DMD Fast Forward. Published on April 4, 2007 as doi:10.1124/dmd.107.015271

POSSIBLE BIOACTIVATION PATHWAYS OF LAMOTRIGINE

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Running Title: Bioactivation of Lamotrigine

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Abbreviations: **BSA**, bovine serum albumin; **CAD**, collision-activated dissociation; **GSH**, glutathione; **LC/MS**, liquid chromatography/mass spectrometry; **MPO**, myeloperoxidase; **NADPH**, nicotinamide adenine dinucleotide phosphate, reduced form; **P450**, cytochrome P450; **PAPS**, 3'-phosphoadenosine-5'-phosphosulfate; **RLM**, rat liver microsomes

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ABSTRACT:

The anticonvulsant, lamotrigine, is associated with idiosyncratic drug reactions, especially skin rashes. Most idiosyncratic reactions are believed to be due to 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 HOCl. The more reactive N-chloro-metabolite forms an adduct with N-acetylhistidine, and covalent binding was observed when radiolabeled lamotrigine was incubated with MPO/H_2O_2/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 prostaglandin 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 [^{14}C]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 (P450) 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; Wittbjer 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 amines. 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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Materials and Methods

Materials. Lamotrigine, lamotrigine N-oxide and [^{14}C]Lamotrigine (155 μCi/mg) were generous gifts from GlaxoSmithKline Inc. [^{14}C]Clozapine (15 μCi/mg) was a generous gift from Sandoz Research Institute. NaOCl, phorbol myristate acetate, sulfur trioxide/pyridine, *m*-chloroperbenzoic acid, arachidonic acid, N-acetylhistidine, N-acetyllysine, N-acetylserine, and mushroom tyrosinase were purchased from Sigma-Aldrich (Oakville, Canada). Purified prostaglandin H synthase was obtained from Cayman Chemicals Co. (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 BCA 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 H_2O_2 per 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 mice used in this study were 6-8 weeks old female C57BL/6 mice supplied by Harlan (Indianapolis, IN, USA). Arriving animals were acclimated for a week before experiments.

HPLC. Analyses were performed with a Shimadzu HPLC system including a 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. LC/MS and tandem mass spectrometry (LC/MS/MS) were performed inline with a Perkin-Elmer Sciex API III triple quadrupole

mass spectrometer (Perkin-Elmer Sciex, Toronto, Ontario, 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 Nonius Kappa-CCD diffractometer using monochromated Mo-Kα radiation and were measured using a combination of ϕ scans and ω scans with κ offsets, to fill the Ewald sphere. The data were processed using the Denzo-SMN package. The structure was solved and refined using SHELXTL V6.1 for full-matrix least-squares refinement that was based on F^2. All H atoms were included in calculated positions and allowed refinement with a riding-motion approximation and U~iso~ 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 (2 mm × 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 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 (4×10^6/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 analyzed by LC/MS using the same conditions as those for products in hypochlorite incubations.

Oxidation of Lamotrigine by the MPO System. Lamotrigine (100 μM), MPO (1 unit/ml) and H_2O_2 (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 dichloromethane (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 in 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, average weight 300 g). After the rats were anesthetised with ketamine and xylazine, the livers were removed, chilled on ice and then minced in 4 volumes of cold sucrose buffer (0.25 M). The liver pieces were homogenized using an

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electric homogenizer at 4°C. The homogenate was then centrifuged at 10,000g for 25 min at 4°C. The pellet was resuspended in 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 100,000 g at 4°C for 90 min. The microsomal pellet was resuspended in 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). [^{14}C]Lamotrigine or [^{14}C]clozapine (10 uM, 0.397 µCi/ml 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 hour. After incubation, 3 volumes of acetonitrile were added at room temperature and vortexed. After chilling on ice for 5 min, the mixture was centrifuged at 9000 g for 7 min and the supernatant was removed. The precipitate was redissolved in buffer (300 ul), and the precipitation and the wash procedures were repeated twice. At the end of the third wash the precipitate was re-dissolved in water (1 ml). One 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 Co (Evans et al., 2004) so that the results could be compared to 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).

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Protein Covalent Binding (Human MPO Incubation). [^{14}C]Lamotrigine or [^{14}C]clozapine (10 uM, 0.397 µCi/ ml or 0.050 µCi/ml, respectively) was added to BSA (1 mg, Sigma) and hydrogen peroxide (100 uM) 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. Again clozapine 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), sulfur trioxide/pyridine (32 mg, 0.2 mM) in anhydrous N,N-dimethylformamide (500 ul) was stirred at room temperature and analyzed by TLC (Rf: 0.6; ethylacetate: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 (McCall et al., 1983).

