Mouse Liver Effects of Cyproconazole, a Triazole Fungicide: Role of the Constitutive Androstane Receptor

Richard C. Peffer,*† Jonathan G. Moggs,‡ Timothy Pastoor,* Richard A. Currie,† Jayne Wright,† Gill Milburn,† Felix Waechter,‡ and Ivan Rusyn§

†Syngenta Crop Protection, Inc., Greensboro, North Carolina 27419; ‡Syngenta Central Toxicology Laboratory, Alderley Park, Cheshire, United Kingdom; §Syngenta Crop Protection AG, Basel, Switzerland; and †Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received March 16, 2007; accepted May 30, 2007

Cyproconazole, a triazole fungicide, causes hepatocellular adenomas and carcinomas in CD-1 mice at dose levels of 100 and 200 ppm. The constitutive androstane receptor (CAR) has been shown to play a significant role in the overall mode of action for several nongenotoxic rodent carcinogens such as phenobarbital. The liver effects of dietary cyproconazole or phenobarbital were investigated after 2, 7, or 14 days in male CD-1, C57BL/6J, and C3H/HeNClBR mice. Cyproconazole produced similar, dose-responsive effects in all three strains of mice, and the response was similar to that of phenobarbital. Subsequently, Car-null and wild-type male mice on a C3H/HeNClBR background were administered 200 or 450 ppm cyproconazole, or 850 ppm phenobarbital for up to 7 days. In wild-type mice, 200 ppm cyproconazole caused liver hypertrophy, increased liver weight and cell proliferation, single-cell necrosis and fat vacuolation, effects generally similar to those caused by 850 ppm phenobarbital. Plasma cholesterol was decreased by both compounds, but cyproconazole had a greater effect. The higher dose (450 ppm) of cyproconazole caused similar changes, but greater evidence of liver damage was observed, including a large increase in plasma transaminases. Induction of CAR target genes Cyp2b10 and Gadd45β was observed with both compounds, whereas the cell cycle regulatory gene Mdm2 was unaffected. In Car-null mice, the effects noted with either cyproconazole or phenobarbital were absent or greatly diminished. These experiments demonstrate that short-term liver effects of cyproconazole in mice are CAR-dependent and similar to those of phenobarbital, a known nongenotoxic rodent liver carcinogen.

Key Words: cyproconazole; triazole; constitutive androstane receptor; Mdm2; Gadd45β; liver.

Cyproconazole is a triazole fungicide that is used agriculturally for protection of crops against a wide variety of fungal pathogens. Like nearly all triazole fungicides, it produces liver toxicity in rodents after subchronic and chronic administration at high dose levels. In lifetime feeding studies, cyproconazole caused hepatocellular adenomas and carcinomas in CD-1 mice at dose levels of 100 and 200 ppm, but no tumor response was observed in Wistar rats at doses up to 350 ppm (Syngenta Crop Protection AG, unpublished data). Studies with other triazole fungicides, which include both agricultural products and human pharmaceuticals such as fluconazole, have demonstrated an induction of cytochrome P450 (CYP) isoenzymes in mice and rats that is very similar to the pattern of induction with phenobarbital (Juberg et al., 2006; Sun et al., 2005, 2006). Phenobarbital causes rodent liver tumors via a nongenotoxic mode of action (Whysner et al., 1996). Activation of the constitutive androstane receptor (CAR), a xenobiotic-sensing nuclear receptor, is a key necessary step in the hepatocarcinogenic mode of action for phenobarbital in mice, based on a lack of effects in Car-null mice (Huang et al., 2005; Yamamoto et al., 2004). Similar lack of a tumor response in Car-null mice has also been reported for the more potent CAR activator 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), either with or without initiation with diethylnitrosamine (Huang et al., 2005).

Recent studies have reported the gene expression profiles of four triazole fungicides (fluconazole, myclobutanil, propiconazole, and triadimefon) in livers of mice and rats after 14 days of treatment (Goetz et al., 2006; Tully et al., 2006). Treatment with triazoles increased the expression of multiple isofoms of CYP enzymes as well as other xenobiotic metabolizing enzymes, many of which are known to be activated by CAR, pregnane X receptor (PXR), or possibly other nuclear receptors. Evidence of cross-talk between CAR and PXR for expression of some genes has been demonstrated (Wei et al., 2002; Xie et al., 2000), but distinct sets of altered genes specific to CAR or PXR have also been identified (Maglich et al., 2002). Indeed, the relative contributions of CAR and PXR in responses to various xenobiotics, and the toxicological consequences of these responses are an active area of research (Stanley et al., 2006).

The major objective of the current work was to determine whether CAR plays a significant role in the liver effects of...
cyproconazole in mice, by comparing the responses in wild-type and Car-null mice. Prior to conducting these experiments, it was necessary to compare the relative effects of cyproconazole and the model CAR activator phenobarbital in CD-1 (outbred) mice, in which all prior cyproconazole studies were conducted, and inbred C57BL/6J and C3H/HeNClrBR mice, strains for which Car-null animals are available. This allowed selection of an appropriate mouse strain that would reflect changes seen previously in CD-1 mice, as well as determining the time of maximal effects for cyproconazole and phenobarbital treatment. Comparative studies in Car-null and wild-type mice were conducted in the C3H/HeNClrBR strain, and the results of the combinations of studies with cyproconazole and phenobarbital are reported here.

