Peroxidase-Mediated Bioactivation of Hydroxylated Metabolites of Carbamazepine and Phenytoin

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ABSTRACT:

Carbamazepine (CBZ) and phenytoin (PHN) are associated with a relatively high incidence of idiosyncratic drug reactions. Most such reactions are believed to be due to reactive metabolites. The reactions associated with these two drugs are similar, and if a patient has a reaction to one, he or she is at increased risk of having a reaction to the other, suggesting that a similar reactive metabolite may be involved. CBZ causes neutropenia in approximately 10% of patients; this suggests that reactive metabolites are formed by myeloperoxidase (MPO), the major oxidative enzyme in neutrophils. Major metabolites of CBZ are the 2- and 3-OH metabolites, and that of PHN is the 4-OH metabolite. We found that both 2-OH-CBZ and 3-OH-CBZ were further oxidized by MPO/H$_2$O$_2$, and the oxidation of 3-OH-CBZ was much faster than the oxidation of 2-OH-CBZ or CBZ itself. Oxidation by MPO formed dimers of 3-OH-CBZ and 4-OH-PHN and, in the presence of N-acetyltyrosine, cross dimers were formed. This strongly suggests free radical intermediates. Bioactivation of 3-OH-CBZ and 4-OH-PHN by MPO/H$_2$O$_2$ led to covalent binding to the tyrosine of a model protein. Free radicals usually generate reactive oxygen species (ROS). We also tested the ability of these metabolites to generate ROS and found that 3-OH-CBZ generated more ROS than 2-OH-CBZ, which was, in turn, greater than that generated by CBZ. These results suggest that bioactivation of 3-OH-CBZ and 4-OH-PHN to free radicals by peroxidases may play a role in the ability of these drugs to cause idiosyncratic drug reactions.
tion of acridan derivatives (Furst and Uetrecht, 1993; Bernus et al., 1996). These metabolites are extensively converted to N- or O-glucuronide conjugates (Lertratanangkoon and Horning, 1982).

For CBZ, several reactive metabolites were proposed (Fig. 1). These include arene oxides generated by cytochromes P450 (Spielberg et al., 1981), a carbocation formed by MPO (Furst and Uetrecht, 1993, 1995), an iminoquinone from 2-OH-CBZ (Ju and Uetrecht, 1999; Pearce et al., 2005), CBZ-10,11-epoxide (Bu et al., 2005), and an o-quinone from the corresponding catechol (Lertratanangkoon and Horning, 1982; Leeder, 1998).

There are two general metabolic pathways for PHN (Fig. 2). One involves hydrolysis to form diphenylhydantoic acid and α-aminodi-phenylacetic acid. The other type involves formation of several hydroxylated compounds: 3-OH-PHN, 4-OH-PHN, 3,4-dihydrodiol, 3,4-diOH-PHN, and a methylated catechol as well as their N- or O-glucuronide conjugates (Chow et al., 1980; Pantarotto et al., 1982).

As with CBZ, the formation of phenolic metabolites suggests the formation of a reactive arene oxide. Also analogous to CBZ, the catechol can be further oxidized to a reactive o-quinone, and there are in vitro data to support this pathway (Munns et al., 1997; Yamazaki et al., 2001). In addition, there is evidence for N-oxidation of PHN by prostaglandin H synthase to a reactive free radical (Fig. 2), which is likely to rearrange to a carbon-centered free radical and a reactive isocyanate (Parman et al., 1998). PHN is also chlorinated to

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**FIG. 1.** Metabolic pathways and proposed reactive metabolites of CBZ. Structures in brackets are inferred from products.

**FIG. 2.** Metabolic pathways and proposed reactive metabolites of PHN. Structures in brackets are inferred from products.

**FIG. 3.** Formation of a dimer from phenoxyl radicals of phenols such as tyrosine.
a reactive metabolite by myeloperoxidase (Fig. 2) (Uetrecht and Zahid, 1988).

