The Hepatocarcinogenic Conazoles: Cyproconazole, Epoxiconazole, and Propiconazole Induce a Common Set of Toxicological and Transcriptional Responses

Susan Hester, Tanya Moore, William T. Padgett, Lynea Murphy, Charles E. Wood, and Stephen Nesnow

Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Received November 2, 2011; accepted February 8, 2012

Conazoles are fungicides used as agricultural pesticides and pharmaceutical products. We investigated whether a common core of toxicological and transcriptional responses underlies the observed carcinogenic effects of three conazoles: cyproconazole, epoxiconazole, and propiconazole. In studies where mice were fed diets of these conazoles for 30 days, we found a common set of toxicological effects altered by these conazoles: hepatomegaly, hepatocellular hypertrophy, decreased serum cholesterol, decreased hepatic levels of all-trans-retinoic acid, and increased hepatic cell proliferation. Microarray-based transcriptional analysis revealed 330 significantly altered probe sets common to these conazoles, many of which showed strong dose responses for cytochrome P450, glutathione S-transferase, and oxidative stress genes. More detailed analyses identified a subset of 80 altered genes common to the three conazoles that were associated with cancer. Pathways associated with these genes included xenobiotic metabolism, oxidative stress, cell signaling, and cell proliferation. A common TGFβ-centric pathway was identified within the 80-gene set, which, in combination with the toxicological and other transcriptomic findings, provides a more refined toxicity profile for these carcinogenic conazoles.

Key Words: conazoles; cyproconazole; epoxiconazole; propiconazole; transcriptional analyses; hepatocarcinogenic; cell proliferation; liver; mice; all-trans-retinoic acid; cholesterol; Ki67.

Conazoles are fungicides used to control and prevent fungal growth on a variety of crops (Zarn et al., 2003) and for the treatment of local and systemic fungal infections (Georgopapadakou and Walsh, 1996). Members of this class of fungicide have a variety of toxicological outcomes in mammals including carcinogenicity, reproductive toxicity, and hepatotoxicity (Georgopapadakou and Walsh, 1996; INCHEM, 1981, 1987, 1992, 1997, 2001; Juberg et al., 2006; Peffer et al., 2007). Conazoles act to inhibit a specific cytochrome P450 (CYP) enzyme, CYP51 (lanosterol-14α-demethylase), which mediates a critical step in the biosynthesis of ergosterol, a steroid required for the synthesis of the fungal cell wall (Georgopapadakou and Walsh, 1996; Zarn et al., 2003). In mammalian systems, many conazoles are known to both induce and inhibit hepatic CYPs. In mice, a number of conazoles have been reported to induce several hepatic CYP isoforms with Cyp2b, Cyp2c, and Cyp3a being the most common forms (Allen et al., 2006; Chen et al., 2009; Georgopapadakou and Walsh, 1996; Juberg et al., 2006; Nesnow et al., 2009; Peffer et al., 2007; Sun et al., 2005, 2006; Ward et al., 2006; Zarn et al., 2003). Induction of these CYPs is mediated through the activation of two primary nuclear receptors, the constitutive androstane receptor and the pregnane X receptor (Maglich et al., 2002; Nallani et al., 2003). Mice treated with a number of these conazoles responded with increased hepatomegaly, increased oxidative stress, increased metabolism of all-trans-retinoic acid (atRA), decreased hepatic levels of atRA, decreased serum cholesterol levels, and increased hepatic cell proliferation (Allen et al., 2006; Bruno et al., 2009; Chen et al., 2009; Juberg et al., 2006; Nesnow et al., 2009, 2011a,b; Ortiz et al., 2010; Peffer et al., 2007; Ward et al., 2006).

Within this chemical class, cyproconazole (Cypro), epoxiconazole (Epoxi), and propiconazole (Propi) each induced hepatocellular carcinomas and hepatocellular adenomas in mice. Cypro was administered in a feed study to male and female CD-1 mice for up to 88 weeks at concentrations of 5, 15, 100, and 200 ppm. In male mice, there were statistically significant positive trends for adenomas and combined tumors. Males also had statistically significant increased incidence of carcinomas at the three highest doses, adenomas at 100 ppm, and combined tumors at the two highest doses (Kidwell, 2007). Epoxi was administered in a feed study to male and female C57Bl/6NCrIBr mice for 79 weeks at feed levels of 0, 1, 5, 200, or 500 ppm. Both male and female mice had significant increasing trends in liver adenomas, carcinomas, and adenomas, carcinomas combined. There was also a statistically significant trend and a significant increase in hepatocellular
adenomas/carcinomas and in combined adenomas/carcinomas for both males and females at the 500-ppm level compared with control (Arthur et al., 2005). Dietary administration of Propi to male CD-1 mice at feed levels of 0, 100, 500, and 2500 ppm for 104 weeks gave a statistically significant increase in the incidence of adenomas and carcinomas in male mice at 2500 ppm (Dewhurst and Dellarco, 2004).