MATERIALS AND METHODS

Animals. Male CD-1, C57BL/6J, and C3H/HeNClrBR mice were supplied by Charles River (Margate, Kent, UK or Raleigh, NC). Male Car-null mice (Yamamoto et al., 2004) on a C3H/HeNClrBR background (Charles River Laboratories, Raleigh, NC) were a kind gift of Drs Maronpot and Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC) and bred at Integrated Laboratory Systems, Inc. (Research Triangle Park). All mice were housed individually in sterilized cages in a facility with a 12-h night/day cycle. Temperature and relative humidity were held at 22 ± 4°C and 30–70%, respectively. All animals were given humane care in compliance with relevant national guidelines, and studies were performed according to protocols approved by the appropriate institutional review board. The animals were acclimated to laboratory conditions for 5–7 days prior to dosing. Purina ProLab Isopro RMH 3000 irradiated meal (Ralston Purina, St Louis, MO) and tap water were available throughout the studies ad libitum. The diet was milled before feed substances were added. Diets were mixed with cyproconazole (97.0% purity, Syngenta Crop Protection, Münchwilen, Switzerland) or phenobarbital (sodium salt, 100% stated purity, Sigma-Aldrich, Milwaukee, WI). The test compound content in experimental diets was analyzed to confirm achieved concentration, homogeneity, and stability (data not shown).

Treatments. In the initial study of CD-1, C57BL/6J, and C3H/HeNClrBR mice, groups of 10 male mice each received cyproconazole at dietary concentrations of 200 and 450 ppm, or phenobarbital at a dietary concentration of 850 ppm for 2, 7, or 14 days. Another 10 animals of each strain (controls) were fed control diet for the same periods of time. Animals were between 5 and 6 weeks of age at the start of treatment. Five animals from each group were implanted with osmotic minipumps (1.0 μl/h, Alzet 1003D, Cupertino, CA) containing bromodeoxyuridine (BrDU, Sigma-Aldrich, 16 mg/ml in phosphate buffered saline pH 7.4) 3 days prior to sacrifice using Temgesic (Schering-Plough) as an analgesic and isofluorane (Abbott Laboratories) as an anesthetic.

Due to limited availability of Car-null mice, a staggered approach was adopted in the studies with wild-type and Car-null mice. Four separate experiments were conducted and in each batch, wild-type and Car-null animals were treated in one of the following ways: 200 or 450 ppm cyproconazole for 7 days, or 850 ppm phenobarbital for 3 or 7 days. Control groups were included in each batch. Animals in all batches were closely matched in age (6–8 weeks of age) during the treatment period. Control and treatment wild-type groups each contained five animals, and control and treatment Car-null groups each contained three to five mice.

During treatment each animal was observed at least once daily. Body weights were recorded daily and food consumption was determined at least every 3 days. Mice were terminated at the end of the study by over exposure to halothane vapor or isofluorane (Abbott Laboratories) followed by exsanguination by cardiac puncture. Blood samples were taken into 1-ml tubes with lithium heparin as an anticoagulant (Techlab) and separated into plasma and packed cells by centrifugation. Plasma samples were frozen and stored at −80°C. The liver was weighed and sections were taken as follows. For histology, samples of the right median, left lateral, and caudate lobes were trimmed, fixed in 10% Neutral Buffered Formalin, embedded in paraffin wax, 5-μm sections were cut, and stained with hematoxylin and eosin. For Oil-Red O (ORO) staining of fat, frozen sections were collected into optimal cutting temperature embedding compound and stored at −80°C. Prepared slides stained with ORO were evaluated by a standard microphotography read and/or by image analysis with BIOQUANT (Nashville, TN) Nova Prime software version 6.9. Microsomes were prepared fresh from samples of the left median, right lateral, and papillary processes and stored frozen. For gene expression, samples of the left lateral lobe were flash frozen in liquid nitrogen and stored at −80°C. The remaining liver was frozen in liquid nitrogen and stored at −80°C for further analyses.

Cell proliferation in the liver. Cell proliferation in liver was assessed by immunohistochemical staining of liver sections for nuclear incorporation of BrdU, or by evaluating nuclear protein levels of Ki67 (Muskhelishvili et al., 2003). BrdU- or Ki67-labeling indices were determined as a ratio of positively stained nuclei to the total number of nuclei counted in a minimum of 10,000 mononuclear hepatocytes across three liver lobes. BrdU incorporation was measured for all strains in the initial strain comparison study, with additional measurement of cell proliferation by Ki67 in two strains. Based on a similar pattern of results, Ki67-labeling indices were used to measure cell proliferation in the studies with wild-type and Car-null mice, to avoid the need for additional groups with surgically implanted minipumps.