For these two drugs, which can form multiple reactive metabolites, it is important to distinguish which one (or combinations of the reactive metabolite(s) is (are) responsible for IDRs. The observation that there is cross-sensitivity between CBZ and PHN suggests these two drugs may share similar IDR mechanisms and possibly share similar reactive metabolites; therefore, our studies focus on the possible reactive metabolites or precursors with similar structural and chemical properties. Three categories of reactive metabolites that might be common to both drugs are arene oxides, quinones/iminoquinones, and free radicals including those formed from the phenolic metabolites. Arene oxides are reactive, but they are produced by cytochromes P450, which are in low concentration in the skin, a major target of the IDRs. Furthermore, a deficiency of epoxide hydrolase is not a risk factor for the IDRs associated with these two drugs (Gaedigk et al., 1994; Green et al., 1995). These two observations make arene oxides less attractive candidates for the reactive metabolites responsible for the IDRs associated with these two drugs. α-Quinones are reactive, and their catechol precursors are much easier to oxidize than the parent drug, and so they are more likely to be formed in skin and bone marrow; however, the circulating levels of the catechols are presumably much lower than those of the parent drugs. 2-OH-CBZ, 3-OH-CBZ and 4-OH-PHN are major metabolites of CBZ and PHN, respectively, and their concentrations are significantly higher than those of the catechols. These phenols have the potential to be oxidized to free radicals by peroxidases. As mentioned earlier, the skin and bone marrow are significant targets of the IDRs associated with these drugs, and the skin contains peroxidases such as prostaglandin synthase, and the major peroxidase in the bone marrow is myeloperoxidase. Therefore, we studied the oxidation of the hydroxy metabolites of CBZ and PHN by peroxidases. In addition, as mentioned above, generalized hypersensitivity reactions associated with CBZ and PHN usually affect the liver; therefore, we studied oxidation of these phenolic metabolites by hepatic nonparenchymal cells, which also contain peroxidase. A typical reaction of such phenoxyl free radicals is dimerization (Heinecke, 2002), as shown in (Fig. 3), and this can be taken as a marker for phenoxyl free radical production.

Materials and Methods

Materials. All reagents and horseradish peroxidase (type II) were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise mentioned. All solvents were from Fisher Scientific (Ottawa, Ontario, Canada) with American Chemical Society certified grade for synthesis and HPLC grade for HPLC. MPO was obtained from Cortex Biochemical (San Leandro, CA). One unit of MPO activity was defined as the amount of enzyme that decomposed 1.0 μmol of H₂O₂ per min at 25°C and pH 6. The concentration of NaOCl was determined spectrophotometrically (Hussain et al., 1970).

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solution was refluxed until most of the ammonia had evaporated, and then the solution was condensed to dryness. This step was repeated 3 times until thin layer chromatography showed completion of the reaction. The residue was fractioned by silica gel column chromatography using a gradient solvent system (hexane: ethyl acetate = 6:1 to 1:2). The yield of 3-methoxy-5H-dibenzo[b,f]azepine-5-carboxamide (3) was 44 mg (90%). 1H-NMR (CDCl3) δ (ppm): 7.26–33 (m, 4H), 7.28 (d, J = 8.5 Hz, 1H), 7.01 (d, J = 2.5 Hz, 1H), 6.90 (d, J = 8.5, 2.5 Hz, 1H), 6.89 (d, J = 11.5 Hz, 1H), 6.83 (d, J = 11.5 Hz, 1H), 4.44 (s, 2H), 3.84 (s, 3H). MS (ESI) m/z: 267.0 (MH+) 284.0 (MNH4)2 288.6 (MNa, 2MH+), 533.2 (2MH+).

Under N2 and with stirring, 3 (9 mg, 0.034 mmol) was dissolved in anhydrous CH2Cl2 (2 ml) and cooled to −78°C. BBr3 (1 M) in CH2Cl2 (240 µl) was added. The temperature was slowly raised to approximately −15°C and kept at that temperature for 2 h. After the reaction mixture was cooled to −78°C, water (1 ml) was added. The pH was adjusted to 6 with NaOH solution (1 N) and the mixture was extracted with CHC14 (5 ml) 4 times. The organic layer was dried over MgSO4, evaporated to dryness, and fractioned by silica gel column chromatography using a gradient solvent system (hexane: ethyl acetate = 2:1 to 1:2). The yield of 3-hydroxy-5H-dibenzo[b,f]azepine-5-carboxamide (3-OH-CBZ) was 8.6 mg (100% yield). 1H-NMR (CDCl3) δ (ppm): 6.91 (d, J = 8.5 Hz, 1H), 7.01 (d, J = 2.5 Hz, 1H), 6.91 (d, J = 11.5 Hz, 1H), 6.80 (d, J = 11.5 Hz, 1H), 6.76 (dd, J = 8.5, 2.5 Hz, 1H), 5.4 (br, 1H), 4.50 (s, 2H); MS (ESI) m/z: 253.4 (MH+) 505.0 (2MH+) 527.2 (3MH+); MS/MS (ESI), m/z (%): 253.4 (25%), 210.2 (100%), 208.2 (50%), 195.0 (10%), 182.0 (25%), 167.2 (25%). The spectra were consistent with the standard provided by Novartis Pharma AG (Basel, Switzerland).

