Possible Bioactivation Pathways of Lamotrigine

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ABSTRACT:

The anticonvulsant lamotrigine is associated with idiosyncratic drug reactions, especially skin rashes. Most idiosyncratic reactions are believed to be caused by reactive metabolites. Previous studies have found evidence that an arene oxide is formed in rats; however, when we incubated radiolabeled lamotrigine with rat liver microsomes virtually no covalent binding was detected, and the expected downstream phenolic metabolites are not observed in humans. Rare cases of agranulocytosis have been associated with lamotrigine therapy, and we found that lamotrigine is oxidized to two different N-chloro products by HOCI. The more reactive N-chloro metabolite forms an adduct with N-acetylcysteine, and covalent binding was observed when radiolabeled lamotrigine was incubated with myeloperoxidase/H2O2/Cl-. Another lamotrigine metabolite is an N-oxide. If this N-oxide were sulfated, it might be sufficiently reactive to bind to protein. The synthetic N-sulfate reacted with N-acetylserine; however, no covalent binding was detected when the radiolabeled N-oxide was incubated with sulfotransferase. We also investigated the possibility that lamotrigine might be oxidized to a free radical by other peroxidases or oxidized by other enzymes such as prostanoid H synthase or tyrosinase, but no evidence of oxidation was found, and lamotrigine did not cause any detectable increase in lipid peroxidation in vivo. In view of the virtual lack of covalent binding to hepatic microsomes and the lack of any other likely pathway leading to metabolic activation in the skin, it is possible that the parent drug rather than a reactive metabolite causes lamotrigine-induced skin rashes.

Lamotrigine is an important anticonvulsant (Fitton and Goa, 1995), but its use is associated with a significant incidence of idiosyncratic drug reactions (Schlienger et al., 1998; Beller and Boyce, 2002). The symptoms can include fever, skin rash, agranulocytosis, and lymphadenopathy (Schlienger et al., 1998). There appears to be an association between the formation of reactive metabolites and the risk that a drug will cause idiosyncratic drug reactions (Maggs et al., 2000). The two major hypotheses that have been used to link the formation of reactive metabolites and idiosyncratic drug reactions are the hapten hypothesis and the danger hypothesis (Uetrecht, 1999). Therefore, we looked for evidence of reactive metabolite generation that might be responsible for lamotrigine-induced idiosyncratic reactions.

The first step in the search for reactive metabolites is to look for clues in the known metabolic pathways. Human [14C]metabolite-profiling studies revealed that the major metabolites of lamotrigine are the N-2 glucuronide, the N-5 glucuronide, the N-2 methyl, and N-2 oxide (Doig and Clare, 1991) (Fig. 1). The N-2 glucuronide is weakly reactive, but the fact that valproic acid inhibits formation of the glucuronide and also increases the risk of idiosyncratic reactions argues against this metabolite as the cause of adverse reactions (Yuen et al., 1992). None of the other metabolites are considered to be reactive, but they could be the precursor of a reactive metabolite.

A previous study found convincing evidence of a glutathione conjugate of lamotrigine in rat bile, which was presumed to be formed from a reactive arene oxide (Maggs et al., 2000). However, no phenol metabolites were detected in humans (Doig and Clare, 1991), and in general phenols are one of the major downstream products of arene oxides. In addition, the amount of cytochromes P450 (P450s) in the skin is limited, and this makes an arene oxide less attractive as a cause of skin rash. The skin does contain other metabolic enzymes such as peroxidases (Strohm and Kulkarni, 1986), sulfotransferases (Kudlacek et al., 1995; Higashi et al., 2004), and tyrosinase (Pomerantz and Ances, 1975; Vijayan et al., 1982; Wittbjörk et al., 1991).

