Peroxidase-Mediated Bioactivation of Hydroxylated Metabolites of Carbamazepine and Phenytoin

Wei Lu\textsuperscript{1} and Jack P. Uetrecht

Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

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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\textsubscript{2}O\textsubscript{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-acetyl tyrosine, cross dimers were formed. This strongly suggests free radical intermediates. Bioactivation of 3-OH-CBZ and 4-OH-PHN by MPO/H\textsubscript{2}O\textsubscript{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.

Carbamazepine (CBZ) and phenytoin (PHN) are effective and widely used anticonvulsants. However, they are associated with a relatively high incidence of idiosyncratic drug reactions (IDR) including a syndrome called anticonvulant hypersensitivity syndrome (Haruda, 1979; Askmark and Wiholm, 1990). The syndrome is similar for both drugs (Vittorio and Muglia, 1995) with fever, skin rash, and lymphadenopathy being most common, and other organs such as the liver are also often affected. The rash can range from a mild maculopapular exanthem to Stevens-Johnson syndrome or toxic epidermal necrolysis. Neutropenia and agranulocytosis can also occur in CBZ- and PHN-treated patients (Turner, 1960; Hart and Easton, 1980; Joffe et al., 1985). In addition to the similarity in the syndromes associated with these two anticonvulsants, there is cross-sensitivity among these anticonvulsants, that is, up to 80% of patients who have an IDR to one of these drugs will also have an adverse reaction to the other, which suggests that a common pathogenic mechanism is involved (Shear and Spielberg, 1988). Such cross-sensitivity has not been observed between these agents and other anticonvulsants such as valproate and benzodiazepines (Hyson and Sadler, 1997).

There is a large amount of circumstantial evidence to suggest that most such IDRs are due to reactive metabolites rather than the parent drug (Park et al., 1995). When a reactive metabolite is found for a drug that causes IDRs it is often assumed that this reactive metabolite is responsible for the IDRs; however, it is very difficult to demonstrate that a reactive metabolite is responsible for an IDR, and most drugs have the potential to form several reactive metabolites. Therefore, we are usually left with circumstantial evidence implicating a specific reactive metabolite as being responsible for a specific IDR.

Reactive metabolites are not usually observed directly in vivo studies because of their chemical reactivity; however, metabolic pathway studies can often provide clues to identify possible reactive metabolites. CBZ is extensively metabolized to over 30 metabolites as detected in human or rat urine (Lertratanangkoon and Horning, 1982). One major metabolic pathway is oxidation to CBZ-10,11-epoxide followed by hydration to CBZ-10,11-trans-diol (Fig. 1). Other major pathways are the oxidations to 2-hydroxy-CBZ (2-OH-CBZ) and 3-hydroxy-CBZ (3-OH-CBZ). The 2-OH-CBZ is further oxidized to an iminoquinone and both the 2- and 3-OH-CBZs can be oxidized to a catechol. Other pathways include ring contraction with the forma-

ABBREVIATIONS: 2D, two-dimensional; CBZ, carbamazepine; COSY, correlation spectroscopy; 3,4-dihydropyridine-5(3,4′-dihydroxy-1′,5′-cyclohexadien-1-yl)-5-phenylhydantoin; 3,4-dioDO-PHN. 5(3′,4′-dihydroxyphenyl)-5-phenylhydantoin; ESI, electron spray ionization; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum correlation; IDR, idiosyncratic drug reaction; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MH\textsuperscript{+}, protonated molecular ion; MPO, myeloperoxidase; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NATyr, N-acetyl tyrosine; NMR, nuclear magnetic resonance; 3-OH-PHN, 3-hydroxyphenytoin; 4-OH-PHN, 4-hydroxyphenytoin; PHN, phenytoin; ROS, reactive oxygen species.
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-aminodiphenylacetic acid. The other type involves formation of several hydroxylated compounds: 3-OH-PHN, 4-OH-PHN, 3,4-dihydriodiol, 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

![Fig. 1. Metabolic pathways and proposed reactive metabolites of CBZ. Structures in brackets are inferred from products.](image1)

![Fig. 2. Metabolic pathways and proposed reactive metabolites of PHN. Structures in brackets are inferred from products.](image2)

![Fig. 3. Formation of a dimer from phenoxyl radicals of phenols such as tyrosine.](image3)
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. o-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 syntheses 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 fractionated 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), carboxamide (3-OH-CBZ, 4-OH-PHN dimer 1 (PP1) (B). The symmetry of the dimer spectrum suggests that the C-C bond was formed ortho to the hydroxy group. The integration of the protons in the dimer represents the relative number of protons; being a dimer, the absolute number of protons is twice the number shown.

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 l), 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%
fragmentation was from the double-charged ion, MH\(^+\).

**LC-Qtrap MS/MS** was performed using the nano spray source. MS/MS parameters: declustering potential was 20 and collision energy was 25.

