Peroxidase-Mediated Bioactivation of Hydroxylated
Metabolites of Carbamazepine and Phenytoin*

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Abbreviations

CBZ, carbamazepine; COSY, correlation spectroscopy; 3,4-dihydrodiol, 5-(3’,4’-dihydroxy-1’5’-cyclohexadien-1-yl)-5-phenylhydantoin; 3,4-diOH-PHN, 5-(3’,4’-dihydroxyphenyl)-5-phenylhydantoin; ESI, electron spray ionization; GSH, glutathione; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; IDR, idiosyncratic drug reaction; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MH+, protonated molecular ion; MPO, myeloperoxidase; MRM, multiple reaction monitoring; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NATyr, N-acetyl Tyrosine; 3-OH-PHN, 3-hydroxyphenytoin; 4-OH-PHN, 4-hydroxyphenytoin; PBS, phosphate buffered saline; PHN, phenytoin; ROS, reactive oxygen species
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, they are at increased risk of having a reaction to the other suggesting that a similar reactive metabolite may be involved. CBZ causes neutropenia in about 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/H₂O₂, 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/H₂O₂ 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.
Introduction

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 anticonvulsant hypersensitivity syndrome (Askmark and Wiholm, 1990; Haruda, 1979). 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 (Hart and Easton, 1982; Joffe et al., 1985; Turner, 1960). In addition to the similarity in the syndromes associated with these two anticonvulsants, there is cross-sensitivity among these anticonvulsants, i.e. 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 *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 formation of acridan derivatives (Bernus et al., 1996; Furst and Uetrecht, 1993). 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 (Leeder, 1998; Lertratanangkoon and Horning, 1982).

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-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 is 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 a reactive metabolite by myeloperoxidase (MPO, 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. ω-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 nonparenchimal cells which also contain peroxidase. A typical reaction of such phenoxylic free radicals is dimerization (Heinecke, 2002) as shown in (Fig. 3), and this can be taken as a marker for phenoxylic free radical production.
Material 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 ACS 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).

Synthesis of 3-OH-CBZ. 3-Methoxy-10,11-dihydro-5H-dibenz[b,f]azepine-5-carbaldehyde (1, Fig 4) was prepared from 4-methoxy-2-nitrotoluene by the six-step method of Jorgensen, et al. (1999).

Under N₂ and with stirring 1,3-dibromo-5,5-dimethylhydantoin (106.4 mg, 0.40 mmol) and benzoyl peroxide (4 mg) were added to 1 (200 mg, 0.80 mmol) in anhydrous CCl₄ (4 ml) and the mixture was heated to 65 °C for 1 h. The reaction mixture was cooled to room temperature and, after the solid was filtered off, water (4 ml) was added and the mixture was extracted with CH₂Cl₂ (4 ml). The organic layer was dried over MgSO₄ and evaporated with a stream of N₂. KOH/ethanol (0.1 g/4 ml) was added and refluxed for 1 h. The reaction mixture was evaporated with a stream of N₂ and water (4 ml) was added and extracted twice with ethylacetate (7 ml). The organic layer was dried over MgSO₄, evaporated, and then fractioned by silica gel column chromatography using a solvent system of hexane/ethylacetate (10:1). The yield of 3-methoxy-5H-
dibenz\[b,f\]azepine (2) was 50 mg (28%). $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 6.75-7.03 (m, 4H), 6.46 (d, $J = 7.5$ Hz, 1H), 6.36 (dd, $J = 7.5$, 2.5 Hz, 1H), 6.23 (d, $J = 11.5$ Hz, 1H), 6.16 (d, $J = 11.5$ Hz, 1H), 6.06 (d, $J = 2.5$ Hz, 1H), 4.91 (s, br, 1H), 3.74 (s, 3H); MS(ESI), m/z: 224.0 (MH$^+$).