Due to the current environmental use of these agents, studies have been undertaken to delineate the mechanism of action of these hepatocarcinogenic conazoles. To determine the potential common key elements involved in their mechanism of action of hepatocarcinogenic activity, we conducted dietary dose-response studies with each of these conazoles using the same feed concentrations used in their chronic bioassays (Arthur et al., 2005; Dewhurst and Dellarco, 2004; Kidwell, 2007) and using an extended dosing time period, 30 days. Liver tissues from these studies were analyzed for histological, immunohistochemical (IHC), transcriptional, and biochemical alterations that could provide more detailed information on their mechanism of action. Previous studies with Propi identified a series of transcriptional, biochemical, molecular, proteomic, and metabolomic changes that provided a framework for these current analyses (Allen et al., 2006; Chen et al., 2008, 2009; Nesnow et al., 2009, 2011a,b; Ortiz et al., 2010; Ross and Leavitt, 2010; Ross et al., 2009; Sun et al., 2005; Ward et al., 2006).

In the studies presented here, we focused on uncovering the toxicological and transcriptional responses common to Cypro, Epoxi, and Propi as we reasoned that a common set of biochemical, molecular, and metabolic changes could provide more detailed information on their mechanism of action. To determine these hepatocarcinogenic conazoles. To determine the potential common key elements involved in their mechanism of action of hepatocarcinogenic activity, we conducted dietary dose-response studies with each of these conazoles using the same feed concentrations used in their chronic bioassays (Arthur et al., 2005; Dewhurst and Dellarco, 2004; Kidwell, 2007) and using an extended dosing time period, 30 days. Liver tissues from these studies were analyzed for histological, immunohistochemical (IHC), transcriptional, and biochemical alterations that could provide more detailed information on their mechanism of action. Previous studies with Propi identified a series of transcriptional, biochemical, molecular, proteomic, and metabolomic changes that provided a framework for these current analyses (Allen et al., 2006; Chen et al., 2008, 2009; Nesnow et al., 2009, 2011a,b; Ortiz et al., 2010; Ross and Leavitt, 2010; Ross et al., 2009; Sun et al., 2005; Ward et al., 2006).

In the studies presented here, we focused on uncovering the toxicological and transcriptional responses common to Cypro, Epoxi, and Propi as we reasoned that a common set of responses from transcriptional and conventional assays may hold clues for their underlying shared hepatocarcinogenic effects. We also present new evidence for cell proliferative responses induced by these three carcinogenic conazoles after an extended treatment period.

**MATERIALS AND METHODS**

**Chemicals.** Cypro (96.7% purity) and Epoxi (96.8% purity) were obtained from LKT Laboratories (St Paul, MN). Propiconazole (Orbit; 94.2% active ingredient) was a gift from Syngenta Crop Protection, Greensboro, NC. Formic acid and acetic acid were purchased from Fluka (Sigma-Aldrich Company, St Louis, MO) and atRA was purchased from Alfa Aesar (Ward Hill, MA).

**Preparation of conazole-adulterated feed.** The general method for the preparation of conazole-adulterated diets has been previously reported (Allen et al., 2006) and used with modification. The following adulterated feeds were prepared: Cypro (0, 50, 100, and 200 ppm), Epoxi (0, 50, 200, and 500 ppm), and Propi (0, 500, 1250, and 2500 ppm). Briefly, cyproconazole epoxiconazole or propiconazole was dissolved in acetone and added drop-wise to Purina Mills Certified Rodent Diet 5001 meal in a Hobart N50 5-Quart Counter Top Mixer (Troy, OH). Feeds were sampled for chemical analyses in 5 g lots. Samples consisted of feed that were sampled from six different locations within the container. Quantitative analyses of the levels of conazoles in the adulterated feeds were accomplished by high-performance liquid chromatography using methanol extracts of the adulterated feed samples and using standard curves of the reference conazoles. For analyses of the Cypro and Epoxi feeds, samples (5 μl) were injected onto a 4.6 × 250 mm, 5 micron, 120 A YMC ODS AQ column (Waters Corp., Milford, MA). The elution solvent was methanol:water (8:2) and the flow rate was 1 ml/min. Cypro eluted as two diastereomers at 7.3 and 8.1 min, the peak at 8.1 min was used for the quantitation. It was detected using a diode array detector at 220 nm. Epoxi eluted at 8.4 min and was detected at 230 nm. For analyses of the propiconazole feeds, samples (10 μl) were injected onto a 4.6 × 250 mm, 3 micron, 120 A YMC ODS AQ column. The elution solvent was methanol:water (9:1) each containing 0.02% formic acid and, the flow rate was 0.75 ml/min. Propiconazole was detected using a diode array detector at 215 nm. The two diastereomers coeluted at 7.3 min.

