Fenbendazole is a broad-spectrum anthelmintic drug widely used to prevent or treat nematode infections in laboratory rodent colonies. Potential interactions between fenbendazole and hepatotoxicants such as acetaminophen are unknown, and this was investigated in this study. Mice were fed a control diet or a diet containing fenbendazole (8–12 mg/kg/day) for 7 days prior to treatment with acetaminophen (300 mg/kg) or phosphate buffered saline. In mice fed a control diet, acetaminophen administration resulted in centrilobular hepatic necrosis and increases in serum transaminases, which were evident within 12 h. Acetaminophen-induced hepatotoxicity was markedly increased in mice fed the fenbendazole-containing diet, as measured histologically and by significant increases in serum transaminase levels. Moreover, in mice fed the fenbendazole-containing diet, but not the control diet, 63% mortality was observed within 24 h of acetaminophen administration. Fenbendazole by itself had no effect on liver histology or serum transaminases. To determine if exaggerated hepatotoxicity was due to alterations in acetaminophen metabolism, we analyzed sera for the presence of free acetaminophen and N-acetyl-aminopyrine (NAPQI) intermediate from acetaminophen via cyp2e1 and cyp3a, and to a lesser extent, cyp1a2, which are predominantly localized in centrilobular regions of the liver (Moyer et al., 2011; Tonge et al., 1998). Under physiological conditions, NAPQI is detoxified by conjugation with glutathione (GSH) (Moyer et al., 2011). However, following acetaminophen overdose, GSH is depleted leading to covalent binding of NAPQI to hepatocyte proteins (Cohen et al., 1997). Findings that agents, such as arsenic, L-buthionine (S,R)-sulfoximine, and 1,3-bis(2-chloroethyl)-1-nitrosourea, which deplete GSH, augment acetaminophen-induced hepatotoxicity demonstrate the importance of this pathway in the removal of NAPQI (Manimaran et al., 2010; Miners et al., 1984; Nakae et al., 1988; Watanabe et al., 2003).

Pinworm infestation (Syphacia obvelata, Syphacia muris, and Aspiculuris tetrapterus) is a common problem in laboratory rodent colonies. Infection can result in alterations in red and white blood cell development, autoimmune dysfunctions, disruptions of the neuroendocrine and digestive systems, and aberrant growth and behavior (Agersborg et al., 2001; Bugarski et al., 2006; McNair and Timmons, 1977; Michels et al., 2006; Wagner, 1988). Fenbendazole (methyl 5-(phenylthio)-2-benzimidazole) is a broad-spectrum anthelmintic agent commonly added to laboratory rodent feed to prevent or treat pinworm infestation (Villar et al., 2007). The efficacy of fenbendazole is attributed to its ability to preferentially inhibit pinworm glucose uptake and tubulin polymerization (Coghlan et al., 1993; Dawson et al., 1984). Fenbendazole clearance is predominantly through metabolism by cyp3a2, cyp2b1, and cyp2c6/11, along with the flavin monooxygenase pathways (Capece et al., 2009; Murray et al., 1992). Despite its widespread use, little is known about the interaction between fenbendazole and other drugs metabolized by cyp450.

Acetaminophen is a mild analgesic that is the major cause of acute liver failure due to accidental and intentional overdose (Bower et al., 2007). Toxicity is characterized by centrilobular hepatic necrosis progressing to fulminant liver failure (Ramachandran and Kakar, 2009). Hepatotoxicity involves the generation of a highly reactive N-acetyl-p-benzoquinoneimine (NAPQI) intermediate from acetaminophen via cyp2e1 and cyp3a, and to a lesser extent, cyp1a2, which are predominantly localized in centrilobular regions of the liver (Moyer et al., 2011; Tonge et al., 1998). Under physiological conditions, NAPQI is detoxified by conjugation with glutathione (GSH) (Moyer et al., 2011). However, following acetaminophen overdose, GSH is depleted leading to covalent binding of NAPQI to hepatocyte proteins (Cohen et al., 1997). Findings that agents, such as arsenic, L-buthionine (S,R)-sulfoximine, and 1,3-bis(2-chloroethyl)-1-nitrosourea, which deplete GSH, augment acetaminophen-induced hepatotoxicity demonstrate the importance of this pathway in the removal of NAPQI (Manimaran et al., 2010; Miners et al., 1984; Nakae et al., 1988; Watanabe et al., 2003).

