Profiling the Hepatic Effects of Flutamide in Rats: A Microarray Comparison with Classical AhR Ligands and Atypical CYP1A Inducers

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Microarray Analysis of Flutamide Hepatic Effects

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Abbreviations used in this paper are:

% LW/BW, percent of liver weight to body weight ratio; ADME, absorption distribution metabolism and distribution; AhR, aryl hydrocarbon receptor;

AhRE, aryl hydrocarbon response element; Alas, aminolevulinic acid synthase;
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ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; ARNT, aryl hydrocarbon receptor nuclear translocator; AST, aspartate aminotransferase; CAR, constitutive androstane receptor; ED50, effective dose for 50% of maximal response; GST, glutathione-S-transferase; HAH, halogenated aromatic hydrocarbon; NQO, NAD(P)H:quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon; SAhRM, selective aryl hydrocarbon receptor modulator; TCDD, 2,3,7,8-tetrachloro-dibenzo-dioxin; XRE, xenobiotic response element.
Abstract

The anti-androgen flutamide (FLU) is used primarily for prostate cancer and is an idiosyncratic hepatotoxicant that sometimes causes severe liver problems. To investigate FLU’s overt hepatic effects, especially on inducible drug clearance-related gene networks, FLU’s hepatic gene expression profile was examined in female Sprague-Dawley rats using ~22,500 oligonucleotide microarrays. Rats were dosed daily for three days with FLU at 500, 250, 62.5, 31.3, and 15.6 mg/kg/day, and hepatic RNA was isolated. FLU resulted in the dose dependent regulation of ~350 genes. Employing a gene response compendium, FLU was compared to three classical aryl hydrocarbon receptor (AhR) ligands, 3-methylcholanthrene (3MC), benzo[a]pyrene (BAP), and beta-naphthoflavone (BNF), and four atypical CYP1A inducers, indole-3-carbinol (I3C), omeprazole (OME), chlorpromazine (CPZ), and clotrimazole (CLO). The FLU gene response was comparable to classical AhR ligands across a signature AhR ligand gene set that included CYP1A1 and other members of the AhR gene battery. Dose-related responses of CYP1 genes established a maximum response ceiling and discerned potency differences in atypical inducers. FLU had a sharp down-regulation of c-fos which was comparable to all compounds except CPZ and CLO. FLU ADME gene expression analysis revealed that FLU as well as I3C and OME induced CYP2B and CYP3A, distinguishing them from the classical AhR ligands. By using a compendium of gene expression profiles, FLU was shown to signal in rats similar to an AhR activator with additional CYP2B and CYP3A effects that most resembled the ADME gene expression pattern of the atypical CYP1A inducers I3C and OME.
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Introduction

Flutamide (FLU) is an oral, non-steroidal anti-androgen used primarily to treat prostate cancer. Despite efficacy, there is concern about idiosyncratic hepatotoxicity, which has an incidence of below 1% to almost 10% (Wysowski and Fourcroy, 1996; Cetin et al., 1999). FLU idiosyncratic hepatotoxicity can result in cholestasis, jaundice and liver necrosis, and has resulted in liver transplant and death (Thole et al., 2004).

Although idiosyncratic hepatotoxicity is a human-specific event, not detectable in experimental animals, many drugs with this problem are known to form reactive metabolites, are metabolized by cytochrome P450 (CYP) enzymes, and are often CYP inducers (Li 2002). We have therefore used microarrays to study FLU’s overt hepatic effects, especially on inducible drug clearance-related gene networks.

FLU was recently shown to be an activator of the aryl hydrocarbon receptor (AhR) in vivo in rats (Hu et al., 2005). The AhR is a cytosolic receptor that is activated by ligand-dependent or ligand-independent mechanisms. In the ligand-dependent mechanism, a xenobiotic ligand enters the cell and binds to AhR, causing it to dissociate from its chaperone protein complex of HSP90 and XAP2, and migrate to the nucleus, where it binds to the aryl hydrocarbon nuclear translocator (ARNT). This heterodimer functions as a transcription factor for target genes that contain the aryl hydrocarbon regulatory element (AhRE, a.k.a. xenobiotic response element (XRE)) in their enhancer region. Responsive genes are collectively known as the AhR gene battery and include the Phase I drug metabolizing enzymes CYP1A1, CYP1A2, CYP1B1, aldehyde dehydrogenase (ALDH3A1), NAD(P)H:quinone oxidoreductase (Nqo1) and the Phase II enzymes glutathione-S-transferase Ya (Gsta1, Ya) and UDP-glucuronosyl transferase.
The AhR is believed to have a binding pocket that accommodates hydrophobic aromatic compounds that contain at least two aromatic rings and can adopt a planar confirmation (Safe, 1995). These “classical” AhR ligands are potent activators and include halogenated aromatic hydrocarbons (HAHs) like the environmental pollutant 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD) and polyaromatic hydrocarbons (PAHs) like 3-methylcholanthrene (3MC), benzo[a]pyrene (BAP), and beta-naphthoflavone (BNF) (Denison and Nagy, 2003).