Preparation of microsomes from mouse liver, enzyme activity assays and Western blotting. Liver samples were weighed and processed for the preparation of subcellular fractions by standard differential centrifugation as described by Green et al. (1997). The washed microsomal pellets were resuspended in 250 mM sucrose/5 mM ethylenediaminetetraacetic acid/20 mM Tris solution, pH 7.4 (1 ml/2 g of tissue weight) and aliquots were stored at −70°C. Protein concentration was determined using a Pierce (Rockford, IL) Protein Assay kit. CYP-mediated monooxygenase activities were measured in freshly thawed microsomes. The dealkylation of 7-benzyloxyresorufin (BROD), which is catalyzed by Cyp2b isoenzymes in mice, was measured as described by Burke et al. (1985). The hydroxylation of coumarin to 7-hydroxycoumarin (COH), which is catalyzed by Cyp2a5 in mice, was measured as described by Aitio (1978).

Furthermore, samples of liver microsomes from each animal (10 μg of protein) were loaded on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (4–20% acrylamide in Tris–HCl). After separation, the proteins were transferred from the gels to polyvinylidene fluoride membranes (Hybond P, GE Healthcare Life Sciences, Piscataway, NJ) and immunodetection was carried out using a primary antibody (goat polyclonal anti-rat CYP2B1, Daiichi Pure Chemicals Co., Tokyo, Japan) followed by a secondary antibody (anti-goat horseradish peroxidase conjugated, Sigma-Aldrich) and detection with ECL Plus Western Blotting Reagents (GE Healthcare Life Sciences). A negative control C1 (liver microsomes isolated from untreated wild-type C3H/HeNClrBR mice) and a positive control C2 (liver microsomes isolated from phenobarbital-treated C3H/HeNClrBR mice) as well as molecular weight standards (Chemichrome Ultimate, Sigma-Aldrich) were included on each gel.

Messenger RNA isolation and gene expression analysis by RT-PCR. While frozen, a small fragment (approximately 10–30 mg) of liver tissue was removed for each sample and homogenized for 30 s using a T8 basic IKA ultra-Turrax dispersion tool (IKA Works, Wilmington, NC) in 600 μl of 80 mM Tris buffer (Qiagen, Valencia, CA) containing 1% β-mercaptoethanol (Sigma-Aldrich). The lysates were centrifuged for 5 min at 13,000 rpm. From the resulting supernatant total RNA was isolated using a RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNA integrity and quantity were assessed using RNA 600 nanoliter LabChip and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s protocol.

Total RNA (10 μg) was reverse transcribed using random primers and the high capacity complementary DNA (cDNA) archive kit (Applied
BioSystems, Foster City, CA) according to the manufacturer’s protocol. The resulting cDNA was diluted to 1:100 with RNase-free H2O, mixed gently, aliquoted, and stored at −20°C until required. The following Taqman (Applied Biosystems) gene expression assays were used for quantitative real-time PCR: Cyp2b10 (Mm00456591_m1), Mdn2 (Mm00487656_m1), Gadd45β (Mm00435123_m1), and Hprt1 (Mm00446968_m1). Reactions were performed in a 96-well assay format. Each plate contained one experimental gene and a housekeeping gene. Each reaction contained 1 μl of cDNA template, 1 μl of Taqman gene expression assay primer, 7.7 μl of RNase-free H2O, 10 μl of 2× FullVelocity SYBR Green QPCR master mix (Stratagene, La Jolla, CA), and 0.3 μl of diluted (1:500 with RNase-free H2O) dye. Reactions were mixed by vortexing and pelleted by centrifugation. Reactions were processed using a run cycle comprised of one cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C on the Mx3000P QPCR Instrument (Stratagene). All reactions were carried out in duplicate. The cycle threshold (Ct) for each sample was determined from the linear region of the amplification plot. The ΔCt value for Mdn2, Cyp2b10, and Gadd45β relative to the control gene Hprt1 was determined. The ΔΔCt values for Mdn2, Cyp2b10, and Gadd45β were calculated using treated group means relative to control group means. Fold change data were calculated from the ΔΔCt values.

Statistical analysis. Numerical data were evaluated using the MIXED procedure in the SAS 2004 (SAS Institute, Cary, NC) statistical package. Analyses were carried out separately for cyproconazole and phenobarbital groups, each experimental batch or strain of mice and for the wild-type mice and Car-null mice. Analysis of variance (ANOVA) was used for statistical analysis, where appropriate. If necessary, data were transformed logarithmically or by a double arcsine transformation prior to analysis. To compare relative liver weights after adjustment for body weight, liver weights were considered by analysis of covariance (ANCOVA) on final body weight. Terminal body weights were evaluated statistically both unadjusted (ANOVA) and by ANCOVA on initial body weight. Using the MIXED procedure in SAS, differences from control were tested statistically by comparing each treatment group least-squares mean with the control group least-squares mean using a two-sided Student’s t-test, based on the error mean square in the initial analysis (ANOVA or ANCOVA). Results were considered statistically significantly different at p < 0.05.