The lamotrigine-N-oxide sulfation product was isolated by preparative HPLC. HPLC was performed using an Ultracarb column (4.6×100 mm). The eluant consisted of water, 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 mmol in 1 ml 25% acetonitrile) was quickly

mixed with methanol (200 µl), or N-acetylhistidine, or N-acetyllysine, or N-acetylserine (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 Sulfotransferase. 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 PAPS-generating system (sodium sulfate, 10 mM; ATP, 7 mM; Mg^{2+}, 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 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-3 column (2 × 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 [^{14}C]Lamotrigine N-oxide. [^{14}C] lamotrigine N-oxide was prepared from [^{14}C]lamotrigine by a method provided by GlaxoSmithKline Inc. A solution of [^{14}C]lamotrigine (3.9×10^{-4} mg, 1.5×10^{-4} mmol) in acetonitrile (50 µl) was added to 2 equivalents of m-chloroperbenzoic acid in acetonitrile (100 µl) and stirred overnight. The [^{14}C]lamotrigine N-oxide was isolated with an open silica gel column and a solvent system consisting of 85% ethyl acetate/15% methanol. Both [^{14}C]lamotrigine and [^{14}C]lamotrigine N-oxide were quantified by HPLC using a standard curve generated from nonradioactive lamotrigine and lamotrigine N-oxide.

[^{14}C]lamotrigine N-oxide or [^{14}C]lamotrigine (as a control) at a concentration of 2.5×10^{-7} M (0.01 µCi) was incubated with cytosol (1.0 mg/ml) and PAPS (0.7 mM; 3'-phosphoadenosine-5'-phosphosulfate 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 acetone was added and vortexed to precipitate the protein. After removing the supernatant, the protein was redissolved in phosphate buffer and the acetone 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 100ºC 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 PM and the control groups were treated with vehicle (water) only. Mouse food was removed after each treatment and refilled at 9 AM the next day. After the last day of treatment, overnight urine (5 PM to 9 AM) was collected and frozen at –80ºC 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 F_{2t}) from different treatment groups was measured using an enzyme immunoassay kit for urinary isoprostane purchased from Oxford Biomedical Research (Oxford, MI, USA) according to the manufacture'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 due to non-specific binding and analyzed at 450 nm by a SPECTRAmax PLUS 384 plate-reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Urinary creatinine concentration in the urine samples was also measured using a creatinine colorimetric microplate assay kit for creatinine purchased from Oxford Biomedical Research (Oxford, MI, USA) according to the manufacture'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 3 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.

In order 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 show). Presumably it was chlorinated at the same positions as the two monochloro lamotrigine isomers.

Chlorination of Lamotrigine by Activated Neutrophils. LC/MS of the supernatant from the lamotrigine/neutrophil incubation shows one product with an M + 1 ion at 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-acetyllysine, N-acetylarginine 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 show) 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. A N-acetylhistidine adduct was observed by LC/MS with an ion M + 1 ion at 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%), 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 while 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 hours) 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 CAD fragments at m/z (relative intensity): 272 (20%), 255 (10%), 254 (10%), 242 (50%), 220 (10%), 214 (10%), 207 (10%), 200 (10%), 199 (10%), 185 (100%), 180 (10%), 173 (30%), 172 (30%), and 165 (50%).

Reaction of Lamotrigine Sulfate with Methanol, Amino Acids, and Lysozyme

1) 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 a 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 CAD fragments at m/z (relative intensity): 286

(20%), 254 (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.

2) Although many amino acids were tested, only N-acetyl serine showed evidence of an adduct, in this case with a MH^+ ion at m/z 401, and like other lamotrigine adducts, the isotope peak pattern of the molecular ion peaks are consistent with the presence of 2 chlorine atoms. The observation that lamotrigine sulfate could form adducts with methanol and N-acetyl serine 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 show). Both of these experiments used cytosolic enzyme. PAPS was used for nitrophenol while 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.

Covalent Binding of [^{14}C]Lamotrigine N-oxide in the Presence of Sulfotransferase. To further test if bioactivation by sulfotransferase could lead to protein covalent binding, [^{14}C]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 cytochromes 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 pmole/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 make this unlikely to be responsible for lamotrigine-induced idiosyncratic reactions. Furthermore, the levels of cytochromes 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-acetylhistidine 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 macrophages 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 sulfate, which would lead to a reactive metabolite, there was a loss of SO_3 to regenerate the N-oxide. However, the sulfate 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 sulfotransferases; however, the synthetic sulfate 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 a 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 are 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 due to 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 felt 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.

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Unnumbered Footnote

This work was supported by a grant from the Canadian Institutes of Health Research and by Glaxo Smith-Kline. JPU is supported by a Canada Research Chair in Adverse Drug Reactions

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Figure Legends

Fig. 1. Lamotrigine metabolites.

Fig. 2. Postulated reactivity of 2-N-lamotrigine sulfate.

Fig. 3. LC/MS chromatogram from lamotrigine incubations. (A) Lamotrigine incubated with human neutrophils, (B) MPO/H_2O_2/Cl^-, and (C) HOCl. The computer extraction of ions are m/z 256 for lamotrigine and m/z 290 for N-chlorolamotrigine. (The HPLC solvent was not the same for the different runs which led to a different retention time. The lamotrigine peak in B is saturated leading to distortion of the peak.)

Fig. 4. X-ray crystallography structure for the stable N-chlorolamotrigine. (A) X-ray structure, (B) simplified structure.

Fig. 5. LC/MS of adduct formed between N-chlorolamotrigine and N-acetylhistidine. (A) Total ion current, (B) Selected ion current of lamotrigine, and (C) Selected ion current of lamotrigine N-acetylhistidine adduct.

