Bioactivation of Minocycline to Reactive Intermediates by Myeloperoxidase, Horseradish Peroxidase, and Hepatic Microsomes: Implications for Minocycline-Induced Lupus and Hepatitis

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ABSTRACT:

Of the tetracyclines, minocycline is unique in causing a significant incidence of a lupus-like syndrome and autoimmune hepatitis. It is also unique among the tetracyclines in having a para-\(\text{N},\text{N}\)-dimethylaminophenol ring. Many drugs that cause autoimmune reactions are oxidized to reactive metabolites by the myeloperoxidase (MPO) system of macrophages. In this study, we showed that minocycline is oxidized to reactive intermediates by MPO/H\(_2\)O\(_2\)/Cl\(^-\), HOCl, horseradish peroxidase/H\(_2\)O\(_2\), or hepatic microsomes. When trapped with N-acetylcysteine (NAC), two adducts with protonated molecular ions at m/z 619 were isolated and analyzed by NMR. One represents attack of the aromatic D ring by NAC meta to the N\(\text{N}\)-dimethylaminophenol group, which implies that the reactive intermediate was a quinone iminium ion. The NMR of the other adduct, which was not observed when minocycline was oxidized by hepatic microsomes, indicates that the NAC is attached at the junction of the B and C rings. In the oxidation by HOCl, we found an intermediate with a protonated molecular ion of m/z 510 that represents the addition of HOCl to minocycline. The HOCl presumably adds across the double bond of the B ring, and reaction of this intermediate with NAC led to the second NAC adduct. We were surprised to find that the same NAC adduct was not observed after oxidation of tetracycline with HOCl, even though this part of the tetracycline structure is the same as for minocycline. We propose that one or more of these reactive metabolites are responsible for the idiosyncratic drug reactions that are specific to this tetracycline.

Minocycline (Fig. 1), a tetracycline antibiotic (Kamel et al., 2002), is used for the treatment of acne (Cullen and Cohan, 1976), infectious diseases, and rheumatoid arthritis. However, minocycline is associated with serious adverse reactions, which include hypersensitivity reactions (Antunes et al., 1999), a serum sickness-like reaction (Puyana et al., 1990), hepatitis (Hardman et al., 1996), and a drug-induced lupus-like reaction (Farver, 1997). The incidence of minocycline-induced lupus is approximately 15 cases/100,000 prescriptions and is more common in women (Borchers et al., 2007). Patients treated with minocycline often develop perinuclear antineutrophilic cytoplasmic antibodies (P-ANCA, usually antimyeloperoxidase) (Dunphy et al., 2000), and in patients with minocycline-induced lupus, P-ANCA is present in 80% of cases (Sturkenboom et al., 1999).

Minocycline-induced hepatitis can lead to hepatic failure requiring liver transplantation (Boudreaux et al., 1993). The incidence of minocycline-induced hepatitis in new users is approximately 1/10,000 patients (Seaman et al., 2001). Reports to the World Health Organization of the hepatic adverse drug reactions associated with the minocycline accounted for 6% (493) of all the minocycline-induced adverse drug reactions (8025) (Lawrenson et al., 2000). The reported cases of minocycline-induced hepatitis are characterized by autoimmune characteristics, including antinuclear antibodies and a very long duration of therapy (more than 1 year) (Lawrenson et al., 2000) before the onset of clinical signs of hepatitis (Knowles et al., 1996; Teitelbaum et al., 1998). There is a large amount of circumstantial evidence to support the hypothesis that most idiosyncratic drug reactions are caused by reactive metabolites (Lavergne et al., 2008). Cytochromes P450 (P450s) are likely responsible for the production of more reactive metabolites than any other enzyme; however, myeloperoxidase (MPO) is also capable of forming reactive metabolites, especially when the drug has an easily oxidized nitrogen (Uetrecht, 1992). The cells that contain MPO include neutrophils, macrophages, and other antigen-presenting cells.