The ligand-independent pathway is best characterized for the benzimidazole, omeprazole (OME). A gastric proton pump inhibitor, OME results in a CYP1A1 up-regulation in human hepatocytes (Diaz et al., 1990) but is incapable of displacing [3H] TCDD from the AhR, despite the use of non-saturating TCDD concentrations (Backlund and Ingelman-Sundberg, 2004). Rather than binding to the AhR, OME is believed to activate a tyrosine kinase signal transduction pathway that translocates AhR into the nucleus, leading to the induction of AhRE-bearing target genes (Lesca et al., 1995; Backlund and Ingelman-Sundberg, 2005). There is still some debate over the existence of this tyrosine kinase pathway, and some argue that an OME metabolite may serve as an AhR ligand (Delescluse et al., 2000). In any event, other imidazoles induce CYP1A1, including thiabendazole (Aix et al., 1994), lansoprazole (Kikuchi et al., 1996), and clotrimazole (CLO) (Navas et al., 2004).

FLU, an isopropylanilide with nitro and trifluoromethyl substituents, is structurally distinct from classical AhR ligands and benzylimidazoles (Figure 1). Some argue that the AhR harbors a promiscuous ligand binding site, perhaps explaining its activation by a diverse array of endogenous and exogenous molecules (Denison and
Nagy, 2003). The AhR can be activated by “non-classical” ligands, whose structure and physicochemical properties differ from the classical HAHs and PAHs, including flavonoids and indole derivatives like the plant dietary constituent, indole-3-carbinol (I3C). Even single-ring aromatic compounds, like some phenylhydrazine analogs (Nagy et al., 2002) and a nitroaniline (Degawa et al., 1998), activate the AhR.

We have used gene expression profiling to elucidate the role of AhR activation in FLU hepatic effects in rats in vivo. FLU-induced changes in hepatic gene expression were compared to those of the classical AhR ligands 3MC, BNF, and BAP; the AhR ligand I3C; the putative tyrosine kinase activator OME; and two atypical CYP1A inducers, chlorpromazine (CPZ) and CLO. Our goals were to: 1) Identify AhR ligand responsive genes, 2) Compare FLU and atypical CYP1A inducers to AhR ligands, and 3) Relate FLU’s gene expression profile to hepatic homeostasis, metabolism, and toxicity.


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**Materials and Methods:**

**Rat in vivo study.** Female Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were allowed to acclimate for approximately one week under a twelve hour light-day cycle during which they were fed a specified amount of chow and supplied water *ad libitum*. At approximately eight weeks of age, rats weighing between 140-215 g were selected for study and were dosed at one animal per dose level according to the paradigm developed by Meador et al. (2002), where a log2 dose escalation of six doses is sufficient to capture general trends related to dose elevation. Compounds were obtained from Sigma/Fluka (St Louis MI) or Aldrich (Milwaukee WI) as follows: 3MC (catalog # 213942, Aldrich); BAP (catalog # B10080, Sigma); BNF (catalog # 70415-5G, Fluka/Sigma); FLU (catalog # F-9397, Sigma); I3C (catalog # I7256, Sigma); OME (catalog # O104, Sigma); CLO (catalog # C6019 Sigma); and CPZ (catalog # C0982-5G, Sigma). Compound doses were chosen based primarily on the RTECS database (Registry of Toxic Effects of Chemical Substances (http://www.cdc.gov/niosh/rtecs/default.html)) and used at an upper dose that was approximately 50-75% of the single dose LD50 by the same route. When RTECS data was not available, the highest known non-lethal *in vivo* doses were derived from the literature as follows: 3MC (Waring et al., 2001); BAP (Environmental Protection Agency, 1986); BNF (Brady et al., 2002); FLU (Robbins GR, 1971); I3C (Brady et al., 2002); OME (Kashfi et al., 1995); CLO (Meneses-Lorente et al., 2003); and CPZ (Wei et al., 2002).

Dosing regimens were as follows: 3MC (125, 62.5, 31.3, 15.6, 7.8, and 3.9 mg/kg/day (MKD)); BAP (31.3, 15.6, 7.8, 3.9, 1.9, and 1.0 MKD); BNF (250, 125, 62.5, 31.3, 15.6, and 7.8 MKD); FLU (500, 250, 125, 62.5, 31.3, and 15.6 MKD); I3C (250, 125, 62.5, 31.3, 15.6,
and 7.8 MKD); OME (500, 250, 125, 62.5, 31.3, and 15.6 MKD); CLO (250, 125, 62.5, 31.3, 15.6, and 7.8 MKD), and CPZ (125, 62.5, 31.3, 15.6, 7.8, and 3.9 MKD). All compounds were administered to rats in corn oil by oral gavage once daily in the morning for three days. Vehicle control animals (N=3) were treated in similar fashion using corn oil only. Compounds were dosed in two separate protocols with their own vehicle controls (protocol 1: 3MC, BAP, BNF, FLU, I3C, OME, and protocol 2: CLO and CPZ).