Fig. 6. MS/MS of lamotrigine - N-acetylhistidine adduct

Fig. 7. Possible structures of the methanol adduct of lamotrigine sulfate.

Fig. 8. Possible structures of the deaminated methanol adduct of lamotrigine sulfate.

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Table 1. Protein covalent binding of lamotrigine and clozapine in RLM and MPO bioactivation systems. [a]Complete: complete system: RLM/NADPH. [b]Complete: complete system: MPO/H_2O_2/Cl^-, [^{14}C] lamotrigine or [^{14}C] clozapine and BSA. [c]Values are the mean ± SD from six experiments. *Different than control (incomplete system) with $p < 0.001$ using the student t test.

		RLM		MPO	
		- NADPH	Complete[a]	- MPO	Complete[b]
Lamo-trigine	protein covalent binding (pmol/mg)[c]	5.43 ± 0.70	17.1 ± 0.86*	7.00 ± 0.86	38.7 ± 4.9*
	% of drug bound	0.054%	0.17%	0.07%	0.39%
Cloza-pine	protein covalent binding (pmol/mg)[c]	20.8 ± 2.3	106 ± 8.1*	31.1 ± 4.8	1290 ± 290*
	% of drug bound	0.21%	1.1%	0.31%	13%

Table 2. Summary of conditions and findings in lamotrigine and lamotrigine N-oxide sulfation experiments.

Compound	Cytosol added?	Lamotrigine sulfate found?
Lamotrigine N-Oxide	Yes	No
Lamotrigine sulfate	Yes	No
Lamotrigine sulfate	No	Yes

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Table 3. Effects of lamotrigine treatment on urinary 8-isoprostane concentrations in mice. Values are the means ± SD from five groups.

Animal treatment	The concentration of urinary isoprostane normalized with the creatinine (ng/mg)	
	Average	SD
Control	17.1	± 4.4
Lamotrigine	16.6	± 4.0