**TABLE 1**

\[\text{HSQC 2D NMR spectrum of 4-OH-PHN dimer (PP1)}\]

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**TABLE 2**

\[\text{HMBC 2D NMR spectrum of 4-OH-PHN dimer (PP1)}\]

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**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 (phenylhydantoin; Fig. 5B). The \(^1\)H-NMR (ppm) (CD\(_3\)OD): 7.37–7.44 (m, 5H); 7.17–7.22 (m, 2H); 6.91 (d, \(J = 9.0\) 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 group. Two-dimensional NMR spectra such as \(^1\)H-\(^1\)H correlation spectroscopy (COSY), \(^1\)H-\(^13\)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 (Heinecke, 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

![Nonparenchymal Cell / H$_2$O$_2$, MH$^+$=503, 3-OH-CBZ dimer](image)

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

![MPO / H$_2$O$_2$, MH$^+$=474, 3-OH-CBZ-NATyr cross dimer (CY1, CY2)](image)

**TABLE 3**

<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>3-OH-CBZ dimer</td>
<td>9.1</td>
<td>503 (CC1)</td>
<td>503.2 (20%), 460.1 (100%), 443.6 (25%), 417.1 (20%), 414.7 (20%)</td>
</tr>
<tr>
<td></td>
<td>12.1</td>
<td>503 (CC2)</td>
<td>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>
</tr>
<tr>
<td></td>
<td>13.9</td>
<td>503 (CC3)</td>
<td>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)</td>
<td>474.0 (50%), 456.2 (10%), 429.2 (100%), 386.5 (35%), 343.3 (15%), 341.4 (15%)</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>474 (CY2)</td>
<td>474.2 (75%), 431.2 (40%), 428.8 (40%), 386.4 (100%), 369.1 (25%), 341.4 (20%)</td>
</tr>
</tbody>
</table>

![FIG. 9. LC/MS detection of peroxidase oxidation products of 3-OH-CBZ (MH$^+$ = 253). Dimers (MH$^+$ = 503) and NATyr cross dimers (MH$^+$ = 474) were formed in MPO or hepatic nonparenchymal cell incubations.](image)
are not sufficient for unambiguous structural identification. When cells or H$_2$O$_2$ or the H$_2$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/H$_2$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/H$_2$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 H$_2$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>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. 10.** LC/MS detection of peroxidase oxidation products of 4-OH-PHN (MH$^+$ = 269). Dimers (MH$^+$ = 535) and NATyr cross dimers (MH$^+$ = 490) were formed in MPO or hepatic nonparenchymal cell incubations.
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 VYIHFPFHL. 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.
cause cell damage. The reduced phenol can continue to redox cycle and regenerate a macromolecule leading to a macromolecule free radical and regenerating 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.

Several lines of evidence suggest that peroxydases can generate phenol free radicals by one-electron oxidation of 3-OH-CBZ or 4-OH-PHN. Theoretical and experimental results with the phenoxyl radical are in agreement with the experimental results. Specifically, 3-OH-CBZ is 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 phenol free radicals formed from these metabolites covalently bind to protein, they could act as haptens. Leeder et al. found that patients with hypersensitivity reactions to CBZ have antibodies with specificity against a sequence found in the human peroxidases prostacyclin synthase and thromboxane synthase (Leeder et al., 1998). Thus, nonmonooxygenase cytochromes P450 are potential human autoantigens in anticonvulsant hypersensitivity reactions. This suggests that oxidation of 3-OH-CBZ by prostacyclin and/or thromboxane synthase followed by binding of the phenol radical to the enzyme could be involved in the induction of anticonvulsant hypersensitivity IDRs. However, in vitro system is artificial, and in vivo the major reaction of phenol radicals is likely to be abstraction of a hydrogen atom rather than covalent binding. Therefore, instead of clarifying the

(Oshino et al., 1975). It is also likely that the murine monocyte/macrophage RAW 264.7 cells used in this study contain peroxidase (Bruno et al., 1999). The hypothesis of bioactivation by peroxydases in different organs to generate reactive metabolites is consistent with the clinical observation that many IDRs associated with CBZ and PHN involve several organs (Asmark and Wholm, 1990). Although peroxydases are much less substrate-specific than cytochrome P450, the binding sites of mammalian peroxydases, such as human MPO, are buried in the center of peroxydases 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 peroxydase-mediated one electron oxidation to form a radical. The phenol 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.

Several lines of evidence suggest that peroxydases can generate phenol 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 peroxydase-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 phenol free radicals formed from these metabolites covalently bind to protein, they could act as haptens. Leeder et al. found that patients with hypersensitivity reactions to CBZ have antibodies with specificity against a sequence found in the human peroxydases prostacyclin synthase and thromboxane synthase (Leeder et al., 1998). Thus, nonmonooxygenase cytochromes P450 are potential human autoantigens in anticonvulsant hypersensitivity reactions. This suggests that oxidation of 3-OH-CBZ by prostacyclin and/or thromboxane synthase followed by binding of the phenol radical to the enzyme could be involved in the induction of anticonvulsant hypersensitivity IDRs. However, in vitro system is artificial, and in vivo the major reaction of phenol radicals is likely to be abstraction of a hydrogen atom rather than covalent binding. Therefore, instead of clarifying the
mechanism of CBZ- and PHN-induced IDR s, these studies provide alternative hypotheses but no clear answers.

Acknowledgments. We thank Novartis Pharma AG for providing the 3-OH-CBZ standard; Dr. Ori D. Rotstein (Toronto General Hospital, University of Toronto) for sharing the RAW 264.7 cell line; and Drs. Ling Xu, Ying Yang, and Lingjie Meng (Mass Spectrometry Center, University of Toronto) for their training and support for LC/MS studies.

References


Address correspondence to: Jack Uetrecht, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, ON, Canada M5S 3M2. E-mail: jack.uetrecht@utoronto.ca