOCl and Activated Neutrophils. DMI was oxidized by HOCl to two major products, with retention times of
16 and 28 min. The molecular weight of the product with a retention time of 16 min was 2 mass units less than that of DMI. The other product, with a retention time of 28 min, showed a molecular weight of 378, corresponding to DMI plus chlorine minus hydrogen. No GSH conjugates were observed when GSH was added to the reaction mixture of DMI and HOCl. When DMI was incubated with activated neutrophils, only the product with a retention time of 16 min was observed. When GSH was included in the incubation mixture, no GSH conjugates were detected.

**Discussion**

DMBI was oxidized by neutrophils to a reactive intermediate that could be trapped by GSH. MPO appears to be the enzyme in activated neutrophils responsible for this oxidation. This is supported by our observations that the MPO/H2O2 system generated the same metabolites as did neutrophils, that the metabolism by neutrophils required activation of the cells (resulting in the release of MPO and the generation of H2O2), and that the metabolism by neutrophils was inhibited by azide (which inhibits MPO) and catalase (which catalyzes the breakdown of H2O2).

The mechanism presumably involved N-chlorination, followed by the loss of HCl to form an iminoquinone, as shown in fig. 8. N-Chloro-DMBI was not directly observed. However, strong evidence for the identity of the reactive iminoquinone intermediate was obtained. The diode-array UV spectrophotometric data showed that DMBI absorbance (λmax = 280 nm) decreased rapidly upon addition of HOCl and a new species, with absorbance at longer wavelengths (suggesting a quinone-like structure), was formed within seconds. When the products of the reaction between DMBI and HOCl were analyzed by MS in the flow system, the iminoquinone intermediate, with a MH+ ion at m/z 204, was observed. This iminoquinone intermediate was reactive with sulfydryl-containing molecules, such as GSH and NAC. The proton NMR data confirmed the structure of the iminoquinone-NAC conjugate, in which the sulfur was substituted in the ortho-position (relative to the hydroxyl group) on the aromatic ring.

When DMBI was oxidized by activated neutrophils, the final stable metabolites, with MH+ ions at m/z 409 and 407, were formed. Both products were generated from the iminoquinone intermediate reacting with another molecule of DMBI. In the case of the product with a MH+ ion at m/z 409, the iminoquinone intermediate reacted with another DMBI molecule in the 4-position, which is ortho to the hydroxyl group. The aromatic carbon ortho to the hydroxyl group of DMBI molecule is expected to be electron-rich and could act as a nucleophile to attack carbon-4 of the iminoquinone intermediate (fig. 5). Another possible mechanism for the formation of the product with a MH+ ion of m/z 409 involves a coproportionation reaction between DMBI and the iminoquinone, to form two radicals that could dimerize. However, because of the solvent cage effect, the free radical might not be able to react with other molecules and contribute to the toxicity; even if a free radical is formed, the major reaction with GSH involves the electrophilic attack of the iminoquinone, rather than the abstraction of a hydrogen atom by a free radical. Presumably the same is true of the reaction between the reactive metabolite and other biological molecules. In the case of the minor product with a MH+ ion at m/z 407, the iminoquinone intermediate reacted with another molecule of DMBI, on the nitrogen of the indole ring, to form a dimer. This is consistent with our observation that the iminoquinone intermediate can react with nitrogen-containing nucleophiles (e.g. N-butyamine), in addition to sulfhydryl-containing nucleophiles. The initially formed dimer can be further oxidized to yield the product with a MH+ ion at m/z 407 (fig. 5).

A product with a MH+ ion at m/z 238 was observed only when DMBI was oxidized by HOCl at high concentrations (presumably yielding an excess of chlorine). When GSH or NAC was added immediately to the reaction mixture of DMBI and HOCl, this product with a MH+ ion at m/z 238 was not observed, which suggested that it was a downstream product when the iminoquinone was the initial reactive metabolite. This product, with a MH+ ion at m/z 238, was not observed when DMBI was oxidized by activated neutrophils or by purified MPO in the absence of added Cl−. This product is probably not a significant in vivo metabolite.

The formation of reactive metabolites is central to most mechanistic theories of drug hypersensitivity reactions (Uetrecht, 1990). Studies from our laboratory have found that several drugs that are associated with a relatively high incidence of hypersensitivity reactions (especially agranulocytosis and lupus) are metabolized to reactive metabolites by activated leukocytes. Similarly, we propose that oxidation of DMBI to the iminoquinone reactive intermediate by activated neutrophils is responsible for hypersensitivity reactions to indomethacin (i.e. agranulocytosis). There is evidence to suggest that other iminoquinones generated by the MPO system or activated neutrophils may cause hypersensitivity reactions (Maggis et al., 1987; Miyamoto et al., 1997; Parrish et al., 1997; Smith et al., 1989; Uetrecht et al., 1994).

In summary, DMBI, a major metabolite of indomethacin in the liver, is metabolized by the MPO enzyme system in activated neutrophils, forming a reactive iminoquinone intermediate. We propose that the iminoquinone intermediate is responsible for some of the idiosyncratic reactions associated with indomethacin, especially agranulocytosis.

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**References**