Analytical. HPLC/UV/MS analyses were carried out on an Agilent 1100 HPLC system (Palo Alto, California) and a PerkinElmer Sci API III triple quadrupole mass spectrometer (PerkinElmer Sciex, Toronto, ON, Canada) with an IonSpray interface. Analyses were in the positive ion mode, unless otherwise stated, with an ionizing voltage of 5000 V and orifice voltage of 55 V for CBZ and 2-OH-CBZ as well as 3-OH-CBZ and 70 V for 4-OH-PHN. The HPLC system was an Ultracarb ODS 30, 2 × 100 mm (Phenomenex, Torrance, CA). The mobile phase consisted of water, acetonitrile, and acetic acid (1%, v/v) with 2 mM ammonium acetate and a gradient of 18.5% acetonitrile to 50% over 10 min and 50% for a further 5 min. HPLC flow rate was 0.2 ml/min, and a splitter was used to introduce 1/10 of the flow into the mass spectrometer. The UV detection wavelength was 254 nm.

All 1H NMR spectra in the process of 3-OH-CBZ synthesis and 1D 1H NMR for 4-OH-PHN and its dimer were obtained with a Varian NMR System 400 spectrometer (Palo Alto, CA) at 400 MHz. NMR spectra for the major 4-OH-PHN dimer characterization were obtained with a Varian Unity-500 spectrometer (Palo Alto, CA) at 500 MHz.

Oxidation of 4-OH-PHN by Horseradish Peroxidase to a Dimer. When H2O2 was added to the reaction mixture of 4-OH-PHN and horseradish peroxidase, the solution turned light brown and the product was found to be a dimer. This dimer has fluorescent properties with an excitation wavelength at 320 nm and emission wavelength at 400 nm. Different concentrations of 4-OH-PHN, horseradish peroxidase, and H2O2 were tested using a fluorescence plate reader (SpectraMAX GeminiXS; Molecular Devices, Sunnyvale, CA), and then the optimized conditions were used for preparation of a larger quantity as described in the next paragraph.

To 4-OH-PHN (268 mg, 1 mmol) in methanol (100 ml) was added horseradish peroxidase (9.2 mg, 2000 units) in phosphate buffer (875 ml, 500 mM, pH 8.5) followed by H2O2 (25 ml, 200 mM). The fluorescence intensity was monitored every minute until there were no longer changes (approximately 10–20 min). The pH was adjusted to 6 to 7, the incubation mixture was extracted with ethyl acetate (1), and the extract was evaporated to dryness. The incubation was performed twice and the products were then fractioned by silica gel column chromatography using a solvent system of CHCl3/methanol (270:20). The isolated product (30 mg) was analyzed by NMR and MS for structural elucidation.
Comparison of the Rates of Oxidation of CBZ, 2-OH-CBZ, and 3-OH-CBZ by MPO/H2O2/Cl−. At 25°C, a mixture of CBZ, 2-OH-CBZ, and 3-OH-CBZ (0.05 mM) was incubated with MPO (0.06 unit/150 μl), H2O2 (0.1 mM), and chloride (108 mM) in 150 μl of buffer A (50 mM phosphate buffer and 100 μM diethylenetriamine pentaacetic acid, pH 7.4). In control incubations, MPO was omitted. Samples (20 μl) were taken at 0, 5, 10, 15, 20, and 25 min from the incubation mixture and frozen in dry ice and analyzed with LC/UV/MS/MS (MRM). Five parallel incubations were performed at the same time. Standard curves of these compounds were linear within the concentration range of the measurement.

Incubations of MPO/H2O2/Cl− with 3-OH-CBZ or 4-OH-PHN in the Presence of N-Acetyltyrosine. At 25°C, 3-OH-CBZ or 4-OH-PHN (0.5 mM) was incubated with MPO (0.1 unit/50 μl) and H2O2 (0.25 mM) with or without N-acetyltyrosine (NATyr; 0.5 mM) in 50 μl of total volume in buffer A (50 mM phosphate buffer and 100 μM diethylenetriamine pentaacetic acid, pH 7.5 or 8.5). In some incubations, 108 mM chloride was added to test its effect on the activity of MPO. In some control experiments MPO was omitted. Samples were taken at 5 or 30 min from the incubation mixture, added to ice-cold acetonitrile, and spun at 1000 rpm for 5 min and then analyzed with LC/UV/MS.