RESULTS

Initial Strain Comparison Studies

The liver responses to cyproconazole and phenobarbital were compared in three strains of mice. Significant increases in hepatocellular proliferation as measured by BrdU incorporation were observed for both phenobarbital and the 450 ppm dose of cyproconazole (Fig. 1). Phenobarbital exhibited an earlier effect with an elevation in cell proliferation evident at 2 days after treatment, while cyproconazole had a maximal effect at around 7 days (Fig. 1). While the response at 200 ppm cyproconazole was not statistically significant, there was a trend toward increases in each of the strains (approximately twofold), and cell proliferation was also maximal at 7 days for this dose level of cyproconazole.

For two of the strains (CD-1 and C3H/HeNClrBR), cell proliferation was also measured by nuclear Ki67 determination, and the trend was similar to that of BrdU data, with a somewhat more pronounced pattern of effects by this technique. In CD-1 mice, significant increases were observed after 450 ppm cyproconazole treatment at 2 days (4.5-fold, p < 0.05) and 7 days of treatment (4.9-fold, p < 0.05). Ki67 labeling was higher in C3H/HeNClrBR mice after 450 ppm cyproconazole treatment, showing increases at 7 days (15.6-fold, p < 0.05), and 14 days (4.2-fold, p < 0.05). With phenobarbital treatment, Ki67 labeling in CD-1 mice was maximal at 2 days (17.3-fold, p < 0.05), whereas it was maximal in C3H/HeNClrBR mice at 7 days (26.8-fold, p < 0.05). At 200 ppm cyproconazole, the Ki67-labeling index was significantly increased in C3H/HeNClrBR mice at 7 days (5.9-fold, p < 0.05), and it showed a similar trend in CD-1 mice as BrdU measurements, with nonstatistically significant increases at 2 days (threefold) and 7 days (1.3-fold). Prior experiments in which CD-1 mice received 200 ppm cyproconazole in the diet for up to 28 days have demonstrated a consistent statistically significant increase in BrdU incorporation after 2, 3, 4, and 7 days of treatment (2- to 12-fold), but not at 14 days or longer (data not shown).

A comparison of a number of other parameters at 7 days is shown in Table 1. Both agents caused hepatomegaly and hepatocyte hypertrophy; the hypertrophy was primarily centrallobular for phenobarbital and centrilocublar/midzonal for cyproconazole. At this time point, cyproconazole, but not phenobarbital,
### TABLE 1
Effects of 7-Day Treatment with Cyproconazole (CCZ) or Phenobarbital (PB) on Clinical Chemistry and Liver Micropathology
Parameters in CD-1, C57BL/6J, and C3H/HeNClBR Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CD-1</th>
<th>C57BL/6J</th>
<th>C3H/HeNClBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose received (mg/kg/day)</td>
<td>0 34.4 88.8 147.0</td>
<td>0 40.8 105.8 198.8</td>
<td>0 51.3 121.3 226.5</td>
</tr>
<tr>
<td>Terminal body weight (g)</td>
<td>36.2 ± 1.3 35.2 ± 1.3 35.4 ± 2.4 37.8 ± 2.2</td>
<td>22.9 ± 0.7 22.1 ± 1.4 20.8 ± 1.1* 23.7 ± 1.5</td>
<td>23.0 ± 2.0 23.1 ± 1.6 21.5 ± 1.4* 23.9 ± 1.2</td>
</tr>
<tr>
<td>(g)</td>
<td>35.8 35.9 35.1 37.5</td>
<td>22.9 22.2* 20.7* 23.7*</td>
<td>23.1 23.2 21.2* 23.6</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>2.2 ± 0.3 2.9 ± 0.2* 3.4 ± 0.5* 3.1 ± 0.5*</td>
<td>1.3 ± 0.3 1.6 ± 0.3 1.8 ± 0.2* 1.8 ± 0.2</td>
<td>1.4 ± 0.2 1.9 ± 0.2* 2.1 ± 0.2* 2.1 ± 0.2*</td>
</tr>
<tr>
<td>Adjusted for terminal body weight (g)</td>
<td>2.1 2.9* 3.4* 2.8*</td>
<td>1.1 1.5* 2.0* 1.5*</td>
<td>1.4 1.9* 2.2* 2.0*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>3.4 ± 0.7 2.2 ± 0.6* 1.6 ± 0.3* 3.1 ± 0.8</td>
<td>1.9 ± 0.3 1.0 ± 0.1* 0.9 ± 0.1* 2.0 ± 0.2</td>
<td>3.8 ± 0.7 1.7 ± 0.2* 1.8 ± 0.2* 3.6 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.1 ± 0.6 2.1 ± 0.9 1.9 ± 0.6 2.8 ± 1.2</td>
<td>1.3 ± 0.4 1.3 ± 0.5 1.6 ± 0.3 1.6 ± 0.5</td>
<td>2.6 ± 1.5 3.3 ± 1.6 4.2 ± 0.7 1.5 ± 0.5</td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/l)</td>
<td>27 ± 5 72 ± 42* 721 ± 363* 72 ± 10*</td>
<td>25 ± 3 79 ± 57* 988 ± 399* 37 ± 10*</td>
<td>32 ± 3 57 ± 16* 271 ± 96* 41 ± 9</td>
</tr>
</tbody>
</table>

*Significantly different from corresponding strain control group (*p < 0.05*).

