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Phenobarbital and propiconazole toxicogenomic profiles in mice show major similarities consistent with the key role that constitutive androstane receptor (CAR) activation plays in their mode of action

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Abstract

Toxicogenomics (TGx) is employed frequently to investigate underlying molecular mechanisms of the compound of interest and, thus, has become an aid to mode of action determination. However, the results and interpretation of a TGx dataset are influenced by the experimental design and methods of analysis employed. This article describes an evaluation and reanalysis, by two independent laboratories, of previously published TGx mouse liver microarray data for a triazole fungicide, propiconazole (PPZ), and the anticonvulsant drug phenobarbital (PB). Propiconazole produced an increase incidence of liver tumors in male CD-1 mice only at a dose that exceeded the maximum tolerated dose (2500 ppm). Firstly, we illustrate how experimental design differences between two in vivo studies with PPZ and PB may impact the comparisons of TGx results. Secondly, we demonstrate that different researchers using different pathway analysis tools can come to different conclusions on specific mechanistic pathways, even when using the same

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Supplementary data description

Supplementary data are available online. Supplementary data consists of: Appendix 1 (Excel file with DEGs for phenobarbital and propiconazole-treated mice), Appendix 2 (TGx Pathway analysis by Plummer (2011)), Appendix 3 (TGx Pathway analysis by Brennan (2010)) and Appendix 4 (five Supplementary figures, described in the text).

Conflicts of interest

R.A.C, R.C.P and A.K.G. acknowledge that they are employees of Syngenta Ltd. or Syngenta Crop Protection, LLC, which sells the fungicide (propiconazole) that is described in this manuscript.

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Transparency document

The Transparency document associated with this article can be found in the online version.

datasets. Finally, despite these differences the results across three different analyses also show a striking degree of similarity observed for PPZ and PB treated livers when the expression data are viewed as major signaling pathways and cell processes affected. Additional studies described here show that the postulated key event of hepatocellular proliferation was observed in CD-1 mice for both PPZ and PB, and that PPZ is also a potent activator of the mouse CAR nuclear receptor. Thus, with regard to the events which are hallmarks of CAR-induced effects that are key events in the mode of action (MOA) of mouse liver carcinogenesis with PB, PPZ-induced tumors can be viewed as being promoted by a similar PB-like CAR-dependent MOA.

Keywords

Conazoles; Phenobarbital; Propiconazole; Toxicogenomics; Constitutive androstane receptor

1. Introduction

Toxicogenomics (TGx) is the application of analytical techniques that focus typically on the parallel (control vs. treated) measurement of mRNAs using microarrays to understand and predict toxicity (Currie, 2012). Additional “omic” methodology, e.g., metabolomics and proteomics, which evaluate small molecules and proteins, respectively, are also employed. However, assessment of mRNAs is, arguably, the most frequently used TGx tool. The goals of TGx include: (1) identifying a consistent pattern of gene-expression changes that can serve as surrogate markers for chemical-induced toxicities of concern, and (2), by identifying the gene expression changes preceding toxicity, TGx can be employed to investigate underlying molecular mechanisms and, thus, become an aid to mechanism or mode of action determination. However, the approach taken for a TGx analysis can profoundly affect its outcome. Various methods of analysis can select different genes as differentially expressed. Those that affect sensitivity might also affect the specificity and/or reproducibility of the results. Thus, the process of independent analyses, reproducibility of results, and interpretation using the same dataset are influenced by the methods of analysis employed (Goetz et al., 2011).

The focus of this article is to illustrate this key point through an evaluation and reanalysis, by two independent laboratories, of two papers in the recent literature. One of these evaluated transcriptional profiles in liver from mice treated with hepatotumorigenic and non-hepatotumorigenic conazole fungicides (Ward et al., 2006). The second addressed changes in gene expression in mouse liver following administration of phenobarbital (PB), a classic nongenotoxic rodent liver carcinogen (International Agency for Research on Cancer [IARC] 2001; Whysner et al., 1996), compared with the mouse liver tumorigenic conazoles, propiconazole (PPZ, a crop protection fungicide sold by Syngenta Crop Protection, LLC) and triadimefon (a crop protection fungicide sold by Bayer Crop Science) (Nesnow et al., 2009). PPZ and its comparisons to PB are the focus of this paper. The transcriptomic data set for the hepatotumorigenic conazoles assessed by Nesnow et al., 2009 was first reported by Ward et al., 2006. In comparing the gene expression data for PB and the conazoles, these analyses focused on differences rather than similarities, and Nesnow et al. (2009) concluded that while the conazoles and PB can both cause mouse hepatocarcinogenesis, the alterations

in gene expression indicated that the mode of action (MOA) of the conazoles is different from that of PB. Additional mechanistic data have been published for PPZ at the excessive (i.e., exceeded the maximum tolerated dose) dose level that is tumorigenic in mice (2500 ppm), and to a lesser extent at the non-tumorigenic dose of 500 ppm PPZ, and a resulting postulated mechanism of action for PPZ including molecular initiating events has been summarized (Nesnow, 2013).

It has been proposed that rodent liver carcinogens that exhibit a PB-like MOA are not relevant to humans (Elcombe et al., 2014; Holsapple et al., 2006). Therefore, it was deemed to be a valuable exercise to re-examine the PB and PPZ datasets to illustrate how the approach to TGx data analysis and the design of experiments to generate these data may influence the conclusions obtained. The reanalysis is also instructive with regard to highlighting factors that are important to consider when comparing sets of TGx data produced and reported at different times. Additional studies described here show that the postulated key event of hepatocellular proliferation is observed in CD-1 mice for both PPZ and PB, and that PPZ is also a potent activator of the mouse CAR nuclear receptor.