Covalent Binding of Phenolic Metabolites to Lysozyme in the Presence of MPO/H2O2. At 25°C, the substrate (2-OH-CBZ, 3-OH-CBZ, or 4-OH-PHN, 0.5 mM final concentration) was incubated with MPO (0.1 unit/50 μl), lysozyme (50 μg/50 μl), and H2O2 (0.5 mM) in buffer A (pH 8.0). The incubation time was 20 min, and substrates and H2O2 were added in four portions at 0, 5, 10, and 15 min. In some control incubations, the substrate, MPO, or H2O2 was omitted. Samples were analyzed immediately by MALDI-TOF (Voyager-DE STR; Applied Biosystems, Foster City, CA) or ESI-Qtrap MS (Q TRAP LC/MS/MS System; Applied Biosystems/MDS SCIEX, Toronto, ON, Canada) or frozen in dry ice for future analysis.

Preparation of Model Peptide (VYIHPFHL). Angiotensin I (ARVYIH-PFHL) (300 μg/100 μl; Sigma-Aldrich) and trypsin (3 μg; Roche, Laval, QC, Canada) were incubated at room temperature overnight in sodium bicarbonate buffer (100 mM, 60 μl, pH 8.0). The completion of digestion was confirmed by MALDI-TOF MS. The resulting polypeptide has better MS ionization properties than angiotensin I.

Incubation of MPO/H2O2 with a Model Peptide (VYIHPFHL). At 25°C, the substrate (3-OH-CBZ or 4-OH-PHN, 0.5 mM final concentration) was incubated with MPO (0.5 unit/50 μl), VYIHPFHL (angiotensin trypsin digest, 60 μg/50 μl), and H2O2 (0.5 mM) in buffer A (pH 8.0) for 20 min. Substrates, MPO, and H2O2 were added in four portions at 0, 5, 10, and 15 min. In some control incubations, the substrate, MPO, or H2O2 was omitted. Samples were taken at 20 min and analyzed immediately by MALDI-TOF MS to check for adduct formation or frozen in dry ice for future analyses.

Isolation of Model Peptide Adducts. 3-OH-CBZ- and 4-OH-PHN-peptide (VYIHPFHL) adducts were isolated using HPLC. The HPLC column was a Luna, 3 μm, 150 × 1 mm (Phenomenex). The flow rate was 50 μl/min with a gradient of 0 to 20% B over 5 min, 20 to 60% B over 40 min, 60 to 100% B over 10 min, and 100% B over 5 min.
fragmentation was from the double-charged ion, MH$_2$

ESI-Qtrap MS/MS was performed using the nano spray source. MS/MS

HPFHHL and MH$_2$

and H$_2$O$_2$ [0.05 mM, or H$_2$O$_2$ generating system: glucose (10 mM)/glucose

The substrates (3-OH-CBZ or 4-OH-PHN; 0.05 mM, omitted in the control)

10 min, 4°C). The pellet was resuspended in Krebs-Henseleit buffer (pH 7.4) to

For 4-OH-PHN adduct. MS/MS parameters: declustering potential was 20 and

A cell concentration of 10$^6$ cells/well; the cell viability was greater than 98%.

TABLE 1

HSQC 2D NMR spectrum of 4-OH-PHN dimer (PP1)

<table>
<thead>
<tr>
<th>C</th>
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<tr>
<td>13</td>
<td>72.58</td>
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<tr>
<td>12d</td>
<td>117.55/117.57</td>
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<td>127.31/127.29</td>
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<tr>
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<td>128.09</td>
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<tr>
<td>9d</td>
<td>128.72/128.77</td>
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<tr>
<td>6d</td>
<td>131.44/131.50</td>
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<tr>
<td>4</td>
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<td>2</td>
<td>158.69</td>
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</table>

TABLE 2

HMBC 2D NMR spectrum of 4-OH-PHN dimer (PP1)

<table>
<thead>
<tr>
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<td>1</td>
<td>177.62</td>
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</table>

B over 15 min, and 100% B for 15 min. Eluant fractions were collected every

The HPLC solvent system consisted of A: 99% water/1% acetic acid and B: 99% acetonitrile/1% acetic acid. Fractions were analyzed by MALDI-TOF MS and peptides or adducts of interest were condensed and subjected to LC-Qtrap MS/MS analyses.

LC-Qtrap MS/MS of Model Peptide Adducts. Samples were analyzed by LC/MS/MS under the following conditions: HPLC flow rate, 200 nl/min with a gradient of 0% B for 5 min, 0 to 70% B over 55 min, and 70 to 90% B over 10 min using a nano pump (Ultimate; LC Packings, Sunnyvale, CA). The HPLC solvent system consisted of A = 99% water/1% acetic acid and B = 99% acetonitrile/1% acetic acid. The column was a simple C8 trapping column. ESI-Qtrap MS/MS was performed using the nano spray source. MS/MS fragmentation was from the double- charged ion, MH$_2$$^+$ m/z: 513.6 for VYI-/H$_{11001}$m

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and B over 15 min, 4°C). The pellet was resuspended in Krebs-Henseleit buffer (pH 7.4) to

for 4-OH-PHN adduct. MS/MS parameters: declustering potential was 20 and

collision energy was 25.

