Short Communication

The Tissue-Specific Toxicity of Methimazole in the Mouse Olfactory Mucosa Is Partly Mediated through Target-Tissue Metabolic Activation by CYP2A5^S

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ABSTRACT:

The antithyroid drug methimazole (MMZ) can cause severe, tissue-specific toxicity in mouse olfactory mucosa (OM), presumably through a sequential metabolic activation of MMZ by cytochrome P450 (P450) and flavin monooxygenases (FMO). The aims of this study were to determine whether CYP2A5, one of the most abundant P450 enzymes in the mouse OM, is involved in MMZ metabolic activation, by comparing *Cyp2a5*-null with wild-type (WT) mice, and whether hepatic microsomal P450 enzymes, including CYP2A5, are essential for MMZ-induced OM toxicity, by comparing liver-*Cpr*-null (LCN) mice, which have little P450 activity in hepatocytes, with WT mice. We showed that the loss of CYP2A5 expression did not alter systemic clearance of MMZ (at 50 mg/kg, i.p.); but it did significantly decrease the rates of MMZ metabolism in the

OM, whereas FMO expression in the OM was not reduced. MMZ induced depletion of nonprotein thiols, as well as pathological changes, in the OM of WT mice; the extent of these changes was much reduced in the *Cyp2a5*-null mice. Thus, CYP2A5 plays an important role in mediating MMZ toxicity in the OM. In contrast, the rate of systemic clearance of MMZ was significantly reduced in the LCN mice, compared to WT mice, whereas the MMZ-induced OM toxicity was not prevented. Therefore, hepatic P450 enzymes are essential for systemic MMZ clearance, but they are not required for MMZ-induced OM toxicity. We conclude that the tissue-specific toxicity of MMZ is mediated by target tissue metabolic activation, and the reaction is partly catalyzed by CYP2A5 in the OM.

Introduction

Methimazole (1-methyl-2-mercaptoimidazole; Tapazole) (MMZ), a drug commonly used for treating hyperthyroidism, and also often used as a model substrate, as well as an inhibitor, for the flavin-containing monooxygenases (FMO), is known to cause tissue-selective toxicity in rodent olfactory mucosa (OM) (Brittebo, 1995; Genter et al., 1995; Bergman and Brittebo, 1999; Bergström et al., 2003; Casallo Blanco et al., 2007). MMZ-induced olfactory deficits have also been reported in human patients (Bandyopadhyay et al., 2002; Woeber, 2002). In mice, cytochrome P450 (P450)-catalyzed MMZ metabolic activation is believed to be involved in MMZ's tissue toxicity, given the observation that pretreatment of mice with P450 inhibitors can suppress MMZ toxicity in both liver and OM (Brittebo, 1995; Mizutani et al., 1999). Intraperitoneally injected MMZ can cause dose-dependent (5–50 mg/kg) decreases of the levels of total nonprotein sulfhydryl [(NPSH) mainly GSH] in the OM (Bergström et al., 2003), suggesting that the olfactory toxicity of MMZ is mediated via its electrophilic

intermediates. Likewise, whereas an oral dose of MMZ (>200 mg/kg) induced only a marginal increase in serum alanine transaminase in mice, with no histopathological changes in the liver, a combined treatment with MMZ (~10 mg/kg, p.o.) and an inhibitor of GSH synthesis resulted in obvious centrilobular necrosis among hepatocytes (Mizutani et al., 1999). Therefore, both the olfactory toxicity and the hepatotoxicity of MMZ depend on the *in vivo* formation of its electrophilic intermediates. It has been proposed that the bioactivation of MMZ is catalyzed sequentially by P450 and FMO, with P450 enzymes catalyzing the epoxidation of the C-4,5 double bond of MMZ, forming *N*-methylthiourea, which is further metabolized by FMO to the reactive sulfenic and sulfinic acid (Mizutani et al., 2000). The sulfenic/sulfinic acid is most likely responsible for the hepatotoxicity (Mizutani et al., 2000), and presumably also OM toxicity, of MMZ. However, the specific P450 enzymes responsible for MMZ metabolic activation have not been identified, and it is unclear whether target-tissue metabolic activation is essential for the OM toxicity.