At 6-9 h after the third daily dose, all animals were euthanatized with isoflurane, weighed, and necropsied. Blood was drawn from the vena cava and used to measure the following clinical chemistry markers: Albumin (g/dL), Albumin/Globulin Ratio, Alkaline Phosphatase (U/L), alanine aminotransferase (ALT) (U/L), aspartate aminotransferase (AST) (U/L), Bile Acids (µmol/L), Total Bilirubin (mg/dL), Calcium (mg/dL), Chloride (mEq/L), Cholesterol (mg/dL), Creatine Kinase (U/L), Creatinine (mg/dL), γ-Glutamyl Transferase (U/L), Globulin (g/dL), Glucose (mg/dL), Phosphorus (mg/dL), Potassium (mEq/L), Sodium (mEq/L), Sorbital dehydrogenase (U/L), Total Protein (g/dL), Triglycerides (mg/dL), and Urea Nitrogen (mg/dL).

Following exsanguination, the liver was excised and weighed. The left lateral lobe of the liver was removed and snap-cap frozen at -70°C. Rank order estimates were derived for hypertrophy by the magnitude of increase in liver weight/body weight ratio by all compounds at a common dose of 31.3 MKD.

All in vivo work as well as the RNA extraction procedure (below) was performed at MPI Research (Mattawan, MI). MPI Research is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). This animal study was approved by both MPI’s and Merck Research Laboratories’ Institutional
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Animal Care and Use Committees (IACUC) and conducted following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

RNA Isolation. Total RNA was isolated by following the protocol for the TRIzol™ Reagent (Invitrogen Life Technologies, Carlsbad, CA). Liver tissue was homogenized in TRIzol™ Reagent (1mL solution per 100mg liver tissue) using a Polytron homogenizer followed by a chloroform extraction. Isolated RNA was subsequently purified using the RNeasy™ RNA Prep Kit (Qiagen Inc., Valencia, CA) and DNAse treated. RNA quantity and purity were assessed using a SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA), and RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). One of three vehicle control animals and the 125 MKD FLU animal failed RNA QC in protocol 1 and were not hybridized.

Expression Profiling. Cy3 and Cy5-labeled cRNA was prepared from treated and control total RNA through cDNA synthesis and in vitro transcription (Hughes et al., 2001). Labeled RNA from compound treatments and from individual vehicle controls was hybridized with fluor reversal on ~22,500 gene Agilent rat oligonucleotide microarrays (Agilent Technologies, Palo Alto, CA) against a RNA-mass-balanced pool made from vehicle-dosed control animals (N=2 in protocol 1 and N=3 in protocol 2). This pair-wise analysis both of treatment and individual vehicle controls to a pool of vehicle treatments on the
same microarray minimizes the potential for significant hepatic gene expression
due to corn oil to appear in the compound specific gene expression profiles.
Genes that are significantly variable in control animals are removed as part of the
gene expression profile extraction process. The array pattern for the microarray
is found on NCBI’s Gene Expression Omnibus
(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL3563), which details
the rat oligonucleotide probe identifier information for gene expression data
browsing, query, and retrieval.

Arrays were scanned using a DNA microarray scanner (Model G2565AA,
Agilent Technologies), and feature intensities were subtracted from background using
QHyb software (Rosetta Biosoftware, an independent business unit of Rosetta
Inpharmatics, LLC, Seattle, WA).

To ensure feature quality or spot success rate, 90% or greater of spots passed a
metrics test. Ratios were reproducible within an intrachip standard deviation of the
log_{10} (ratio) < 0.0607 and a fluor reverse pair standard deviation of the log_{10} (ratio) <
0.0792. The mean observed intrachip ratio assessed ratio accuracy and was not biased
by more than 50% of expected ratio. Spiked cRNA transcripts of known concentrations
were used to ensure ratio sensitivity across the array chip and considered successful
when at least half of these transcripts met their expected ratio within 50%. Gene
expression changes relative to the vehicle are expressed as log ratio data with a range of
-2 to +2 (-100 to +100 fold). Thus a log ratio of -1 or +1 corresponds to a ±10 fold change
relative to vehicle control. Similarly, a log ratio of -0.3 to +0.3 corresponds to a ± 2 fold change relative to vehicle control.

**Gene Expression Analysis.** Data analysis was performed using the Rosetta Resolver Version 5.0 software (Rosetta Biosoftware, Seattle, WA). Drug responsive genes (p < 0.01) were selected using ANOVA analysis and MATLAB-based in-house statistical analysis tools. Pathway analysis was done using Ingenuity Software (Ingenuity™ Systems, Mountain View, CA), a literature curated application that relates gene expression array data to relevant biological pathways.