**Animal treatments and necropsy.** Groups of male CD-1 mice (6–8 weeks) were obtained from Charles River Laboratories (Raleigh, NC) and acclimated for 7 days in the animal facility prior to commencing exposure for 30 days. Mice were ear tagged with unique identifying numbers and randomly assigned and housed two per polycarbonate cage with Alpha-Dry bedding in a room under a 12:12-h light:dark cycle, with controlled temperature (22°C) and humidity (45%). Water and feed were provided ad libitum in open-top stainless steel containers. The following feed concentrations were administered: Cypro (0, 50, 100, and 200 ppm), Epoxi (0, 50, 200, and 500 ppm), Propi (0, 500, 1250, and 2500 ppm). These feed concentrations were selected based on the feed levels used in previous chronic studies with each of these conazoles. The feed concentrations which have been shown to induce a carcinogenic response were: Cypro, 200 ppm; Epoxi, 500 ppm; and Propi, 2500 ppm (Arthur et al., 2005; Dewhurst and Dellarco, 2004; Kidwell, 2007). Animals were monitored daily, and cages and bedding were changed twice/week. Containers of feed were weighed at regular intervals to determine feed and chemical consumption. Mice were individually weighed at the start of the experiment and regularly thereafter. After 30 days of treatment, the animals were euthanized by CO2 asphyxiation. After livers were removed from mice, they were weighed and either immediately snap frozen in liquid nitrogen or placed in 10% neutral buffered formalin. Blood samples were also obtained. All animals were housed in an AAALAC, International accredited U.S. Environmental Protection Agency animal facility, and all procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee.

**Histopathological examination.** Three sections (right medial, left lateral, and caudate) of livers from mice fed diets containing Cypro (0, 50, 100, and 200 ppm) or Epoxi (0, 500, 1250, and 2500 ppm) for 30 days were taken from each of five mice per group for fixation in 10% neutral buffered formalin and processed by routine methods to 5-μm paraffin sections stained with hematoxylin and eosin, and analyzed with light microscopy. Liver alterations were scored as hepatocyte hypertrophy, which was the only alteration present in the majority of samples.

**Cell proliferation.** Liver cell proliferation was determined via Ki67 IHC staining by Experimental Pathology Laboratories, Inc., Durham, NC, on liver samples from mice fed diets of Cypro (0, 50, 100, and 200 ppm) or Epoxi (0, 500, 1250, and 2500 ppm) for 30 days at feed levels of 0 and 2500 ppm. Tissue samples in paraffin blocks were sectioned, deparaffinized, and hydrated. Samples were incubated in 1:20 citrate buffer for 7 min under pressure (decoloring) and then cooled. Blocking steps included quenching of endogenous peroxides with 3% H2O2 and avidin block, a biotin block, and incubation with blocking serum. Sections were labeled with rat anti-mouse Ki67 antibody (1:25 dilution) and a rabbit anti-rat IgG secondary antibody (1:300). Slides were developed using an avidin-biotin complex method following an application of 3,3-diaminobenzidine as the chromogen. The percentage labeling indices (LIs) were determined by counting the number of positive-stained Ki67 nuclei and the total number of cell nuclei with a diameter > 4 mm from photographic images taken at ×40 from three hepatic lobes on slides from each of five mice. Each image was scored and then reviewed by a board certified veterinary pathologist.

**Serum cholesterol levels.** Serum samples from mice fed diets of Cypro (0, 50, 100, and 200 ppm) or Epoxi (0, 50, 200, and 500 ppm) were analyzed for total cholesterol. Blood was collected by cardiac puncture at necropsy and

Downloaded from https://academic.oup.com/toxsci/article-abstract/127/1/54/1639806 by guest on 06 March 2019
placed in serum separation tubes (Becton Dickinson), allowed to clot, centrifuged at 1000 × g, at 4°C for 20 min, and stored in 2 ml microtubes. Serum cholesterol was measured by LabCorp, Research Triangle Park, NC, using a Roche Hitachi 717 Chemistry Analyzer.