In the present study, we have determined whether CYP2A5, one of the most abundant P450 enzymes in the mouse OM, is involved in MMZ metabolic activation, and whether hepatic microsomal P450 enzymes, including CYP2A5, are essential for MMZ-induced OM toxicity. We studied MMZ metabolism and toxicity in two knockout mouse models: 1) a *Cyp2a5*-null mouse (Zhou et al., 2010), which had been used recently for demonstration of the role of CYP2A5 in the metabolic activation and OM-specific toxicity of the herbicide 2,6-

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ABBREVIATIONS: MMZ, methimazole; FMO, flavin-containing monooxygenases; OM, olfactory mucosa; P450, cytochrome P450; NPSH, nonprotein sulfhydryl; DCBN, 2,6-dichlorobenzonitrile; LCN, liver-*Cpr*-null; WT, wild type; B6, C57BL/6; HPLC, high-performance liquid chromatography; AUC, area under the concentration-time curve; CPR, NADPH-cytochrome P450 reductase.

dichlorobenzonitrile (DCBN) (Xie et al., 2010), and 2) the liver-*Cpr*-null (LCN) mouse (Gu et al., 2003), which has been found valuable for differentiating between hepatic and extrahepatic P450 contributions to systemic drug clearance and target tissue toxicities (e.g., Gu et al., 2005; Weng et al., 2007; Fang et al., 2008; Xie et al., 2010). We first compared wild-type (WT) and *Cyp2a5*-null mice, for rates of MMZ metabolism by OM microsomes, and extents of MMZ-induced depletion of nonprotein thiols, as well as histopathological changes. As controls, we examined whether the loss of CYP2A5 expression altered systemic clearance of MMZ, or was associated with a decrease in the expression of FMO proteins in the OM. We then compared WT and LCN mice, for rates of systemic MMZ clearance and extents of MMZ-induced toxicity, to further determine whether hepatic P450 enzymes (including CYP2A5) are required for MMZ-induced OM toxicity. Our findings indicate that the tissue-specific OM toxicity of MMZ is mediated by target-tissue metabolic activation, and the reaction is partly catalyzed by CYP2A5 in the OM.

Materials and Methods

Chemicals and Animal Treatments. MMZ (>99% pure) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center (Albany, NY). LCN [nearly congenic strain after backcrossing to the C57BL/6 (B6) strain for 10 generations], their corresponding WT littermates, *Cyp2a5*-null on B6 background, and WT B6 mice were from breeding stocks maintained at the Wadsworth Center. Animals were normally maintained at 22°C with a 12-h on/12-h off light cycle and were allowed free access to water and a standard laboratory diet. Mice were treated with a single intraperitoneal dose of MMZ at 50 mg/kg. MMZ was dissolved in pure, sterile water at 10 mg/ml. Control mice received injections of vehicle only. Animals were dosed at 10:00 to 11:00 AM, local time. Tail blood samples (10 or 20 μ l each) were collected from individual mice at various times after dosing (5, 30 min, and 1, 2, 4, 8, and 24 h) using heparin-coated capillaries. Each blood sample was diluted with one volume of saline and then centrifuged at 4°C and 9000g for 10 min. The plasma was stored at -80°C until use. At 24 h after MMZ injection, the mice were euthanized by CO₂ overdose, and noses were dissected for histopathological examination. For determination of tissue levels of NPSH, mice were fasted overnight, and liver and OM were dissected at 2 h after MMZ injection; tissue samples were stored frozen at -80°C before analysis.

