MECHANISM-BASED INACTIVATION OF MOUSE HEPATIC CYTOCHROME P4502B ENZYMES BY AMINE METABOLITES OF MUSK XYLENE

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ABSTRACT:
Musk xylene (2,4,6-trinitro-1-t-butylxylene; MX) is a synthetic nitromusk perfume ingredient that induces and inhibits mouse cytochrome P4502B (CYP2B) enzymes in vivo. The purpose of the present work was to determine whether amine metabolites of MX contributed to the enzyme inhibition and, if so, to define the nature and kinetics of this inhibition. When dosed orally to phenobarbital (PB)-treated mice, MX (200 mg/kg) inhibited >90% of the PB-induced O-dealkylation of 7-pentoxyresorufin (PROD), and [14C]MX equivalents bound covalently to microsomal proteins. However, when this experiment was repeated in mice pretreated with antibiotics to eliminate the gastrointestinal flora, no decrease in PB-induced PROD activity and no covalent binding to microsomal proteins were observed. Thus, the ability of antibiotic treatment to eliminate the enzyme inhibition and covalent binding implicated amine metabolites of MX formed by nitroreduction in anaerobic intestinal flora as obligatory for these effects. Two monoamine metabolites of MX were synthesized to study enzyme inhibition directly. These metabolites were 2-amino-4,6-dinitro-1-t-butylxylene and 4-amino-2,6-dinitro-1-t-butylxylene, referred to as o-NH2-MX and p-NH2-MX, respectively, reflecting the position of the amine substitution relative to the t-butyl function. In the in vitro studies with PB-induced mouse liver microsomes, both amines inhibited PROD activity when preincubated in the absence of NADPH. However, only p-NH2-MX caused a time- and NADPH-dependent loss of PROD activity, and the inactivation rate was a pseudo-first-order process that displayed saturation kinetics. These results indicate that p-NH2-MX is a mechanism-based inactivator of mouse CYP2B enzymes. From kinetic analyses, the K_i was calculated to be 10.5 μM and the k_inact was 1.2 min⁻¹. As final confirmation of the inhibitory effects of p-NH2-MX on mouse CYP2B enzymes, the amine (0.67 mmol/kg) was dosed orally to PB-induced mice. At 2 hr after dosing, p-NH2-MX inhibited essentially all of the PB-induced PROD activity, whereas an equimolar dosage of parent MX had no effect at this early time. Thus, although MX is an inducer of mouse CYP2B enzymes, an amine metabolite of MX is a mechanism-based inactivator of mouse CYP2B10. Furthermore, it is likely that the amine is responsible for the lack of functional CYP2B enzyme activity associated with induction of this enzyme by MX.

Cytochrome P450s constitute a superfamily of heme-containing proteins that function in the biotransformation of a wide variety of xenobiotics and endogenous chemicals. They are classified according to structure into gene families and subfamilies, and the gene products have been identified and isolated from a variety of plant and animal species (1). Many of these enzyme families can be induced by exposure to exogenous chemicals, a process usually, but not exclusively, mediated by transcriptional activation of the respective gene (2, 3). The functional activity of these enzymes can also be inhibited by a diverse group of chemicals and by a variety of biochemical mechanisms (4–6).

The most specific inhibitors of cytochrome P450s are mechanism-based inactivators. These compounds are substrates for the target enzyme that with metabolism are converted to an intermediate or product that inactivates the enzyme. Inactivation is mediated by covalent modification of a pyrrole nitrogen in the prosthetic heme group or by direct modification of the heme moiety or apoprotein (4, 7, 8). This mode of inactivation is highly specific because the substrate must bind both to and be metabolized by the enzyme, and it is readily distinguished from reversible inhibition, in which a substrate must be present to exert the inhibitory effect, in that decreased enzyme activity persists after the compound has been cleared from the body.