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ABBREVIATIONS: P-ANCA, perinuclear antineutrophilic cytoplasmic antibodies; P450, cytochrome P450; MPO, myeloperoxidase; HRP, horseradish peroxidase; NAC, N-acetyl-L-cysteine; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography interfaced with mass spectrometry; PBS, phosphate-buffered saline; LC/MS/MS, liquid chromatography interfaced with fragmentation mass spectrometry; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; amu, atomic mass unit.
presenting cells is central to initiation of an immune response. A
generalized activation of macrophages has the potential to cause an
autoimmune syndrome, and many of the drugs that cause drug-
induced lupus, including procainamide, hydralazine, propylthiouracil,
and sulfamethoxazole, are oxidized to reactive metabolites by MPO
(Uetrecht, 2005).

The observation that minocycline forms dark pigment in the thyroid
is probably because of its oxidation to reactive species by thyroid peroxidase, which is very similar to MPO (Doerge et al., 1997).
Minocycline is the only tetracycline that is associated with a relatively
high incidence of lupus and autoimmune hepatitis, and it is the only
tetracycline that has a dimethylamino group para to the aromatic
phenol (Fig. 1). This structure is likely to be oxidized to a reactive
quinone iminium ion. The goal of this work was to characterize the
oxidation of minocycline by MPO, HOCl (which is the major MPO-
generated oxidant), other peroxidases [horseradish peroxidase
(HRP)], and P450s as a first step in the investigation of the mechanism
of minocycline-induced autoimmunity.

Materials and Methods

Materials. Minocycline, N-acetylcysteine (NAC), NaOCl, H2O2 (30% w/v), HRP type VI (250–330 U/mg protein), and human MPO (50 U/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO). The concentration of NaOCl was determined by a spectrophotometric method (Hussain et al., 1970). All the solvents used for high-performance liquid chromatography (HPLC), and liquid chromatography interfaced with the mass spectrometry (LC/MS) analysis were of HPLC grade. Phosphate-buffered saline (PBS; 1×) was obtained from Invitrogen (Carlsbad, CA).

Analytical Methods. The HPLC analyses were carried out using a Hewlett Packard (Palo Alto, CA) series 1050 HPLC system with the detector wave
diagram at 254 nm. The HPLC columns used were Gemini C18, 5 μm (Phenomenex, Torrance, CA), 150 × 2 mm with a flow rate of 0.2 ml/min for analytical work and 150 × 10 mm with a flow rate of 5 ml/min for semi
preparative work. The mobile phase used for analytical work was acetonitrile/water/acetic acid (10:90:0.7, v/v) with 2 mM ammonium acetate, pH = 4, unless otherwise stated.

LC/MS and LC interfaced with tandem mass spectrometry (LC/MS/MS) were performed using a Shimadzu (Kyoto, Japan) HPLC LC-10AD coupled with a PE Sciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Concord, CA) using electrospray ionization in the positive ion mode. Analyses were carried out with an ionizing voltage of 5 kV and an orifice voltage of 65 V. The peaks obtained in all the LC/MS experiments for less than 2 min correspond to the solvent front. For LC/MS/MS experiments, the collision energy used was 40 eV. UV spectrometry was performed using a Varian, Inc. Cary model 50 UV-visible spectrophotometer in the scanning kinetics mode. 1H NMR and 13C-1H coupling heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC) experi
ments were recorded at 500 MHz with a Varian, Inc. Unity Plus 500 spectrometer with D2O or CD3OD as the solvent.

Oxidation of Minocycline by HOCl and Trapping the Reactive Inter
mediates with Various Nucleophiles. To minocycline dissolved in water
(10 μl, 10 mM) was added 0.1 M phosphate buffer (75 μl, pH = 7), followed

FIG. 1. Chemical structures of minocycline, tetracycline, oxytetracycline, and chlorotetracycline.

FIG. 2. a, LC/MS total ion current spectrum from the reaction of minocycline with
HOCl. b, LC/MS/MS of the peak at m/z = 510 amu (M2) corresponding to
minocycline + HOCl with a retention time of 10 min.
by the addition of aqueous NaOCl (10 μl, 10 mM). In some experiments, an aliquot of the reaction mixture (2 μl) was immediately analyzed by LC/MS.