**Determination of atRA levels in mouse liver.** Liver samples from Cypro (0, 50, 100, and 200 ppm), Epoxi (0, 50, 200, and 500 ppm), and Propi (0, 500, 1250, and 2500 ppm) treatments for 30 days were analyzed for the hepatic levels of atRA. All extraction procedures were carried out on ice using solvents precooled on ice. Additionally, the extractions were carried out under yellow light to minimize photooxidation. Liver was homogenized in water at a concentration of 100 mg/ml (wt/vol) using a Microson ultrasonic cell disruptor XL2000 (Misonix, Inc. Farmingdale, NY) at 10 watts for 20 s. Acitretin (100 µl, 100 pg/µl dissolved in acetonitrile) was added as the internal standard to 2 ml of the homogenate followed by 3 ml of 0.025M potassium hydroxide dissolved in ethanol, and the mixture was vortexed. Hexane (10 ml) was added, and the mixture was shaken vigorously for 2 min and centrifuged to separate the layers. The organic layer was removed and discarded, and the aqueous mixture was vortexed to resuspend any material and acidified with 180 µl of 4N HCl, then allowed to stand for 10 min on ice. Hexane (10 ml) was added, and the mixture was shaken vigorously for 2 min and centrifuged to separate the layers. The organic layer was placed in a conical vial and evaporated with a stream of nitrogen without heating. The residue was dissolved in 100 µl of acetonitrile and transferred to an amber autosampler vial for Liquid Chromatograph/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) analysis. LC/MS/MS was performed on an Applied Biosystems QStarElite mass spectrometer with an Atmospheric Pressure Chemical Ionization source operating in the positive mode. Samples were introduced to the mass spectrometer through an Agilent 1200 LC system including a cooled autosampler. Samples were cooled to 4°C for injection. The injection volume was 40 µl. Samples were chromatographed on a Supelco ABZ+plus column (150 × 4.6 mm, 3 µm) using a flow rate of 1 ml/min. A ternary solvent system was employed: Solvent A, water containing 0.1% formic acid; Solvent B, methanol containing 0.1% formic acid; and Solvent C, acetonitrile containing 0.1% formic acid. The initial concentration of A:30%, B:40%, and C:30% was held for 2 min, followed by a linear gradient to A: 20%, B:50%, and C:30% at 25 min.; then A:0%, B:70%, and C:30% at 26 min which was held to 30 min, followed by reequilibration from 30.5 min to 45 min. Runs were monitored at the following ions: m/z 301 (M + 1) for atRA and m/z 327 (M + 1) for acitretin. The transition from m/z 301 to m/z 205 was used to quantitate the amount of atRA, and the transition from m/z 327 to m/z 177 was used for acitretin. A standard curve atRA and acitretin was constructed to calibrate the atRA levels. An atRA check standard (25 pg/µl dissolved in acetonitrile) was run with each set of samples to verify the standard curve. Two additional quality control samples were run with each sample set to assess extraction efficiency. Water (2 ml) was spiked with acetonitrile solution of atRA (40 µl, 100 pg/µl) and an acetonitrile solution of acitretin (100 µl, 100 pg/µl) and extracted as outlined above. Control liver (2 ml, 100 mg/ml) that had previously exhibited no atRA was spiked in the same manner with atRA and acitretin and extracted as outlined above. Extraction efficiency from the water was quantitative, whereas the extraction efficiency from liver was in the range of 90–95%.

**RNA isolation.** The frozen mouse liver tissues from the Cypro (0, 50, 100, and 200 ppm) or Epoxi (0, 50, 200, and 500 ppm) treatments were ground in Tri Reagent (Molecular Research Center, Cincinnati, OH) with IKA Ultra-Turrax T 25 basic tissue grinder (IKA Works, Wilmington, NC). The total RNA pellet was resuspended in 100 µl water and purified by RNeasy kit from Qiagen (Valencia, CA). Purified total RNA was eluted in water, and the concentration was measured by NanoDrop ND-1000 spectrometer (NanoDrop, Wilmington, DE). The quality of RNA was assessed by an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA).

**Affymetrix microarray hybridization.** Gene expression analysis was conducted using Affymetrix Mouse Genome 430_2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) using one animal per gene chip with five animals per group (total of 20 GeneChips for Cypro and 20 GeneChips for Epoxi). This whole mouse genome array interrogates 34,000 variants and 39,000 transcripts from well-characterized mouse genes using 45,000 probe sets. Total RNA (4 µg) for each sample was amplified and labeled by Enzo BioArray RNA Amplification and Biotin labeling System (Enzo Life Sciences, Farmingdale, NY). For each array, 15 µg of amplified biotin-RNAs was fragmented and hybridized to the array for 16 h at 45°C in a rotating hybridization oven using the Affymetrix eukaryotic target hybridization controls and protocol. Slides were washed with streptavidin/phycoerythrin using a double-antibody staining procedure and washed using the EukGE-WS2v5 protocol of the Affymetrix Fluidics Station FS450 for antibody amplification. Arrays were scanned with an Affymetrix Scanner 3000.

**Microarray data analysis.** Microarray analyses were carried out using the 20 Cypro and 20 Epoxi GeneChips combined with 12 previously processed Propi GeneChips reported in Allen et al. (2006), which were generated using the RNA from livers of male CD-1 mice fed diets of Propi (0, 100, 500, and 2500 ppm) for 30 days. The raw signal intensities from each scan (.cel files) were imported into the gene expression analysis software Rosetta Resolver version 7.2 software (Rosetta Inpharmatics, Kirkland, WA). This software was used to determine which probe sets in a treatment group differed from the control group. The scans were processed into profiles within this software, and the data were normalized using Rosetta error model (Weng et al., 2006). Statistically filtered sequences were identified using one-way ANOVA with a false discovery rate (Benjamini-Hochberg test) of p ≤ 0.05 for significance.