2. Materials and methods

2.1. Chemicals

Propiconazole (CAS No. 60207-90-1) was obtained from Syngenta AG (Basel, Switzerland) or its predecessor companies, with a purity in the range of 87.2–95.2%. Sodium phenobarbital (Article No. 04710) was obtained from Fluka Chemika AG, Switzerland, with a purity of 99.0%. Clotrimazole (CLOT) was obtained from Sigma (St Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was obtained from the Environmental Chemistry Laboratory at the University of Washington (Seattle, WA). Meclizine was purchased from MP Biomedicals (Solon, OH). All other materials were of reagent grade or higher.

2.2. Treatment in 104-week carcinogenicity study in CD-1 mice

A 104-week carcinogenicity study in mice was conducted as described previously (Dewhurst and Dellarco, 2006). Briefly, groups of 52 male and female CD-1 mice were treated with propiconazole (purity 87.2–91.9%) for two years at dietary concentrations of 0, 100, 500 and 2500 ppm, equivalent to a mean daily intake of 10.0, 49.4 and 344mg/kg/day in males and 10.8, 55.6 and 340 mg/kg/day in females, respectively. Additional satellite groups of 12 individuals were used for laboratory examinations and interim sacrifice after one year of treatment. Survival was monitored during the study, and all surviving mice were sacrificed after 104 weeks. Microscopic examination of tissues from all animals was conducted, and a re-evaluation of the liver by an independent pathologist was performed (Dewhurst and Dellarco, 2006). The 104-week carcinogenicity study was conducted in accordance with US EPA Guideline 832(B) - Oncogenicity, and dose levels were selected to fulfill the US EPA requirements in force at that time, which mandated that the high dose should produce marked toxicity, without resulting in a large decrease in overall survival.

2.3. 1- to 60-Day cell proliferation study in CD-1 mice

Groups of 5 young adult male CD-1 mice (Charles River Germany) each received propiconazole at dietary concentrations of 0,850 or 2500 ppm for 1–60 consecutive days (1, 2, 3, 4, 7, 14, 28 and 60 days). Additional groups of 5 male mice received phenobarbital in the diet at 850 ppm for the same time intervals. The in-life experiment was carried out under standard laboratory conditions. The mice were housed individually in standard cages (macrolon type 2) on soft wood bedding at 22 ± 2 °C, at a relative humidity of 45–65%, and a 12 h light/dark cycle. The animals were acclimatized to laboratory conditions for 11 days.

All mice had free access to tap water via a water bottle. A pelleted standard rodent chow (NAFAG 8900 for GLP, Nafag, Gossau SG, Switzerland) was provided during the treatment period with the respective concentrations of propiconazole and phenobarbital ad libitum. Homogenous blends of propiconazole or phenobarbital were prepared into pelleted diet; the admixtures were stored at room temperature throughout the experimental period. Body weights were recorded daily throughout the study. Two hours before sacrifice, each animal received a single injection (i.p.) of 100 mg/kg body weight bromodeoxyuridine (BrdU) dissolved in 0.9% saline. Prior to scheduled sacrifice, the animals were not fasted overnight. For scheduled sacrifices, all survivors were bled under ether anesthesia. The carcass weight was taken after bleeding. The livers of all animals were quickly removed and weighed.

After weighing the liver, one tissue slice from the left lobe and two from the right medial and lateral liver lobes (3 liver samples per animal) and one sample of the small intestine were taken from all animals. All samples were fixed in neutral buffered 4% formalin, processed for paraffin embedding and mounted in one paraffin block. Serial sections were prepared from paraffin blocks and stained via hematoxylin and eosin or BrdU immunohistochemical staining to detect nuclear incorporation of BrdU. The labeling index (LI) for BrdU-positive hepatocytes was calculated as the percentage of labeled nuclei over the total number of nuclei. Depending on the available section area, between 17,028 and 59,867 hepatocyte nuclei per animal were evaluated.

For body and liver weight, Dunnett's pairwise comparison (Dunnett, 1955) was used to assess differences between treated groups and their respective controls. Mann-Whitney Rank Tests (Mann and Whitney, 1947; Wilcoxon, 1945,1947) were performed to assess differences between treated groups and their respective controls for BrdU labeling indices.

2.4. CAR3 reporter assays with PPZ and model activators

A hallmark feature of CAR that distinguishes it from other nuclear receptors lies in its ligand-independent activation state, one that exhibits high constitutive activity. In the liver or in primary hepatocytes, CAR is typically tethered in a cytoplasmic complex. Following chemical activation, CAR translocates to the cell nucleus thereby free to regulate gene transcriptional responses. (Kawamoto et al., 1999). However, in established cell lines used more standardly in research assays, when CAR is delivered intracellularly by transfection, CAR spontaneously transfers directly to the nucleus. (Swales and Negishi, 2004). To establish a more sensitive method for the detection of CAR modulators, we developed a sensitive reporter assay to test for direct activation of CAR constructs from mouse and

human (Omiecinski et al., 2011). These approaches were used in the current investigation as previously described (Omiecinski et al., 2011). In the human splice variant CAR3, the presence of a 5 amino acid (APYLT) insertion in CAR3 functionally blocks the constitutive activity of CAR, but yet appears to enable the receptor to retain the same ligand binding properties and subsequent receptor activation as the native form of CAR. As described previously, the corresponding amino acids present in human CAR3 were engineered into the mouse CAR receptor and its ligand activation profiles with model substrates have been characterized using a luciferase reporter assay in primate-derived COS-1 cells. A similar assay using the natural CAR3 splice variant of the human receptor has also been tested. The assay involves transactivation following ligand binding to CAR3 to activate a CYP2B6 transcriptional response element fused to a firefly luciferase reporter. The light emissions occur in direct proportion to the strength of the CAR3 promoter activation. The output from the COS-1 cells was measured with a luminometer (Veritas, Turner/Promega) for each experimental condition using a Dual Luciferase Reporter Assay (Promega). Final outputs are expressed as normalized luciferase activity, comparing the firefly luciferase activity to a *Renilla* luciferase activity that is present as a measure of transfection efficiency.