There have been a few reports of neutropenia/agranulocytosis associated with lamotrigine treatment (Solvason, 2000; Lambert et al., 2002; LeDrew et al., 2005); this suggests the possible involvement of myeloperoxidase (MPO). Another possibility is that, analogous to other N-oxides such as minoxidil (Hamamoto and Mori, 1989; Johnson et al., 1992; Baker et al., 1994), the N-oxide of lamotrigine could be a substrate for sulfotransferases leading to a N-sulfate, which might have sufficient chemical reactivity to bind to protein (Fig. 2). Although the amount of N-oxide in urine is less than that of glucuronide, this may underestimate the amount formed because N-oxides can be reduced back to amine. In addition, lamotrigine might be oxidized to a free radical that could lead to redox cycling and/or covalent binding that might not be reflected in the observed metabolites. With this background, we performed studies in search of reactive metabolites that might be responsible for lamotrigine-induced idiosyncratic reactions.

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ABBREVIATIONS: P450, cytochrome P450; MPO, myeloperoxidase; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RLM, rat liver microsome; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.
Materials and Methods

Materials. Lamotrigine, lamotrigine N-oxide, and [14C]lamotrigine (155 μCi/mg) were gifts from GlaxoSmithKline Inc. (Uxbridge, Middlesex, UK). [14C]Clorazepine (15 μCi/mg) was a gift from Novartis (Basel, Switzerland). NaOCl, phorbol myristate acetate, sulfur trioxide/pyridine, and mushroom tyrosinase were purchased from Sigma-Aldrich (Oakville, ON, Canada). Purified prostaglandin H synthase was obtained from Cayman Chemical (Ann Arbor, MI). MPO was obtained from Cortex Biochemical (San Leandro, CA). The concentration of NaOCl was determined spectrophotometrically (Hussain et al., 1970). Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. One unit of MPO activity was defined as the amount of enzymes that decomposed 1.0 μmol of H2O2/min at 25°C and pH 6.

Animals. Animal studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care. All the mice used in this study were 6- to 8-week-old female C57BL/6 mice supplied by Harlan (Indianapolis, IN). Arriving animals were acclimated for 1 week before experiments.

High-Performance Liquid Chromatography. Analyses were performed with a Shimadzu high-performance liquid chromatography (HPLC) system, including an SPD-6A UV detector set at 254 nm (Shimadzu, Kyoto, Japan). The HPLC columns were supplied by Phenomenex (Torrance, CA), and the specifications of the column are indicated for each different type of analysis. Liquid chromatography (LC)/mass spectrometry (MS) and LC/tandem MS (LC/MS/MS) were performed in line with a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Toronto, ON, Canada) with an IonSpray interface. Analyses were carried out in the positive ion mode unless otherwise stated with an ionizing voltage of 5000 V and orifice voltage of 55 V.

X-Ray Crystallography. Data were collected on a Bruker-Nonius (Madison, WI) κ-CCD diffractometer using monochromated Mo-Kα radiation and were measured using a combination of φ scans and ω scans with k offsets to fill the Ewald sphere. The data were processed using the Denzo-SMN package. The structure was solved and refined using SHELXTL version 6.1 (Bruker-Nonius) for full-matrix least-squares refinement that was based on F2. All the H atoms were included in calculated positions and allowed refinement with a riding-motion approximation and Uiso-tied to the carrier atom. The raw data are available as Supplemental Data.

Oxidation of Lamotrigine by HOCl. Lamotrigine (1 mM final) in aqueous acetic acid (pH 4) was mixed with aqueous NaOCl (1 mM final) for 1 min. The products were analyzed by LC/MS. A Prodigy column (Phenomenex) (2 × 100 mm, 3-μm particle size) was used for HPLC, and the mobile phase consisted of water, acetonitrile, and acetic acid (59:40:1, v/v) containing 1 mM ammonium acetate. One stable product was isolated and recrystallized in ethanol for X-ray crystallographic analysis.

Metabolism of Lamotrigine by Activated Neutrophils. Neutrophils and peripheral blood mononuclear cells were isolated from human blood by differential centrifugation in ice-cold Ficoll-paque (Pharmacia, Uppsala, Sweden) as described previously (Uetrecht et al., 1988). The cells had a viability of greater than 95% as determined by trypan blue exclusion. Lamotrigine (100 μM) was added to neutrophils (4 × 10⁷/ml) in Hanks’ balanced salt solution (without phenol red). The cells were activated with phorbol myristate acetate (40 ng/ml) and incubated for 45 min at 37°C. The mixture was centrifuged, and the supernatant was analyzed by LC/MS using the same conditions as those for products in hypochlorite incubations.