Determination of Tissue NPSH Levels. NPSH was determined using a reported method (Tonge et al., 1998), with modifications in the homogenization step as reported (Xie et al., 2010), to accommodate the small amount of nasal tissues available from a single mouse. Liver was homogenized on ice with a Polytron (model GT 10-35; Kinematica Inc., Bohemia, NY), in 100 mM Tris-acetate buffer, pH 7.4, containing 1.0 mM EDTA and 150 mM potassium chloride (buffer H), at a tissue-to-buffer ratio of ~1:10. OM (~20 mg wet weight) from individual mice was homogenized with use of a Bullet Blender (Next Advance, Averill Park, NY) in an 1.5-ml Eppendorf centrifuge tube containing 400 μ l of buffer H and two stainless steel beads (0.5-mm diameter). The samples were homogenized in the blender at a speed setting of 4, continuously for 4 min, at 4°C. Reduced GSH was used as the standard.

Histopathological Examination. Tissue blocks containing the nasal passages were dissected and soaked in Bouin's solution for fixation and decalcification for 2 weeks, as described previously (Gu et al., 2005). Each tissue block was cut into smaller blocks at four anterior-rostral levels, as described by Young (1981). Paraffin sections (4 μ m) from levels 1 to 4 were stained with hematoxylin and eosin for pathological examination. For semiquantitative assessment of the extent of tissue toxicity, the severity of lesions in the OM was graded, as described previously (Gu et al., 2005); for each treatment group, 4 to 5 mice were examined (4 blocks/mouse; 1 section/block). For quantitative analysis of the extent of epithelial injury, the total length of intact olfactory epithelium in the dorsal medial meatus was measured (1 section/mouse). Level-3 sections of the dorsal medial meatus of each nasal cavity (Young, 1981) were photographed at 4 $\times$ magnification and printed (8 $\times$ 10 inch prints). Length measurements were made with a MapWheel (Scalex,

Carlsbad, CA), calibrated to a stage micrometer photographed at the same magnification. Tissue sectioning and staining were performed at the Wadsworth Center Pathology core facility. Images were obtained using a Nikon model 50i light microscope (Nikon Instruments, Melville, NY), fitted with a digital camera, at the Wadsworth Center Light Microscopy core.

Determination of MMZ in Blood. A HPLC-UV protocol was established, based on a method described previously (Hoffman et al., 2002), for the determination of plasma concentrations of MMZ. An Agilent model 1100 (Agilent Technologies, Santa Clara, CA) HPLC system with a diode array UV detector was used. Two volumes of methanol were added to the plasma samples to precipitate proteins. Aliquots of the supernatant fraction (5 μ l) were analyzed using a 4- μ m Nova-Pak C18 column (3.9 $\times$ 150 mm; Waters, Milford, MA) and an isocratic mobile phase consisting of 10 mM ammonium acetate, pH 4.0 (96.5%, v/v) and acetonitrile (3.5%), at a flow rate of 0.8 ml/min. MMZ was detected by UV at the wavelength of 254 nm. Standard curves were prepared by spiking authentic MMZ into blank mouse plasma at various concentrations (400–4000 ppb), before precipitation with methanol. The recovery of MMZ from plasma samples was ~50%.

Assay for MMZ In Vitro Metabolism. OM microsomes were prepared as reported (Gu et al., 1998), from pooled tissues from 6 to 8 mice per sample. Activity was determined by measuring rates of MMZ disappearance in microsomal reaction mixtures containing 50 mM phosphate buffer, pH 7.4, 5 μ M MMZ, 0.2 mg/ml microsomal protein from 2- to 3-month-old male WT or *Cyp2a5*-null mice, and 1 mM NADPH, in a final volume of 0.1 ml. Reactions without NADPH addition were used as negative controls. The reactions were carried out at 37°C and were stopped by the addition of an equal volume of methanol. After centrifugation at 9000g for 10 min, the supernatant was analyzed using HPLC-UV for levels of residual MMZ, as described under *Determination of MMZ in Blood*. A calibration curve was prepared by spiking authentic MMZ into complete incubation mixtures containing boiled microsomes, to give a series of standard calibrating samples with final concentrations ranging from 0 to 40 μ M.