PPZ was evaluated at 1, 3, 10, and 30 μ M concentrations for each construct, including the negative empty vector control. Meclizine was also evaluated at 1, 3, 10 and 30 μ M, as a substrate that had been tested for concentration-response in prior experiments (Omiecinski et al., 2011). DMSO was used as a solvent control. Positive control assays with model direct CAR activators were used at a single concentration. These consisted of CITCO at a concentration of 5 μ M (model substrate for human CAR3), TCPOBOP at a concentration of 0.5 μ M (model substrate for mouse CAR3), and clotrimazole at a concentration of 10 μ M (model substrate for rat and mouse CAR3).

2.5. Toxicogenomics data analysis

The in-life portion of in vivo toxicogenomics studies of the liver were performed as described originally in Ward et al. (Ward et al., 2006) for control and 2500 ppm PPZ treatments of male CD-1 mice, and as described in Nesnow et al. (Nesnow et al. (2009)) for control and 850 ppm PB treatments of male CD-1 mice, plus comparisons between compounds. The 850 ppm phenobarbital and 2500 ppm PPZ expression data along with data for their respective control groups from GSE16777 were downloaded directly from GEO and analyzed using Genedata Analyst 2.2 (Genedata AG, Basel Switzerland). The current analysis focused on the control and treated samples derived from the separate PB and PPZ experiments reported in Nesnow et al. (2009). The triadimefon treated samples were not analyzed. To explore the differences between experiments a series of scatter diagrams were constructed that plot the expression values of the control samples between samples from day 4 and day 30. To identify the major sources of variance in the data a principle component (PC) Analysis was performed using both the control and PPZ or PB treated samples from both experiments.

2.6. Toxicogenomics pathway analyses

The control, PB and PPZ expression data from GSE16777 were analyzed using Rosetta Resolver™ (Centre for Medical Biology Systems, Leiden Univ., Netherlands). Generation

of signature lists of differentially expressed genes (DEGs) was accomplished by 1-way ANOVA with Benjamini Hochberg multiple test correction on the 4-day and 30-day PB- and PPZ-treated expression values against their respective controls. The list of DEGs generated by this process is provided in Appendix 1.

A focused analysis of the DEGs was conducted using Ingenuity Pathway Analysis (IPA) to explore PB and PPZ mouse liver carcinogenesis pathways, based on the major pathways or key events that had been proposed for PPZ by Nesnow et al. (2009) and were further summarized in Nesnow (2013). These major IPA pathways were: CAR/PXR regulated genes, oxidative stress response genes, DNA damage signaling, cell proliferation, lipid homeostasis, retinoic acid (RA) signaling/metabolism, endoplasmic reticulum (ER) stress, cholesterol biosynthesis/metabolism, and apoptosis. An unbiased analysis using IPA canonical pathways analysis of PB and PPZ 4- and 30-day DEG data was also performed. Further methodological details and the full analysis are in Appendix 2.

In a further evaluation of the DEGs derived in Appendix 2, sets of DEGs were subjected to enrichment analysis across the biological ontologies in MetaCore and ToxHunter v.6.3, including Canonical Pathway Maps and GeneGo Processes, as described in Dezso et al., 2008 (Brennan, 2010; Dezso et al., 2008). The hypergeometric distribution was used to evaluate the statistical significance of overlap between DEG sets, or genes comprising particular interaction networks or pathway maps, and components of the ontologies, and to rank them by *p*-value. The hypergeometric distribution was also used to evaluate and rank the significance of connectivity between DEG's and particular transcription factors with multiple-testing correction using false discovery rate filtering (FDR). Transcription factor associations were made using the MetaCore database of confirmed transcription factor targets. The MetaCore database of manually curated information on protein-protein interactions was used to construct directed interaction networks to investigate signaling, metabolism and biological functions relating to DEG's or to specific genes of interest. The Analyze Networks algorithm was used to generate sub-networks enriched with root nodes from DEG lists or genes of interest and rank sub-networks by *p*-value, G-score, and gene ontology processes. Statistical significance for networks was estimated using the *p*-value of the hypergeometric intersection. Further methodological details and the full analysis are in Appendix 3.

3. Results and discussion

3.1. In vivo studies in CD-1 mice

In a 104-week study in CD-1 mice, PPZ caused an increased incidence of hepatocellular adenomas and carcinomas in male mice only at a dose of 2500 ppm that exceeded a Maximum Tolerated Dose (MTD) (Table 1) (United States Environmental Protection Agency [US EPA] 2006a,b). Survival in the 2500 ppm males (25.5%) was statistically significantly lower than the control group, and the cumulative body weight gain was 42% lower than the control values at week 13. In addition, relative liver weights were >2-fold larger than control values at both the interim sacrifice (week 53) and the terminal sacrifice (week 104). Moderate effects on body weight gain and liver weight were observed at 500 ppm, but there was no increase in tumors. Based on statistically significant decreases in

survival, large deficits in body weight gain, and clear liver toxicity, the US Environmental Protection Agency has previously concluded that 2500 ppm PPZ administered to male CD-1 mice is an excessive dose (United States Environmental Protection Agency [US EPA] 2006a,b).

In a 60-day study in male CD-1 mice, a similar response and time-course of cell proliferation in the liver was observed with doses of 850 ppm and 2500 ppm PPZ and with 850 ppm PB (Fig. 1). Cell proliferation as assessed by BrdU incorporation was increased during Days 1–7 of treatment, but was no different from control values from Day 14 onward. Carcass weights and liver weights from this study are summarized in Table 2. The excessive dose of 2500 ppm PPZ caused a statistically significant lower carcass weight on Day 4 (8% lower than controls), whereas carcass weights were unaffected by 850 ppm PPZ and 850 ppm PB. In addition, the absolute and relative liver weights at 2500 ppm were up to 2-fold higher than control values (156–209%). The liver weights following treatment with 850 ppm PPZ or 850 ppm PB were statistically significantly increased (131–162%), and this change was lower in magnitude than at the high dose of PPZ.