Under N$_2$ and with stirring, 2 (40 mg, 0.18 mmol) was dissolved in anhydrous toluene (800 µl), and phosgene (20% in toluene, 800 µl) was added. The reaction mixture was refluxed for 1 h, cooled to room temperature, and evaporated to dryness. Ammonia (7N) in methanol (1 ml) was added, the 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 TLC showed completion of the reaction. The residue was fractioned by silica gel column chromatography using a gradient solvent system (hexane : ethylacetate = 6:1 to 1:2). The yield of 3-methoxy-5$H$-dibenz[b,f]azepine-5-carboxamide (3) was 44 mg (90%). $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 7.26-33 (m, 4H), 7.28 (d, $J = 8.5$ Hz, 1H), 7.01 (d, $J = 2.5$ Hz, 1H), 6.90 (dd, $J = 8.5$, 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 (MNH$_4^+$), 288.6 (MNa$^+$), 533.2 (2MH$^+$).

Under N$_2$ and with stirring, 3 (9 mg, 0.034 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (2 ml) and cooled to −78 °C. BBr$_3$ (1 M) in CH$_2$Cl$_2$ (240 µl) was added. The temperature was slowly raised to about −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 (1N) and the mixture was extracted with CHCl$_3$ (5 ml) 4 times. The organic layer was dried over MgSO$_4$, evaporated to dryness, and fractioned by silica gel column chromatography using a gradient solvent system (hexane: ethylacetate = 2:1
to 1:2). The yield of 3-hydroxy-5H-dibenz[b,f]azepine-5-carboxamide (3-OH-CBZ, 4) was 8.6 mg (100% yield). $^1$H-NMR (CDCl$_3$) $\delta$ (ppm): 7.36-7.48 (m, 4H), 2.33 (d, J=8.5 Hz, 1H), 6.98 (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$^+$); 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 Perkin-Elmer Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Toronto, Ontario, 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, 2-OH-CBZ as well as 3-OH-CBZ and 70 V for 4-OH-PHN). The HPLC column 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 $^1$H NMR spectra in the process of 3-OH-CBZ synthesis and 1D $^1$H 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 H$_2$O$_2$ 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 H$_2$O$_2$ were tested using a fluorescence plate reader (SpectraMAX GeminiXS, Molecular Devices) 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 H$_2$O$_2$ (25 ml, 200 mM). The fluorescence intensity was monitored every min until there were no longer changes (about 10-20 min). The pH was adjusted to 6-7, the incubation mixture was extracted with ethylacetate (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 (CHCl$_3$: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/H$_2$O$_2$/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), H$_2$O$_2$ (0.1 mM), and chloride (108 mM) in...
150 µl 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, 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-acetyltirosine.** 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-acetyltirosine (NATyr; 0.5 mM) in 50 µl total volume in Buffer A (50 mM phosphate buffer and 100 uM diethylenetriamine pentaacetic acid, pH 7.5 or 8.5). In some incubations, 108 mM chloride was added to test its affect on the activity of MPO. In some control experiments MPO was omitted. Samples were taken at 5 min 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 (The Applied Biosystems Voyager - DE STR, Foster City,
Preparation of model peptide (VYIHPFHL). Angiotensin I (ARVYIHPFHL) (300 µg/100 µl, Sigma-Aldrich) and trypsin (3 µg, Roche, Laval, Quebec, 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, Torrance, CA). 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 15 min and 100% B for 15 min. Eluant fractions were collected every min. 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% B to 90% B over 10 min using a nano pump (Ultimate, by 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, \( \text{MH}^2^+\ m/z: 513.6 \) for VYIHPFHL and \( \text{MH}^2^+\ m/z: 638.4 \) for 3-OH-CBZ adduct and \( \text{MH}^2^+\ m/z: 646.5 \) 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, Montreal, Quebec, 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 \( \text{H}_2\text{O}_2 \) (0.05 mM, or \( \text{H}_2\text{O}_2 \) generating system: glucose (10 mM)/glucose oxidase (1 unit/ml) (Moridani et al., 2001) were added. The mixtures were incubated at 37 °C for 60 min. After the incubation, the incubation mixtures were extracted with ethylacetate and spun down. The organic layer was dried with a stream of nitrogen and redissolved with acetonitrile/water (1:1, in 1/10 of the original volume) and analysed using LC/UV/MS.
Oxidative stress induced by phenolic metabolites in RAW 264.7 cells. The murine monocyte/macrophage cell line, RAW 264.7 (ATCC) was provided by Dr. Ori Rotstein, Toronto General Hospital. Preliminary experiments confirmed that the experimental method in the reference paper was appropriate; therefore, all procedures followed the protocols described in their paper (Garle et al., 2000). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamine (2 mM), pyruvic acid (1 mM), fetal bovine serum (10% v/v), penicillin/streptomycin (10,000 IU/l), and fungizone (2 mg/l). Cells were incubated with air/CO₂ (5%) at 37 °C.