Other Methods. Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Immunoblot analysis of FMO expression was carried out using goat anti-FMO1, FMO2, and FMO5 antibodies (Genter and Ali, 1998). The levels of calnexin were also determined, as a loading control, using rabbit anti-human calnexin (GenScript, Piscataway, NJ). Pharmacokinetic parameters, including area under concentration-time curve (AUC), maximum plasma concentration (C_{max}), the time when C_{max} is reached (T_{max}), elimination half-time ($t_{1/2}$), and clearance, were calculated using the WinNonlin software (Pharsight, Mountain View, CA). Statistical significance of differences between two groups in various parameters was examined using the Student's *t* test, with use of the SigmaStat software (SPSS, Chicago, IL).

Results

CYP2A5 Plays an Important Role in Mediating MMZ Toxicity in the OM. WT and *Cyp2a5*-null mice were treated with MMZ at 50 mg/kg i.p., a dose known to induce observable tissue toxicity in the OM of WT mice (Brittebo, 1995). The MMZ-induced histopathologic changes in the OM of WT mice were mainly characterized as diffuse degeneration of the olfactory epithelium, at 24 h after the treatment; the epithelium was often absent, with only a denuded basement membrane remaining. In some areas of the nasal cavity (dorsal, caudal, and lateral), epithelium was still present, though markedly abnormal. The olfactory epithelium, when still present, was often detached from the basement membrane, fractured and disorganized (Fig. 1). In the *Cyp2a5*-null mice, there was less congestion in the submucosa as well as less glandular necrosis, compared to the WT mice; in addition, considerably more reserve cells remained attached to the basement membrane. The total length of intact olfactory epithelium in the dorsal medial meatus, an area that can be reproducibly sampled in the nasal cavity, was also measured, to provide a quantitative assessment of the extents of epithelial injury. The results showed that the fraction of intact olfactory epithelium (mean $\pm$ S.D.) in *Cyp2a5*-null (60.9 $\pm$ 15.9%) was significantly larger than that in WT (14.6 $\pm$ 17.4%; $P < 0.01$; $n = 5$).

FIG. 1. Histopathological analysis of nasal mucosal damages caused by MMZ treatment in WT, *Cyp2a5*-null, and LCN mice. Two- to 3-month-old male mice were treated with a single intraperitoneal injection of either sterile water or MMZ in water at 50 mg/kg. Tissues were obtained for histopathological examination at 24 h after the treatment. Representative cross-sections of the nasal cavity, obtained at the level 3 of Young (1981) and stained with hematoxylin and eosin, are shown. Vehicle-treated WT B6 mice had normal ethmoidal turbinates (ET) and nasal septum (SP), as well as a clear nasal passage. Both WT and LCN mice showed extensive injury to the olfactory epithelium, evident as epithelial necrosis, detachment, sloughing, and ulceration, after the MMZ treatment. The extent of lesion was significantly reduced in the *Cyp2a5*-null mice, which showed less congestion in the submucosa, less glandular necrosis, and ample reserve cell attachment to the basement membrane, compared with the WT mice. LP, lamina propria; BG, Bowman's glands. Boxed areas (the dorsal medial meatus) in the left panels (40 $\times$) are shown at 400 $\times$ in the right panels. Scale bar, 50 μ m in 40 $\times$; 20 μ m in 400 $\times$.

MMZ treatment (at 50 mg/kg i.p.) induced NPSH depletion in the OM of WT mice, at 2 h after dosing (by $51.8 \pm 3.4\%$, compared to vehicle-treated mice; $n = 3-6$, $P < 0.01$, Student's t test). MMZ also induced NPSH depletion in the OM of the *Cyp2a5*-null mice (by $41.5 \pm 6.9\%$; $n = 3$, $P < 0.01$). The extent of NPSH depletion was significantly lower for the *Cyp2a5*-null mice than for the WT mice ($P < 0.05$, Student's t test).