In order to apply molecular profiling to *in vivo* toxicogenomic prediction of hepatic liabilities early in drug discovery, we adapted the compound-sparing and animal-sparing ascending lethal dose toxicity study design described by Meador et al. (2002) to this study. Unlike conventional study designs that typically use multiple animals per dose group and a limited number of dose groups, this design uses only one animal for each dose in an ascending 6 dose (log2) dose escalation. Commonly used statistical methods that require replication, such as the t-test or ANOVA test, cannot be used to distinguish treatment effects from random variance in expression within the expression profiles. However, we propose that various statistical tests for dose trends in genes that are significantly regulated at p<0.01 relative to a vehicle control over the six animals identifies genes significantly regulated by each compound (He et al., unpublished). A union approach was taken using the following battery of tests: a test for gene expression changes that trend with dose (a correlation test); a test for gene expression changes that do not trend with dose to capture genes saturating at lower
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doses (an ANOVA test); a test for gene expression changes that trend with clinical chemistry measurements (a correlation test); and a test for irregular gene expression either by a two-population test (Kolmogorov-Smirnov test) or by an enrichment of signature genes in the treated group (a hyper-geometric test). A given gene was declared significantly regulated by a compound if any one of p-values resulting from the above combination of tests was less than 0.01. Individual compound gene expression profiles were compared and a consensus signature for AhR ligands was derived from the intersection of four ligands (3MC, BAP, BNF and I3C). Atypical ligands such as FLU were then compared by overlap to the consensus signature.

Relative ligand potency was ranked for some bellwether genes that had a log linear dose response and a definable maximum response. Ranking was based on the dose that first attained the visually estimated maximum response.
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Results:

*Dose related effects on clinical chemistry and liver weight*

Compound treated rats were dosed in singlicate over a 6 dose log2 dose escalation for three days before necropsy, and clinical chemistry analysis were performed. Clinical chemistry data are included in the supplementary tables. ALT levels, as an indicator of liver damage, were mostly within the range of vehicle controls and displayed no obvious dose-dependent relationship for any compound. CLO (31.3 MKD, a mid-range dose) was an apparent outlier with the highest rise in ALT at 66 U/L, which was approximately 4-fold greater than the upper limit of normal in control treated animals (17 U/L). 3MC and BAP are known to perturb clinical chemistry and/or liver histopathology in rats at time points longer than the three day dose schedule employed here (Knuckles et al., 2001; Kondraganti et al., 2005).

The most consistent effect of most of these inducers, including FLU, was liver hypertrophy. Dose related increases in the percent of liver weight to body weight ratio (% LW/BW) were observed in all compounds except I3C and CPZ when compared to the mean of six vehicle control rats (3.2 ± 0.2) (Figure 2). Potent AhR ligands are known to increase liver weight (Knuckles et al., 2001; Waring et al., 2001; Waring et al., 2002), and all three classical AhR ligands demonstrated a modest rise in % LW/BW, although due to the singlicate design, significance was not measured. CLO (250 MKD) resulted in the largest increase of % LW/BW at 5.2, a 62% increase from vehicle control animals. FLU (500 MKD) led to a % LW/BW of 4.7, a 44% increase. At a common dose of 31.3 MKD, relative potency for weight gain effects, based on a singlicate observation, was approximately CLO, FLU, BAP, 3MC > BNF, I3C, OME, CPZ. Liver weight gain may in
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part be explained by an expansion of the endoplasmic reticulum due to enzyme induction.

**FLU dose responsive genes and their functional classification**

Dose responsive genes due to FLU treatment in rat across five doses were identified. At a p value threshold of < 0.01, 343 genes were significantly regulated for FLU treatment compared to three corn oil vehicle controls. These genes were agglomeratively clustered using a Cosine correlation in Figure 3, where yellow and blue represent up and down-regulated genes with color saturation at +10 or -10-fold, respectively. The highest FLU dose (500 MKD) led to an up-regulation of 179 genes and a down-regulation of 164 genes. Fold change data for annotated genes of FLU’s 343 gene set are included in a supplemental table. Functional analysis of FLU’s genes was performed using Ingenuity, an application capable of identifying literature curated biological pathways related to gene expression changes (Table 1). The major cell pathways associated with FLU gene expression are related to control of gene expression, cell signaling, cell compromise, inflammation, and cellular growth and proliferation, and may be associated with the liver weight gain induced by FLU.

**Comparison of AhR ligand responsive genes to FLU and atypical CYP1A inducers**

In order to investigate FLU as an AhR activator, genes responsive to AhR ligand activation were identified. Responsive genes were determined for all classical AhR ligands and atypical CYP1A inducers at a p value threshold of < 0.01. At the doses employed, changes were greatest for CLO (721 genes) and smallest for CPZ (49 genes).
3MC, compared to the other classical AhR ligands, had the greatest effect on gene expression with 604 responsive genes. The dose range chosen for each compound may influence the extent of these gene changes. The Venn diagrams in Figure 4 display how an AhR ligand responsive gene set was determined by using each compound’s set of dose responsive genes. The gene overlap of the three classical AhR Ligands 3MC, BAP, and BNF yielded 100 commonly regulated genes, collectively called the “Planar AhR Ligand Consensus Set”. Fold change data for the annotated genes of the AhR Ligand Consensus across each compound’s highest dose is found in a supplemental table. The overlap of this consensus set upon the non-classical AhR ligand I3C’s profile reduced the Planar AhR Ligand Consensus Set to 40 genes, collectively called the “AhR Ligand Consensus Set”. Gene expression profiles of FLU and the remaining atypical CYP1A inducers were compared to the AhR Ligand Consensus. FLU and OME had a greater ratio of gene overlap than CPZ or CLO. The gene expression profile of CPZ over the dose range 3.9-125 MKD was very small and may in part account for the limited overlap.