Cells (2×10⁶ cells/ml) were placed in 24-well plates at 2×10⁶ cells/well and incubated for 2 h to allow adherence to the plate. Then cells were washed with Hanks’ balanced salt solution (without phenol red), and dichlorofluorescin diacetate (20 µM) in Hanks’ balanced salt solution containing the compounds to be tested (0-100 µM) was added. Cells were incubated for 4 h and fluorescence was analyzed at wavelengths of 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (SpectraMax GeminiXS, Molecular Devices). No H₂O₂, which could produce artifacts, was added in this experiment.
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); 7.17-7.19 (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 to 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 as that 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/H₂O₂, 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 N-acetyltirosine (NATyr) cross dimers with MH⁺ ions at m/z 474 when NATyr was included in the MPO/H₂O₂ incubation (Fig. 9 and Table 3). Consistent with the previous observation that 3-OH-CBZ was more easily oxidized by MPO/H₂O₂ 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/H₂O₂ incubation (Fig. 10 and 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 H₂O₂.** 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 \( \text{H}_2\text{O}_2 \) or a \( \text{H}_2\text{O}_2 \) 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 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.** In order 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 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, 13, and 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 dichlorofluorescin 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 about 30% of fluorescent intensity compared to 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 if a reactive metabolite(s) is responsible for a specific IDR and, if so, to determine which reactive metabolite is involved and how the reactive metabolite causes the IDR so that we can determine which type of reactive metabolite is most likely to cause IDRs. Unfortunately, it is difficult to definitively determine what reactive metabolites are responsible for IDRs.

The IDRs associated with CBZ and PHN have been studied for many years in an attempt to understand the mechanism involved and many possible reactive metabolites have been proposed; however, there is still no clear evidence of which reactive metabolite(s) is(are) 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/H₂O₂ analogous to the reactions reported for tyrosine (Malencik et al., 1996).
We also found that these dimers were formed in incubations with MPO/H₂O₂. In co-incubations 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 if the same oxidation can 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 H₂O₂. 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 H₂O₂ largely as a by-product of P450-mediated oxidations. One study found that H₂O₂ production was 82 nmol/min/g in perfused rat liver (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 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 (Askmark and Wiholm, 1990). Although peroxidases are much less substrate specific than 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 (Heinecke, 2002; Zeng and Fenna, 1992). 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.

Several lines of evidence suggest that peroxidases can generate phenoxylic 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, while 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. If the phenoxylic 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, non-monoxygenase 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 phenoxy radical to the enzyme could be involved in the induction of anticonvulsant hypersensitivity IDRs. However, our in vitro system is artificial and in vivo the major reaction of phenoxy 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 IDRs these studies provide alternative hypotheses but no clear answers.

Acknowledgements

We wish to 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; Drs. Ling Xu, Ying Yang and Lingjie Meng (Mass Spectrometry Center, University of Toronto) for their training and support for LC/MS studies.
References


Footnotes

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Figure Legends

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.

Fig. 4. Scheme for the synthesis of 3-OH-CBZ (DBDMH: 1,3-dibromo-5,5-dimethylhydantoin).

Fig. 5. Characterization of the 4-OH-PHN dimer. The excitation and emission spectra are shown in panel A and the MS/MS spectrum is shown in panel B.