3.2. CAR3 reporter assay results

A strong concentration-dependent activation of mouse CAR3 by PPZ was observed from 3 μ M, with up to 40-fold activation of mouse CAR3 (Fig. 2). In contrast, the human CAR3 response was only statistically significant at 30 μ M, the highest dose tested, and this response only represented a 3-fold activation above solvent control. The model activators CITCO and TCPOBOP produced robust responses in human and mouse CAR3 constructs, respectively, and demonstrated the characteristic species-specific responses that were reported previously for these CAR activators (Omiecinski et al., 2011). Clotrimazole produced a modest activation in both mouse and human CAR3. Meclizine also was tested and produced a concentration-dependent response that was much more marked with mouse CAR3 than with human CAR3.

Based on these results, it was concluded that PPZ is a potent activator of mouse CAR. PB is an indirect activator of both mouse and human CAR (Huang et al, 2005; Yamamoto et al., 2004). The CAR3 reporter assay that is described here does not respond to indirect activators of CAR (such as PB), because the second messenger systems that regulate indirect activation are not present in COS-1 cells (Kawamoto et al., 1999; Swales and Negishi, 2004).

3.3. Toxicogenomics comparisons

3.3.1. Comparing experiments performed at different times—An examination of the Methods used to generate the Ward et al. (2006) data (PPZ-treatment for 4 or 30 days) and Nesnow et al. (2009) data (PB-treatment for 4 or 30 days) revealed three key experimental differences. These fundamental differences are depicted in Fig. 3. First, the number of mice (male CD-1) per group used for the PPZ experiment was 3 (Ward et al., 2006), while the number used for the PB experiment was 5 (Nesnow et al., 2009). Second, while all mice were permitted to acclimate for 7 days prior to starting the experiments, the age of the animals used in the two studies was substantially different. Mice used for the PPZ

experiment were 30 days old when received (Ward et al, 2006), whereas the mice used for the PB experiment were 42–56 days old when received (Nesnow et al., 2009). Therefore, the age of the animals at the different sacrifice and liver sampling times (4-day and 30-day) were different, as shown in Fig. 3. Third, different reagents were used for the critical RNA amplification (cDNA synthesis) and biotin labeling steps. Ward et al, 2006 used an Affymetrix kit for RNA amplification (cDNA synthesis) and biotin labeling while Nesnow et al. (2009) used an Enzo BioArray RNA Amplification and Biotin Labeling System (Enzo Life Sciences, Farmingdale, NY).

A stated rationale for comparing the PPZ study performed in 2005 and reported in 2006 (Ward et al., 2006) with the PB study performed 3 years later (Nesnow et al., 2009) was that a comparison of the control groups from each study (based on error-weighted sequence intensities) at each time point showed that the average Pearson product-moment correlation test for PB controls and PPZ controls at 4- and 30-days were $r^2=0.97875$ and 0.9666 , respectively (Nesnow et al., 2009). However, in light of the fact that many thousands of data points were compared in these analyses, one can obtain an r^2 value that appears highly statistically significant even though the data sets actually exhibit major differences. Indeed, an analysis of the control PPZ data compared with control PB data, at both the 4- and 30-day time points revealed substantial differences. A comparison of the control group signal intensities at 4 days (Supplemental Fig. S1) and 30 days (Supplemental Fig. S2) reveals that in both cases the line of best fit differs substantially from the main diagonal. The dynamic range of expression values was lower in the PPZ controls compared to the PB controls at both the 4- and 30-day time points (Supplemental Fig. S3). Additionally, a Principal Component Analysis revealed that the difference between the two experiments' control groups is greater than the differences between a control group and its respective treated group (Supplemental Fig. S4). Furthermore, there might be differences in gene expression between the control groups used for the PPZ experiment and the PB experiment due to the differences in their ages (Fig. 3). Indeed, a difference of 2 weeks in age can make a difference in gene expression in control mice, probably due to changes associated with maturation (Supplemental Fig. S5). These analyses serve to illustrate confounding factors that come into play when comparing sets of gene array data obtained at different times. Considering that differences between the data sets are likely to have contributed markedly to the observed differences in gene expression it is, in our opinion, most appropriate to focus on similarities between the datasets when re-evaluating the results. In cases where confounding experimental design differences exist there is greater certainty associated with results that are similar, because an unknown proportion of the differences observed would be due to the confounding factors. In conclusion, comparing experiments performed at different times with different study designs complicates the ability to discern actual differences in the transcriptomic pathways for PPZ and PB.

3.3.2. Hypothesis generation from the analysis of toxicogenomic data—The analysis of TGx data can be used in two different ways depending on the level of understanding of the causes, consequences and relationships of gene expression changes. When analysing and interpreting TGx data, it is important to clearly distinguish whether you are using the results of pathway analysis for either: (1) hypothesis generation or (2) as

evidence for the operation of previously established regulatory relationships. As an example of this latter use, if a considerable body of experimental evidence provides support to the view that a stimulated transcription factor consistently alters the expression of a number of identified target genes, then TGx measurements of consistent alterations of those target genes provides evidence for the activation of that transcription factor. However, as discussed below, this level of certainty in the relationships between a set of genes and their control pathways is not common, and therefore toxicogenomic analysis usually is of more use for hypothesis generation

It is instructive to reflect on important limitations of gene expression data, i.e., measuring changes in mRNA levels. It is the protein encoded by the gene that carries out its function, and the level of the mRNA is not necessarily directly proportional to the level of the protein (Ideker et al, 2001). Furthermore, posttranslational modification plays a key role in determining the activity of particular proteins (Tate, 2008). Some genes can have many upstream inputs. Most genes do not have established transcriptional regulators. Therefore, usually TGx measurements are used to generate hypotheses for either the cause or consequence of the gene expression changes. These hypotheses rely on a number of implicit assumptions: for instance, that changes in expression of genes associated with particular pathways or biological processes co-occur in a consistent direction, and furthermore, that changes in expression of genes associated with a pathway or process imply a change in signaling through that pathway or an alteration to that process. Other potential regulatory interactions (e.g., feedback inhibition/stimulation) should also be considered as possible causes for any gene expression change, and these might need to be excluded or at least acknowledged as an uncertainty in a TGx analysis. Although these assumptions provide the basis for the interpretation of gene expression data, it is not known if they are valid for all genes, processes and pathways. However, as a hypothesis generating tool, TGx data can provide very useful information, which when used in combination with other experimental approaches, can aid in discerning the MOA of a chemical.