MX2 (fig. 1) is a synthetic nitromusk that has been widely used as a perfume ingredient and fixative in a variety of household products. Recent experiments have shown that MX treatment causes a PB-like induction of mouse hepatic cytochrome P450 enzymes. Specifically, a single dose of MX increased CYP2B10 mRNA in a manner similar to PB and, with repeated dosing, MX treatment markedly increased liver weight, caused hepatocellular hypertrophy, and increased immunoreactive CYP2B10 protein ~25-fold over control levels. Despite these changes, however, there was no corresponding increase in CYP2B enzyme activity associated with increased CYP2B mRNA and protein (9, 10).

The lack of functional CYP2B activity suggested that MX, despite inducing the enzyme, inhibited its catalytic function in some way. When dosed to PB-induced mice, MX decreased CYP2B activity by ~90%, further confirming that MX inhibited mouse CYP2B activity. However, in vitro experiments indicated that parent MX was not a mechanism-based inactivator of the CYP2B enzymes (10).

In rodents, an important pathway in the metabolism of many

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2 Abbreviations used are: MX, musk xylene; PB, phenobarbital; CYP2B, cytochrome P4502B; PROD, 7-pentoxyresorufin; p-NH2-MX, 4-amino-2,6-dinitro-1-t-butylxylene metabolite; k_inact, maximum rate constant for inactivation; o-NH2-MX, 2-amino-4,6-dinitro-1-t-butylxylene metabolite.
The structure of MX (2,4,6-trinitro-1-t-butylylene) and the monoamine metabolites used in this study. Amine metabolites were synthesized by iron-catalyzed acidic reduction of MX.

CYP2B INACTIVATION BY MUSK XYLENE METABOLITES

Materials and Methods

Structures of MX and monoamine metabolites.

The major objective of the present work was to test the hypothesis that monoamine metabolites of MX were central to the loss of CYP2B enzyme activity in MX- and PB-induced mice. If observed, the second objective was to characterize the nature and kinetics of this inhibitory effect. To this end, monoamine metabolites of MX were synthesized and their ability to inhibit and inactivate CYP2B enzymatic activity was determined directly.

Materials and Methods

Chemicals and Reagents. MX was obtained from Polarea (Jersey City, NJ) and was essentially 100% pure as determined by GC/MS analysis. [Methyl-3H]MX (18.2 mCi/mmol; synthesized by New England Nuclear, Boston, MA) with a radiochemical purity of 97% as determined by HPLC-radiometric analysis. Amine metabolites used in this work were synthesized by iron-catalyzed reduction of MX in the presence of hydrochloric acid and benzene (12). The isomeric monoamine products were separated by preparative HPLC on silica gel with a solvent system of hexane/ethyl acetate (90/10) eluent. Identity and purity of the monoamines were confirmed by GC/MS analysis.

PB (sodium salt), neomycin sulfate, tetracycline hydrochloride, bacitracin, resorufin and 7-pentoxresorufin were from Sigma Chemical Co. (St. Louis, MO). A polyclonal antibody to rat CYP2B1 (Xenotech, Kansas City, KS) was used to detect homologous mouse CYP2B proteins that were visualized with a goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad, Hercules, CA). All other reagents used for immunoblotting procedures were electrophoresis grade.

Animals. Male B6C3F1 mice (Charles River Laboratories, Portage, MI; 20–25 g) were used throughout. Animals were housed in humidity- and temperature-controlled rooms and allowed free access to food (Purina Laboratory Rodent Chow,Ralston-Purina, St. Louis, MO) and water.

PB-induced microsomes used for in vitro experiments were prepared from male B6C3F1 mice previously allowed ad libitum exposure to PB in drinking water (0.05% as the sodium salt) for 5 days. At the end of this treatment period, animals were fasted overnight before livers were removed, weighed, and microsomes were prepared by differential centrifugation according to procedures outlined by Guengerich (13). Microsomes were pooled and stored at −80°C pending biochemical analyses.