The 40 gene AhR Ligand Consensus Set contained 15 annotated genes, presented as a heat map in Figure 5. The subset includes members of the AhR gene battery (CYP1A1, CYP1A2, CYP1B1, NQO1, and UGT1A6) as well as other ADME-related genes (CYP3A11 (a.k.a. CYP3A2, a growth hormone responsive, male-specific gene (Thangavel et al., 2006)), and GSTM2). The dose response of FLU and the atypicals OME and I3C are remarkably consistent with the classical AhR ligands and clearly indicates relative potency. The other two atypicals, CPZ and CLO, are less potent, with CYP1A response only at the highest dose, and no down-regulation of the upper clade comprised of Chn1,
Cyr61, Nr4a1, c-fos, and CYP3A11 (CYP3A2). Table 2 displays the log ratio data for the
15 annotated genes at each compound’s highest dose.

Log dose response curves for selected genes compare the relative in vivo potency of compounds for AhR responsive genes (Figure 6). For the classical AhR ligands 3MC, BNF, and BAP and for FLU, CYP1A1, the bellwether AhR-responsive gene, has a large fold up-regulation and is saturated for 3MC and BNF at the lowest dose. It is important to note that some ADME gene responses saturate at the lowest dose for some ligands. Many other genes were on scale through the dose range, and many did not respond at all. Thus, no perfect dose range exists in this 6 dose log2 dose escalation that will capture a dose response for all genes.

The apparent maximum fold up-regulation of CYP1A1, an indicator of intrinsic activity based on our log dose response plot, is about 30-40 fold (log ratio 1.5-1.6). BAP increases CYP1A1 expression in a log linear fashion to achieve the maximum fold change at its top dose of 31.3 MKD. I3C and OME dose response curves are shifted to the right; however, these less potent AhR activators still resemble full agonists by attaining a near maximal response.

A relative in vivo potency can be very roughly estimated from this simple singlicate experimental design, where log linear data can be used to extract the effective dose for 50% of maximal response (ED50) of approximately 100 MKD for OME. Defining the potency of AhR ligands as an ED50 from singlicate data, based on bellwether AhR gene dose responses, is an approximation made possible only when dose responses of AhR responsive ADME genes were log linear with a definable maximum fold change (as for omeprazole and CYP1A1). In Figure 6, the 31.3 MKD I3C-
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dosed animal illustrates that data are not always so well behaved in the singlicate dose escalation design, and as such, visually extracted relative measures of potency are perhaps best. CYP1A1 induction rank order potency is established as: 3MC, BAP, BNF > FLU, I3C > OME > CPZ, CLO. This rank order remains relatively consistent for the other genes of the AhR gene battery (CYP1A2, CYP1B1, and UGT1A6) and for Alas2. OME is unique in its CYP1B1 response where it resembles a partial agonist by saturating at approximately 2-fold.

FLU, I3C, OME, and the classical AhR ligands all cause a sharp down-regulation of the transcription factor c-fos, which distinguishes these compounds from the atypical CYP1A inducers CPZ and CLO.

FLU ADME gene expression and evidence for the participation of other orphan nuclear receptors

ADME genes found in FLU’s 343 gene expression profile were extracted and clustered on a heat map against all compounds at all dose levels (Figure 7). FLU along with I3C and OME were clearly similar to the classical AhR ligands in the up-regulation of the AhR related genes CYP1A1 and CYP1A2. Nevertheless, in a clade of genes to the far left, FLU, I3C, and OME all had ADME gene induction distinct from the classical AhR ligands, and similar to CPZ and CLO. These genes include CYP3As, CYP2Bs, and Abcc3, which are known to be inducible by phenobarbital and responsive to constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) orphan nuclear receptor activation. In mice, CLO is a weak activator of both PXR and CAR, while CPZ is a moderate activator of CAR only (Honkasoki et al., 2003). Though PXR activation is commonly associated with CYP3A induction and CAR activation with CYP2B induction,
both CYP isoforms can also be induced by ligands that interact predominantly with the other nuclear receptor. This crosstalk makes it difficult to assign induction for a given gene/ligand combination that is exclusive to one pathway (Honkakoski et al., 2003).