Incubation of 3-OH-CBZ or 4-OH-PHN with Rat Liver Nonparenchymal Cells. Total liver cells were isolated from a Sprague-Dawley rat (Charles River Laboratories, Montreal, QC, Canada) by collagenase perfusion of the liver as described in the literature (Moldeus et al., 1978). After gravity "settlement," the supernatant of total liver cell suspension was spun at 60 g/574 rpm (3 min, 4°C), and the supernatant was spun again at 500 g/1657 rpm (5 min, 4°C). The pellet was resuspended in Krebs-Henseleit buffer (pH 7.4) to a cell concentration of 10 $\times$ 10$^6$/ml; the cell viability was greater than 98%.

The substrates (3-OH-CBZ or 4-OH-PHN; 0.05 mM, omitted in the control)

and H$_2$O$_2$ [0.05 mM, or H$_2$O$_2$ generating system: glucose (10 mM)/glucose

The substrates (3-OH-CBZ or 4-OH-PHN; 0.05 mM, omitted in the control)

the phenolic ring. This structure was identified as being due to

6. Proton NMR signals A, B, and C were assigned to hydrogens on the

that the experimental method in the reference paper was appropriate; therefore,

TABLE 1

2-OH-CBZ or CBZ.

FIG. 8. Relative rates of oxidation in an equimolar mixture of CBZ, 2-OH-CBZ, and 3-OH-CBZ by MPO/H$_2$O$_2$. Oxidation of 3-OH-CBZ was much faster than that of 2-OH-CBZ or CBZ.

Results

Characterization of the Major 4-OH-PHN Dimer. Similar to dityrosine, which has excitation/emission wavelengths of 320/400 nm (Heinecke et al., 1993), the major 4-OH-PHN dimer formed by oxidation with horseradish peroxidase also has excitation/emission wavelengths of 320/400 nm (Fig. 5A). This suggests that the dimer of 4-OH-PHN has an aromatic conjugation system similar to that of dityrosine. The MS (ESI) spectrum of the 4-OH-PHN dimer consists of m/z 535 (MH$^+$), which indicates that it is a dimer and the major fragment ion in the MS/MS spectrum is m/z 175 (phenylhydantoïn; Fig. 5B). The $^1$H-NMR (ppm) (CD$_3$OD): 7.37–7.44 (m, 5H); 7.17–7.19 (m, 2H); 6.91 (d, J = 8.8 Hz, 1H) of the dimer is shown in Fig. 6. Proton NMR signals A, B, and C were assigned to hydrogens on the benzene ring and signals D, E, and F were assigned to hydrogens on the phenolic ring. This structure was identified as being due to C3’-C3’ bond formation ortho to the phenol. This is based on proton NMR and 2D NMR, which demonstrated that the structure is symmetrical; if it were not, the spectrum would be much more complex. Compared with the $^1$H-NMR spectrum of 4-OH-PHN, the “missing” aromatic H is farthest upfield, which is expected to be the one closest to the phenolic ring. Two-dimensional NMR spectra such as $^1$H-$^1$H correlation spectroscopy (COSY), $^1$H-$^1$C heteronuclear single quantum correlation (HSQC; Fig. 7; Table 1), and heteronuclear multiple
bond correlation (HMBC; Table 2) NMR spectra were also acquired, which confirmed the structure. Complete $^1$H and $^{13}$C signal assignments are also presented in Fig. 7. NMR data were consistent with a free radical mechanism because the dimer is formed between the carbons likely to have the most radical character. This is analogous to the dimerization of tyrosine to form o,o'-dityrosine, which has been extensively studied (Heincke, 2002).

Comparison of the Rates of Oxidation of CBZ, 2-OH-CBZ, and 3-OH-CBZ by MPO. In an equimolar mixture of CBZ, 2-OH-CBZ, and 3-OH-CBZ incubated with MPO/H$_2$O$_2$, the disappearance of each compound was measured using LC/MS/MS (MRM). The relative rates of oxidation were 3-OH-CBZ $>$ 2-OH-CBZ $>$ CBZ (Fig. 8).