Determination of CYP2B Enzyme Activity. O-Dekylation of PROD, used to assess CYP2B activity, was determined according to the fluorometric method described by Burke et al. (14). Microsomal protein used in this assay was typically 25–50 μg and, unless otherwise noted, the substrate concentration was 5 μM. Reactions were conducted at 37°C in fluorometric cuvettes using a Perkin-Elmer LS50-B luminescence spectrometer (Norwalk, CT) equipped with 4-position, thermostat-controlled, stirred cell holder. The assay was calibrated with resorufin, and reaction rates, monitored over a 2-min interval (excitation and emission wavelengths of 530 and 585, respectively), were determined directly with the fluorescence data manager (Perkin-Elmer). Microsomal protein levels were adjusted on an as-needed basis to ensure that all reaction rates were linear (r² > 0.95) over the 2-min data collection interval.

In Vivo Enzyme Inactivation Experiments. A two-stage incubation procedure was used to determine the ability of the amine metabolites to inactivate CYP2B and to define the kinetics of the inactivation process. In the first stage, the amines (2.5–15 μM) were incubated (37°C) with PB-induced microsomes (5 mg/ml) and 200 mM phosphate buffer (pH 7.4) in the presence or absence of an NADPH-generating system. The total volume of the first stage metabolism reaction was 1 ml, and amines were added in 1 μl of dimethylsulfoxide. At 0.25, 0.5, 1, 1.5, 2, 3, 5, and 10 min, an aliquot was removed, diluted 100-fold into second-stage incubation (the PROD assay components as described previously), and the residual enzyme activity was determined.

In Vivo Analysis of Inhibition of CYP2B Enzyme Activity. Two experiments were conducted to evaluate the in vivo effects of MX on PB-induced CYP2B enzyme activity. In the first experiment, the effect of antibiotic treatment on CYP2B enzyme activity and covalent binding of [3H]MX equivalents to microsomal protein were determined. Four groups of mice (N = 5/group) were exposed to PB in drinking water (0.05%) for 5 days and, during this time, two groups were dosed twice daily (7 a.m. and 5 p.m.) by gavage with antibiotics (neomycin, tetracycline, and bacitracin at 400, 200, and 200 mg/kg/day, respectively), whereas the remaining mice received saline as pretreatment control (10 ml/kg). On the afternoon of the fourth day of PB treatment, mice were dosed with PB by gavage with [3H]MX (0 or 200 mg/kg; 150 μCi/kg), and microsomes were prepared from fasted animals 18 hr after dosing MX. PB and antibiotic treatment continued until necropsy.

In the second experiment, mice were allowed ad libitum exposure to PB in drinking water (0.05% as the sodium salt) for 5 days, after which they received a single oral dosage of MX or p-NH₂-MX (0, 0.67 mmol/kg; structure shown in fig. 1), and microsomes were prepared from fasted animals 2 or 18 hr later (N = 5/group). The 18-hr treatment groups were dosed with MX or p-NH₂-MX on the afternoon of the fourth day of PB treatment so that microsomes from animals in the 2- or 18-hr groups were prepared on the same day. From these samples, microsomal protein, total cytochrome P450 content, PROD activity, and immunoreactive CYP2B protein levels were determined.

Other Biochemical Assays. To determine covalent binding, an aliquot of microsomal protein from the PB-induced mice dosed with [3H]MX (2 mg; −5000 dpm) was precipitated in 3 volumes of methanol. The precipitate was washed and resuspended in water and precipitated two additional times with methanol. After the third precipitation, 1 ml of 1 N NaOH was added and the pellet was dissolved by heating at 60°C for 30 min. The dissolved protein pellets were dialyzed overnight against a 3 kDa molecular cutoff membrane (Pierce, Rockford, IL), after which aliquots were taken for liquid scintillation counting (Packard, 2500 TR, Meriden, CT) and protein determination.

Microsomal protein was determined by the method of Bradford (15), with bovine serum albumin as standard. The concentration of cytochrome P450 was determined by the method of Omura and Sato (16), from the carbon monoxide difference spectrum of dithionite-reduced microsomes with an extinction coefficient of 91 mM⁻¹ cm⁻¹.