Log dose response curves were prepared for selected PXR/CAR related genes from the FLU ADME heat map (Figure 8). For CYP3A1 and Abcc3, the rank order potency is CLO >> CPZ, FLU, I3C > OME >> 3MC, BAP, BNF. For CYP2B15 and P450b, expression follows the rank order potency of CLO > I3C > OME, CPZ, FLU >> 3MC, BAP, BNF. Based on these genes, FLU, I3C, and OME all appear to crosstalk into PXR/CAR pathways, especially at higher doses. Down-regulation of male-specific, growth hormone-responsive CYP3A11 (CYP3A2) by FLU, I3C, and OME is in contrast to CLO-mediated up-regulation of this gene. This difference is interesting since we profiled female rats, but may be exaggerated in magnitude due to negligible basal expression.
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Discussion

A singlicate log2 dose escalation design for rodent in vivo toxicogenomic assessments

This study adapts an ascending LD50 in vivo toxicology screening paradigm (Meador, 2002) to a toxicogenomic investigation. The paradigm is a standardized “snapshot-in-time” and ignores the complex temporal aspects of the induction and damage/repair processes. This design is resource-sparing, in animals, drug and microarrays, an essential to early application of toxicogenomics to drug industry pipeline compounds. Meador (2002) states: “Statistical evaluation may not be a useful tool for evaluating small datasets, but the toxicologist with expertise and a set of guidelines for interpretation can minimize risk by making educated decisions.” We agree with this statement and also took the analysis two steps further. First, leveraging our ability to determine statistically significant changes in gene expression on a given microarray pair, we defined compound specific signatures as the union of a battery of statistical tests that define trends in gene expression in the six dose log2 dose escalation (He, et al., unpublished). Second, individual compound gene expression profiles were compared and a consensus signature for AhR ligands was derived from the intersection of four ligands (3MC, BAP, BNF and I3C). Atypical ligands such as FLU were then compared by overlap to the consensus signature.

The study duration (3 day dosing) and sacrifice time (six hour post-last-dose) was specifically chosen to capture early changes in hepatic gene expression that coincide with drug exposure in the liver (6 h after the last dose) and predict, rather than diagnose, the onset of overt liver toxicity that may manifest later as altered ALT and/or
histopathology. Histopathology and clinical chemistry still remain the most cost-effective way to diagnose overt hepatotoxicity.

An AhR-responsive gene set demonstrates FLU is an AhR activator

FLU is an activator of the AhR in vivo in rat liver, based on gene expression profile comparison to CYP1A inducers. We identified genes responsive to ligand-mediated AhR activation using the classical AhR ligands 3MC, BAP, and BNF, and the atypical CYP1A inducers I3C and OME, across a 6 dose log2 dose escalation. Fifteen annotated, dose-dependent genes were regulated by all the AhR ligands. These included CYP1A1 and other members of the AhR gene battery. Dose response relationships of these genes were used to compare FLU and the other atypical CYP1A inducers to the classical AhR ligands. For FLU, CYP1A1 induction saturates at 31.3 MKD with a potency between the classical AhR ligands and the atypicals. FLU had a similar expression pattern for the AhR battery genes CYP1A2, CYP1B1, NQO1, and UGT1A6, and for down-regulated genes that included CYP3A11 (CYP3A2) and c-fos. CLO and CPZ, which induce CYP1A1 only at the top dose, and do not down-regulate CYP3A11 and c-fos, are different from FLU and the other CYP1A1 inducers. Therefore, based on gene expression profiling, FLU has similar potency and spectrum to classical AhR activators, though it is not currently known if this is related to AhR binding by FLU.

Gene responses to AhR activation is dependent upon a number of biological and experimental variables including cellular concentration and localization of drug, available Phase II enzymes, cell type, tissue specific-context, and pharmacokinetics (Nebert et al., 2004). Therefore, in our “snapshot-in-time” analysis at 6-9 h after three
daily doses, the magnitude and direction of a gene response is dependent upon the
temporal interplay between the effects of sustained drug exposure and developing
repair processes.

Interesting responses for Alas2 and c-fos are noted. Aminolevulinic acid
synthase 2 (Alas2), the erythroid-specific isoform involved in the rate-limiting step of
heme biosynthesis, is up-regulated by all inducers except CLO. Its role in liver heme
biosynthesis remains unclear. The ubiquitous isoform, aminolevulinic acid synthase 1
(Alas1), is inducible by phenobarbital in mouse liver and elevates heme levels to
compensate for a rise in CYP content (Kakizaki et al., 2003); however, no literature was
found that related Alas2 to CYP induction. Alas2 is consistently up-regulated among
potent CYP1A inducers, but Alas1 induction occurs only for OME and CLO (data not
shown).

The down-regulation of c-fos distinguishes FLU and other CYP1A inducers from
CLO and CPZ. Functionally, c-fos and c-jun dimerize into the AP1 DNA binding motif
and initiate transcription of the late-response gene cyclinD, resulting in cell cycle
progression past the G1 checkpoint. C-fos is up-regulated to form AP-1 in rats treated
with BAP after 24 hrs (Parrish et al., 1998). Our observed down-regulation of c-fos may
be due to feedback inhibition after three days of sustained compound pressure, or
perhaps a compensatory response to the liver hypertrophy caused by most of the
CYP1A inducers. AhR crosstalk with c-fos is possible, considering the overlap of
invariant core nucleotides shared by each transcription factor’s response element
(Nebert et al., 2000). In rodent uterine and mammary gland tissue and human breast
cancer cell lines, TCDD-activated AhR directly binds to the inhibitory pentanucleotide
(GCGTG) dioxin response element in promoters of c-fos and its target genes, disrupting their transcriptional activation (Safe et al., 2000; Pocar et al., 2005). Though a similar effect has not been delineated in liver, our array data supports an intrinsic relationship between AhR activity in the cell nucleus and c-fos down-regulation at 6-9 h after three daily doses of AhR ligands.