RESULTS

**Effects of Conazoles on Mouse Liver Weight, Serum Cholesterol Levels, and Histological Analyses**

The effects of conazole treatments for 30 days on animal body weights, histology, and serum cholesterol levels are shown in Table 1. Mice fed diets containing Cypro at the 50, 100, and 200 ppm feed levels showed statistically significant increases in liver weight, liver/body weight ratios, and liver cell hypertrophy compared with control mice. Mice fed Epoxi at the 50, 200, and 500 ppm feed levels had statistically significant increases in liver weights, liver/body weights ratios, and liver cell hypertrophy. These results were compared with previously reported studies of mice fed diets containing Propi at the 0, 100, 500, and 2500 ppm feed levels. In these studies, Propi at 2500 ppm induced statistically significant increases in liver weights, liver/body weights ratios, and liver cell hypertrophy compared with control mice (Allen et al., 2006).
Liver cell hypertrophy was also increased at 500 ppm Propi. Serum cholesterol levels were significantly lower with feed concentrations of Cypro to 52% of control at the 200 ppm feed level. Significant lower serum cholesterol levels by Epoxi treatment were observed at the 200 and 500 ppm feed concentrations compared with the untreated controls with 500 ppm feed level. Significant lower serum cholesterol levels by Epoxi concentrations compared with control (Allen et al., 2006). As previously reported, Propi induced similar decreases in serum cholesterol to 52% of control at the 200 ppm feed level. Significant lower serum cholesterol levels by Epoxi concentrations compared with the control group as shown in Table 3. Cypro (Table 3). atRA levels were lower in most treatment groups compared with the control group as shown in Table 3. Cypro vs. control.

### Hepatic Cell Proliferation Using Ki67 Immunohistochemistry

Cell proliferation was determined by Ki67 IHC staining of slides from the livers of mice fed Cypro (0, 50, 100, and 200 ppm), Epoxi (0, 200, and 500 ppm), or Propi (0, 500, 1250, or 2500 ppm) for 30 days (Table 2). The Propi slides were obtained from stored reference blocks from a previously reported study where male CD-1 mice were fed Propi for 30 days at feed levels of 0 and 2500 ppm (Allen et al., 2006). At each feed level, at least 5000 cells were counted to obtain the LI. Cypro (200 ppm), Epoxi (200 and 500 ppm), and Propi (2500 ppm) resulted in significantly higher LIs of 1.97-fold or greater compared with respective control groups.

### Levels of Hepatic atRA

The levels of atRA were determined by LC/MS/MS in the livers of mice fed Cypro (0, 50, 100, and 200 ppm), Epoxi (0, 200, and 500 ppm), or Propi (0, 500, 1250, or 2500 ppm) (Table 3). atRA levels were lower in most treatment groups compared with the control group as shown in Table 3. Cypro vs. control.

### Table 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Target concentrations (ppm)</th>
<th>Actual concentrations (ppm), (mean ± SD)</th>
<th>Consumed dose (mg/kg/d), (mean ± SD)</th>
<th>Liver weight (g), (mean ± SD)</th>
<th>Liver:BWT (%), (mean ± SD)</th>
<th>Liver:BWT T/C, (mean ± SD)</th>
<th>Serum cholesterol (mg/dl), (mean ± SD)</th>
<th>Histology, mice with lesions/mice scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypro</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.08 ± 0.29</td>
<td>5.92 ± 0.45</td>
<td>1</td>
<td>185.3 ± 33.8</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48.6 ± 0.98</td>
<td>9.42 ± 0.43</td>
<td>2.37 ± 0.26&lt;</td>
<td>6.67 ± 0.56</td>
<td>1.13</td>
<td>140.5 ± 33.8</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.3 ± 0.77</td>
<td>17.5 ± 0.83</td>
<td>2.65 ± 0.5&lt;</td>
<td>7.58 ± 1.59</td>
<td>1.28</td>
<td>120.9 ± 25.7</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>204.1 ± 0.52</td>
<td>36.5 ± 1.41</td>
<td>2.8 ± 0.32&lt;</td>
<td>7.92 ± 0.79</td>
<td>1.34</td>
<td>96.8 ± 15.7</td>
<td>5/5</td>
</tr>
<tr>
<td>Epoxi</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.03 ± 0.21</td>
<td>5.42 ± 0.47</td>
<td>1</td>
<td>155.6 ± 33.4</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.6 ± 0.77</td>
<td>9.9 ± 0.52</td>
<td>2.36 ± 0.22&lt;</td>
<td>6.21 ± 0.84</td>
<td>1.15</td>
<td>162.5 ± 26.4</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>196.6 ± 5.42</td>
<td>40.2 ± 1.9</td>
<td>2.56 ± 0.29&lt;</td>
<td>7.05 ± 0.37</td>
<td>1.3</td>
<td>119.3 ± 32.7</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>495.3 ± 7.94</td>
<td>94.5 ± 4.2</td>
<td>3.15 ± 0.30&lt;</td>
<td>8.53 ± 0.57</td>
<td>1.57</td>
<td>97.1 ± 41.46</td>
<td>5/5</td>
</tr>
<tr>
<td>Propi</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.1 ± 0.4</td>
<td>5.3 ± 0.03</td>
<td>1</td>
<td>134.6 ± 12.7</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>102.0 ± 4.3</td>
<td>15.1 ± 1.9</td>
<td>2.0 ± 0.2</td>
<td>5.6 ± 0.3</td>
<td>0.95</td>
<td>ND</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>72.5 ± 5.8</td>
<td>72 ± 5.8</td>
<td>2.4 ± 0.2</td>
<td>6.0 ± 0.3</td>
<td>1.2</td>
<td>ND</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2617 ± 92.5</td>
<td>349.6 ± 46.7</td>
<td>4.0 ± 0.8&lt;</td>
<td>10.4 ± 1.3&lt;</td>
<td>2.0</td>
<td>75.6 ± 20.7&lt;</td>
<td>5/5</td>
</tr>
</tbody>
</table>

**Note.** ND, not determined.

*a* = 5 per group.