In other experiments, after 2 min, various nucleophiles (5 μl, 200 mM) were added to the reaction mixture, and then after 5 min an aliquot of the reaction mixture (2 μl) was injected into the LC/MS for analysis. For this study, the nucleophiles NAC, NAC methyl ester, glutathione, KCN, N-acetylycysteine, and N-acetylycysteine were used. These nucleophiles (KCN, glutathione, NAC methyl ester) were dissolved in 0.2 M phosphate buffer at pH 7 or pH 8.5 (N-acetylycysteine and N-acetylycysteine). In one set of experiments, minocycline was replaced by tetracycline.

**Oxidation of Minocycline by MPO/H2O2/Cl− and Trapping the Reactive Intermediates with NAC.** To minocycline dissolved in water (20 μl, 10 mM) were added PBS (163 μl, pH = 7.4), NAC (5 μl, 200 mM) in 0.5 M phosphate buffer, pH = 8.5, and MPO (2 μl, 1 U/μl). The reaction was initiated by the addition of 30% H2O2 (5 μl, 8.8 mM). After 30 s at 25°C, NAC (5 μl, 200 mM) in 0.5 M phosphate buffer, pH = 8.5, was added. The reaction mixture was incubated for 30 min and then analyzed by LC/MS. In control experiments, NAC was omitted.

**Oxidation of Minocycline by HRP/H2O2/Cl− and Trapping of Reactive Intermediates with NAC.** To minocycline dissolved in water (100 μl, 10 mM) were added PBS buffer (700 μl, pH = 7.4), NAC (100 μl, 200 mM, in 0.5 M phosphate buffer, pH = 8.5), and HRP (2.5 mg, 1080 U/ml). The reaction was initiated by the addition of 30% H2O2 (100 μl, 8.8 mM). After 5 min at 25°C the reaction mixture was analyzed by LC/MS. In the control experiments, NAC was omitted.

**Metabolism of Minocycline by Rat Liver Microsomes in the Presence of NAC.** Rat liver microsomes were prepared from male Brown Norway rats (average weight, 300 g). The rats were sacrificed by cervical dislocation, and their livers were homogenized in 20 ml of ice-cold isotonic KC1 solution (1.15%). The homogenate was centrifuged at 10000g for 10 min at 4°C. The supernatant was centrifuged again at 100,000g for 50 min at 4°C. The microsomal pellet was added to 2.25 ml of glycerol/phosphate/KCl buffer (50 mM KH2PO4 buffer, pH 7.4, containing 20% glycerol and 0.4% KCl). The mixture was then homogenized briefly and stored at −78°C until further use. Total microsomal protein concentrations were determined according to the bicinchoninic acid method (Smith et al., 1985). P450 content was confirmed by its reduced carbon monoxide difference spectrum (Omura and Sato, 1964).

To phosphate buffer (223 μl, 0.1 M, pH = 7.4) were added minocycline (2 μl, 30 mM), NAC (2.5 μl, 1 mM in 0.5 M phosphate buffer, pH = 8.5), and rat liver microsomes (9.2 μl, 27 mg/ml). This was followed by the addition of an NADPH-generating system consisting of reagent A (10.5 μl, NADP+ (31 mM), glucose 6-phosphate (66 mM), and MgCl2 (66 mM)) and reagent B (2.5 μl, glucose-6-phosphate dehydrogenase (40 U/ml)). The suspension was incubated at 37°C for 1 h in a shaking water bath, after which an equal volume of ice-cold acetonitrile was added to precipitate the proteins, and the mixture was centrifuged at 5600g for 10 min. The supernatant was evaporated under a stream of nitrogen; the residue was redissolved in water; and the residue was analyzed by LC/MS. In control experiments, NAC was omitted.