Oxidation of Lamotrigine in the MPO System. Lamotrigine (100 μM), MPO (1 unit/ml), and H2O2 (100 μM) in phosphate buffer at pH 6 were incubated at 25°C for 45 min. The products were analyzed by LC/MS using the same conditions as those in analysis for products in neutrophil incubations except that the HPLC and the mobile phase contained 35% acetonitrile instead of 40%.

HOCl Oxidation Product of Lamotrigine Trapped with N-Acetylhistidine. Lamotrigine (1 mM final concentration in 1-ml final volume) in aqueous acetic acid (pH 4) was mixed with NaOCl (2 mM final concentration) for 1 min. The reaction mixture was then rapidly extracted with dichlormethane (3 ml), and the combined extracts were washed with water to remove NaOCl. The dichloromethane was then evaporated with a stream of nitrogen. The residue was redissolved in methanol and added to N-acetylhistidine (5 mM in 1 ml of phosphate buffer at pH 7). The products were analyzed by LC/MS/MS using the same conditions as those in analysis of the products from neutrophil incubations except that the mobile phase contained 20% acetonitrile instead of 40%.

Preparation of Rat Liver Microsomal and Cytosolic Fractions. Rat liver microsomal (RLM) and cytosolic fractions were prepared from male Sprague-Dawley rats (Charles River, Wilmington, MA) (average weight, 300 g). After the rats were anesthetized with ketamine and xylazine, the livers were removed, chilled on ice, and then minced in 4 volumes of ice-cold sucrose buffer (0.25 M). The liver pieces were homogenized using an electric homogenizer at...
4°C. The homogenate was then centrifuged at 10,000g for 25 min at 4°C. The pellet was resuspended in ice-cold sucrose buffer and centrifuged at 10,000g for 25 min. The supernatant from the second centrifugation was added to that from the first and centrifuged at 100,000g at 4°C for 90 min. The microsomal pellet was resuspended in ice-cold storage buffer (100 mM phosphate, 1 mM EDTA, and 10% glycerol). The cytosolic fraction, which is the supernatant from the 100,000g centrifugation, was also isolated during this procedure and used as a source of sulfotransferase.

**Protein Covalent Binding (RLM Incubation).** [14C]lamotrigine or [14C]clozapine (10 μM, 0.397 or 0.050 μCi/ml, respectively) was incubated with RLM (1 mg) in HEPES-buffered medium (pH 7.4; final volume, 1 ml). The mixtures were incubated with NADPH (1 mM, omitted in control incubations) at 37°C for 1 h. After incubation, 3 volumes of acetonitrile was added at room temperature and vortexed. After chilling on ice for 5 min, the mixture was centrifuged at 9000g for 7 min, and the supernatant was removed. The precipitate was redissolved in buffer (300 μl), and the precipitation and the wash procedures were repeated twice. At the end of the third wash, the precipitate was redissolved in water (1 ml). Half of the sample was used to determine the radioactivity, and the other half was used to determine the protein concentration. These conditions are similar to those standardized at Merck (Whitehouse Station, NJ) (Evans et al., 2004) so that the results could be compared with other drugs known to cause idiosyncratic reactions. Clozapine was used as a control because it is known to covalently bind to hepatic microsomes and neutrophils (Maggs et al., 1995; Pirmohamed et al., 1995).

**Protein Covalent Binding (Human MPO Incubation).** [14C]lamotrigine or [14C]clozapine (10 μM, 0.397 or 0.050 μCi/ml, respectively) was added to bovine serum albumin (1 mg, Sigma) and hydrogen peroxide (100 μM) in buffer A (pH 7.4; final volume, 1 ml) containing sodium chloride (108 mM) (Heinecke et al., 1993). The mixtures were incubated with MPO (1 unit, omitted in control incubations) at 37°C for 1 h. Covalent binding was determined after exhaustive solvent extraction similar to the procedure described above for RLM. Protein concentrations at the end of the experiments were also measured to ensure that no significant protein loss occurred during the washes. Clozapine again was used as a control because it is known to be oxidized to a reactive metabolite by the MPO system (Liu and Uetrecht, 1995).