Products of 3-OH-CBZ and 4-OH-PHN Oxidation by MPO. LC/MS detected three major and several minor chemical dimers of 3-OH-CBZ with MH$^+$ ions at m/z 503 and NATyr cross dimers with MH$^+$ ions at m/z 474 when NATyr was included in the MPO/H$_2$O$_2$ incubation (Fig. 9; Table 3). Consistent with the previous observation that 3-OH-CBZ was more easily oxidized by MPO/H$_2$O$_2$ than 2-OH-CBZ, the quantity of dimer formed with 3-OH-CBZ was much greater than that from 2-OH-CBZ (data not shown).

In the case of 4-OH-PHN oxidation, LC/MS detected one major and one minor chemical dimer of 4-OH-PHN with MH$^+$ ions at m/z 535 and three major NATyr cross dimers with MH$^+$ ions at m/z 490 when NATyr was included in the MPO/H$_2$O$_2$ incubation (Fig. 10; Table 4). More dimer was generated at pH 8.5 than at pH 7.5 (data not shown). This is consistent with a one-electron oxidation of the phenolate anion.

Oxidation of 3-OH-CBZ and 4-OH-PHN by Rat Liver Nonparenchymal Cells in the Presence of H$_2$O$_2$. 3-OH-CBZ or 4-OH-PHN dimers were also found when 3-OH-CBZ or 4-OH-PHN was incubated with freshly isolated rat nonparenchymal cells (mostly Kupffer cells) and H$_2$O$_2$ or a H$_2$O$_2$ generating system (Figs. 9 and 10). The retention times and fragmentation patterns of the major peaks of the 3-OH-CBZ and 4-OH-PHN dimers formed by nonparenchymal cells were quite similar to those from MPO incubations, and this suggests that the structures are the same; however, the mass spectra

### Table 3

<table>
<thead>
<tr>
<th>Compound or Possible Structure</th>
<th>Retention Time</th>
<th>Molecular Ion (MH$^+$) Fragment Pattern m/z (Percentage of Intensity)</th>
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<tr>
<td>3-OH-CBZ dimer</td>
<td>9.1</td>
<td>503 (CC1) 503.2 (20%), 460.1 (100%), 443.6 (25%), 417.1 (20%), 414.7 (20%)</td>
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<td>12.1</td>
<td>503 (CC2) 503.4 (90%), 486.2 (20%), 485.4 (30%), 469.0 (50%), 460.5 (100%), 443.4 (100%), 441.4 (75%), 417.0 (25%), 414.9 (30%), 213.1 (25%)</td>
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<td></td>
<td>13.9</td>
<td>503 (CC3) 503.0 (100%), 486.1 (25%), 459.9 (75%), 457.9 (75%), 442.8 (30%), 415.3 (25%), 386.8 (25%), 208.6 (50%)</td>
</tr>
<tr>
<td>3-OH-CBZ NATyr cross dimer</td>
<td>9.0</td>
<td>474 (CY1) 474.0 (50%), 456.2 (10%), 429.2 (100%), 386.5 (35%), 343.3 (15%), 341.4 (15%)</td>
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<tr>
<td></td>
<td>9.6</td>
<td>474 (CY2) 474.2 (75%), 431.2 (40%), 428.8 (40%), 386.4 (100%), 369.1 (25%), 341.4 (20%)</td>
</tr>
</tbody>
</table>

Nonparenchymal Cell / H$_2$O$_2$, MH$^+$=503, 3-OH-CBZ dimer

MPO / H$_2$O$_2$, MH$^+$=503, 3-OH-CBZ dimer (CC1, CC2, CC3)

MPO / H$_2$O$_2$, MH$^+$=474, 3-OH-CBZ-NATyr cross dimer (CY1, CY2)
are not sufficient for unambiguous structural identification. When cells or \( \text{H}_2\text{O}_2 \) or the \( \text{H}_2\text{O}_2 \)-generating system was omitted from the incubation, no such dimers were formed, which suggests that the enzyme present in the nonparenchymal cells that catalyzes the oxidation is a peroxidase.

**Covalent Binding of 3-OH-CBZ and 4-OH-PHN to Lysozyme in the Presence of MPO/\( \text{H}_2\text{O}_2 \).** The formation of cross dimers in the presence of NATyr suggested the possibility that the phenoxyl free radicals could bind to tyrosyl residues on protein. Incubation of both 3-OH-CBZ and 4-OH-PHN with MPO/\( \text{H}_2\text{O}_2 \) and a model protein (lysozyme, molecular mass 14317 Da) led to protein adducts that were detected by MALDI-TOF MS (Fig. 11). The difference in molecular mass of these adducts is consistent with the addition of 3-OH-CBZ or 4-OH-PHN, respectively. Lysozyme contains 3 tyrosines, which fits with the number of 4-OH-PHN that bind to lysozyme, but the number of 3-OH-CBZ molecules that bind to lysozyme appear to exceed the number of tyrosines. However, 3-OH-CBZ can form dimers before binding to lysozyme and/or 3-OH-CBZ can bind to another 3-OH-CBZ already bound to lysozyme. No lysozyme adduct was observed when 3-OH-CBZ, 4-OH-PHN, MPO or \( \text{H}_2\text{O}_2 \) was omitted from the incubations.