The data shown are mean ± SD (*n* = 10).

Micropathology:

- Inflammatory cell foci: minimal 3 5 5 2 7 7 6 7 2 1 2 3
- Single cell necrosis: Minimal 0 3 2 4 0 3 4 2 0 1 9 0
- Slight 0 0 7 1 0 0 5 0 0 0 0 0
- Hepatocyte hypertrophy: slight 0 7 10 10 0 9 9 10 0 5 10 10
- Hepatocyte lipid content (ORO): Minimal 3 0 0 1 0 0 0 1 0 0 0 3
- Slight 1 1 0 0 0 2 2 0 1 1 0 2
- Moderate 0 1 0 1 0 3 1 0 0 1 4 0
- Marked 0 3 5 1 0 0 0 1 0 2 1 0

Note. *Significantly different from corresponding strain control group (*p < 0.05*).

The data shown are mean ± SD (*n* = 10).

Data from 10 animals per group, except where noted otherwise.

Data from five animals per group.
caused a decrease in plasma cholesterol levels and exhibited a marked effect on fat accumulation in liver parenchymal cells (Table 1). Both of these effects were observed in phenobarbital-treated mice at day 2 (data not shown). There was an increased incidence and severity of single cell necrosis in the high dose cyproconazole group (450 ppm) compared to lesser effects in the low dose cyproconazole (200 ppm) and phenobarbital (850 ppm) groups. This was accompanied by a greater elevation in plasma alanine aminotransferase at 450 ppm cyproconazole (Table 1).

Cyproconazole produced similar effects in a dose-related manner in all three strains of mice, and the response was largely similar, but not identical, to that of phenobarbital, a known activator of CAR. Compared to phenobarbital, cyproconazole produced a larger and more sustained decrease in plasma cholesterol levels, and a greater effect on fat deposition in the liver. Other than these specific parameters and slight differences in time to peak effects for changes such as cell proliferation (~2 days for phenobarbital; ~7 days for cyproconazole), the response to phenobarbital was generally the same as that to cyproconazole. A slightly less pronounced effect on markers of cell proliferation was observed for CD-1 mice compared to the two inbred strains, depending in part on the method of analysis (BrdU or Ki67). Wider interindividual variability in an outbred strain (CD-1) may be a possible explanation for these minor observed differences. However, the overall pattern of response in C57BL/6J mice or C3H/HeNClrBR mice was comparable to that in CD-1 mice following cyproconazole treatment.

Studies with Car-Null and Wild-Type C3H/HeNClrBR Mice. Based on the similar responses in all three strains in the initial study, Car-null and wild-type male mice on a C3H/HeNClrBR background were tested with cyproconazole or phenobarbital for up to 7 days. Results at the previously established time of peak effects for cyproconazole (7 days) and phenobarbital (3 days) are presented (Table 2). In wild-type mice, 200 ppm cyproconazole caused liver hypertrophy, increase in liver weight (absolute and adjusted for body weight), and increases in cell proliferation, single cell necrosis, and fat vacuolation—effects generally similar to those caused by 850 ppm of phenobarbital (Table 2 and Fig. 2). Hepatocyte fat vacuolation in hematoxylin and eosin–stained hepatocytes was more pronounced after cyproconazole treatment than phenobarbital (Table 2), but staining of frozen sections with ORO revealed a similar increase in hepatocellular lipids with both compounds (Fig. 3 and Table 2). Plasma cholesterol levels were decreased by both compounds, but 200 ppm cyproconazole had a greater effect (Table 2). The higher dose (450 ppm) of cyproconazole caused similar changes in a dose-related manner, but greater evidence of hepatocellular perturbation at this dose was indicated by increases in plasma alanine aminotransferase and a slightly greater severity of single cell necrosis (Table 2). Plasma triglycerides were decreased in a dose-related manner by cyproconazole treatment, but no effect on this parameter was observed with phenobarbital treatment. In Car-null mice, the effects noted with either cyproconazole or phenobarbital were absent or greatly diminished, including no effects on absolute liver weight, and no effects on liver micropathology other than a minor increase in ORO staining in the Car-null mice.

To determine whether molecular changes in liver in response to cyproconazole are similar to those of phenobarbital and are dependent upon CAR, protein, and/or messenger RNA (mRNA) levels of several CAR target genes were determined. Marked induction of both protein (Fig. 4) and mRNA (Table 3) levels for Cyp2b10 (147- to 294-fold) was observed in cyproconazole- and phenobarbital-treated wild-type mice. mRNA for Gadd45β, an antiapoptotic/proliferative gene, was also increased with both compounds (two to sevenfold). In contrast, the cell cycle regulatory gene Mdm2 was largely unaffected in mice treated with either compound. Similarly to other phenotypic measurements, these effects of either cyproconazole or phenobarbital were absent or greatly diminished in Car-null mice.