**Sulfation of Lamotrigine N-Oxide by Chemical Reagents.** A mixture of lamotrigine N-oxide (27 mg, 0.1 mM) and sulfur trioxide/pyridine (32 mg, 0.2 mM) in anhydrous N,N-dimethylformamide (500 μl) was stirred at room temperature and analyzed by thin layer chromatography (RI: 0.6; ethyl acetate/methanol, 8:1) and LC/MS. Minoxidil is an N-oxide that is known to be sulfated in the skin. Minoxidil sulfate was also prepared by this method, and the product was separated by precipitation with water (McCull et al., 1983).

The lamotrigine–N-oxide sulfation product was isolated by preparative HPLC. HPLC was performed using an Ultracarb column (4.5 x 100 mm). The eluant consisted of water and acetonitrile (75:25, v/v) containing 1 mM ammonium acetate. The flow rate was 1.0 ml/min.

**Reaction of Lamotrigine Sulfate with Methanol and Amino Acids.** Lamotrigine sulfate isolated by HPLC (0.01 mM in 1 ml of 25% acetonitrile) was quickly mixed with methanol (200 μl) or N-acetylhistidine, N-acetylcysteine or N-acetylseryne (3 mg solid for the amino acids), and solid sodium carbonate was added to adjust the pH to between 7 and 8. The mixture was stirred overnight and subjected to LC/MS analysis.

**Incubation of Lamotrigine N-Oxide with Sulfortransferase.** Rat liver cytosolic fraction containing sulfotransferase was isolated from the livers of two female Sprague-Dawley rats as described above, and the concentration of protein was determined to be 28 mg/ml. Lamotrigine N-oxide was incubated with sulfotransferase at 35°C; the incubation mixture contained 0.5 mM lamotrigine N-oxide or minoxidil (positive control), sulfotransferase (final protein concentration of 5 mg/ml), and a 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-generating system (sodium sulfate, 10 mM; ATP, 7 mM; MgCl2, 7 mM; glycine-NaOH buffer, final pH 8.0) with a final volume of 100 μl. Negative controls did not contain the PAPS-generating system (Stohrer et al., 1972; Mulder et al., 1978; Johnson et al., 1982). After 30 min, the incubation mixture (50 μl) was added to 50 μl of ice-cold acetonitrile to stop the reaction. After centrifugation, the supernatant was analyzed by HPLC (conditions, see analytical methods section below) and LC/MS. In some incubations, the chemically synthesized lamotrigine sulfate was added to the incubation mixture to test its stability under the conditions of the incubation.

HPLC analysis of lamotrigine, minoxidil, and their related compounds such as N-oxides or sulfates was performed using a Phenyl-H column (2 x 100 mm; Phenomenex). The eluant consisted of water, acetonitrile, and acetic acid (79:20.1, v/v) containing 1 mM ammonium acetate (pH 6.0). The flow rate was 0.2 ml/min. For sulfates, the LC/MS was performed in the negative ion mode.

**Enzymatic Sulfation and Covalent Binding of [14C]lamotrigine N-Oxide.** [14C]lamotrigine N-oxide was prepared from [14C]lamotrigine by a method provided by GlaxoSmithKline Inc. A solution of [14C]lamotrigine (3.9 x 10^-4 ng, 1.5 x 10^-4 mmol) in acetonitrile (50 μl) was added to 2 equivalents of m-chloroperbenzoic acid in acetonitrile (100 μl) and stirred overnight. The [14C]lamotrigine N-oxide was isolated with an open silica gel column and a solvent system consisting of 85% ethyl acetate/15% methanol. Both [14C]lamotrigine and [14C]N-methylation products were quantified by HPLC using a standard curve generated from nonradioactive lamotrigine and lamotrigine N-oxide.