**Preparation of NAC Adducts for NMR Studies.** HOCl reaction. To minocycline dissolved in water (300 μl, 10 mM) was added 0.1 M phosphate buffer (2.25 ml, pH = 7), followed by the addition of aqueous NaOCl (300 μl, 10 mM). After 2 min, NAC (150 μl, 200 mM) in 0.5 M phosphate buffer, pH = 8.5, was added, and after another 5 min, the reaction mixture (3 ml) was separated by semipreparative HPLC. The mobile phase used was isocratic acetonitrile/water (20:80) with 2 mM ammonium formate, pH = 6.5. The peak at 2.77 min was collected; the acetonitrile was removed under a stream of nitrogen; and the aqueous layer was freeze-dried overnight to remove ammonium formate. This procedure was repeated 25 times to give approximately 3.3 mg of the NAC adduct. The overall yield for the reaction was 7.1%. The purity of the NAC adduct was 89%. There was a coeluting product with a retention time of 2.6 min that was not readily separable. This NAC adduct had a retention time of 3.7 min on the analytical LC/MS system. The product obtained as a powder was stored at −75°C until NMR analysis. Isolation of a minor product at 2.8 min, which appeared to be a diastereomer, was not successful.

**HRP reaction.** To minocycline dissolved in water (200 μl, 10 mM) were added PBS buffer (1.4 ml, pH = 7.4), NAC (200 μl, 100 mM in 0.5 M phosphate buffer, pH = 8.5), and HRP (5 mg, 290 U/ml). The reaction was initiated by the addition of 30% H2O2 (200 μl, 8.8 mM), and after another 5 min, the reaction mixture (2 ml) was separated by semipreparative HPLC. The...
FIG. 5. a, $^1$H NMR spectrum of the minocycline. b, $^1$H NMR spectrum of the minocycline-NAC adduct M5, which has a retention time of 3.7 min on LC/MS.
ions were at m/z 492 (2.5%; MH+ - H2O), 475 (80%; MH+ - H2O-NH3), 439 (100%; MH+ - H2O-NH3-HCI), 411.2 (6.5%; MH+ - H2O-NH3-HCl-O), 368.2 (53%; MH+ - H2O-NH3-HCl-CO-NH(CH3)2), 154.0 (21%, MH+ - HCl-NH2H2O-C6H5NO3), and 58 (0.37%, CH2 = N+(CH3)2). The loss of HCl from the 475.2 peak suggests that the chloro group is not attached to the aromatic ring. That a fragment at m/z 154, corresponding to the retro-Diels-Alder product of ring A, was formed implies that the oxidation did not involve the A ring.

The UV spectrum of the mixture before addition of HOCl and then at 10-s intervals (Fig. 3) showed that the reaction was essentially over in the first 10 s with a shift from the λmax of minocycline at 245 nm (spectrum a) to a new λmax at 278 nm (spectrum b). The decrease in absorption at 245 nm is likely because of the loss of enol conjugated with one carbonyl group in ring B. By using HPLC, it was found that the product M2 decreased with a half-life of approximately 28 min to form M1 and traces of parent drug minocycline, whereas M3 remained unchanged. The fact that M2 was unstable and spontaneously formed M1 precluded its isolation for NMR studies.

Results

Oxidation of Minocycline by HOCl. When minocycline (M) was oxidized by HOCl and the reaction mixture immediately analyzed by LC/MS, a major product corresponding to minocycline + HOCl [M2, 510 atomic mass units (amu)] at 10 min and minor products corresponding to minocycline-H + OH (M1, 474 amu) at 2.5 min and minocycline -H + Cl (M3, 492 amu) at 21 min, along with other minor products, were observed (Fig. 2a).

The LC/MS/MS of M2 is shown in Fig. 2b. The major fragment ions were at m/z 492.2 (2.5%; MH+ - H2O), 475.2 (80%; MH+ - H2O-NH3), 439.2 (100%; MH+ - H2O-NH3-HCI), 411.2 (6.5%; MH+ - H2O-NH3-HCl-O), 368.2 (53%; MH+ - H2O-NH3-HCl-CO-NH(CH3)2), 154.0 (21%, MH+ - HCl-NH2H2O-C6H5NO3), and 58 (0.37%, CH2 = N+(CH3)2). The loss of HCl from the 475.2 fragment suggests that the chloro group is not attached to the aromatic ring. That a fragment at m/z 154, corresponding to the retro-Diels-Alder product of ring A, was formed implies that the oxidation did not involve the A ring.