The results from both the histopathological analysis and the NPSH determination indicated that the *Cyp2a5* gene deletion led to a reduction in the extent of MMZ-induced cytotoxicity in the OM. This result suggested that CYP2A5 is partly responsible for the metabolic activation of MMZ in vivo. Consistent with this idea, the rates of MMZ metabolism by OM microsomal P450 were decreased in the *Cyp2a5*-

null mice, compared to WT mice. To assay the in vitro metabolism of MMZ, we measured the rates of MMZ disappearance in microsomal incubations. With MMZ at 5 μ M, $58.7 \pm 5.6\%$ ($n = 3$) of added MMZ was metabolized after a 15-min incubation with OM microsomes from WT mice; however, only $28.7 \pm 1.7\%$ ($n = 3$; $P < 0.01$, significantly lower than the WT value) of the added MMZ was metabolized after a 15-min incubation with OM microsomes from *Cyp2a5*-null mice.

The pharmacokinetic profile (Fig. 2A) and pharmacokinetic parameters (Table 1) for plasma MMZ were not significantly different between the *Cyp2a5*-null and the WT B6 mice, indicating that the loss of CYP2A5 expression did not alter systemic clearance of MMZ. As an additional control, we confirmed by immunoblot analysis that the expression of FMO1 and FMO2 proteins (the major FMOs detected) in OM microsomes was not changed in the *Cyp2a5*-null mice, compared to WT mice (Supplemental Fig. 1). Thus, the observed decrease in microsomal activity toward MMZ did not result from a decrease (which is unexpected) in FMO expression.

Hepatic P450 Enzymes Are Essential for MMZ Clearance, but Not for MMZ-Induced OM Toxicity. To further determine whether hepatic P450 enzymes (including CYP2A5) are required for MMZ-induced OM toxicity, we compared WT and LCN mice, for extents of MMZ-induced OM toxicity. As shown in Fig. 1, the nasal lesion caused by MMZ treatment in the LCN mice was not less (rather, it was actually slightly more) severe than the lesion found in MMZ-treated WT mice, as evidenced by a more noticeable denudement of the basement membranes. The fraction of intact olfactory epithelium in the dorsal medial meatus (mean $\pm$ S.D.) in WT littermates ($39.8 \pm 22.5\%$) was actually greater than that in LCN mice (0% ; $P < 0.05$; $n = 4$). Likewise, significant decreases in NPSH levels were detected in both WT and LCN mice at 2 h after the MMZ injection, compared to the corresponding vehicle-treated mice (by 51.8 ± 3.4 and $48.2 \pm 3.7\%$, respectively; $n = 3-6$, $P < 0.01$, Student's t test). Thus, hepatic P450 enzymes (including CYP2A5) are not required for MMZ-induced OM toxicity.

To confirm that hepatic MMZ metabolism was indeed suppressed in the LCN mice, compared with the WT mice, we further determined the impact of hepatic CPR loss on MMZ clearance in vivo. In contrast to the consequence of *Cyp2a5* gene deletion, the loss of hepatic *Cpr* expression led to substantial decreases in the rates of systemic clearance of MMZ (Fig. 2B; Table 1). The AUC values, which represent total exposure amounts, as well as the maximal plasma MMZ concentrations, were significantly higher in the LCN mice than those in the WT mice. Thus, tissues of the LCN mice were exposed to greater amounts of the unmetabolized MMZ (and lesser amounts, if any, of hepatic P450-produced MMZ metabolites) than did tissues of the WT mice.

Discussion

Our results of the current study with the LCN mouse model indicated that hepatic P450s play a large role in MMZ clearance, because loss of hepatic microsomal P450 activity significantly reduced the systemic clearance rate for MMZ. However, 24 h after the single intraperitoneal injection at 50 mg/kg, plasma MMZ was decreased to an undetectable level in the LCN as well as WT mice, indicating that pathways other than hepatic microsomal P450s (e.g., FMO, extrahepatic P450s) are capable of clearing MMZ, although less efficiently. The reduced clearing rate of MMZ in LCN mice led to an increased total exposure to MMZ (by ~ 4 -fold) in these mice than in the WT mice, which explains the increased (rather than comparable) OM toxicity in the LCN mice.