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Key Words: fenbendazole; acetaminophen; cytochrome P450; glutathione; liver.
Fenbendazole has been reported to induce cytochrome P450 (cypl1a2, cypl2b1, and cypl3a1) in rats, as well as cytochrome P450 (cypl1a2) in rabbits (Gleizes et al., 1991; Shoda et al., 1999). These findings suggest that fenbendazole may modify the response to liver toxins such as acetaminophen, and this was investigated in the present studies.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma Chemical Company, St Louis, MO, unless otherwise specified.

Animals and treatments. C57BL/6J mice (7–8 weeks, 20–25 g) were obtained from the Jackson Laboratory (Bar Harbor, ME). Male mice were used as they exhibit a greater toxic response to acetaminophen than female mice (Masubuchi et al., 2011). Mice were provided with food and water ad libitum and housed in microisolation cages. Animals were placed on the Laboratory Rodent Diet 5001 (LabDiet, Saint Paul, MN) or Laboratory Rodent Diet 5001 containing 150 mg/kg fenbendazole (Bio-serv, Frenchtown, NJ), which corresponds to 8–12 mg/kg/day. The variation in the dose of fenbendazole is due to the variation in daily food intake. Seven days later, mice were fasted overnight to reduce hepatic GSH levels (Walker et al., 1982) and then administered a single dose of acetaminophen (300 mg/kg) or PBS control (ip). Food was returned 0.5 h following acetaminophen treatment. Animals were sacrificed 30 min to 24 h following acetaminophen administration. Serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined using a diagnostic assay kit (ThermoElectron, Pittsburgh, PA). All animals received humane care in compliance with the institution’s guidelines, as outlined in the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

Histology. Sections of the left lateral liver lobe were fixed overnight at 4°C in PBS containing 3% paraformaldehyde and 2% sucrose, washed three times with 2% sucrose/PBS, transferred to 50% ethanol (Pharmco-Aaper, Brookfield, CT), and then paraffin embedded. Six-micron tissue sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). Three liver sections per mouse were examined.

Measurement of hepatic GSH. Samples of liver (25 mg) were minced in ice cold 5% metaphosphoric acid containing 2% sucrose, washed three times with 2% sucrose/PBS, transferred to 50% ethanol (Pharmco-Aaper, Brookfield, CT), and then paraffin embedded. Six-micron tissue sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). Three liver sections per mouse were examined.

Acetaminophen metabolism. Blood samples (0.5 ml) were centrifuged (3000 × g, 4°C, 10 min) and serum frozen in liquid nitrogen and stored at −80°C until analysis. Free acetaminophen and acetaminophen-glucuronide were analyzed as previously described (Brunner and Bai, 1999). Briefly, samples were thawed and deproteinized with 6% perchloric acid containing 1.0 M NaOH and 1.0 M sucrose, washed three times with 2% sucrose/PBS, transferred to 50% ethanol (Pharmco-Aaper, Brookfield, CT), and then paraffin embedded. Six-micron tissue sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). Three liver sections per mouse were examined.