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Oxidation of Minocycline by HOCl and Trapping of the Reactive Intermediates with Various Nucleophiles. Various nucleophiles were added to the products formed by the oxidation of minocycline with HOCl. The thiol nucleophiles were able to trap the reactive metabolite, with NAC providing the best yield (18%, assuming that the extinction coefficient of the adduct is the same as that of minocycline; glutathione, 16%; and NAC methyl ester, only 4%). A small amount of product was obtained with cyanide (3% yield), but no products were observed when N-acetylhistidine or N-acetylslysine was used to trap the reactive intermediate.

Analysis of the products by LC/MS after adding NAC revealed five peaks (Fig. 4a): a major NAC adduct corresponding to minocycline -2H + NAC (M5, 619 amu) at 3.7 min, residual minocycline (M1), and three minor peaks corresponding to minocycline + HOCl (residual M2, 510 amu), minocycline -H + OH (M1, 474 amu), and minocycline -H + Cl (M3, 492 amu). When M2 was isolated and

### TABLE 1

<table>
<thead>
<tr>
<th>NMR Proto/Carbon Numbers</th>
<th>M (Minocycline)</th>
<th>M5</th>
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<tr>
<td>δH ppm (Multiplicity†)</td>
<td>δC ppm</td>
<td>δH ppm (Multiplicity†)</td>
</tr>
<tr>
<td>1</td>
<td>196.23</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>97.71</td>
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</tr>
<tr>
<td>3</td>
<td>188.69</td>
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<tr>
<td>4</td>
<td>4.05 (d, 1H, J = 1.5Hz)</td>
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<tr>
<td>4a</td>
<td>2.90–2.97 (m, 1H)</td>
<td>35.92</td>
</tr>
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<td>5</td>
<td>1.63 (dt, 1H, J = 11.5, 14 Hz)</td>
<td>35.08</td>
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<tr>
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<td>2.16–2.23 (m, 2H)</td>
<td>31.12</td>
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</tr>
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<td>6a</td>
<td>3.42 (dd, 1H, J = 4.5, 15.5 Hz)</td>
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</tr>
<tr>
<td>7</td>
<td>—</td>
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<tr>
<td>8</td>
<td>7.43 (d, 1H, J = 9 Hz)</td>
<td>129.70</td>
</tr>
<tr>
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<td>117.00</td>
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<tr>
<td>16</td>
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</tr>
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<td>2.79 (dd, 1H, J = 9, 13 Hz)</td>
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<td>18</td>
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<td>19</td>
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<td>4.51 (dd, 1H, J = 4, 9 Hz)</td>
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<tr>
<td>20</td>
<td>—</td>
<td>1.83 (s, 3H)</td>
</tr>
<tr>
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<td>—</td>
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</table>

Multiplicity: b, broad; s, singlet; d, doublet; q, quartet; dt, double triplet; dd, double doublet; m, multiplet; —, peaks absent in HMBC spectra. N.S., no peak was observed, presumably because these are quaternary carbons.

† NMR acquired in CD3OD.

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reacted with NAC, **M5** was the major product. When tetracycline (**T**) was oxidized with HOCl followed by addition of NAC, no NAC adducts were observed (Fig. 4b).

**NMR of the Minocycline-NAC Adduct M5.** The proton NMR spectra of **M5** (Fig. 5; Table 1) shows an almost complete disappearance of the H-4 proton at 4.1 ppm, presumably because the H-4 proton is acidic and exchangeable with deuterium of the NMR solvents. This result was confirmed by the exchange of the H-4 proton of minocy-
cline with D₂O at pH > 7 (data not shown). Except for the addition of the NAC protons, there were only minor changes in the proton NMR of M₅ when compared with that of minocycline. This effect implies that the proton that was lost in forming M₅ was an exchangeable proton. An attempt was made to obtain the spectrum in dimethyl sulfoxide, but M₅ was found to be unstable in this solvent.