3.3.3. Similarities and differences in three analyses of the PPZ and PB toxicogenomics data—To initiate this re-analysis, we downloaded the gene list from Nesnow et al. (2009) from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/accession> number: GSE16777), and then created a list of differentially expressed genes (DEGs) from the Affymetrix data using 1-way ANOVA ($p < 0.01$) for the PPZ and PB experiments separately. These gene lists are provided in Supplemental Appendix 1, and the details on creation of the DEG lists are provided in Supplemental Appendix 2. The DEGs served as the basis for the subsequent independent analyses by two different analysts (Brennan, 2010; Plummer, 2011). These investigators used two different and commonly used commercial pathway analysis tools: Plummer, 2011 used Ingenuity Pathway Analysis (IPA) and Brennan, 2010 used MetaCore™ incorporating MetaTox™ (GeneGo), currently known as Systems Toxicology Module™ (Thomson Reuters), for biological interpretation of these pathway analyses. In the original analysis of Nesnow et al. (Nesnow et al, 2009), IPA was used to evaluate the canonical pathways that were affected, and the “Compare Experiments” function within GeneGo MetaCore was used to create significant networks from differentially expressed genes.

Table 3 summarizes our comparison of the findings of each analyst plus a summation of Table 3 and 5 from Nesnow et al. (2009), as well as observations made about conazoles in Ward et al. (2006). These results illustrate that, although each analyst started with the Affymetrix data, there were both differences and points of agreement in the major pathways that were affected by PPZ and/or PB. Additionally the same findings were sometimes described by different names due to differences in annotation between the three analysis tools employed. Table 3 provides a comparison using a common set of names. We extracted the salient data from the reports of the two independent analysts, as presented in Table 3, and used this information for the discussion below. However, we also provide access to the full reports (Brennan, 2010; Plummer, 2011) in order to provide readers the opportunity to peruse the full packages and perhaps draw their own conclusions.

For all 3 analysts the most strongly altered DEGs and pathways for both PPZ and PB were those related to CAR/PXR activation. Car- and Pxr-related pathways are illustrated in Appendix 3, Fig. 6 (p. 24) and 7 (p. 25), respectively. These CAR/PXR-responsive genes included large increases in expression of Cyp2b10, Gadd45 β and Cyp2c65. A recent publication that compared full gene expression profiles in wild-type, CAR-null and PXR-null mice treated with model CAR or PXR activators also reported that Gadd45(3 expression is CAR-mediated and strongly induced by a model CAR activator (Tojima et al., 2012). Therefore, the first pattern to emerge is that at the doses tested, PPZ and PB share a common pathway via CAR/PXR activation.

None of the analysts identified evidence of a response to DNA damage in the TGx data.

There was evidence that both PB and PPZ altered genes associated with the cell cycle, and the current analyses replicated the finding reported by Nesnow et al. (2009) that the differentially expressed genes related to the cell cycle were different between PB and PPZ. However, differences at the individual gene level do not necessarily imply that the underlying processes are distinct. The cell cycle is a dynamic progression and the evaluation of one point in time (4 or 30 days) via TGx may reflect an examination of different snapshots of gene changes for PPZ or PB that are part of the same overall continuum of change. Given this backdrop, it is therefore unreasonable to expect two different compounds to produce exactly the same biological effects. Additionally, the systematic experimental confounding factors described above (Fig. 3) might mask our ability to observe changes in common genes related to cell cycle. It should be noted that the International Life Sciences Institute's Health and Environmental Sciences Institute (ISLI/HESI) and the Microarray Quality Control Consortium (MAQC) both recommend pathway-level rather than individual gene analyses when interpreting microarray data (Pennie et al., 2004; Shi et al., 2006). Nevertheless, and importantly, the TGx analysis clearly indicates that both PPZ and PB would be expected to alter cell cycle and proliferation in mouse liver. Indeed, both PB (850 ppm) and PPZ (850 ppm or 2500 ppm) administered in the diet to male CD-1 mice caused a large increase in cell proliferation as measured by bromodeoxyuridine (BrdU) incorporation during days 1–7 of treatment (Fig. 1). The level of BrdU incorporation returned to background levels after 14 days of treatment for both compounds. This result is similar to the transient cell proliferation response reported for PB in earlier studies (Kolaja et al, 1996; Whysner et al., 1996).

In the work of Ward et al. (2006) and Nesnow et al. (2009), the TGx data were used to propose additional mechanistic hypotheses involving alterations in cholesterol metabolism, retinoic acid metabolism and oxidative stress. For these pathways, the results of analyses by Plummer, 2011 (Appendix 2) and Brennan (2010) (Appendix 3) were not completely concordant with one another or with those of Nesnow et al., 2009 (Table 3).