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Fig 1

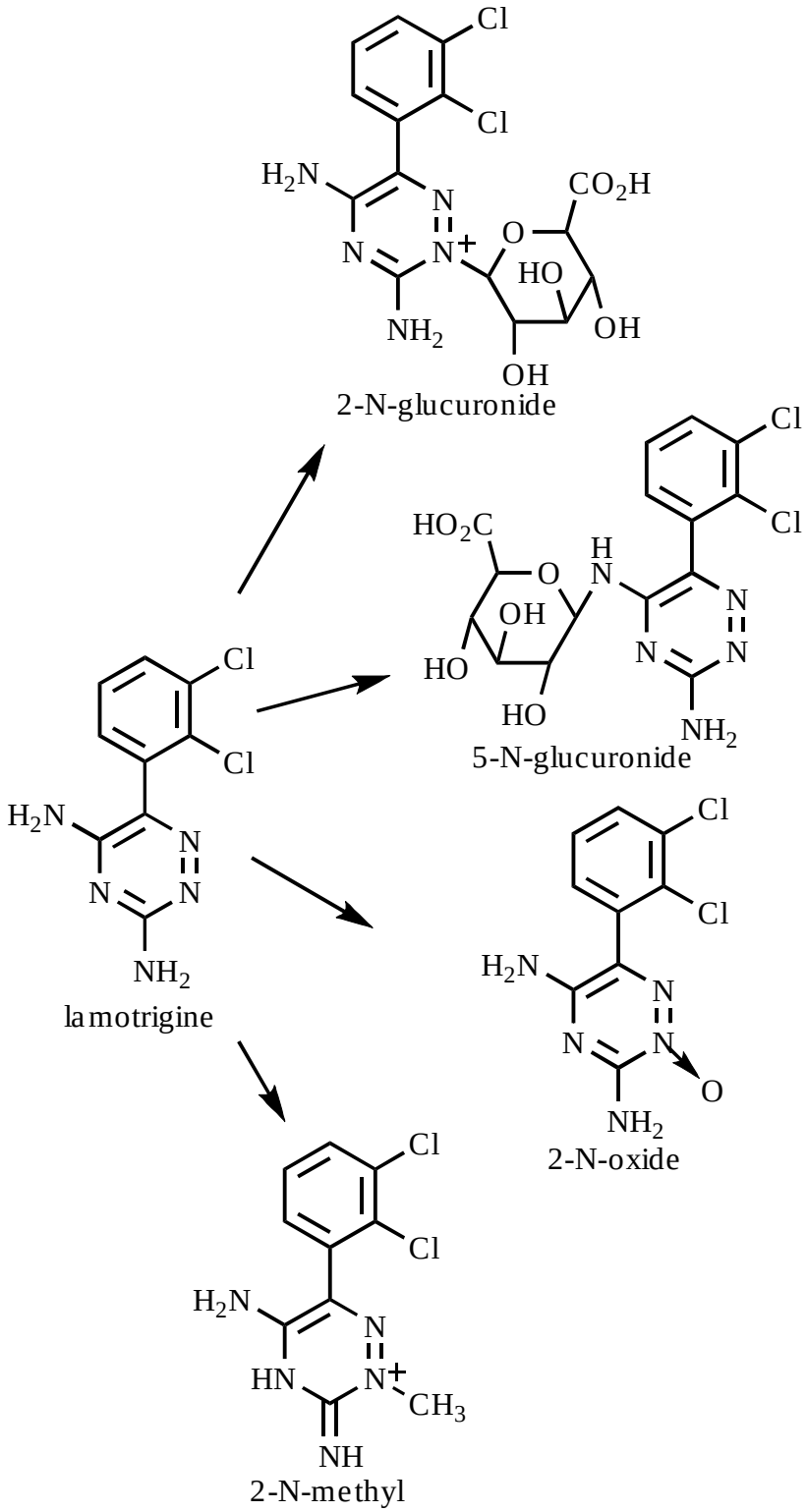

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Fig 2

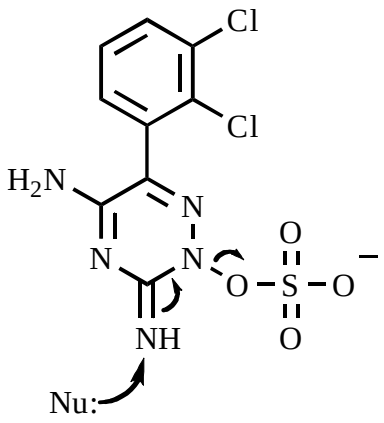

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Fig 3