*b* = 10–16 per group.

*c* = 10–12 per group for the Cypro dose groups, and n = 10–14 for the Epoxi dose groups.

*b* The lesion score was hepatocellular hypertrophy.

*b* Statistically different compared with the Cypro control group by the Holm-Sidak multiple comparisons method, *p* < 0.05.

*b* Statistically different compared with the Epoxi control group by the Holm-Sidak multiple comparisons method, *p* < 0.05.

*b* Data from Allen et al. (2006). Different from concurrent control by Student’s *t*-test (*p* < 0.05).

*b* Data from Allen et al. (2006). Different from concurrent control by Dunnett’s multiple comparison test (*p* < 0.05).

### Table 2

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Feed level (ppm)</th>
<th>Number of cells counted (mean ± SD)</th>
<th>LI (mean ± SD)</th>
<th>Treated/control, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypro</td>
<td>0</td>
<td>7198</td>
<td>1.71 ± 0.49</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6404</td>
<td>1.95 ± 0.23</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5274</td>
<td>3.37 ± 0.40&lt;</td>
<td>197</td>
</tr>
<tr>
<td>Epoxi</td>
<td>0</td>
<td>8151</td>
<td>1.54 ± 0.54</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5921</td>
<td>3.38 ± 1.24&lt;</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>5097</td>
<td>3.54 ± 1.33b</td>
<td>230</td>
</tr>
<tr>
<td>Propi</td>
<td>0</td>
<td>7945</td>
<td>1.33 ± 0.24</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>5246</td>
<td>2.76 ± 0.66c</td>
<td>207</td>
</tr>
</tbody>
</table>

**Note.** ND, not determined.

*b* Statistically different compared with the Cypro control group by the Holm-Sidak multiple comparisons method, *p* < 0.05.

*b* Statistically different compared with the Epoxi control group by the Holm-Sidak multiple comparisons method, *p* < 0.05.

*b* Statistically different compared with the Propi control group by the Student’s *t*-test, *p* < 0.05.
Carcinogenic conazoles. (see Supplementary file: Excel spreadsheet of 80 common genes and their fold values). These 80 genes were subjected to IPA analysis to identify a set of 330 probe sets common to the three conazoles–induced cell proliferation at their carcinogenic doses, and these are shown in a heatmap divided into six panels to facilitate visualization (Fig. 1). This figure identifies a general trend of probe sets with strong intensity induction as well as those with repression, and the magnitude of the intensity response mirrors increasing dose range for each conazole.

### Analyses of the Effect of Conazoles on the Responses of Specific Genes

CYPs are a class of genes that are strongly induced by conazoles. The responses of four CYPs (Cyp1a2, Cyp2b, Cyp2c55, and Cyp2c65) to conazole treatment are presented in Figure 2. At the highest and tumorigenic exposure level, each conazole produced significant changes in the Cyp2b and Cyp2c isoforms with maximal fold induction of up to 100-fold, and very strong increasing responses to increasing exposure levels of conazole were observed. Cyp1a2 response was increased by conazoles but to a much lower extent. The responses of a series of glutathione S-transferases (GSTs) (Gstm1, Gstm3, and Gstm4) were increased with increasing conazole treatment levels (Fig. 3). Gstm3 was the most responsive of all GST genes with increases of up to 12-fold in Epoxi-treated mice, whereas Gstm1 was the least responsive to each conazole. The effects of conazole treatment on a group of genes associated with oxidative stress were examined (Fig. 4). These genes, Abcc4, Akr1b7, Ephx1, and Gpx4 also exhibited strong responses to conazole treatment. In Propi-treated mice, maximal induction was over 15-fold for Abcc4 and over 10-fold for Akr1b7.

#### Pathway and Network Analyses of Common Cancer-Related Genes

Since our results indicated that three carcinogenic conazoles–induced cell proliferation at their carcinogenic doses, we reasoned that further inspection of the 330 probe sets common to the carcinogenic conazoles would reveal a core transcriptional pattern that would provide a biological basis for the observed exposure-related carcinogenic responses. Using the 330 common probe sets shared by all the carcinogenic conazoles tested, we investigated whether this core probe set contained an inherent biologic cohesiveness. IPA software identified 80 genes from the 330 common probe sets that were annotated as participating in cancer (see Supplementary file: Excel spreadsheets of 80 common genes and their fold values). These 80 genes were subjected to IPA network analysis, which yielded six networks, four major (≥13 genes represented in each network) and two minor (nine genes or less represented in each network). The four major networks were merged into one network by the IPA software and the result is presented as an interaction map (Fig. 5). A number of salient of Tox Lists were overlaid on this map. It was recognized that a TGFα-centric pathway was present in these 80 genes, and a TGFα-based cell proliferation pathway was further developed from this 80-gene set using IPA software (Fig. 6). TGFα was over expressed by each conazole and was linked by the IPA software to RRas, Mapk2k3k, Myc,
DISCUSSION