Electrophoresis and Immunoblotting Procedures. Microsomal proteins (5 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 7.5% gel for CYP2B1 and 10% gel for CYP2B2 (1 nmol/mg of protein) and transferred electrophoretically to nitrocellulose with a mini trans-blot apparatus. The nitrocellulose was treated with blocker (5% nonfat milk in Tris-buffered saline) and the membranes were incubated overnight at 4°C with the primary antibody. After incubation, membranes were washed several times with 0.1% bovine serum albumin and 0.05% Tween 20 (in TBS) and incubated for 30 min at room temperature with an HRP-conjugated secondary antibody. Membranes were washed several times and subsequently exposed to ECL reagents (Amersham) and developed using X-Omat film (Eastman, Rochester, NY). The resulting signals were analyzed using the Fluor-S Multimager (Bio-Rad). A standard curve of 2 µg of microsomal protein was prepared for each of the CYP2B isoforms.
TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antibiotics</th>
<th>Total P450</th>
<th>PROD</th>
<th>Covalent Binding</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/mg</td>
<td>pmol/min/mg</td>
<td>nmol [14C]MX/mg</td>
</tr>
<tr>
<td>PB/corn oil</td>
<td>-</td>
<td>1.94 ± 0.16</td>
<td>10 22 ± 60</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.74 ± 0.11</td>
<td>10 98 ± 67</td>
<td>ND</td>
</tr>
<tr>
<td>PB/MX</td>
<td>-</td>
<td>1.86 ± 0.04</td>
<td>92 ± 16*</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.86 ± 0.07</td>
<td>1355 ± 13**</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Results represent the means ± SE of 5 mice. ND, not determined; N/D, not detected. *p values are as follows: a statistically different from respective PB/corn oil control (p < 0.05); ** statistically different from respective PB/corn oil control and PB/MX group not receiving antibiotics (p < 0.05).

All mice were exposed to PB in drinking water (500 ppm) for 4 days and then dosed with corn oil (10 ml/kg) or [14C]MX (200 mg/kg; 150 μCi/kg).

Mice were treated twice daily with a regimen of antibiotics (neomycin, tetracycline, and bacitracin at 400, 200, and 200 mg/kg/day, respectively) for the duration of the experiment. Control animals received saline on the same dosing schedule.

Radioactivity levels were not above instrument background.

Data Analysis. Where appropriate, data were analyzed for statistical significance by analysis of variance, followed by Dunnett’s multiple comparison test. Rate constants of inactivation were determined by linear regression analysis of the natural logarithm of the residual activity as a function of the preincubation time. All analyses were conducted with StatView statistical application (Abacus Concepts, Berkeley, CA).

Results

MX is a trinitroaromatic compound, and the two monoamine metabolites synthesized and used in this work are shown in fig. 1. Throughout this study, the 2-amino and 4-amino derivatives are referred to as o-NH₂-MX and p-NH₂-MX, respectively; thus reflecting the location of the amine substitution relative to the t-buty1 function on MX. The amine metabolites used in these studies were synthesized and isolated with a purity of essentially 100%.

The major PB-inducible enzyme in mice is CYP2B10 (18). We have previously shown that MX treatment inhibits CYP2B10 activity in vivo (10). When [14C]MX was dosed to PB-induced mice, MX reduced PROD activity by 90% of control values, and [14C]MX equivalents were bound covalently to microsomal protein. However, spectral determination of total cytochrome P450 levels was not affected (table 1). In contrast, with antibiotic treatment, PROD activity in the PB-induced mice exposed to MX was increased relative to the PB-induced level, and no covalent binding of [14C]MX equivalents to microsomal proteins was detected. These results suggest that amine metabolites of MX are responsible for both the covalent binding to microsomal protein and inhibition of CYP2B enzyme activity.

To determine whether the monoamine metabolites inactivated CYP2B activity, the compounds were preincubated with PB-induced microsomes in the presence or absence of NADPH. In the absence of NADPH, both amines reduced PROD activity relative to control (fig. 2). However, although o-NH₂-MX inhibited PROD activity, no further loss of enzyme activity was observed when preincubated with NADPH, indicating that this metabolite does not inactivate CYP2B10. In contrast, p-NH₂-MX caused a time-dependent loss of PROD activity during preincubation with NADPH. The inactivation profile exhibited a pseudo–first-order loss of activity, reaching maximum inactivation at 5 min when ~85% of the enzyme activity had been lost.