[14C]lamotrigine N-oxide or [14C]lamotrigine (as a control) at a concentration of 2.5 x 10^-7 M (0.01 μCi) was incubated with cytosol (1.0 mg/ml) and PAPS (0.7 mM; PAPS lithium salt, Sigma) in phosphate buffer (0.1 M, pH 7.8) at 37°C for 30 min. The final incubation volume was 1 ml. After incubation, 3 ml of acetonitrile was added and vortexed to precipitate the protein. After removing the supernatant, the protein was redissolved in phosphate buffer, and the acetyl wash procedure was repeated twice. At the end of the third wash, the precipitate was redissolved in water, and the radioactivity was determined by scintillation counting. Protein concentrations at the end of the experiments were also measured to ensure no significant protein loss in the process of the experiments. In one set of control experiments, the PAPS solution was replaced by water, and in another set of control experiments, the cytosol was preheated at 100°C for 15 min before use.

**Oxidation of Lamotrigine by Prostaglandin H Synthase.** Lamotrigine (0.5 mM), arachidonic acid (0.1 mM), and prostaglandin H synthase (1000 units/ml) in phosphate buffer (100 mM, 1.0 ml) at pH 7.4 were incubated at 37°C for 30 min. In some incubations, glutathione (5.0 mM) was added to test for adduct formation. The products were analyzed by LC/MS.

**Oxidation of Lamotrigine by Tyrosinase.** Lamotrigine (1.0 mM) and mushroom tyrosinase (50 unit/ml) in phosphate buffer (50 mM, pH 7.4, 1.0 ml) were incubated at 25°C for 45 min. In some incubations, glutathione (5.0 mM) was added to test for adduct formation. The products were analyzed by LC/MS.

**Lipid Oxidation Study.** Groups of female C57BL/6 mice were treated with 50 mg/kg/day lamotrigine by gavage for 4 days at 5:00 PM, and the control groups were treated with vehicle (water) only. Mouse food was removed after each treatment and refilled at 9:00 AM the next day. After the last day of treatment, overnight urine (5:00 PM to 9:00 AM) was collected and frozen at -80°C until analysis. Each urine sample was a pool of urine from three mice with the same treatment.

Urinary 8-isoprostane (also known as 15-isoprostane F2t) from different treatment groups was measured using an enzyme immunoassay kit for urinary isoprostane purchased from Oxford Biomedical Research (Oxford, MI) according to the manufacturer’s protocol. Different dilutions (1:4 or 1:8) of the urine samples (100 μl) were mixed with an enhanced dilution buffer that decreases interference caused by nonspecific binding and analyzed at 450 nm by a SPECTRAmax PLUS 384 plate reader (Molecular Devices Corporation, Sunnyvale, CA). Urinary creatinine concentration in the urine samples was also measured using a creatinine colorimetric microplate assay kit for creatinine purchased from Oxford Biomedical Research according to the manufacturer’s protocol. The results of the urinary 8-isoprostane concentration determination were normalized with the corresponding urinary creatinine concentrations.

**Results**

**Oxidation of Lamotrigine by HOCl.** The major products formed by the oxidation of lamotrigine by HOCl were two species with protonated molecular ions at m/z 290 (Fig. 3) and a pattern of isotope peaks, indicating that three chlorines were present. The isomer with the shorter retention time was quite reactive. Both N-chlorinated
products were reduced by ascorbic acid, which suggests that they are N-chlorinated species.

To identify the more stable chlorinated product, a small crystal was grown, and X-ray crystallography showed that the chlorine was on the 5-amino group (Fig. 4). The structure of the other N-chlorolamotrigine could not be determined because it was too reactive to be isolated. There was also a significant amount of dichlorolamotrigine formed, which had a retention time of 38 min (data not shown). Presumably, it was chlorinated at the same positions as the two monochlorolamotrigine isomers.