**Specificity of 3-OH-CBZ and 4-OH-PHN Binding.** To determine the site of binding of the free radicals to protein, a smaller peptide and LC-Qtrap MS/MS were used. Both 3-OH-CBZ and 4-OH-PHN also

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**TABLE 4**

<table>
<thead>
<tr>
<th>Compound or Possible Structure</th>
<th>Retention Time</th>
<th>Molecular Ion (MH(^+))</th>
<th>Fragment Pattern m/z (Percentage of Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OH-PHN dimer</td>
<td>11.4</td>
<td>535 (PP1)</td>
<td>535.3 (25%), 518.0 (30%), 500.5 (15%), 464.5 (20%), 175.0 (100%), 103.9 (25%)</td>
</tr>
<tr>
<td></td>
<td>12.7</td>
<td>535 (PP2)</td>
<td>535.2 (50%), 475.8 (100%), 175.4 (75%)</td>
</tr>
<tr>
<td>4-OH-PHN NATyr cross dimer</td>
<td>8.5</td>
<td>490 (PY1)</td>
<td>489.9 (25%), 447.8 (25%), 444.0 (20%), 401.9 (100%), 385.3 (25%), 228.3 (15%), 175.2 (20%)</td>
</tr>
<tr>
<td></td>
<td>9.3</td>
<td>490 (PY2)</td>
<td>489.6 (10%), 449.1 (10%), 402.6 (25%), 385.2 (100%), 210.9 (15%), 175.2 (10%)</td>
</tr>
</tbody>
</table>
FIG. 11. MALDI-TOF MS of 3-OH-CBZ- and 4-OH-PHN-lysozyme adducts. A, incubation of lysozyme (14,317 Da) with MPO/H2O2 alone. B, in the presence of 3-OH-CBZ, a mixture of products was formed consistent with the addition of 2, 3, 4, 5, and 6 mol of 3-OH-CBZ (MW = 253). C, in the presence of 4-OH-PHN, the mixture of the products was consistent with addition of 1 and 2 molecules of 4-OH-PHN (MW = 269).

FIG. 12. ESI-Qtrap MS/MS detection of covalent binding of 3-OH-CBZ to the peptide VYIHPFHHL. MS/MS fragmentation revealed the site of covalent binding is the tyrosyl residue (Y). A, native peptide. B, after incubation with 3-OH-CBZ/MPO/H2O2, fragments containing tyrosine + 3-OH-CBZ (MW = 253).
formed adducts with a model peptide (VYIHPFHL) in the presence of MPO/H$_2$O$_2$. MS/MS fragmentation showed that the site of covalent binding is on the tyrosyl residue because all fragments containing tyrosine have a molecular weight shift, which is consistent with the addition of 3-OH-CBZ or 4-OH-PHN, respectively (Figs. 12 and 13; Table 5).

**Oxidative Stress Induced by OH-CBZ Metabolites.** Incubation of the monocyte/macrophage cell line RAW 264.7 with 2- and 3-OH-CBZ (0–100 μM) stimulated the generation of ROS as detected by increased oxidation of dichlorofluorescein diacetate to a fluorescent form. 3-OH-CBZ generated much more fluorescence than 2-OH-CBZ, which was, in turn, greater than that generated by CBZ (similar to blank control; Fig. 14). At the same concentration, 3-OH-CBZ can induce approximately 30% of fluorescent intensity compared with the positive control, capsaicin.

**Discussion**

Although it is widely believed that reactive metabolites are responsible for most IDRs, the detailed mechanisms are unknown. It would be ideal to develop drug candidates that do not generate any reactive metabolite; however, that would be virtually impossible. Therefore, it is important to determine whether a reactive metabolite(s) is responsible or even if a reactive metabolite is involved in the mechanism. The clinical observation of cross-sensitivity between CBZ and PHN suggests that, if reactive metabolites are involved, the two drugs form similar reactive metabolites. Furthermore, the fact that the skin and bone marrow are common targets of these IDRs suggests that the reactive metabolites can be formed by enzymes present in these tissues.