Because PPZ inhibits lanosterol-14 α -demethylase (CYP51) to produce its fungicidal effects, and CYP51 is conserved in mammals as a cholesterol synthetic enzyme, Nesnow et al. (2009) had hypothesized that altered cholesterol metabolism might also play a role in the liver mode of action. One independent analyst, Plummer (2011) (Appendix 2) observed some changes in cholesterol metabolism genes at day 4 with PB and on both day 4 (up regulation like Ward et al, 2006) and day 30 (down regulation, and not reported by Nesnow et al., 2009) with PPZ. In contrast, the second independent analyst, Brennan (2010), (Appendix 3) identified only very limited and inconsistent effects on the cholesterol biosynthetic pathways with both PB and PPZ. In addition, using the TGx data he tested the hypothesis that inhibition of cholesterol biosynthesis and metabolism should alter oxysterol and bile acid levels and therefore systematically change SREBP2 and LXR/FXR signaling. Using the target gene annotations in MetaCore, SREBP2 was not activated by PPZ, and no consistent effects were noted for LXR/FXR target genes. These results were in contrast to the activation of these LXR/FXR pathways as a hallmark of CYP51 inhibition that was postulated to occur for conazoles by Ward et al. (Brennan, 2010; Ward et al., 2006).

Both analysts agreed with Ward et al., 2006 that retinoic acid (RA) metabolism genes were altered by PPZ. In addition Brennan (2010) (Appendix 3) considered that RA metabolism was also altered by PB, similar to the results of Ward et al. (2006) and Nesnow et al. (2009). Plummer (2011) (Appendix 2) concluded that RA metabolism genes were altered by PPZ, but not by PB. The basis of this difference was due to the different weight of evidence assigned to changes in Cyp26a1 alone by Plummer (2011), versus the entirety of the RA metabolism metabolic map in MetaCore by Brennan (2010) (Appendix 3; Fig. 21). The CYP changes in the MetaCore RA metabolism map are principally caused by CAR/PXR-mediated enzyme induction. Again, it is not clear whether the lack of PB effect on Cyp26a1 expression in this case is a genuine treatment-related difference or is the consequence of systematic confounders (Fig. 3).

Ward et al. (2006) highlighted the importance of NRF2 and oxidative stress in the TGx response, and Nesnow et al. (2009) showed consistent effects of NRF2 mediated oxidative stress for PB and PPZ pathways. Again the two independent analysts arrived at different conclusions with regards to the TGx data providing evidence for oxidative stress. By placing particular weight on the AKR1B7 gene change [a homolog in humans has been shown by gene knockdown to be NRF2-dependent (MacLeod et al., 2009)], Plummer (2011) (Appendix 2) considered that NRF2 target genes had been induced and that this provided evidence for oxidative stress. In addition, Plummer (2011) (Appendix 2) noted that upregulation of GSTs, glutathione metabolism, and other genes related to metabolism of reactive intermediates, were considered to be regulated by NRF2. However, Brennan (2010) (Appendix 3) suggested that his analysis provided equivocal evidence for oxidative stress because most of the genes changed (GSTs and glutathione metabolism) have also been

described as CAR/PXR target genes, and as such a robust alternative hypothesis (that already explains many other gene changes within this dataset) also exists. Brennan (2010) (Appendix 3) noted that the GST and glutathione metabolism genes were significantly upregulated by both PB and PPZ, but these were not assigned by GeneGo to NRF2-mediated oxidative stress. Therefore the TGx data can be used to hypothesize that there is increased oxidative stress for both PB and PPZ, but does not provide consistent pathway-based evidence for it.

In the analysis by Nesnow et al. (2009), a common pathway that was significant for PB and PPZ at 30 days, but not at 4 days, was apoptosis signaling. The genes involved in this pathway and the direction of change were not described further. Plummer (2011) (Appendix 2) noted that only a few genes related to apoptosis were altered by PB or PPZ; however, the genes involved were different. PB caused up-regulation of CDC2 (4 days) and tBID (30 days), whereas at 4 days PPZ produced a down-regulation of Bcl-XL and caspase 6 and up-regulation of TNFR. Plummer (2011) (Appendix 2) concluded that it was unclear whether these mRNA expression changes were sufficient to affect hepatocyte apoptosis. Finally, Brennan (2010) (Appendix 3) conducted a more detailed study of PPZ expression data using ToxHunter Toxic Pathology Biomarkers, which indicated dysregulation of midzonal necrosis genes that was fairly unique to the 4-day PPZ treatment. Further interrogation of these probe sets in MetaCore led to a significant number of nodes involved in regulation of apoptosis and cell death. The network was centered around Bcl-XL, a key regulator of apoptosis, which was highly down-regulated, suggesting a pro-apoptotic state in the PPZ-treated liver at 4 days. Overall, the genomic analyses by three different analysts differed somewhat in terms of regulation of apoptosis, but a trend was observed suggesting an increase in apoptosis with PPZ treatment at 4 days. This is discussed further in the next section.

3.3.4. Comparison of excessive and non-excessive dose levels—Toxicity data demonstrated that 2500 ppm PPZ administered to CD-1 mice was a dose that exceeded the Maximum Tolerated Dose, resulting in statistically significant decreases in survival, large deficits in body weight gain, and liver toxicity (United States Environmental Protection Agency [US EPA] 2006a,b). Data from the 104-week study are summarized in Table 1. Survival in the 2500 ppm males (25.5%) was statistically significantly lower than the control group, and the body weight gain was 42% lower than the control values at Week 13. At the dose of PB that was utilized (850 ppm), there is no report of a decrease in body weight (Nesnow et al., 2009; Pepper et al., 2007), or hepatocyte necrosis (Nesnow et al., 2009), and no increased mortality in long-term studies either at this dose or at similar dose levels in mice of various strains (Jones et al., 2009; Whysner et al., 1996). In the 60-day study comparing PPZ and PB (Table 2), a statistically significant decrease in body weight was observed at 2500 ppm PPZ, but not at 850 ppm PPZ or 850 ppm PB. The dose level of 850 ppm produced similar increases in liver weight for PB and PPZ (131–152%), whereas 2500 ppm PPZ resulted in a larger effect on relative liver weight (up to 200%). Thus, the toxicogenomic analyses were conducted at a dose of PPZ that exceeded a maximum tolerated dose, and are compared to results obtained using a dose of PB that was not excessive.