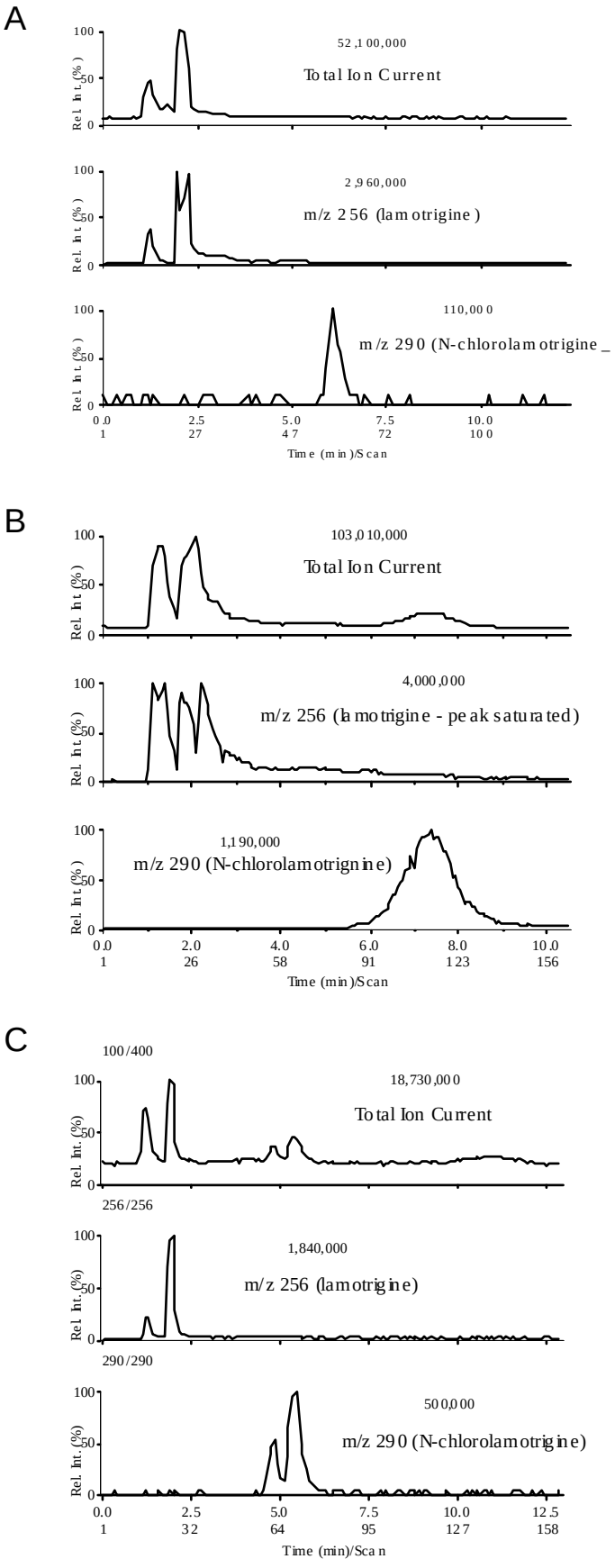

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Fig 4

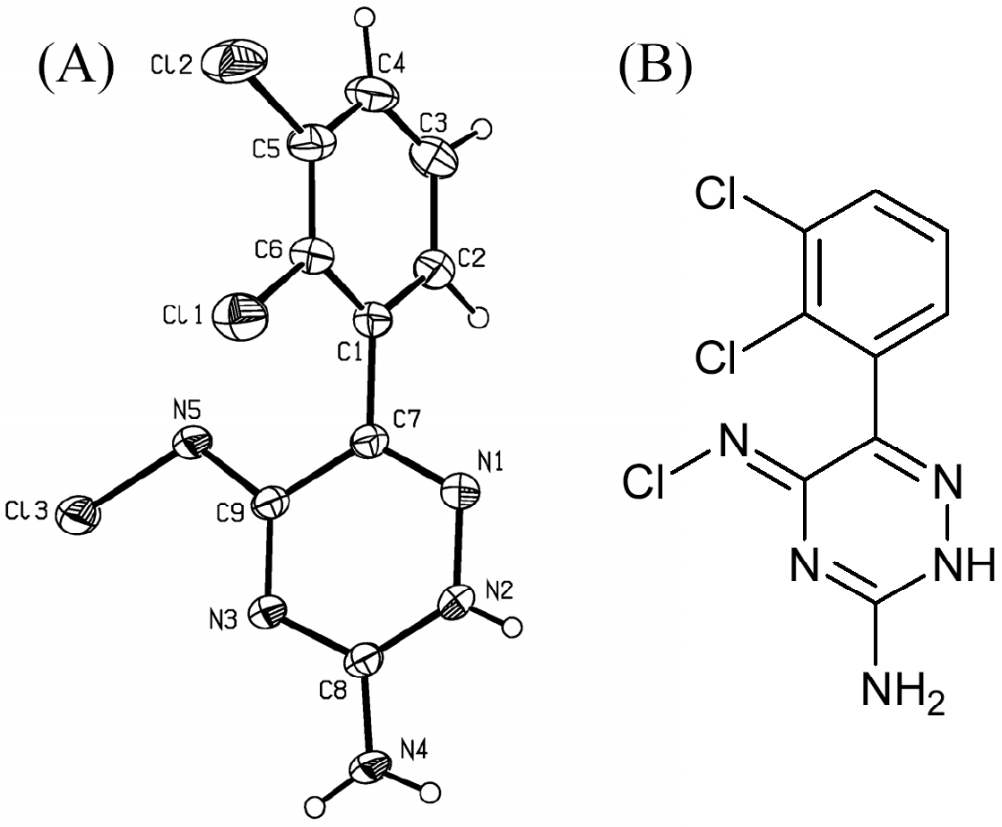

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Fig 5

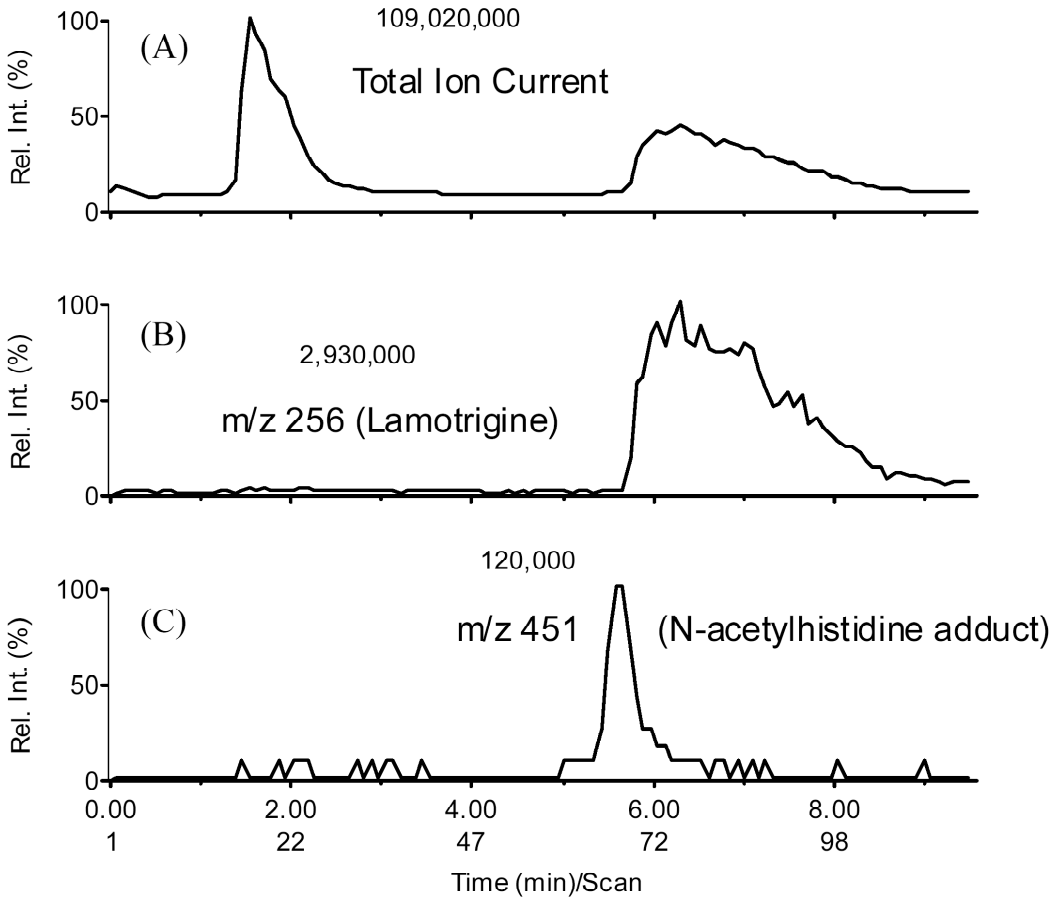