The mechanism of conazole-induced liver carcinogenesis in mice has been the subject of extensive investigations (Allen et al., 2006; Bruno et al., 2006; Chen et al., 2008, 2009; Juberg et al., 2006; Nesnow et al., 2008, 2009; Peffer et al., 2007; Ross and Leavitt, 2010; Ross et al., 2009, 2010; Sun et al., 2005, 2006; Ward et al., 2006). Studies on two tumorigenic conazoles (Propi and Triadimefon) have reported effects on hepatic nuclear receptor activation, increased hepatic hypertrophy, CYPs induction, increased hepatic cell proliferation, decreased hepatic atRA levels, and decreased serum cholesterol levels (Allen et al., 2006; Arthur et al., 2005; INCHEM, 1987; Kidwell, 2007; Nesnow et al., 2009; Peffer et al., 2007). These observations suggested that the carcinogenic conazoles shared a core of common critical events associated with the development of liver tumors.

Liver cancer development is a progressive multistep process that results from dysregulation of oncogenes and tumor suppressor genes including growth factors that frequently

FIG. 1. Heatmap of 330 significantly altered probe sets common to Cypro, Epoxi, and Propi. In this matrix, rows are intensity ratios (treated/control), and columns are the three conazoles with each of the three exposures each. The heatmap is divided into six panels to facilitate visualization. This figure relates the expression changes for each individual probe set. The intensity of the red coloration represents the extent of probe set induction, and the intensity of the green coloration represents the extent of probe set repression, relative to control treatment. [Please see the online issue of the journal for the full color figure.]

c-Jun, Cdc34/Cdc42, and E2f, all of which are known to be involved in cell proliferation.
upregulate hepatocyte cycling in signaling pathways such as TGFα (Jhappan et al., 1990; Thorgeirsson and Grisham, 2002). A number of studies in transgenic mice have shown that over-expression of TGFα results in mouse hepatic tumor development (Jhappan et al., 1990; Kaufmann et al., 1992; Murakami et al., 1993; Presnell et al., 1997; Thorgeirsson and Grisham, 2002) leading to malignant transformation, and increased levels of TGFα protein have been documented in premalignant foci of hepatocytes in carcinogen-fed rats (Kaufmann et al., 1992). Moreover, the c-myc and c-myc/TGFα transgenic mouse models share similar genetic and molecular mechanisms with human liver cancer development suggesting that these models are relevant to the oncogenic events of human hepatocellular carcinoma (Calvisi and Thorgeirsson, 2005; Vucur et al., 2010).
In the present study, 80 significantly expressed genes common to Cypro, Epoxi, and Propi were found to be associated with the cancer processes. Their interconnectivity is shown in Figure 5. This interaction map reflects the transcriptional complexity on a toxicological process level, which is at the core of these carcinogenic conazoles. The mapping features nuclear receptor activation and Phase I, II, and III xenobiotic metabolism (CYPs, GSTs, and transporters, respectively) and is consistent with positive effects of the carcinogenic conazoles on xenobiotic metabolism. This result is often seen and can be understood because many of the xenobiotic-metabolizing genes CYPs, GSTs, and transporters are also involved in the carcinogenic process. In addition to xenobiotic metabolism, other processes highlighted included Nrf-2–mediated oxidative stress response and a number of other signaling processes including NF-κB and liver proliferation. NF-κB is a transcription factor commonly activated under environmental stress conditions or by inflammatory cytokines (Limuro et al., 1998) and has been reported to be involved in the regulation of hepatocyte proliferation and apoptosis in vitro and in vivo (Jones and Czaja, 1998). The role of NF-κB in rat hepatocyte proliferation was assessed by introducing an adenovirus repressor for NF-κB activity into their livers and subjecting the rats to partial hepatectomy. This resulted in increased apoptosis and a failure of hepatocyte proliferation, thus providing evidence for the requirement of NF-κB for normal hepatocyte proliferation processes (Limuro et al., 1998). When considered collectively, these cancer genes and cancer pathways could contribute to an environment leading to liver tumor development, and these results are supported by the demonstrated cell proliferation and carcinogenic effects induced by these three conazoles.

atRA controls a wide-range of physiological processes including cell growth, differentiation, embryonic development, and immune function (Goodman, 1984). Epidemiological studies have shown that individuals with lower dietary vitamin A intake are at an elevated risk to develop cancer (Sun and Lotan, 2002). atRA is known to suppress cell proliferation and carcinogenesis in a variety of tissue types, and it has been used as an anticancer or cancer-preventative therapy regimen (Hofmann, 1992; Okuno et al., 2004). We previously conducted a toxicogenomic study in mice exposed to carcinogenic conazoles showing alteration of a number of metabolic and signaling pathways (Nesnow et al., 2009; Ward et al., 2006). One of the over expressed pathways noted was retinoic acid metabolism. A follow-up study was conducted to investigate the effects of a series of conazoles (Propi, Triadimefon, and Myclobutanil) on hepatic microsomal metabolism of atRA and on hepatic atRA levels. In that study, mice were treated for 4 days with each of these conazoles. The results showed that these conazoles showed a consistent pattern of increased atRA metabolism-associated P450 enzymes, increased hepatic atRA metabolism, and decreased hepatic atRA levels in mouse liver (Chen et al., 2009).