As further confirmation of induction of xenobiotic metabolism in liver, the activity of CYP-mediated monoxygenases was measured. BROD activity (catalyzed by Cyp2b isoenzymes in mice) and COH activity (catalyzed by Cyp2a5 in mice) were markedly induced by both cyproconazole and phenobarbital in wild-type mice (Fig. 5). In Car-null mice, BROD activity was unaffected by treatment with either compound. COH activity was completely unaffected by phenobarbital in Car-null mice, while the effect of cyproconazole was blunted by ~65–75% as compared to the response in wild-type mice.

**DISCUSSION**

Nearly all triazole fungicide agents, including cyproconazole, have marked effects on the liver in rodents (www.cdpr.ca.gov/docs/toxsums/toxsumlist.htm) and many are hepatocarcinogenic in mice, but not rats (Goetz et al., 2006). It has been suggested that the triazole fungicide fenbuconazole may operate by a mode of action similar to that of the classical CAR activator phenobarbital, a known nongenotoxic rodent liver carcinogen (Juberg et al., 2006). In accord with this, the data presented here demonstrate that the short-term liver effects of cyproconazole in mice are CAR dependent and similar to those of phenobarbital. Furthermore, to our knowledge this is the first published report where a triazole fungicide was tested in Car-null mice and shown not to produce most of the hallmarks of liver toxicity that occur in wild-type mice. Of particular note is the lack of adverse effects in Car-null mice on liver micropathology (hepatocyte hypertrophy, single cell necrosis, or fat vacuoles), or absolute liver weight. In combination with the absence of liver tumor formation following longer-term treatment of Car-null mice with the prototypical CAR
### TABLE 2
Effects of the 7-Day Treatment with Cyproconazole (CCZ) or 3-Day Treatment with Phenobarbital (PB) on Clinical Chemistry and Liver Micropathology Parameters in Wild-Type (C3H/HeNClBr) and Car-null Mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment, duration</th>
<th>C3H/HeNClBr</th>
<th>Car-null</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCZ (7 days)</td>
<td>PB (3 days)</td>
<td>CCZ (7 days)</td>
</tr>
<tr>
<td>Dose, ppm</td>
<td>0 200 0 450 0 850</td>
<td>0 200 0 450 0 850</td>
<td></td>
</tr>
<tr>
<td>Dose received (mg/kg/day)</td>
<td>0 38.6 0 71.5 0 140.4</td>
<td>0 34.0 0 54.7 0 117.1</td>
<td></td>
</tr>
<tr>
<td>Number of animals</td>
<td>5 5 5 5 5 5</td>
<td>5 5 5 5 5 5</td>
<td></td>
</tr>
<tr>
<td>Terminal body weight (g)</td>
<td>24.2 ± 0.6 23.7 ± 1.3 24.4 ± 0.8 23.1 ± 0.7* 23.3 ± 1.2 23.4 ± 0.8</td>
<td>25.6 ± 0.9 24.7 ± 1.0 27.0 ± 0.9 25.1 ± 1.7 26.7 ± 2.2 25.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Terminal body weight, adjusted for initial wt (g)</td>
<td>24.1 23.8 24.4 23.0* 23.5 23.1</td>
<td>25.4 24.9 26.9 25.1 26.6 25.0*</td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.4 ± 0.1 1.8 ± 0.1* 1.4 ± 0.1 1.9 ± 0.2* 1.4 ± 0.1 1.9 ± 0.1*</td>
<td>1.5 ± 0.1 1.6 ± 0.1 1.5 ± 0.1 1.5 ± 0.2 1.5 ± 0.2 1.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Liver weight adjusted for terminal body weight (g)</td>
<td>1.4 1.8* 1.3 2.0* 1.4 1.9*</td>
<td>1.5 1.6 1.4 1.6* 1.5 1.4*</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>133 ± 2 55 ± 5* 142 ± 11 41 ± 10* 136 ± 7 110 ± 9*</td>
<td>120 ± 7 123 ± 5 128 ± 6 94 ± 12* 133 ± 6 114 ± 15*</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>88 ± 12 70 ± 1* 93 ± 42 38 ± 15* 85 ± 28 62 ± 17</td>
<td>97 ± 20 98 ± 21 110 ± 5 43 ± 24* 128 ± 26 132 ± 43</td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/l)</td>
<td>56 ± 22 111 ± 26* 50 ± 13 504 ± 142* 56 ± 25 140 ± 56*</td>
<td>53 ± 22 50 ± 20 153 ± 185 81 ± 42 57 ± 20 79 ± 46</td>
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</tbody>
</table>

**Micropathology**

| Hepatocyte vacuolation | Minimal | 0 2 0 1 0 0 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
|                        | Slight  | 0 0 0 4 0 0 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
| Single cell necrosis   | Minimal | 0 5 0 3 0 0 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
|                        | Slight  | 0 0 0 2 0 0 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
| Hepatocyte hypertrophy | Minimal | 0 5 0 5 0 5 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
|                        | Slight  | 0 5 0 5 0 5 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |
| Hepatocyte lipid content (ORO) | Minimal | 4 0 4 0 5 0 | 4 1 1 0 5 3 | 1 0 0 0 0 0 |
|                        | Slight  | 1 0 0 0 0 0 | 0 4 0 4 0 2 | 0 4 0 4 0 2 |
|                        | Moderate| 0 5 0 5 0 5 | 0 0 0 0 0 0 | 0 0 0 0 0 0 |

**Note.** * Significantly different from corresponding strain’s control group (*p < 0.05*).

*The data shown are mean ± SD.*
activators TCPOBOP or phenobarbital (Huang et al., 2005; Yamamoto et al., 2004), these data suggest that cyprocona-
zole’s mode of action is likely to be largely dependent on initial
activation of CAR to produce liver tumors in mice.