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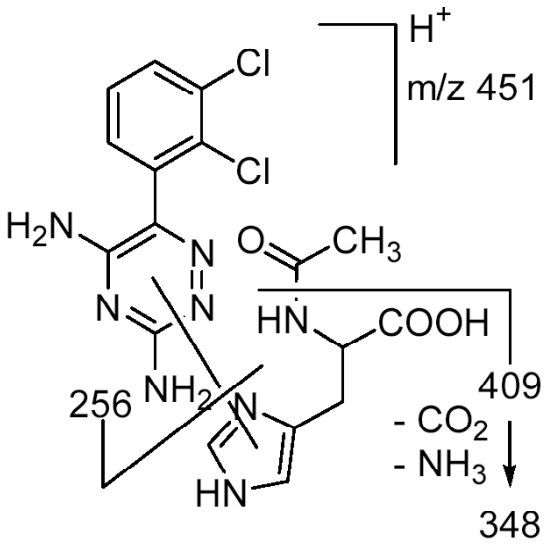
Fig 6



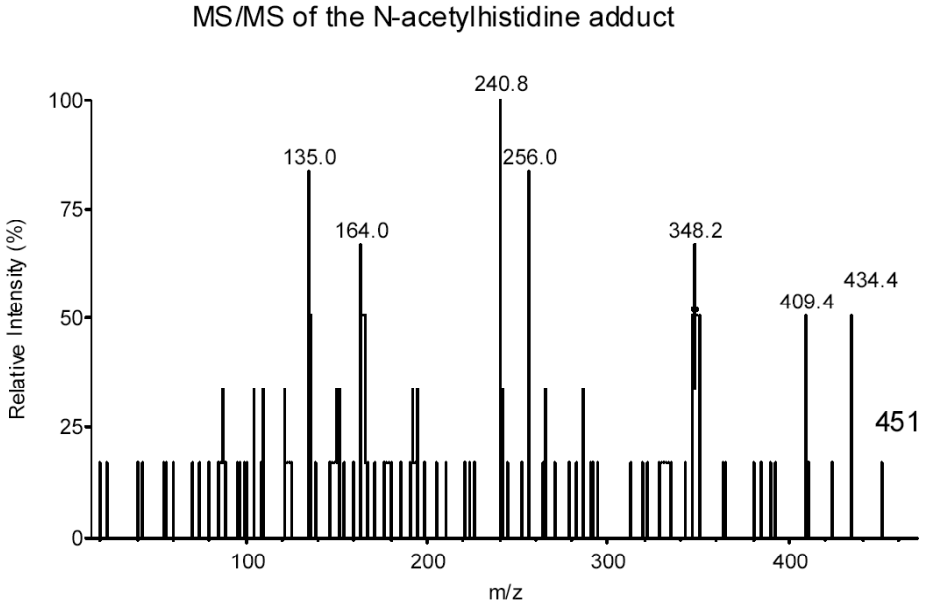


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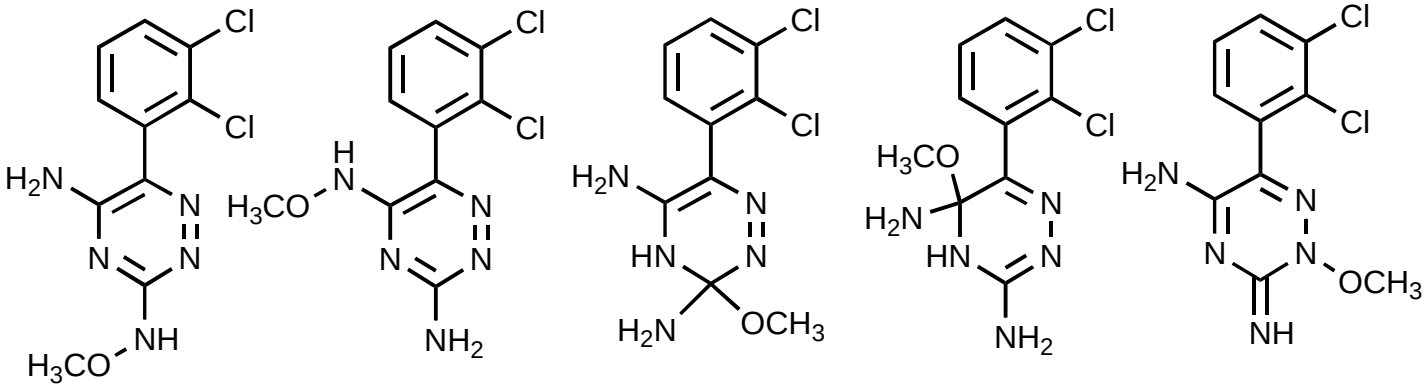
Fig 7