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Measurement of hepatic cytochrome P450 activity. To prepare microsomes, liver samples (1 g) were homogenized at 4°C in 2 volumes (wt/vol) of 10 mM Tris-base (pH 7.4) containing 1.5% KCl using a Teflon-glass homogenizer. Homogenates were centrifuged at 1000 × g (10 min, 4°C), supernatants collected and centrifuged at 12,000 × g (20 min, 4°C) to remove cellular debris, and then at 105,000 × g (1.5 h, 4°C). Microsomes were resuspended in homogenization buffer containing 0.5 mM phenylmethanesulfonyl fluoride and centrifuged at 105,000 × g (90 min, 4°C). Pellets were resuspended in 0.25 M sucrose containing 10 mM Tris-base (pH 7.4) and stored at −80°C until analysis (Cooper et al., 1993). Cytochrome P450 activity was measured by the formation of p-nitrotoecatechol from p-nitrophenol (Koepf, 1986). Microsomes were incubated with p-nitrophenol (200 μM) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (500 μM) at 37°C for 30 min, followed by trichloroacetic acid (20%, wt/vol) to stop the reaction. Microsomes were then centrifuged (10,000 × g, 5 min, 4°C), supernatants collected and mixed with 2 M NaOH. Changes in absorbance were measured spectrophotometrically at 535 nm. Concentrations of p-nitrotoecatechol in the samples were calculated based on a standard curve generated using authentic product (Chang et al., 2006; Wolf et al., 2004). Cytochrome P450 activity was measured as previously described (van Beusekom et al., 2010) with some modifications. Briefly, liver microsomes were incubated with 0.1 M potassium phosphate buffer (pH 7.4) containing 1.0 mM MgCl2, 0.1 mM EDTA, 0.5 mM NADPH, and 0.005 mM 7-methoxyresorufin for cytochrome P450 CYP2C18 or 7-benzyloxysresorufin for cytochrome P450 CYP3A. Relative fluorescence units were recorded over a 10-min interval at an excitation wavelength of 530 nm and an emission wavelength of 590 nm on a Spectromax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA). Rates of product formation were calculated using SoftMaxPro5 software (Molecular Devices). The concentration of the reaction product, resoruﬁn, was calculated based on a standard curve generated using authentic product. Each determination was then repeated in triplicate for all animals. To assess cytochrome P450 isozyme speciﬁcity, enzyme activities were measured after the addition of 1 μM of the cytochrome P450 inhibitor, rutecarpine, or the cytochrome P450 inhibitor, ketoconazole (Stresser et al., 2000; Ueng et al., 2002). Whereas rutecarpine inhibited cytochrome P450 activity by 97%, ketoconazole blocked cytochrome P450 activity by 87% (Table 1).

Statistics. All experiments were performed using 3–8 mice/group. Data were analyzed using a Student’s t-test.

RESULTS

Effects of Fenbendazole on Acetaminophen-Induced Hepatotoxicity

In initial studies, we analyzed the effects of feeding mice a diet containing fenbendazole on acetaminophen-induced liver injury. The results are presented in Table 1.

TABLE 1

<table>
<thead>
<tr>
<th>Enzyme Activities</th>
<th>Control</th>
<th>Fenbendazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp2e1</td>
<td>1.02 ± 0.05</td>
<td>1.02 ± 0.04</td>
</tr>
<tr>
<td>Cyp1a2</td>
<td>133.5 ± 33.0</td>
<td>47.1 ± 0.6*</td>
</tr>
<tr>
<td>Cyp1a2 + rutecarpine</td>
<td>5.8 ± 1.1b</td>
<td>1.7 ± 0.1a,b</td>
</tr>
<tr>
<td>Cyp3a</td>
<td>8.7 ± 0.6</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>Cyp3a + ketoconazole</td>
<td>1.0 ± 0.04b</td>
<td>0.7 ± 0.06b</td>
</tr>
</tbody>
</table>

Notes. Mice were fed a standard control diet or a diet containing fenbendazole for 7 days. Liver microsomes were then prepared and assayed for cytochrome P450 activities as described in the “Materials and Methods” section. In some experiments, rutecarpine (1 μM) or ketoconazole (1 μM) was added to the assay mixtures to confirm enzyme specificity. Data are presented as picomoles per minute per milligram protein. Each value is the mean ± SEM of triplicate determinations from three mice.*Significantly different (p ≤ 0.05) from control fed mice. **Significantly different (p ≤ 0.05) from enzymes assays in the absence of inhibitor.
injury. In mice fed a control diet, acetaminophen caused a rapid induction of hepatotoxicity with significant elevations in serum ALT and AST within 12 h (Fig. 1). This was associated with histological alterations in the liver characterized by hepatocellular cytoplasmic degeneration, bridging necrosis, and severe congestion (Fig. 2). At this time, greater than 50% of the liver lobules contained necrotic regions. Acetaminophen-induced hepatotoxicity was significantly increased in mice fed a diet containing fenbendazole, as measured by increases in serum transaminase levels and by more extensive structural alterations in the liver (Figs. 1 and 2). Additionally, while administration of acetaminophen to mice on the control diet resulted in 0% mortality at 24 h, mortality was 63% in mice fed the diet containing fenbendazole (Table 2). The higher mortality of the fenbendazole-fed mice was due to extensive liver damage. The fenbendazole-containing diet by itself had no effect on serum transaminases, liver histology, or mortality in control animals.