Fig. 6. Comparison of the $^1$H-NMRs of 4-OH-PHN (A) and a 4-OH-PHN dimer (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.

Fig. 7. HSQC $^{13}$C-$^1$H 2D NMR spectrum of 4-OH-PHN major dimer product.
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.

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.

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/H$_2$O$_2$ 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 moles 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 VYIHPFHL. 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) are labelled with *.

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/H₂O₂. Fragments containing tyrosine + 4-OH-PHN (MW = 269) are labelled with *.

Fig. 14. Approximate rate of ROS formation generated by CBZ, 2-OH-CBZ and 3-OH-CBZ in RAW 264.7 cells.

Fig. 15. Comparison of the resonance structures of the (A) 3-OH-CBZ, (B) 2-OH-CBZ, and (C) 4-OH PHN free radicals.
Table 1. HSQC 2D NMR spectrum of 4-OH-PHN dimer (PP1)

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

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Table 3. LC/MS of products of MPO/H$_2$O$_2$ oxidation of 3-OH-CBZ

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<th>Molecular ion (MH$^+$)</th>
<th>Fragment Pattern m/z (percentage of intensity)</th>
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<td>3-OH-CBZ dimer</td>
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<td>503</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>
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<td>13.9</td>
<td>503</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>
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<td>3-OH-CBZ NATyr cross dimer</td>
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Table 4. LC/MS of products of MPO/H$_2$O$_2$ oxidation of 4-OH-PHN

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<td>NATyr cross dimer</td>
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Table 5. ESI-Qtrap MS/MS of products formed by reaction of 3-OH-CBZ and 4-OH-PHN radicals to the peptide VYIHPFHL. Theoretical and experimental m/z for b and y ions of (A) VYIHPFHL, (B) 3-OH-CBZ-modified and (C) 4-OH-PHN-modified. The modified tyrosine residue is designated Ya (for 3-OH-CBZ modification) and Yb (for 4-OH-PHN).

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Fig 1

trans-CBZ-diol → CBZ-10,11-epoxide → acridan derivative → carbamazepine (CBZ) → CYP3A4, CYP2C8 → P450 → iminoquinone → I → carbocation

CBZ arene oxide → 3-OH-CBZ → 2-OH-CBZ → 2,3-diol-CBZ → o-quinone
Fig 3

2
\[ \text{precursor} \rightarrow \text{products} \]

other products
Fig 4

1. 3-Methoxy-10,11-dihydro-5H-dibenz[b,f]azepine-5-carbaldehyde
2. 3-Methoxy-5H-dibenz[b,f]azepine
3. 3-Methoxy-5H-dibenz[b,f]azepine-5-carboxamide
4. 3-Hydroxy-5H-dibenz[b,f]azepine-5-carboxamide (3-OH-CBZ)
**Fig 5**

A

![Graph A](Image)

Ex=320nm  Em=400nm

B

![Graph B](Image)

Relative Intensity (%) vs m/z

m/z: 104, 175, 518, 535
Fig 6

A

4-OH-PHN

B

4-OH-PHN dimer 1
Fig 7
Fig 9

Nonparenchymal Cell / H₂O₂
MH⁺=503, 3-OH-CBZ dimer

MPO / H₂O₂,
MH⁺=503, 3-OH-CBZ dimer
(CC1, CC2, CC3)

MPO / H₂O₂, MH⁺=474,
3-OH-CBZ-NATyr cross dimer
(CY1, CY2)
Fig 10

Nonparenchymal Cell / H₂O₂
MH⁺=535, 4-OH-PHN dimer

MPO / H₂O₂,
MH⁺=535, 4-OH-PHN dimer
(PP1, PP2)

MPO / H₂O₂, MH⁺=490,
4-OH-PHN-ÑATyr cross dimer
(PY1, PY2)
Fig 11

A

B

C

% Intensity

Mass (m/z)
Fig 14

Fluorescence intensity ~ ROS Concentration

Concentration (µM)

- CBZ
- 2-OH-CBZ
- 3-OH-CBZ