HMBC data for M₅ (Fig. 6) revealed that the carbon peak for C-11a at 110.65 ppm in minocycline is missing and was replaced by a peak.

Fig. 7. a, ion extraction LC/MS of m/z = 619 amu (M₅ and M₆) corresponding to the NAC adducts of minocycline at 3.7 and 14.5 min generated by HOCl. b, LC/MS/MS of the peak at m/z = 619 amu (M₅) corresponding to NAC adduct of minocycline with a retention time of 3.7 min.

Fig. 8. a, ion extraction LC/MS of m/z = 619 amu (M₄, M₅, and M₆) corresponding to the NAC adduct of minocycline at 2.6, 3.3, and 14.7 min generated by MPO in presence of NAC. b, LC/MS/MS of the peak at m/z = 619 amu (M₆) corresponding to NAC adduct of minocycline at 14.7 min.
at 68.98 ppm. Furthermore, this carbon interacts with the protons of H-18 (NAC-CH$_2$ hydrogens), along with H5, H5a, and H6. There is a correlation of H4a with C-3 at 187.5 ppm, implying NAC is not attached at C-3. The chemical shift of C-4 (74 ppm with correlations to H-13 and H-14) did not change significantly, implying that substitution did not occur at this position. Taken together, the carbon shift at 68.98 ppm was assigned to C-11a, and hence we conclude that NAC is attached at this position. From the NMR, it appears that the attack of NAC was stereospecific, probably leading to the more stable trans ring junction, but we do not have definitive evidence to support this assignment.

The extracted ion chromatogram of the NAC adduct, M5 (Fig. 7a), and the LC/MS/MS of M5 generated by HOCl oxidation are shown in Fig. 7b. The major fragment ions were at m/z 602.4 (60%; MH$^-$-NH$_3$), 473.3 (5.9%; MH$^-$-NH$_3$-C$_6$H$_5$NO$_3$), 441.2 (100%; MH$^-$-NH$_3$-C$_6$H$_5$NO$_3$S-H$_2$O), 423.2 (1.1%; MH$^-$-NH$_3$-C$_6$H$_5$NO$_3$S-H$_2$O-CO), 395.1 (1.19%; MH$^-$-NH$_3$-C$_6$H$_5$NO$_3$S-H$_2$O-CO), 162.1 (55%, C$_5$H$_8$NO$_3$), and 154 (3.9%, MH$^-$-NH$_3$-C$_6$H$_5$NO$_3$S-C$_16$H$_17$NO$_4$). The peak at 154 implies that NAC is not attached to the A ring, and the fragment at m/z 162 implies that NAC is not attached to the aromatic D ring. The fragmentation pattern is consistent with the structure proposed in Figs. 5 and 6. A trace amount of a minor NAC adduct (M6, 619 amu) at 14.5 min is also seen in the extracted ion chromatogram (Fig. 7a).

**Oxidation of Minocycline by MPO/H$_2$O$_2$/Cl$^-$ and Trapping the Reactive Intermediates with NAC.** Minocycline was readily oxidized by MPO, and in the presence of NAC, three NAC adducts were observed.
FIG. 11. a, $^{1}$H NMR spectrum of the minocycline M. b, $^{1}$H NMR spectrum of minocycline-NAC adducts M6 and M7, which have retention times of 14.5 and 16.9 min, respectively, on LC/MS. The primed signal assignment of proton chemical shifts correspond to diastereomer M7.
corresponding to minocycline $\cdot$H + NAC (m/z 619) were observed (M4, M5, and M6). The major M5 adduct had a retention time of 3.3 min, and the other minor adducts, M4 and M6, had retention times of 2.6 and 14.7 min, respectively (Fig. 8a). The MS/MS spectrum of the NAC adduct M4 is essentially the same as M5. Because it also has a similar retention time as M5, it could be concluded that M4 is a diastereomer of M5, formed by slow epimerization of M5, most likely at C4. At pH 4, we saw a change in the ratio of M5/M4 from 27:1 to 0.3:1 over 44 h.