In the current study, Cypro, Epoxi, and Propi each upregulated a set of CYPs that are known to be involved in the metabolism of atRA: Cyp2b10, Cyp2c55, and Cyp2c65 (Marill et al., 2000, 2003). After 30 days of treatment at carcinogenic and lower doses, Cypro, Epoxi, and Propi also reduced hepatic atRA levels. These data suggest that the
decrease in atRA levels would provide a tumor-promoting environment (Wang, 2003). As reduced levels of atRA are associated with increased cell proliferation, we determined the cell proliferative responses to Cypro, Epoxi, and Propi using Ki67 IHC in livers of mice treated with these agents after 30 days. We found that cell proliferation was increased by each of these conazoles at their carcinogenic doses. These biochemical and molecular events support and reinforce our findings that increased proliferation could be a result of decreased hepatic atRA levels.

A previously reported study on the effects of Cypro treatment in male CD-1 mice revealed that Cypro treatment did not induce hepatic cell proliferation as measured by Ki67 IHC at the carcinogenic 200 ppm feed concentration in a 14 days study (Peffer et al., 2007). Our results suggest that mice treated with Cypro at a longer treatment time respond with an increase in cell proliferation, which is consistent with the observed transcriptional changes. We also found an increase in hepatic cell proliferation measured by Ki67 IHC in mice treated with Propi for 30 days at the 2500-ppm dose level. This study used the tissue blocks from an earlier published study on the effects of Propi on hepatic cell proliferation in mice treated for 30 days at the 2500-ppm feed level (Allen et al., 2006). However, in the Allen et al. (2006) study, we found no increase in cell proliferation using proliferating cell nuclear antigen IHC (Allen et al., 2006). These differences in results can be explained by the increased sensitivity of the cell proliferation–counting method (by increasing the numbers of cells counted by over fivefold) and use of the more sensitive Ki67 IHC method (Muskhelishvili et al., 2003; Peffer et al., 2007). The findings that Cypro, Epoxi, and Propi can induce cell proliferative responses after extended treatment times is counter to the suggestions that in general, conazoles are like phenobarbital, which only induces a short burst of cell proliferation (Peffer et al., 2007; Smith et al., 1991; Tharappel et al., 2008; Whysner et al., 1996). Moreover, these findings are supported by previous transcriptional analyses that compare two conazoles with phenobarbital and find that these agents are transcriptionally different (Nesnow et al., 2009).

The ability of a cell to clonally expand into a tumor may be related to an increased sensitivity to stimulatory growth factor
signals such as TGF\(\alpha\) or a diminished capacity to respond to inhibitory signals, which allow altered cells to proliferate beyond basal levels of normal cells. Figure 6 depicts a proposed TGF\(\alpha\)-centric cell proliferation–signaling pathway with the transcriptomic data derived from this study. We observed overexpression of TGF\(\alpha\) in all three conazoles with Epoxi showing the strongest induction. One of the early effects of TGF\(\alpha\) overexpression in the liver is the persistent induction of hepatocyte proliferation (Murakami et al., 1993). TGF\(\alpha\) is known to signal through Rras to mitogen-activated protein kinase to initiate a cascade of signals to Myc/Jun and E2f (Chen et al., 2003). Enhanced E2f activity during hepatocarcinogenesis has been reported to play a role in murine hepatocyte proliferation (Murakami et al., 1998) along with the induction of E2f target genes Cdc2 and c-Myc. E2f has also been reported to be involved in murine hepatocyte proliferation in vitro (Zellmer et al., 2010). The signaling cascade depicted in Figure 6 shows gene signaling induced by conazoles and taken collectively may explain, in part, the observed proliferative effects associated with these compounds.

In conclusion, we have identified a set of toxicological responses and 330 common probe sets in the livers of mice treated Cypro, Epoxi, and Propi at their carcinogenic doses. In addition, we identified a set of 80 core genes that represent a series of metabolic and growth pathways associated with the development of cancer and containing a prominent TGF\(\alpha\)-centric pathway. We have also demonstrated for the first time that these three conazoles can induce cell proliferative responses after an extended treatment time. Our transcriptomic findings were also consistent with the toxicological results. Taken together, these toxicological and transcriptomic data form a toxicity profile for carcinogenic conazoles and provide a model for future exploration.

**REFERENCES**