**FLU is most similar in ADME gene expression to the atypical CYP1A inducers OME and I3C**

FLU ADME gene expression distinguishes FLU, OME, and I3C from the classical AhR ligands, and implicates other orphan nuclear receptors. These compounds all induce expression of PXR/CAR-related genes, including CYP3As, CYP2Bs, and Abcc3 (mrp3). Induction occurs at higher doses of FLU and is lower than for the PXR/CAR activator CLO. Both I3C and OME are known to be mixed CYP inducers. I3C elevates CYP1A, CYP2B, and CYP3A activity after three day administration to rats (Renwick et al., 1999). Likewise, OME at 300 MKD elevates CYP1A, CYP2B, and CYP3A activity after seven days in rats (Masubuchi and Okazaki, 1997). OME activates the hPXR receptor in transfected HepG2 cells at 200uM (Raucy, 2003). FLU induction of CYP2Bs and CYP3As has not been demonstrated, but the structural analogue bicalutamide is a CYP3A inducer in laboratory animals at higher doses (Cockshott, 2004). Though OME and bicalutamide are metabolized by CYP3A, the clinical relevance of CYP3A induction is uncertain considering the elevated doses required to induce *in vivo* in rat, or in human hepatocytes. The same is probably true for FLU, which elevates CYP2B and CYP3A expression only at higher doses. The involvement of other orphan nuclear receptors, which possess a promiscuous ligand binding domain, is probably a consequence of
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sustained high doses allowing for binding of low affinity ligands. This may also explain CYP1A1 induction noted for CLO and CPZ, which are not known as potent inducers of the AhR-responsive genes, and were shown here to induce CYP1A1 only at higher doses. Ultimately, the FLU ADME gene response in rat liver is most similar to the atypical CYP1A inducers I3C and OME and supports the conclusion that FLU works as a pleiotropic inducer in rats, presumably through AhR/PXR/CAR activation in response to a substantial dose.

Relevance of FLU AhR activation to its metabolism and toxicity

Historically, AhR has been associated with xenobiotic-induced toxicity. AhR is responsible for the CYP-mediated bioactivation of PAHs to potent carcinogens (Miller and Ramos, 2001). AhR null mice are protected from TCDD mediated tissue damage and teratogenicity (Fernandez-Salguero et al., 1996; Mimura et al., 1997), and BAP-dependent tumor formation (Shimizu et al., 2000). Nevertheless, some say AhR ligands and selective aryl hydrocarbon receptor modulators (SAhRMs) are potential breast and prostate cancer therapies (Safe et al., 2000; Safe and McDougal, 2002; Loaiza-Perez et al., 2004; Morrow et al., 2004). Thus, relevance of AhR activation to toxicity is system-dependent.

Drug-mediated CYP induction is one factor of many that may contribute to IDRs (Li, 2002); however, the relationship of CYP1 induction to toxicity remains complicated and can be overstated (Nebert et al., 2004). This was especially evident after OME was shown to induce CYP1A. After demonstrating elevated CYP1A1 and CYP1A2 activities in livers of patients taking OME, Diaz et al. (1990) speculated that AhR activation by
OME places patients taking OME at greater risk for phenacetin- and acetaminophen-induced hepatotoxicity and PAH- and aflatoxin-B1-mediated carcinogenesis (Diaz et al., 1990). Such speculation proved unfounded (Petersen, 1995), and after over twenty years of clinical use, OME was safe enough to be approved for over-the-counter use.

Idiosyncratic hepatotoxicity is a human-specific event that is not currently detectable in experimental models, thus any potential relevance of FLU’s AhR activation to idiosyncratic hepatotoxicity was not elucidated here. However, autoinduction of pathways that form reactive metabolites may be a factor. Human CYP1A2 metabolizes FLU into its major \textit{in vivo} and active metabolite 2-hydroxyflutamide (Shet et al., 1997) and has been shown to be induced in rats upon FLU treatment (Wang et al., 2005). Both hCYP1A1 and hCYP1A2 enhance FLU covalent binding \textit{in vitro} (Berson et al. 1993), and primary rat hepatocytes pretreated with BNF are more susceptible to FLU toxicity, based on LDH release and covalent protein binding (Fau et al. 1994).

Our data shows that FLU induces the AhR gene battery and that CYP1A induction occurs in rat liver at all doses tested. We used this \textit{in vivo} design to monitor ADME gene induction and the early manifestations of the overt hepatotoxicity of FLU in rats. The lowest dose used (15.6 MKD) exceeded the human clinical dose of FLU (750 mg/day p.o. or \~{}10 MKD). As such, these data should not be considered directly predictive of FLU’s hepatic effects in humans.