FIG. 2. Pharmacokinetic analysis of MMZ clearance in various mouse strains. Two- to 3-month old male *Cyp2a5*-null and WT B6 mice (A) or LCN and WT littermates (B) were given a single injection of MMZ (50 mg/kg i.p.). Tail blood samples from individual mice were used for determination of plasma concentrations of MMZ, as described under *Materials and Methods*. Data represent means $\pm$ S.D., $n = 4$ –5. *, $P < 0.05$; **, $P < 0.01$ (LCN compared with WT littermates or *Cyp2a5*-null compared with WT B6); Student's t test. The structure of MMZ (C) and a representative HPLC-UV chromatogram for the detection of MMZ (400 nmol injected) are also shown (D).

TABLE 1

Pharmacokinetic parameters for MMZ clearance in various mouse strains

Plasma levels of MMZ (see Fig. 2, A and B) were used to calculate various pharmacokinetic parameters. Values represent means $\pm$ S.D. ($n = 4$ for each strain).

Strains	$T_{1/2}$	T_{max}	C_{max}	AUC_{INF}	CL
	h	h	µg/ml	µg · h/ml	ml/h
WT	1.5 ± 0.4	0.9 ± 0.3	48.4 ± 8.5	149 ± 38	8.9 ± 2.8
LCN	4.3 ± 1.7^a	0.6 ± 0.3	69.5 ± 13.1^a	585 ± 169^b	2.3 ± 0.6^b
WT B6	1.3 ± 0.2	0.6 ± 0.2	67.2 ± 12.7	128 ± 26	10.7 ± 2.8
<i>Cyp2a5</i> -null	1.4 ± 0.3	0.6 ± 0.2	76.8 ± 7.5	154 ± 15	8.7 ± 0.6

^a $P < 0.05$ and ^b $P < 0.01$, compared to WT in the same treatment group; Student's t test.

Loss of hepatic microsomal P450 did not protect the LCN mice from MMZ-induced olfactory toxicity, suggesting that locally produced toxic MMZ intermediates are responsible for causing OM toxicity. Thus, MMZ is similar to two other olfactory toxicants, acetaminophen and DCBN, which have been found to cause olfactory toxicities through extrahepatic (mostly likely in situ) bioactivation (Gu et al., 2005; Xie et al., 2010). However, MMZ differ from DCBN in that whereas CYP2A5 was largely responsible for DCBN's OM toxicity, it was only partially responsible for the OM toxicity of MMZ. Thus, this is the first study to show that CYP2A5, which is one of the most abundant P450 isoforms in the mouse OM (Gu et al., 1998) and is known to be active toward a number of nasal toxicants and procarcinogens (Jalas and Hecht, 2003; Xie et al., 2010), is able to metabolize MMZ, as indicated by the decreased MMZ metabolism in *Cyp2a5*-null OM microsomes. Previous studies have shown that several isoforms of FMO are active in metabolizing MMZ (Siddens et al., 2008). Furthermore, NADPH-supported formation of *N*-methylimidazole from MMZ by rat hepatic microsomes was largely dependent on the P450 system (Lee and Neal, 1978). However, the specific P450 enzymes responsible for microsomal MMZ metabolism in either liver or OM have not been identified. In that connection, FMO1 and FMO2, as well as many P450s, are known to be expressed in the OM

(Genter et al., 1995; Shehin-Johnson et al., 1995; Ling et al., 2004). Further studies are needed to determine the contributions of these FMOs and P450 enzymes (other than CYP2A5) to MMZ toxicity in the OM. In addition, it remains to be determined whether human orthologs of CYP2A5, CYP2A6 and CYP2A13, are also active toward MMZ, which may have important implications for understanding potential MMZ nasal toxicity in human patients.

In summary, the present study demonstrated that hepatic P450s are important in systemically clearing MMZ, a model FMO substrate. The olfactory toxicity of MMZ does not require hepatic P450-catalyzed activation but partly depends on olfactory CYP2A5-catalyzed activation. This study also exemplified the use of the LCN mouse model for delineating the relative contributions of hepatic microsomal P450 versus other biotransformation enzymes, such as FMO, to systemic clearance of xenobiotics.

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Authorship Contributions

Participated in research design: Xie, Genter, Gu, and Ding.

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