Effects of Fenbendazole on Acetaminophen Metabolism

To determine if exaggerated hepatotoxicity and mortality in mice fed the fenbendazole-containing diet was due to altered acetaminophen metabolism, we measured serum levels of free acetaminophen and acetaminophen-glucuronide. HPLC analysis of serum samples collected 30 min to 2 h after acetaminophen administration revealed a time-related decrease in serum acetaminophen and acetaminophen-glucuronide in mice fed a standard control diet (Fig. 3). The addition of fenbendazole to the diet had no significant effects on plasma clearance of glucuronide-conjugated or unconjugated acetaminophen.

We next measured the activities of cyp2e1, cyp3a, and cyp1a2, enzymes involved in the metabolism of acetaminophen to the toxic intermediate, NAPQI (Moyer et al., 2011; Tonge et al., 1998). Similar rates of cyp2e1-mediated hydroxylation of p-nitrophenol were observed in liver microsomes from mice fed a standard diet and a diet containing fenbendazole (Table 1). Additionally, no differences were noted in microsomal cyp3a activity between mice fed the different diets. In contrast, cyp1a2 metabolism of 7-methoxyresorufin was suppressed by 65% in mice fed the fenbendazole-containing diet when compared with mice fed the control diet.

Effects of Fenbendazole on Hepatic GSH Content

In further studies, we determined if enhanced acetaminophen sensitivity in mice fed the fenbendazole-containing diet was associated with alterations in hepatic GSH. In both control and fenbendazole fed mice, acetaminophen administration caused

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tbody>
<tr>
<td>Effects of Fenbendazole on Acetaminophen-Induced Liver Damage and Mortality</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diet</th>
<th>Time following APAP</th>
<th>Number of mice</th>
<th>Liver damage (% necrosis)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 h</td>
<td>5</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>8</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>PBS</td>
<td>3</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>5</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>8</td>
<td>n.d.</td>
<td>63</td>
</tr>
</tbody>
</table>

Notes. Mice were fed a standard control diet or a diet containing fenbendazole for 7 days and then treated with acetaminophen (APAP) or PBS control. Sections of the left lateral liver lobe were fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Three liver sections per mouse were examined. n.d., not determined due to high mortality.
a rapid (within 1 h) decrease in reduced GSH (Fig. 4). No significant differences were noted between the diets. Whereas GSH levels returned to control by 12 h in mice fed the standard diet, they remained suppressed in mice fed the fenbendazole-containing diet (Fig. 4).

DISCUSSION

Fenbendazole is a member of the benzimidazole carbamate class of drugs commonly used as anthelmintics (Velik et al., 2004; Villar et al., 2007). Due to strain and species differences in sensitivity, the dose of fenbendazole used therapeutically varies (Hunter et al., 2007; Ramp et al., 2010; Short et al., 1988). In mice, fenbendazole is typically delivered in food at a dose of 8–12 mg/kg for 7 days. The present studies demonstrate that treatment of mice with this clinical dose regimen of fenbendazole exacerbates the hepatotoxicity of acetaminophen resulting in significant mortality within 24 h. These findings are novel and suggest that there should be more controlled use of this anthelmintic agent in laboratory rodent colonies.

In mice, glucuronidation is the major pathway by which acetaminophen is detoxified (Gregus et al., 1988; Laine et al., 2009). Consistent with previous reports (Gardner et al., 2010; Yoon et al., 2006), we found high levels of acetaminophen-glucuronide, as well as free acetaminophen in serum within 30 min of acetaminophen administration, which rapidly declined toward control. Our findings that there were no differences in the response of control, and fenbendazole-treated mice suggest that the rate of acetaminophen clearance via glucuronidation does not contribute to exaggerated hepatotoxicity.