Chlorination of Lamotrigine by Activated Neutrophils. LC/MS of the supernatant from the lamotrigine/neutrophil incubation shows one product with an \( m/z \) 290 as shown in Fig. 3. The isotope peaks are consistent with N-chlorolamotrigine, and this product corresponds to the more stable N-chlorinated product formed by HOCl oxidation; presumably, the more reactive species reacted with the neutrophils.

Oxidation of Lamotrigine by the MPO System. Oxidation of lamotrigine in the MPO system gave the same N-chlorinated product as that produced by activated neutrophils as shown in Fig. 3. The retention time was at 7.4 min because the HPLC solvent system was different.

Reaction of N-Chlorolamotrigine with Nucleophiles. The major products produced by the reaction of sulfhydryl and amine-containing nucleophiles (glutathione, N-acetylcysteine, N-acetylylsine, N-acetyl-arginine, and butylamine) with N-chlorolamotrigine (crude product formed by oxidation of lamotrigine with NaOCl) were oxidation products of the nucleophiles, i.e., glutathione was converted to its oxidized form (data not shown), and no adducts were observed. However, S-N and N-N bonds are relatively unstable, and so it is more likely that N-chlorolamotrigine would react with a nucleophile in which a C-N bond is formed. Histidine is a nucleophile in which either the nitrogen or carbon of the imidazole ring can act as a nucleophile. An N-acetylhistidine adduct was observed by LC/MS with an ion \( m/z \) 451 (Fig. 5). The LC/MS/MS of the lamotrigine N-acetylhistidine adduct showed major fragments at \( m/z \) (relative intensity): 451 (20%), 434.4 (50%), 409.4 (5%), 348.2 (50%), 256.0 (80%), 240.8 (100%), 164.0 (70%), and 135.0 (80%). Fragmentation is consistent with fragments of lamotrigine (MH\(^+\) of \( m/z \) of 256) and N-acetylhistidine (Fig. 6). Thus, one possible mechanism for the
N-acetylhistidine adduct is analogous to the postulated mechanism for the reactivity of the N-sulfate shown in Fig. 2, in which a chloride replaces the sulfate and the nucleophile is the carbon of the histidine ring.

Covalent Binding of Lamotrigine. Covalent binding of \([^{14}C]lamotrigine\) in the MPO incubation was 38.7 pmol/mg protein, whereas it was only 17.1 pmol/mg in the RLM incubation (Table 1). It is not possible to directly compare the MPO activation with that of RLM, but this result suggests the importance of bioactivation of enzymes other than P450. The level of clozapine protein covalent binding in RLM system is comparable with that reported in the literature (Maggs et al., 1995), which provides a level of confidence in the lack of significant binding of lamotrigine.

Sulfation of Lamotrigine N-Oxide by Chemical Reagents. The sulfation products of lamotrigine N-oxide and minoxidil were synthesized, and their identities were confirmed by MS. Under anhydrous conditions, the lamotrigine or minoxidil sulfates were stable for days. At room temperature, these products were not stable in aqueous solution and readily hydrolyzed back to lamotrigine N-oxide (within 18 h) and minoxidil, respectively. HPLC fractions of these sulfate products were collected, frozen immediately in liquid nitrogen, and stored in the freezer for future study. No matter how quickly the fraction of lamotrigine sulfation product from preparative HPLC was reinjected into an analytical HPLC, there was always a lamotrigine N-oxide peak, indicating that the sulfation product hydrolyzed during the process of HPLC purification. The LC/MS/MS of lamotrigine sulfate showed major collision-activated dissociation fragments at \(m/z\) (relative intensity): 272 (20%), 255 (10%), 254 (10%), 242 (50%), 220 (10%), 219 (10%), 199 (100%), 190 (25%), 184 (20%), 172 (20%), 166 (50%), 163 (20%), 157 (20%), and 138 (10%).