In this context, we proposed that altered expression of stress response genes would be more prominent in the 2500 ppm PPZ samples. Both Plummer (2011) (Appendix 2) and Brennan (2010) (Appendix 3) found evidence for altered endoplasmic reticulum (ER) stress or unfolded-protein stress response that was more noticeable with PPZ treatment than with PB treatment. Plummer (2011) (Appendix 2) described up-regulation of HSPA5 (BiP), PERK, and IRE1 with PPZ treatment at 4 days, but no effect on these markers of ER stress with PB at 4 days. However, IRE1 was up-regulated by both PB and PPZ at 30 days. Brennan (2010) (Appendix 3) expanded upon the ToxHunter Toxic Pathology Biomarkers analysis for 4-day PPZ treatment and described a unique pattern of up-regulation of heat-shock proteins (HSPs), a likely signal of response to ER stress. Significant responses were observed in HSP70, HSP90, and the stress signaling sensors IRE1 and ATF-6, which activate the chaperone system and initiate the unfolded protein response. However, in the 30-day data of Brennan (2010) (Appendix 3), PB produced significant differences in the pathways “Protein folding ER and cytoplasm” and “Protein folding Response to unfolded protein”. In the analysis of Nesnow et al. (2009), a significant pathway that was common to PB and PPZ at both 4 days and 30 days was “Endoplasmic reticulum stress pathway”. No further elaboration on the genes involved in this pathway or the comparative response of PB and PPZ was included. Overall, the TGx data can be used to hypothesize that there are increased ER stress responses for PPZ at an early timepoint of 4 days, which also matches an increase in pro-apoptotic gene signals and greater hepatocyte toxicity in vivo with PPZ than with PB at this time point. However, the change in Gadd45 β gene expression showed a large and consistent up-regulation with PB treatment at 4 days (27.5-fold) and with PPZ treatment at 4 days and 30 days (22.4 and 43.1-fold), which provides evidence of a suppression of apoptosis within the liver. In addition, pathway analysis by different analysts identified increases in ER stress signals at 30 days for both PB and PPZ.

In summary, the TGx analysis of PPZ at an excessive dose level of 2500 ppm and of PB at a non-excessive dose of 850 ppm appears to be reflected in a greater tendency toward ER stress and possibly altered apoptosis with PPZ at the 4-day and/or 30-day time interval. Some evidence for these pathways in the PB data was observed, but the exact nature of the differences between PB and PPZ in these pathways is complicated by the different dose levels, the specific pathway analysis tools used, and the experimental design differences between the PB and PPZ studies.

4. Conclusion

There are major experimental design and analysis differences (Fig. 3) between the Ward et al. (2006) and Nesnow et al. (2009) publications involving a TGx evaluation of liver profiles for PPZ and PB. These differences, as well as the use of an excessive dose of 2500 ppm for PPZ, are expected to contribute markedly to the observed incongruities in the results of pathway analysis for these compounds. The reanalysis provided in this article highlights factors that need to be considered when comparing sets of TGx data generated at different times, and serves as a good test case for a reanalysis by separate analysts acting independently of each other (Brennan, 2010; Plummer, 2011).

While there are some differences between analysts and compounds in the degree of response for specific pathways or processes, when the gene expression data are viewed in the context of signaling pathways and cell processes affected (Table 3), there is a considerable degree of similarity between PB and PPZ and, importantly, a high degree of congruence between Nesnow et al. (2009), Plummer (2011) and Brennan (2010) regarding PPZ exhibiting PB-like effects. While there are some differences between the results presented in Table 3 regarding the “Cell Cycle/Cell Proliferation” category, it is noteworthy that all three of the analyses indicated effects in pathways associated with cell cycle/cell proliferation. The degree of similarity is actually quite remarkable when one considers that no two compounds, especially those as structurally different as PB and PZ, are expected to produce identical biological effects. It is especially instructive to note that PB and PPZ exhibit similar key events with regard to the consequences of CAR/PXR signaling, namely Cyp induction and cell cycle/proliferation (Table 3). In addition and consistent with these gene expression changes, experiments described here with PPZ have shown it to be a potent direct activator of mouse CAR (Fig. 2) and able to produce a transient increase in cell proliferation in CD-1 mice that was parallel to the response seen with PB (Fig. 1). In this context, it is reasonable to highlight the fact that the short-term liver effects of Cyp2b10 induction, increased liver weight and cell proliferation for cyproconazole (a triazole fungicide that also causes liver tumors in CD-1 mice) have been shown to be CAR-dependent and similar to PB (Peffer et al., 2007). Thus, with regard to the events which are hallmarks of CAR-induced effects that are key events in the MOA of mouse liver carcinogenesis with PB (Elcombe et al., 2014; Holsapple et al., 2006; Lake, 2009), PPZ-induced tumors can be viewed as being promoted by a similar PB-like CAR-dependent MOA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2014.03.003>.

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Fig. 1. Time-course of hepatocellular proliferation response to dietary treatment with propiconazole (850 and 2500 ppm) or phenobarbital (850 ppm) in male CD-1 mice. Values are mean $\pm$ standard error. BrdU labeling index values were statistically significantly higher than the control value on Days 1–4 (850 ppm propiconazole) or Days 1–7 (2500 ppm propiconazole, 850 ppm phenobarbital) by Mann-Whitney rank test ($p < 0.05$) and then similar to controls thereafter.

Fig. 2.

Mouse CAR3 and human CAR3 reporter assay results with PPZ and model activators values are mean±standard deviation ($n = 4$) for the normalized firefly luciferase activity relative to *Renilla* luciferase activity. Concentrations are in μM . * $p < 0.01$, Dunnett's test.

Fig. 3.

An examination of the experimental design employed to generate the gene expression data following treatment of mice for 4- or 30-days with propiconazole (PPZ) (Ward et al., 2006) or phenobarbital (PB) (Nesnow et al., 2009). Three key experimental differences affect the ability to draw precise conclusions from a comparison of the PPZ and PB gene expression data: (1) the number of mice per experimental group differed; (2) the age of the mice differed; and (3) the preparation and processing of RNA was different. These points are elaborated upon in the text.