The cell proliferation data in the current studies with
cyproconazole and phenobarbital are consistent with those
reported for fenbuconazole (1300 ppm) and phenobarbital
(1000 ppm) by Juberg et al. (2006). In these earlier studies, cell
proliferation in female CD-1 mice by BrdU staining was
significantly increased after 1 week of treatment for both
fenbuconazole (8.5-fold) and phenobarbital (11.7-fold), but
was no different from control values after 4 weeks of treatment.
In the current study, the fold induction above controls by BrdU
staining was not as large as in Juberg et al. (2006) for both
200–450 ppm cyproconazole (twofold increase) or 850 ppm
phenobarbital (fivefold increase), but this could reflect experi-
mental differences such as the use of female mice, a higher
dose of phenobarbital (1000 vs. 850 ppm), and a longer period
of BrdU minipumping (4 vs. 3 days) in the earlier work. Larger
fold-increases in cell proliferation labeling indices were
observed using Ki67-staining techniques in both CD-1 male
mice and C3H/HeNClBR male mice after cyproconazole and
phenobarbital treatment.

Cyproconazole and other triazole fungicides exert their
fungicidal effect by inhibition of sterol 14α-demethylase,
a xenobiotic metabolizing enzyme in fungi that has a homolog
in mammals known as CYP51 (Georgopapadakou, 1998).
CYP51 is involved in the synthesis of cholesterol, and it has been suggested that the liver toxicity of triazoles in mammals may be related to inhibition of cholesterol synthesis via inhibition of CYP51 (Ward et al., 2006). There is strong evidence that changes in lipid/cholesterol metabolism in rodents, particularly mice, correlates with an increase in liver tumors. All of the major statins, a family of cholesterol-lowering drugs, are rodent carcinogens, with the mouse being the more sensitive species (Gerson et al., 1989; MacDonald et al., 1988; Newman and Hulley, 1996; von Keutz and Schluter, 1998). The statins cause inhibition of cholesterol biosynthesis and lower cholesterol levels mainly by inhibition of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. In the current studies, a clear decrease in plasma cholesterol as a result of cyproconazole treatment, and to a lesser extent after phenobarbital treatment, represents an early effect of these agents in the mouse liver. Interestingly, data from our studies in Car-null mice suggest that the cholesterol-lowering effect of cyproconazole is a function of activation of CAR, because the lower cholesterol levels in wild-type mice were not observed in Car-null mice at 200 ppm cyproconazole treatment, and were greatly attenuated at 450 ppm cyproconazole treatment. Accordingly, CAR activation may play a more significant role than inhibition of CYP51 in the liver toxicity and eventual formation of tumors in mice following cyproconazole treatment.

In support of this observation, comparisons of relative potencies for CYP51 inhibition by various triazole and imidazole fungicides have shown that cyproconazole and other triazole fungicides are less potent inhibitors (228-fold difference for cyproconazole) of mammalian compared to yeast CYP51 (Trosken et al., 2006). Potencies for relative CYP51 inhibition vary considerably for different chemical structures, and the ability to activate CAR in different species also varies in relation to chemical structure across a wide range of chemical classes (Stanley et al., 2006). Therefore, the relative contribution of CAR activation, CYP51 inhibition, and possibly other mechanisms to the development of liver tumors in mice is still untested for most triazole fungicides, but a major role for CAR activation has been established for cyproconazole.

Of note is the prevailing theory that phenobarbital is an indirect CAR activator (Honkasoski et al., 1998; Moore et al., 2000). This is based on lack of ligand binding in various reporter constructs with phenobarbital, whereas in whole cell systems and in vivo systems, phenobarbital produced potent induction of CAR-mediated effects including translocation of the CAR receptor complex to the nucleus (Honkasoski et al., 1998; Kawamoto et al., 1999). In contrast, TCPOBOP is a potent, direct activator of mouse CAR, but it does not activate human CAR (Tzameli et al., 2000). The nature of the interaction between cyproconazole and the CAR receptor complex is not yet established, and is a possible avenue for further research.
Not all liver effects of cyproconazole were ameliorated completely in Car-null mice, yet a small but significant increase was observed with 450 ppm cyproconazole. It is possible that CAR-independent stress responses to high doses of cyproconazole, as indicated by the observed small increase in Gadd45β expression in Car-null mice, may play a partial role in producing a proproliferative/antiapoptotic response at the 450 ppm dose. In fact, the 450 ppm dose of cyproconazole produced an overall loss of body weight in wild-type (−0.3 g) and Car-null mice (−0.8 g) during the 7-day treatment period, and it is therefore plausible that this stress may have played a partial role in changes in Gadd45β expression and resulting cell proliferation.