Reaction of Lamotrigine Sulfate with Methanol, Amino Acids, and Lysozyme. On reaction with methanol, the majority of lamotrigine sulfate was hydrolyzed to lamotrigine N-oxide. In addition to lamotrigine N-oxide, LC/MS showed a 7.9-min peak with an MH\(^+\) ion at \(m/z\) 286, which could be one of the structures shown in Fig. 7. LC/MS/MS of this adduct showed major collision-activated dissociation fragments at \(m/z\) (relative intensity): 286 (20%), 255 (10%), 243 (10%), 226 (10%), 219 (10%), 199 (100%), 190 (25%), 184 (20%), 172 (20%), 166 (50%), 163 (20%), 157 (20%), and 138 (10%).

Also, two LC/MS peaks with MH\(^+\) at 273 were found. Possible structures are shown in Fig. 8.
showed evidence of an adduct, in this case with an MH$^+$. FIG. 8. Possible structures of the deaminated methanol adduct of lamotrigine sulfate.

Although many amino acids were tested, only N-acetylserine showed evidence of an adduct, in this case with an MH$^+$ ion at m/z 401, and like other lamotrigine adducts, the isotope peak pattern of the molecular ion peaks is consistent with the presence of two chlorine atoms. The observation that lamotrigine sulfate could form adducts with methanol and N-acetylserine indicated that the hydroxy group is a good nucleophile for lamotrigine sulfate.

Enzymatic Sulfation of Lamotrigine N-Oxide. In these experiments, products from possible enzymatic sulfation reactions were sought by LC/MS using information from chemically prepared sulfation standards. The sulfation product of lamotrigine N-oxide was not found in incubations containing sulfotransferase. As positive controls, the sulfation products of nitrophenol and minoxidil were detected by LC/MS (data not shown). Both of these experiments used cytosolic enzyme. PAPS was used for nitrophenol, whereas a PAPS generation system was used for minoxidil. As another control, synthetic lamotrigine sulfate was added to the cytosolic sulfation system. After incubation, this sulfate could not be detected. However, in a parallel experiment without using cytosol, lamotrigine sulfate that had been added to the incubation could be detected (Table 2).

This indicated that cytosolic proteins probably played a role in the lamotrigine sulfate decomposition. Studies have shown that minoxidil and minoxidil sulfate can be converted to each other as a reaction cycle by sulfotransferase and sulfatase (Johnson et al., 1982). Therefore, lamotrigine sulfate may have been generated, but immediately hydrolyzed, either spontaneously or catalyzed by sulfatase.

**TABLE 1**
Protein covalent binding of lamotrigine and clozapine in RLM and MPO bioactivation systems

<table>
<thead>
<tr>
<th></th>
<th>RLM</th>
<th>MPO</th>
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<tr>
<td></td>
<td>NADPH</td>
<td>Completea</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Protein covalent binding (pmol/mg)$^c$</td>
<td>5.43 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>% of drug bound</td>
<td>0.054%</td>
</tr>
<tr>
<td>Clozapine</td>
<td>Protein covalent binding (pmol/mg)$^c$</td>
<td>20.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>% of drug bound</td>
<td>0.21%</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D. from six experiments.

**TABLE 2**
Summary of conditions and findings in lamotrigine and lamotrigine N-oxide sulfation experiments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytosol Added?</th>
<th>Lamotrigine Sulfate Found?</th>
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</thead>
<tbody>
<tr>
<td>Lamotrigine N-oxide</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lamotrigine sulfate</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lamotrigine sulfate</td>
<td>No</td>
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**Covalent Binding of [14C]Lamotrigine N-Oxide in the Presence of Sulfotransferase.** To further test whether bioactivation by sulfotransferase could lead to protein covalent binding, [14C]lamotrigine N-oxide was incubated with liver cytosol and a PAPS-generating system; however, no significant protein covalent binding was observed.

**Oxidation of Lamotrigine by Prostaglandin H Synthase or Tyrosinase.** No oxidation product or glutathione adduct was observed in incubations of lamotrigine with prostaglandin H synthase or tyrosinase.

**Lipid Oxidation Study.** We did not observe a significant increase in urinary 8-isoprostane levels in lamotrigine-treated animals (Table 3).