The LC/MS/MS of the NAC adduct M6 generated by MPO is shown in Fig. 8b. The major fragment ions were at m/z 602.3 (100%; MH$^+\cdot$NH$_3^+$), 584.2 (4.4%; MH$^+\cdot$NH$_3\cdot$H$_2$O), 556.4 (4.8%; MH$^+\cdot$NH$_3\cdot$H$_2$O-CO), 512.9 (4.8%; MH$^+\cdot$NH$_3\cdot$H$_2$O-CO-NH(CH$_3$)$_2$), 473.3 (56%; MH$^+\cdot$NH$_3\cdot$C$_5$H$_8$NO$_3$), 153.9 (5.2%; MH$^+\cdot$NH$_3$-C$_5$H$_8$NO$_3$S, C$_5$H$_8$NO$_3$), and 129.9 (14%; C$_5$H$_8$NO$_3$S$^-$). The prominent fragments of M6 are the result of loss of NH$_3$ at 602.4 and 473.3 caused by the loss of NAC portion of the molecule, with the sulfur still attached to the parent ion, and this is confirmed by the fragment peak at 129.9 amu. The absence of a significant peak at 441.2 or 162.1 amu, corresponding to breaking the bond between the NAC sulfur and minocycline, also suggests that the NAC is bound to an aromatic carbon.

The ratio of the NAC adducts M5 and M6 formed by the MPO reaction are pH-dependent (Fig. 9). At the lower pH of 5.6, the ratio of the NAC adducts at 3.3 min to 14.7 min was 19:1, and at a higher pH of 7.8, the ratio changes to 2:1.

**Oxidation of Minocycline by HRP in the Presence of NAC.**

Oxidation of minocycline by HRP was performed to determine whether other peroxidases, which have a lower oxidation potential than MPO, would also form the same reactive metabolites. In addition, it made it easier to generate sufficient NAC adduct to obtain an NMR spectrum of M6. Oxidation of minocycline by HRP/H$_2$O$_2$ in the presence of NAC produced four NAC adducts corresponding to minocycline $\cdot$H + NAC (M4, M5, M6, and M7). The major M6 adduct had a retention time of 14.5 min, and the other minor adducts (M4, M5, and M7) had retention times of 2.9, 3.5, and 16.9 min, respectively (Fig. 10a). The LC/MS/MS spectra of M4 and M5 were similar to that of M5 produced by the MPO oxidation, and presumably M5 is the same as the NAC adduct formed by MPO, and M4 is a diastereomer of M5.

The LC/MS/MS of M6 generated by HRP is shown in Fig. 10c. The major fragment ions were at m/z 602.4 (100%; MH$^+\cdot$NH$_3^+$), 584.2 (25%; MH$^+\cdot$NH$_3\cdot$H$_2$O), 556.3 (51%; MH$^+\cdot$NH$_3\cdot$H$_2$O-CO), 513.2 (2.5%; MH$^+\cdot$NH$_3\cdot$H$_2$O-CO-NH(CH$_3$)$_2$), 473.3 (21%; MH$^+\cdot$NH$_3\cdot$C$_5$H$_8$NO$_3$), 441.2 (11%; MH$^+\cdot$NH$_3\cdot$C$_5$H$_8$NO$_3$S$^-$), 162.1 (10%; C$_5$H$_8$NO$_3$S$^-$), 153.8 (8%; MH$^+\cdot$NH$_3$-C$_5$H$_8$NO$_3$S-C$_5$H$_8$NO$_3$), and 130.1 (4.7%; C$_5$H$_8$NO$_3$S$^-$). This MS/MS spectrum is very similar to that of the NAC adduct M6 obtained by oxidation of minocycline with HRP. Because it has almost identical retention time and MS/MS as M6, we concluded that M7 is a diastereomer of M6, formed by slow epimerization of M6, most likely at C4. We saw a change in the ratio of M6/M7 from 73:1 to 3:1 over 24 h at pH 4 as seen by HPLC and confirmed by LC/MS. At pH 8, the ratio of M6/M7 changed from 86:1 to 8:1 over 24 h.