m/z 286

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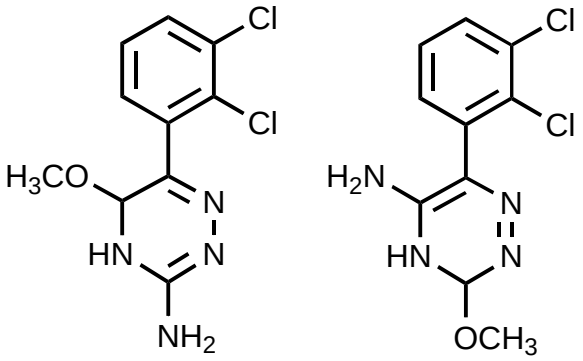
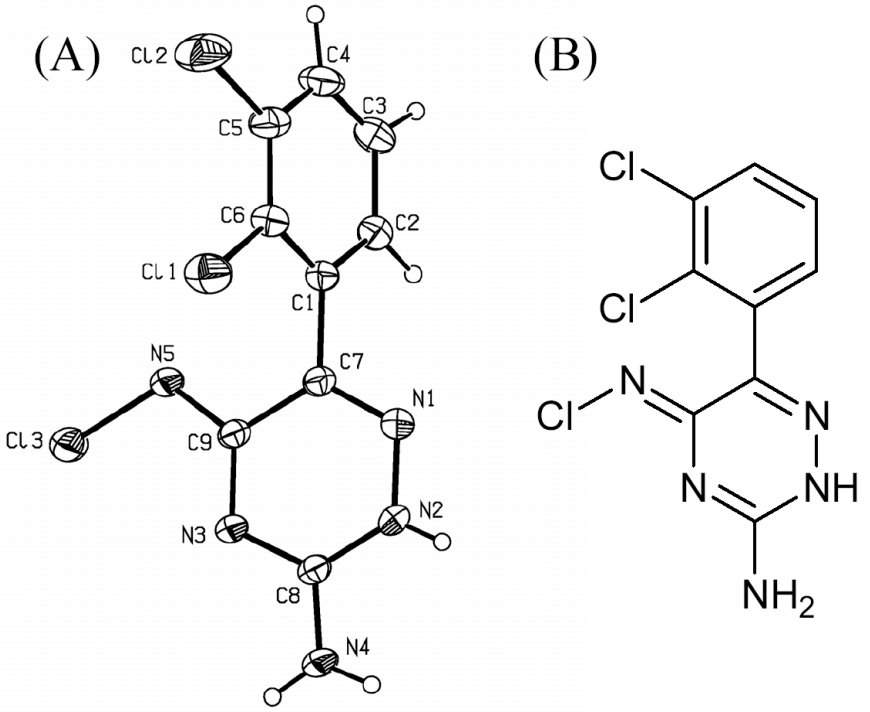
Fig 8



m/z 272

Lamotrigine Supplemental Material

Figure A1. X-ray crystallography data and structure refinement for stable N-chlorolamotrigine. (A) X-ray crystallography, and (B) Proposed structure.



X-ray crystallography data were collected on a Nonius Kappa-CCD diffractometer using monochromated Mo-Kα radiation and were measured using a combination of ϕ scans and ω scans with κ offsets, to fill the Ewald sphere. The data were processed using the Denzo-SMN package[1]. The structure was solved and refined using SHELXTL V6.1[3] for full-matrix least-squares refinement that was based on F^2. All H atoms were included in calculated positions and allowed to refine in riding-motion approximation with U~iso~ tied to the carrier atom. Crystallographic data for the compounds is given in Table 1.

1. Otwinowski, Z. & Minor, W. (1997). Methods in Enzymology, Vol. 276, Macromolecular Crystallography, Part A edited by C. W. Carter & R. M. Sweet pp. 307-326. London: Academic press.

2. Sheldrick, G. M. (2001). SHELXTL/PC. Version 6.1 Windows NT Version. Bruker AXS Inc., Madison, USA.

Table A1. Crystal data and structure refinement for K9703 (stable N-chlorolamotrigine).

Identification code	k9703	
Empirical formula	C9 H6 Cl3 N5	
Formula weight	290.54	
Temperature	293(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	$P2_1/n$	
Unit cell dimensions	a = 9.1842(10) Å	α= 90°.
	b = 12.0629(10) Å	β= 108.176(10)°.
	c = 11.7378(10) Å	γ = 90°.
Volume	1235.5(2) $Å^3$	
Z	4	
Density (calculated)	1.562 Mg/m^3	
Absorption coefficient	0.725 mm^{-1}	
F(000)	584	
Crystal size	0.20 x 0.18 x 0.16 mm^3	
Theta range for data collection	2.47 to 30.38°.	
Index ranges	-12<=h<=12, -16<=k<=16, -15<=l<=15	
Reflections collected	6159	
Independent reflections	3216 [R(int) = 0.0214]	
Completeness to theta = 30.38°	86.4 %	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3216 / 0 / 179	
Goodness-of-fit on F^2	0.978	
Final R indices [I>2sigma(I)]	R1 = 0.0393, wR2 = 0.1009	
R indices (all data)	R1 = 0.0770, wR2 = 0.1104	
Extinction coefficient	0.0009(15)	
Largest diff. peak and hole	0.310 and -0.388 $e.Å^{-3}$	