**Discussion**

The most common screens for reactive metabolites involve bioactivation by P450, either from liver microsomes or hepatocytes. The amount of covalent binding observed in our studies is unlikely to be significant, and it is well below the 50 pmol/mg target used by some companies (Evans et al., 2004). Although it is possible that human P450s would be more active, the fact that phenolic metabolites have not been observed in humans makes this unlikely to be responsible for lamotrigine-induced idiosyncratic reactions. Furthermore, the levels of P450 are quite low in the skin, and this makes it unlikely that this pathway is responsible for lamotrigine-induced skin rashes.

Lamotrigine is oxidized by hypochlorous acid to two different N-chlorinated products. One is less reactive, and its structure was determined. The other was much more reactive, and this precluded determination of its exact structure. By analogy with the other reactions of lamotrigine, the most likely structure of the more reactive...
isomer is 2-N-chlorolamotrigine. This N-chlorolamotrigine did not form a stable adduct with glutathione or N-acetyllysine, but it did form an adduct with N-acetylhistidinamide and lysozyme; the latter was used as a model protein. Cysteine and lysine are commonly used amino acids capable of trapping "soft" and "hard" electrophiles, respectively. The observation that only N-acetylhistidine formed a stable adduct with N-chlorolamotrigine is presumably because it acted as a carbon nucleophile forming a stable C-N bond, in contrast to the S-N or N-N, which would likely be formed from cysteine or lysine, respectively. Thus, standard screening methods of trapping reactive metabolites would not have detected this reactive metabolite. The reactive N-chlorolamotrigine is likely to be formed by activated neutrophils and monocytes and covalently bind to macromolecules, such as protein, presumably on a histidyl residue. This could be responsible for rare reports of agranulocytosis associated with lamotrigine, and covalent binding to macromolecules might cause a generalized hypersensitivity reaction; however, the amount of binding was far less than observed with clozapine, a drug known to be associated with a relatively high incidence of agranulocytosis. This pathway is even less likely to be responsible for idiosyncratic reactions confined to the skin.

One metabolic enzyme that is present in the skin in significant abundance is sulfotransferase. We speculated that sulfation of the N-oxide, a significant human metabolite, might lead to a reactive metabolite. Synthesis of this molecule and a study of its reactions revealed that, in most reactions, instead of loss of sulfite, which would lead to a reactive metabolite, there was a loss of SO3 to regenerate the N-oxide. However, the sulfite was observed to form an adduct with alcohols such as methanol or serine. We did not find evidence that the N-oxide is a substrate for sulfotransferase; however, the synthetic sulfite was found to rapidly hydrolyze in the sulfotransferase system. We also did not observe significant covalent binding when radiolabeled lamotrigine N-oxide was incubated in a system containing rat sulfotransferase, which argues against this pathway being responsible for lamotrigine-induced skin rashes. However, it is possible that human skin has different substrate specificity (Yamazoe et al., 1994).

In addition to MPO and sulfotransferase, we also tried other oxidation systems, such as prostaglandin H synthase, tyrosinase, and chemical oxidation with agents such as sodium persulfate. We did not observe any oxidation products or adduct formation. It is possible that products of one-electron oxidation would be missed, but we also could not detect any increase in lipid peroxidation as measured by isoprostane production. There are other methods for detecting free radical metabolism, and it is possible that it was missed, but at least lipid oxidation produced by other anticonvulsants was detected. Therefore, it is unlikely that prostaglandin H synthase or tyrosinase is responsible for bioactivation of lamotrigine. With lack of evidence of significant reactive metabolite formation in the skin, we have to consider the possibility that idiosyncratic drug reactions caused by lamotrigine may be caused by the parent drug instead of a reactive metabolite. Certainly, evidence of bioactivation would not be detected in any standard screen of reactive metabolite formation that is currently in use. Although we searched hard for evidence of a reactive metabolite that would be a likely candidate for mediating lamotrigine-induced skin rash and did not find any that we believed were likely candidates, it does not preclude the possibility that we missed something or that our assessment of the reactive metabolites that we did find is wrong.

References