To define the kinetics of inactivation of PROD activity by p-NH₂-MX, the first-order rate constants for inactivation were determined with various concentrations of the amine metabolite (table 2). The double-reciprocal plot of the first order rate constants for inactivation as a function of the inhibitor concentration is shown in fig. 3. From this analysis, k<sub>inact</sub> was calculated to be 1.2 min⁻¹, and the concentration at which the rate constant for inactivation was half-maximal (K<sub>i</sub>) was 10.5 μM. The k<sub>inact</sub> of 1.2 min⁻¹ yields a maximum rate of inactivation corresponding to a half-life of ~35 sec.

The potential for o-NH₂-MX to inactivate the enzyme was determined up to a maximum concentration of 25 μM. This concentration inhibited ~80% of the enzyme activity at zero time, but no change in activity was determined in the presence of NADPH (results not shown), thereby confirming the selectivity of the enzyme inactivation for the p-NH₂-MX metabolite.

As final confirmation that p-NH₂-MX was responsible for CYP2B enzyme inactivation, the amine metabolite was dosed to PB-induced mice (fig. 4). At 2 hr after dosing, p-NH₂-MX treatment eliminated virtually all PROD activity. In contrast, MX treatment had no effect on PROD activity at this time. At 18 hr after dosing, loss of PROD activity was seen in both the p-NH₂-MX- and MX-treated animals. Despite the loss of enzyme activity, there was no loss of CYP2B protein as determined by Western blotting (fig. 5) and no change in the spectral detection of total cytochrome P450 content (results not shown).

Discussion

MX is a PB-like inducer of mouse cytochrome P450 enzymes. However, despite increasing hepatic CYP2B10 mRNA and protein levels, MX treatment does not increase CYP2B enzyme activity (9, 10). The results of the present studies indicate that when incubated with PB-induced microsomes, p-NH₂-MX caused a time- and NADPH-dependent loss of PROD activity, and the inactivation rate was a pseudo-first-order process that displayed saturation kinetics. These characteristics are the hallmarks of mechanism-based inactivation (4, 19). When dosed to PB-induced mice, p-NH₂-MX produced a rapid, but prolonged loss of CYP2B activity. Collectively, these results indicate that p-NH₂-MX is a mechanism-based inactivator of mouse CYP2B enzymes, and this inactivation is likely to explain the absence of increased CYP2B activity in MX-treated mice.

A second monoamine metabolite, o-NH₂-MX, was also evaluated in these experiments. This metabolite inhibited CYP2B activity, but did not inactivate the enzyme. With this compound, the amine function is adjacent to the t-buty1 group, and it is possible that the bulky t-buty1 moiety provides sufficient steric hindrance to prevent or slow the metabolism of this amine. Alternatively, these compounds may be oriented in the substrate binding site of the CYP2B10 enzyme in a manner that precludes oxidation of the amine substitution of o-NH₂-MX, while favoring oxidation of the p-NH₂-MX metabolite. In either case, the orientation of the amine substitution on MX is a major determinant of the ability to inactivate the CYP2B enzyme. This observation has important implications with respect to the possibility that other nitromusk perfume ingredients may also inactivate cyto-
chrome P450 enzymes. Specifically, MX is the only trinitrosubstituted compound in this class, and no other member of the nitromusk class (structures shown in ref. 20) possesses a nitro function in the orientation apparently needed for inactivation. Therefore, among the nitromusk perfume ingredients, it is likely that the inactivation of mouse CYP2B10 is unique to MX and more specifically to the \( p^\text{-NH}_2 \)-MX metabolite of MX.