Table A2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for K9703. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
Cl(1)	6358(1)	1155(1)	5409(1)	65(1)
Cl(2)	9508(1)	2318(1)	6734(1)	85(1)
Cl(3)	3258(1)	496(1)	7854(1)	67(1)
N(1)	2615(2)	2906(1)	4317(1)	44(1)
N(2)	1205(2)	2427(2)	3903(2)	46(1)
N(3)	1689(2)	1079(1)	5410(1)	38(1)
N(4)	-570(2)	1093(2)	3843(2)	55(1)
N(5)	3905(2)	1475(1)	7050(1)	46(1)
C(1)	5040(2)	3055(2)	5812(2)	39(1)
C(2)	5086(2)	4140(2)	6195(2)	47(1)
C(3)	6478(3)	4653(2)	6739(2)	57(1)
C(4)	7820(2)	4089(2)	6909(2)	60(1)
C(5)	7796(2)	3014(2)	6515(2)	52(1)
C(6)	6405(2)	2489(2)	5950(2)	44(1)
C(7)	3520(2)	2504(2)	5298(2)	37(1)
C(8)	791(2)	1529(2)	4394(2)	38(1)
C(9)	3012(2)	1627(2)	5950(2)	35(1)

Table A3. Bond lengths [Å] and angles [°] for K9703.

Cl(1)-C(6)	1.726(2)
Cl(2)-C(5)	1.730(2)
Cl(3)-N(5)	1.7286(17)
N(1)-C(7)	1.287(2)
N(1)-N(2)	1.361(2)
N(2)-C(8)	1.337(3)
N(3)-C(8)	1.335(2)
N(3)-C(9)	1.355(2)
N(4)-C(8)	1.324(2)
N(5)-C(9)	1.309(2)
C(1)-C(2)	1.380(3)
C(1)-C(6)	1.392(3)
C(1)-C(7)	1.493(2)
C(2)-C(3)	1.384(3)
C(3)-C(4)	1.367(3)
C(4)-C(5)	1.374(3)
C(5)-C(6)	1.395(3)
C(7)-C(9)	1.464(3)
C(7)-N(1)-N(2)	115.91(16)
C(8)-N(2)-N(1)	123.81(15)
C(8)-N(3)-C(9)	115.68(15)
C(9)-N(5)-Cl(3)	114.71(12)
C(2)-C(1)-C(6)	119.49(16)
C(2)-C(1)-C(7)	118.94(16)
C(6)-C(1)-C(7)	121.53(16)
C(1)-C(2)-C(3)	120.3(2)
C(4)-C(3)-C(2)	120.4(2)
C(3)-C(4)-C(5)	120.10(19)
C(4)-C(5)-C(6)	120.29(19)
C(4)-C(5)-Cl(2)	119.37(15)
C(6)-C(5)-Cl(2)	120.34(17)
C(1)-C(6)-C(5)	119.38(18)
C(1)-C(6)-Cl(1)	119.77(14)

C(5)-C(6)-Cl(1)	120.84(16)
N(1)-C(7)-C(9)	121.15(15)
N(1)-C(7)-C(1)	117.08(16)
C(9)-C(7)-C(1)	121.42(14)
N(4)-C(8)-N(3)	120.03(18)
N(4)-C(8)-N(2)	117.62(17)
N(3)-C(8)-N(2)	122.34(15)
N(5)-C(9)-N(3)	126.00(17)
N(5)-C(9)-C(7)	114.38(14)
N(3)-C(9)-C(7)	119.60(14)

Symmetry transformations used to generate equivalent atoms:

Table A4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for K9703. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + \ldots + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
Cl(1)	56(1)	50(1)	86(1)	-18(1)	20(1)	1(1)
Cl(2)	37(1)	98(1)	118(1)	-10(1)	22(1)	1(1)
Cl(3)	56(1)	87(1)	45(1)	22(1)	-4(1)	-26(1)
N(1)	39(1)	41(1)	44(1)	4(1)	4(1)	-7(1)
N(2)	35(1)	48(1)	42(1)	10(1)	-6(1)	-5(1)
N(3)	30(1)	43(1)	35(1)	3(1)	2(1)	-5(1)
N(4)	37(1)	69(1)	43(1)	14(1)	-9(1)	-17(1)
N(5)	37(1)	53(1)	38(1)	8(1)	-2(1)	-13(1)
C(1)	36(1)	39(1)	38(1)	1(1)	7(1)	-6(1)
C(2)	45(1)	40(1)	51(1)	1(1)	9(1)	-3(1)
C(3)	62(1)	39(1)	66(1)	-9(1)	11(1)	-15(1)
C(4)	45(1)	61(2)	69(2)	-8(1)	11(1)	-23(1)
C(5)	35(1)	62(1)	58(1)	-3(1)	12(1)	-7(1)
C(6)	40(1)	44(1)	48(1)	-4(1)	13(1)	-6(1)
C(7)	33(1)	36(1)	37(1)	-2(1)	5(1)	-2(1)
C(8)	30(1)	45(1)	35(1)	2(1)	4(1)	-2(1)
C(9)	29(1)	37(1)	36(1)	-1(1)	3(1)	-1(1)

Table A5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^{-3}) for K9703.

	x	y	z	U(eq)
H(2B)	720(30)	2690(20)	3310(20)	85(9)
H(4C)	-1080(30)	1391(19)	3230(20)	58(7)
H(4B)	-890(30)	550(20)	4140(20)	62(7)
H(2A)	4110(20)	4510(17)	6019(18)	49(6)
H(3A)	6420(30)	5410(20)	6920(20)	62(7)
H(4A)	8800(30)	4384(19)	7330(20)	66(7)