Our studies suggest that both 3-OH-CBZ and 4-OH-PHN are substrates of a variety of different peroxidases. In preliminary experiments, we observed dimer formation when 3-OH-CBZ or 4-OH-PHN was incubated with horseradish peroxidase/H$_2$O$_2$ analogous to the reactions reported for tyrosine (Malencik et al., 1996). We also found that these dimers were formed in incubations with MPO/H$_2$O$_2$. In coincubations with NATyr, cross dimers of 3-OH-CBZ or 4-OH-PHN with NATyr were also found. These drugs also cause liver toxicity and therefore it was important to determine whether the same oxidation could occur in the liver. LC/MS spectra showed that the retention times and relative intensity of some of these 3-OH-CBZ or 4-OH-PHN dimers were the same as those from incubations of 3-OH-CBZ or 4-OH-PHN with rat liver nonparenchymal cells (mostly Kupffer cells) in the presence of H$_2$O$_2$. This suggests that a peroxidase in rat liver can utilize 3-OH-CBZ or 4-OH-PHN as a substrate. Although tissue macrophages are usually believed to lack MPO, it now appears that Kupffer cells contain catalytically active MPO (Brown et al., 2001). It also appears that the liver generates H$_2$O$_2$ largely as a by-product of cytochrome P450-mediated oxidations. One study found that H$_2$O$_2$ production was 82 nmol/min/g in perfused rat liver

![Fig. 13. ESI-Qtrap MS/MS detection of covalent binding of 4-OH-PHN to the peptide VYIHPFHL. MS/MS fragmentation revealed the site of covalent binding is the tyrosyl residue (Y). A, native peptide. B, after incubation with 4-OH-PHN/MPO/H$_2$O$_2$. **, fragments containing tyrosine + 4-OH-PHN (MW = 269).](image-url)
Several lines of evidence suggest that peroxidases can generate phenoxyl free radicals by one-electron oxidation of 3-OH-CBZ or 4-OH-PHN. Formation of chemical dimers or a cross dimer with NATyr is strong evidence of free radical generation. The free radical formed from 3-OH-CBZ is likely more stable than those formed from 4-OH-PHN and 2-OH-CBZ as seen from the number of resonance structures that can be formed (Fig. 15). This is consistent with the relative rates of peroxidase-mediated oxidation and the observation that the 3-OH-CBZ was more active in the generation of ROS by RAW 264.7 cells. Also, the free radical mechanism can predict the numbers of dimers that can be formed by 3-OH-CBZ or 4-OH-PHN, which is in agreement with the experimental results. Specifically, 3-OH-CBZ can form many dimers, whereas 4-OH-PHN preferentially forms two dimer products.

There are at least two potential mechanisms by which the free radicals produced by oxidation of phenolic metabolites could lead to toxicity: by generating ROS or by covalent binding. These mechanisms are not mutually exclusive. Although ROS has been proposed as being responsible for many types of toxicity and could generate a danger signal and stimulate an immune response, there is little strong evidence for its involvement in the induction of IDRs. The phenoxyl free radicals formed from these metabolites covalently bind to protein, they could act as haptons. Leeder et al. found that patients with hypersensitivity reactions to CBZ have antibodies with specificity as being responsible for many types of toxicity and could generate a danger signal and stimulate an immune response, there is little strong evidence for its involvement in the induction of IDRs. If the phenoxyl radicals can then abstract a hydrogen atom from a macromolecule leading to a macromolecule free radical and regenerating the phenol. The reduced phenol can continue to redox cycle and cause cell damage.

(Oshino et al., 1975). It is also likely that the murine monocyte/macrophage RAW 264.7 cells used in this study contain peroxidases (Bruno et al., 1999). The hypothesis of bioactivation by peroxidases in different organs to generate reactive metabolites is consistent with the clinical observation that many IDRs associated with CBZ and PHN involve several organs (Askmak and Wiholm, 1990). Although peroxidases are much less substrate-specific than cytochrome P450, the binding sites of mammalian peroxidases, such as human MPO, are buried in the center of peroxidases so they can only oxidize low molecular mass substrates and not macromolecules (Zeng and Fenna, 1992; Heinecke, 2002). A phenol substrate has the potential to undergo a peroxidase-mediated one electron oxidation to form a radical. The phenoxyl radical can then abstract a hydrogen atom from a macromolecule leading to a macromolecule free radical and regenerating the phenol. The reduced phenol can continue to redox cycle and cause cell damage.

(FIG. 14. Approximate rate of ROS formation generated by CBZ, 2-OH-CBZ, and 3-OH-CBZ in RAW 264.7 cells.
mechanism of CBZ- and PHN-induced IDR s, these studies provide alternative hypotheses but no clear answers.

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References


FIG. 15. Comparison of the resonance structures of the (A) 3-OH-CBZ, (B) 2-OH-CBZ, and (C) 4-OH-PHN free radicals.


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