Liver tumor incidence and evidence of excessive dosing at the high dose in a 2-year carcinogenicity study with propiconazole in male CD-1 mice.

Table 1

Males	Dietary level (ppm)				
	0	100	500	2500	
Survival	Week 104	45.3%	37.7%	41.2%	25.5%*
Cumulative Body wt. gain - grams (% control)	Week 13	14.78	13.97	12.95**	8.55** (58%)
	Week 52	20.29	19.29	18.45*	13.87** (68%)
	Week 53	5.1	5.4	6.3*	11.2** (220%)
Relative liver wt. (%) (% of control)	Week 104	6.9	6.7	7.3	18.2** (264%)
<i>Micropathology - liver neoplastic findings</i>					
Adenoma		12/64^	7/64	13/62	23/62*
Carcinoma		16/64^	9/64	13/62	25/62
Adenoma and carcinoma		28/64^	16/64*	26/62	48/62**

Mean values or total incidence/total animals examined (for micropathology) are shown.

Survival: * $p < 0.05$ (Cox's test).

Cumulative body weight gain and relative liver wt. (%): * $p < 0.05$, Student's t -test. ** $p < 0.01$, Student's t -test.

Micropathology: Positive trend by Peto trend test (Groups 1-4).

* $p < 0.05$, pairwise Fisher's Exact test, ** $p < 0.01$, pairwise Fisher's Exact test.

Table 2

Carcass weights and liver weights of male cd-1 mice following dietary treatment with propiconazole or phenobarbital for up to 60 days.

	Day	Control	Propiconazole 850 ppm	Propiconazole 2500 ppm	Phenobarbital 850 ppm
Carcass weight (g) (including liver)	4	35.54 ± 2.06	36.44 ± 1.46(103%)	32.58 ± 1.65* (92%)	37.64 ± 1.34(106%)
	60	39.22 ± 2.69	40.92 ± 0.88 (104%)	41.00 ± 1.28 (105%)	39.32 ± 1.50(100%)
Liver weight (g)	4	1.96 ± 0.14	2.67 ± 0.30** (136%)	3.06 ± 0.08** (156%)	3.18 ± 0.22** (162%)
	60	2.24 ± 0.29	3.06 ± 0.17** (137%)	4.67 ± 0.20** (209%)	3.27 ± 0.30** (146%)
Relative liver weight (%)	4	5.54 ± 0.55	7.33 ± 0.67** (132%)	9.42 ± 0.64** (170%)	8.44 ± 0.37** (152%)
	60	5.70 ± 0.59	7.48 ± 0.32** (131%)	11.39 ± 0.23** (200%)	8.32 ± 0.63** (146%)

Values are mean (% of control) ± st. dev. in grams, or as % of carcass wt. for relative liver weight; five animals per group.

* $p < 0.05$, Dunnett's test.

** $p < 0.01$, Dunnett's test.

Table 3
Re-evaluation of toxicogenomic pathways following phenobarbital or propiconazole treatment.

Pathway analysis software: Pathway or process	Nesnow et al. (2009) and Ward et al., (2006)			Plummer, (2011) (Appendix 2)			Brennan, (2010) (Appendix 3)		
	Ingenuity pathways analysis and MetaCore			Ingenuity pathway analysis			MetaCore		
	PB	PPZ		PB	PPZ		PB	PPZ	
CAR/PXR signaling ^a	Yes	Yes		Yes	Yes		Yes	Yes	
DNA damage response	No	No		No	No		No	No	
Cell cycle/proliferation	Yes, Day 4 and 30 limited overlap of genes between PB and PPZ	Yes, Day 4 and 30 limited overlap of genes between PB and PPZ		Yes genes different from PPZ	Yes genes different from PB		Yes genes different from PB and many genes from Nesnow et al. "Cell Cycle" were not DEGs in our analysis	Yes genes different from PB and many genes from Nesnow et al. "Cell Cycle" were not DEGs in our analysis	
Cholesterol pathway	No	Yes, Day 4		Some changes Day 4	Several gene changes, Day 4 and Day 30		Minimal changes	Minimal changes	
Retinoic acid pathway	Yes retinol metabolism Day 4 and Day 30	Yes; RAR activation Day 30 only and retinol metabolism Day 4 and Day 30		No, based on CYP26a1	Yes, based on CYP26a1		Yes; based on RA metabolism pathway map, but no effect on RA signaling, likely CAR-mediated	Yes; based on RA metabolism pathway map, but no effect on RA signaling, likely CAR-mediated	
Oxidative Stress	Yes NRF2 canonical pathway and Tox pathway	Yes, NRF2 canonical pathway and Tox pathway		Yes, NRF2 genes	Yes, NRF2 genes		Weak signal, GST and GSH metabolism genes	Weak signal, GST & GSH metabolism genes	
Apoptosis	Yes Day 30	Yes Day 30		Yes minor changes e.g. intBID	Yes some changes e.g. Bcl-XL and Caspase-6		No	Yes Pro-apoptotic necrosis biomarkers centered on Bcl-XL network	
Endoplasmic Reticulum Stress	Yes, 4 and 30 days	Yes, 4 and 30 days		No evidence at 4 days; weak signal via IRE1 at 30 days	Yes ER-stress sensors and heat shock protein increased		No evidence at 4 days; protein folding pathway altered at 30 days	Yes ER-stress sensors and heat shock protein induction	

^aBased on the IPA analysis by Plummer (2011), there are 15 differentially expressed genes related to CAR/PXR activation in at least one of the two PB time points and one of the two PPZ time points, with a fold change of at least |1.5|: ABCC3, AHR, ALDH1A7, Cyp1a2, Cyp2a5, Cyp2b10, Cyp2c37, Cyp2c65, GSTA5 (human ortholog, refer to Appendix 2), GSTM5, POR, DIO1, SUL1T1D1, ALAS1, and GSTM1.