The molecular functions of Gadd45β and Mdm2 have previously been shown to be associated with different pathways involved in the regulation of proliferation and/or apoptosis in the rodent liver (Daujat et al., 2001; De Smaele et al., 2001; Locker et al., 2003). In one study, the cell cycle regulatory gene Mdm2 was shown to be induced in mouse liver following treatment with TCPOBOP in a CAR-dependent manner, and Mdm2 also was induced by phenobarbital in transgenic mice containing the human CAR gene (Huang et al., 2005). In our studies, neither phenobarbital nor cyproconazole resulted in a robust increase in Mdm2 expression; the only difference from control values was a marginal increase after 3 days of phenobarbital treatment in Car-null mice, but not in wild-type mice. Although the lack of effect could be attributable to differences in the background mouse strain or different potencies of CAR activators (TCPOBOP being a stronger activator than phenobarbital), the current data make it uncertain whether this particular gene is truly responsive to CAR activation in mouse liver as reported in (Huang et al., 2005).

In contrast, consistent increases in expression of Gadd45β were observed for 200 and 450 ppm cyproconazole and 850 ppm phenobarbital in C3H/HeNClBR mice. In Car-null mice, no increase in Gadd45β expression was observed with 200 ppm cyproconazole. At the higher dose of 450 ppm cyproconazole in Car-null mice, the gene expression response was still observed but was somewhat attenuated (4.3- vs. 6.1-fold in wild-type mice). A similar pattern of increased expression, but to a lesser extent than in wild-type mice, was observed for phenobarbital in Car-null mice (two- vs. sevenfold in wild-type mice). While the model CAR activator TCPOBOP has been shown to increase expression of Gadd45β in a CAR-dependent manner (Columbano et al., 2005), alternative pathways other than CAR activation can lead to increased expression of Gadd45β. For example, Gadd45β expression has been shown to be regulated by the transcription factors nuclear factor-kappaB (Jin et al., 2002) and SMAD (Takekawa et al., 2002). In the current studies with cyproconazole, complete absence of Gadd45β induction at 200 ppm versus partial attenuation of Gadd45β induction at 450 ppm in Car-null mice correlated with greater body weight effects and liver toxicity seen at the higher dose of 450 ppm cyproconazole in wild-type mice.
Therefore, it is possible that the alternate pathways of Gadd45β induction are operative at the higher dose, in addition to CAR-mediated Gadd45β induction. These data indicate that expression of this proliferative/antiapoptotic gene is regulated by CAR to a significant extent during cyproconazole or phenobarbital administration.

A cyproconazole-induced increase in fat vacuolation and hepatocellular lipid content in liver of wild-type mice was either absent (vacuolation) or greatly diminished (lipid content) in Car-null mice. Also, COH activity, an indicator of Cyp2a5 induction in mice, was not increased at all in Car-null mice treated with phenobarbital, whereas the response in Car-null mice was still present, though markedly diminished, after cyproconazole treatment. It is possible that cyproconazole may interact with other nuclear receptors that cause induction of Cyp2a5, e.g., PXR. Indeed, evidence of cross-talk between CAR and PXR for regulating the expression of some genes has been demonstrated (Maglich et al., 2002; Wei et al., 2002; Xie et al., 2000). However, the magnitude of difference in overall liver effects between Car-null mice treated with cyproconazole and wild-type mice treated with cyproconazole confirm that CAR activation is the predominant pathway leading to liver changes with this compound.

Recent publications have also indicated that CAR and PXR may act as a backup mechanism to the nuclear receptor farnesoid X receptor in regulating and preventing bile acid accumulation and toxicity (Stanley et al., 2006). Bile acid feedback regulates a wide array of liver metabolic pathways, and the regulation of bile acids and cholesterol is interrelated. Only a small portion of the possible endpoints related to cholesterol and bile acid homeostasis were assessed in the current studies with cyproconazole. Both CAR and PXR have been shown to upregulate hepatic bile acid transport systems as well as phase I and phase II enzymes involved in bile acid metabolism in mice, although data suggest that CAR plays a greater role than PXR in this response (Stanley et al., 2006). Of further interest is the observation that no decrease in plasma cholesterol levels occurs in rats treated with cyproconazole; in fact, high doses (700–1400 ppm) in subchronic studies have been shown to cause an increase in plasma cholesterol in rats (Syngenta Crop Protection AG, unpublished data). Rats do not develop liver tumors after cyproconazole administration, so the species differences in the biochemical events that lead to lower cholesterol levels may play a role in the mouse-specific formation of liver tumors. Indeed, other key events related to liver tumor formation in mice do not occur in rats treated with cyproconazole, particularly single cell necrosis, large increases in plasma transaminases, and cell proliferation (Syngenta Crop Protection AG, unpublished data).

Collectively, these experiments demonstrate that CAR plays an essential role in the development of liver effects in mice following cyproconazole administration, and that the pattern of effects is very similar to the pattern produced by phenobarbital.