Previous studies in mice and rats showed no effects of fenbendazole on total hepatic cyp450 content or on its activity following therapeutic dosing for 5 days (Dalvi et al., 1991). In accord with these findings, we observed no significant differences between control and fenbendazole-treated mice in the activities of cyp2e1 or cyp3a, enzymes key in the metabolism of acetaminophen to NAPQI (Moyer et al., 2011). These data demonstrate that the major metabolic pathways involved in the generation of this cytotoxic acetaminophen metabolite are unaffected by fenbendazole. In contrast, increased cyp1a2, cyp2b1, and cyp4a1 activity has been noted in liver microsomes from rats fed a fenbendazole-containing diet for 8 weeks (Shoda et al., 1999). Similar increases in cyp1a2 were reported in pigs and rabbits fed a diet-containing fenbendazole for 5 days or a related derivative, oxfendazole for 10 days (Gleizes et al., 1991; Savılık et al., 2006). Interestingly, we found that cyp1a2 activity was suppressed in liver microsomes isolated from mice fed the fenbendazole-containing diet for 7 days, relative to control diet. Differences between our findings and previous reports may be due to animal strain or species differences and/or the dosing schedule. Cyp1a2 is constitutively expressed at relatively high levels in rodent livers and reportedly plays a role in metabolizing endogenous substrates such as estradiol, as well as a wide variety of xenobiotics, including toxic doses of acetaminophen to NAPQI (Laine et al., 2009; Omiecinski et al., 1999; Snawder et al., 1994; Villar et al., 2007). The fact that hepatotoxicity is increased in fenbendazole fed mice, despite reduced levels of cyp1a2, indicate that this isozyme is not key to acetaminophen metabolism in this model.

Metabolites of fenbendazole, including oxfendazole and 4′-hydroxyfenbendazole are potent inhibitors of the cyp1a enzyme family in rat microsomes in vitro (Murray et al., 1992). Decreased levels of cyp1a2 in mice treated with fenbendazole may be due to increased production of fenbendazole metabolites.

GSH is known to be present at high levels in hepatocytes (Yuan and Kaplowitz, 2009). In addition to scavenging reactive oxygen species, GSH plays a key role in the detoxification of NAPQI (Jaeschke, 1990). In accord with previous studies (Chiu et al., 2003; Gardner et al., 2010), we found that acetaminophen intoxication resulted in a rapid (within 1 h) decline in reduced GSH levels in livers of mice fed the control diet. Similar results were noted in mice fed the fenbendazole-containing diet.
FENBENDAZOLE POTENTIATES APAP TOXICITY

The authors declare that they have no conflicts of interest.

REFERENCES


Dewa, Y., Nishimura, J., Muguruma, M., Matsumoto, S., Takahashi, M., Jin, M., and Mitsumori, K. (2007). Gene expression analyses of the liver in mice fed the standard diet (CTL) or a diet containing fenbendazole (Fen) for 7 days and then treated with acetaminophen (APAP) or PBS control. Livers were collected 1 and 12 h later and analyzed for GSH content as described in the ‘‘Materials and Methods’’ section. Data are mean ± SE (n = 3–5 mice). aSignificantly different (p < 0.05) from no APAP (PBS control). bSignificantly different (p ≤ 0.05) from CTL.

Whereas by 12 h post-acetaminophen, GSH levels were at control levels in mice fed the standard diet, they remained suppressed in mice fed the fenbendazole-containing diet. GSH levels were also suppressed in control mice fed fenbendazole. Fenbendazole is known to undergo sulfoxidation to generate oxfendazole, which can act as a carbamylating agent for reduced GSH (Schuphan et al., 1981; Short et al., 1988). This leads to the formation of oxfendazole GSH conjugates, effectively depleting GSH from the tissue (Schuphan et al., 1981). Oxfendazole and fenbendazole have also been reported to upregulate glutathione peroxidase and glutathione-S-transferase in rats (Dewa et al., 2007); this may also contribute to persistent GSH depletion.

In summary, the present studies demonstrate that fenbendazole exacerbates the hepatotoxicity of acetaminophen in part by reducing hepatic GSH levels. At present, it is not known if there are other components of the xenobiotic clearance system, such as drug transporters, that are altered by fenbendazole. Fenbendazole treatment of rodents has been shown to be an effective method for controlling nematode infections. However, in view of its ability to suppress hepatic GSH, as well as cyp1a2 enzyme activity, care should be taken in utilizing this anthelmintic drug in rodent studies.

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