The inactivation of the CYP2B enzymes by \( p^\text{-NH}_2 \)-MX was characterized by a \( K_i \) in the micromolar range and a high maximal rate constant. Present data also suggest that \( p^\text{-NH}_2 \)-MX likely inactivates CYP2B10 by covalent modification of the apoprotein. MX or \( p^\text{-NH}_2 \)-MX treatment did not alter the spectral properties of cytochrome P450 enzymes or produce the characteristic green pigment associated with porphyrin alkylation. Therefore, it is unlikely that \( p^\text{-NH}_2 \)-MX modifies the prosthetic heme group of the CYP2B10 enzyme by either metabolic intermediate complexation (21) or alkylation of a pyrrole nitrogen (22, 23). On the other hand, \([^{14}C]\)MX equivalents bound covalently to microsomal proteins, and this binding was abolished when nitreduction was prevented by antibiotic treatment. Thus, although we do not have direct proof, the results of the present studies indicate that covalent binding does occur, and because the heme moiety is not altered, modification of the apoprotein is the likely mode of inactivation. Although we have not identified the nature of the reactive intermediate, \( N \)-oxidation to a reactive hydroxylamine intermediate (24, 25) may be the inactivating species.

A variety of chemical structures are known to be mechanism-based...
inactivators of cytochrome P450 enzymes (8), and several have been shown to specifically or preferentially inactivate CYP2B enzymes in different species. For example, chloramphenicol (26), N-methylcarbazole (27), secobarbital (28), phenacyclidine (29), and acetylenes [such as 9-ethyl-phenanthrene (30) and N-aralkylated derivatives of 1-aminobenzotriazole (31)] are mechanism-based inactivators of CYP2B enzymes. Although mouse liver microsomes were used in the present studies, preliminary experiments suggest that p-NH$_2$-MX also inactivates rat CYP2B1 (unpublished data). Whereas a variety of substituted aromatic amines have been shown to form metabolic intermediate complexes with cytochrome P450 enzymes (32), the identification of an aromatic amine with specificity to inactivate CYP2B enzymes represents a new chemical class of mechanism-based inactivators of this enzyme family. Additional studies will need to be conducted to determine whether p-NH$_2$-MX specifically inactivates CYP2B enzymes or whether it is capable of inactivating other cytochrome P450 families.

It is well-established that nitroreduction to primary amines is a major metabolic pathway for a variety of nitroaromatic compounds, and the role of anaerobic intestinal bacteria in this reductive metabolism is recognized (11). In fact, intestinal bacteria play an obligatory role in the metabolic activation and genetic toxicity of the hepatocarcinogen, 2,6-dinitrotoluene (33, 34). In contrast, despite the importance of bacterial nitroreduction in the enzyme inactivation seen with MX, MX has been characterized as a nongenotoxic compound (35). Therefore, despite similar routes of biotransformation, the fate of amine metabolites of MX seems to be somewhat different from amine metabolites of other nitroaromatic compounds.

This work was initiated because of the original observation that MX induced a nonfunctional CYP2B10 enzyme. Based on the present findings, it seems that MX is a direct inducer of CYP2B enzymes, which, at the same time, can be metabolized to p-NH$_2$-MX, a potent inactivator of the induced enzymes. The complexity of this response underscores the need to confirm the analysis of enzyme activity with the evaluation of corresponding protein or mRNA levels. That is, accurate determination of the effects of MX can only be achieved when the enzyme activity data are compared with at least one additional level of enzyme regulation. In this regard, previous studies characterizing the effects of MX on rat cytochrome P450 enzymes have concluded, based on enzyme activity data alone, that MX had no effect on CYP2B1 (20, 36–38). Because p-NH$_2$-MX is a major metabolite in rats (12) and because preliminary data suggest that it also inactivates CYP2B1, the possibility that MX can induce and inactivate CYP2B enzymes in rats should be considered.

In summary, the results of the present study have identified an amine metabolite of MX as a mechanism-based inactivator of mouse CYP2B10, and it is likely that this amine is responsible for the lack of functional CYP2B enzyme activity associated with induction of this enzyme by MX. However, because of the specificity of the inactivation to the p-NH$_2$-MX metabolite, it is unlikely that other, structurally similar nitromusks will cause a similar inactivation. Collectively, the results confirm that MX treatment will both induce and inactivate the CYP2B enzymes.

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References