**NMR of the Minocycline-NAC Adduct M6.** From a comparison of the proton NMR spectra of M6 (Fig. 11; Table 2) and minocycline (Table 1), it is clear that the aromatic H-9 proton at 6.8 ppm is gone. Instead, there are several singlets close to 7.52 ppm. This implies that
**M6** is a mixture of diastereomers, and NAC substitution occurred at the H-9 position of the aromatic ring. HSQC data (Fig. 12; Table 2) of the aromatic region of the spectrum confirm this observation because of the absence of the carbon signal at 117 ppm corresponding to H-9. The peak corresponding to 128.15 ppm corresponds to the minor diastereomer of the NAC adduct with substitution at the H-9 position. Likewise, there are peaks with chemical shifts for the minor diastereomer **M7** close to the major diastereomer **M6** in the proton region.
and HSQC data. In the nuclear Overhauser effect spectroscopy spectrum of M6 there is a correlation between H-15/16 protons and the H-8 proton, confirming that the NAC adduct is substituted at the H-9 position rather than the H-8 position (data not shown). Other proton and carbon chemical shifts of M6 remained similar to that of minocycline (Table 1). Taken together, we conclude that M6 represents the NAC adduct in which the NAC is attached at the H-9 position of the aromatic ring. Similar assignments could be made for M7 (Fig. 11; Table 2).

**Metabolism of Minocycline by Rat Liver Microsomes in the Presence of NAC.** The major NAC adduct formed by the oxidation of minocycline by microsomes in presence of NAC corresponds to M6 that was formed by MPO and HRP oxidation, along with its minor diastereomer M7. In particular, the HPLC retention time of M6 formed from oxidation by hepatic microsomes (Fig. 10b) and its LC/MS/MS are virtually the same. No NAC adduct with a retention time of 3.3 min corresponding to M5 was observed.

**Discussion**

Minocycline is oxidized by the peroxidases MPO and HRP or just HOCl to generate reactive intermediates. One reactive intermediate can be detected by LC/MS and has the formula minocycline + HOCl (M2, 510 amu). Evidence from the MS/MS spectrum and the structure of the product formed by reaction with NAC (M5) indicated that the HOCl adds to the C11a-C12 double bond (Fig. 13). It is surprising that the analogous NAC adduct was not observed when tetracycline was oxidized by HOCl followed by addition of NAC even though this part of the tetracycline structure is the same. Unlike MPO, HRP does not form HOCl, and M2 was not observed; however, a reactive metabolite was formed by HRP that also reacted with NAC to form M5. In this case, the reactive metabolite could be the peroxy ketone or epoxide, although a peak corresponding to this mass was not observed by LC/MS. The third reactive metabolite was also not directly observed; however, the product formed by reaction with NAC (M6) implies that it is the quinone iminium ion formed by oxidation of ring D (Fig. 13).
It is somewhat surprising that we were only able to detect trace amounts of the quinone that would be formed by the hydrolysis of the iminium ion (data not shown). M6 was the major adduct formed after oxidation by HRP and the only product formed by hepatic microsomes. This adduct was also formed in small quantities by MPO and oxidation by HRP and the only product formed by hepatic microsomes. This adduct was also formed in small quantities by MPO and oxidation by HRP.

Results provide a likely explanation for why minocycline is the only tetracycline that causes a lupus-like syndrome and autoimmune hepatitis. It is also consistent with the pattern that many drugs that cause autoimmune syndromes are oxidized to reactive metabolites by the MPO of macrophages. It could also explain why P-ANCA, antibodies against MPO, are observed in patients with minocycline-induced lupus because MPO would likely be a major target for covalent binding. However, it cannot be determined from these studies which reactive metabolite is more important or whether formation by P450 or MPO is more likely to be responsible for the idiosyncratic reactions associated with minocycline. Even though it is usually assumed that reactive metabolites formed by P450 are responsible for liver toxicity, reactive metabolites formed by the MPO of Kupffer cells may play an important role, especially in the case of drug-induced autoimmune hepatitis.

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References


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