In summary, we demonstrated by microarray analysis that FLU is an AhR activator \textit{in vivo} in rat liver, based on similar gene expression across an AhR ligand gene set. FLU ADME gene expression indicates that FLU is most similar in profile to the atypical CYP1A inducers OME and I3C rather than the classical AhR ligands,
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distinguished by CYP2B and CYP3A induction at higher doses. However, the role of AhR activation in initiating human idiosyncratic reactions (if any) is not clear. Better models and improved human adverse event reporting are both needed to resolve the conundrum of causality among autoinduction, covalent binding, and human idiosyncratic hepatotoxicity.
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Acknowledgements:

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References


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Footnotes

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Legends for Figures

Figure 1. Structure of classical AhR ligands, atypical CYP1A inducers, and FLU. 3MC, BAP, and BFN represent classical PAH AhR ligands. Structurally distinct atypical CYP1A Inducers, include the benzimidazoles OME and CLO, the indole I3C, and the phenothiazine CPZ. FLU is an isopropylanilide that is structurally distinct from the PAHs and tested atypical ligands.

Figure 2. Dose related effects of CYP1A inducers on liver weight to body weight ratio. A three day, six dose, log2 dose escalation was performed in singlicate in rats p.o. for all compounds, a study design modeled after the toxicology screening paradigm of Meador et al. (2002). Animals were euthanized for tissue and biofluid collection at 6-9 h after the last dose. The percent of liver weight to body weight ratio (% LW/BW) was determined for each animal. The vehicle control value of 3.2 ± 0.2% represents the % LW/BW mean of six corn oil treated rats.

Figure 3. Dose escalation heat map of FLU responsive genes. The hepatic gene expression of FLU treated rats (500, 250, 62.5, 31.3, and 15.6 MKD) was compared to three corn oil vehicle control rats. Two color labeled cRNA was hybridized in fluor reverse pairs onto an Agilent 22,500 oligonucleotide array. A global analysis of gene expression using Rosetta Resolver 5.0 and in-house MAT-LAB algorithms identified 343 FLU dose responsive genes (p < 0.01). These genes were agglomeratively clustered using a Cosine correlation onto a heat map, where yellow and blue represent up and down-regulated genes with color saturation at +10 and -10 fold, respectively.

Figure 4. Gene expression profile comparison of CYP1A inducers. To discern AhR responsive genes, gene expression profiles were extracted for each compound and overlaps were examined. The number of genes contained in each compound’s gene expression profile is boxed. The Venn diagram overlaps define the number of genes in common among gene sets. The Planar AhR Ligand Consensus Set
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represents the 100 genes commonly regulated by the classical AhR ligands 3MC, BAP, and BNF. The AhR Ligand Consensus represents the 40 genes commonly regulated by the AhR ligands I3C, 3MC, BAP, and BNF. Gene expression profiles for FLU and the other atypicals are compared to these 100 and 40 gene consensus sets.

Figure 5. Heat Map Comparing the Dose Response of AhR Ligand Responsive Genes for Classical and Atypical CYP1A Inducers.
Fifteen well-annotated genes were extracted from the 40 gene AhR Ligand Consensus Set and were agglomeratively clustered using a Cosine correlation against all compound treatments onto a heat map. Yellow and blue represent up- and down-regulated genes with color saturation at +10 and -10 fold, respectively. Black triangles (top) represent the log2 decline in dose from left to right for each compound.

Figure 6. Log dose response curves for AhR ligand-responsive genes.
Log dose response curves for selected genes from the AhR Ligand Consensus Set were compared among compounds. Gene expression changes relative to the vehicle are expressed as a log ratio, with a range of -2 to +2 (-100 to +100 fold). Thus a log ratio of -1 or +1 corresponds to a ±10 fold change relative to vehicle control. Similarly, a log ratio of -0.3 to +0.3 corresponds to a ±2 fold change relative to vehicle control.

Figure 7. Effect of FLU on ADME gene expression.
Fifty ADME genes found in FLU’s 343 gene expression profile were agglomeratively clustered using a Cosine correlation against all compound treatments, where yellow and blue represent up and down-regulated genes, with color saturation at +3.2 and -3.2 fold, respectively. Black arrows (right) represent dose elevation from top to bottom for each compound.

Figure 8 Log dose response curves for selected CAR- and PXR-responsive genes
Log dose response curves were prepared for some CAR- and PXR- responsive genes in the FLU gene expression profile. CYP3A2 (a.ka CYP3A11) was up-regulated by CLO in
this study on female rats. The literature indicates this is a growth hormone-sensitive, male-specific gene (Thangavel et al., 2005). The y-axis represents the log fold change of a gene’s expression due to treatment. Gene expression changes relative to the vehicle are expressed as a log ratio, with a range of -2 to +2 (-100 to +100 fold). Thus a log ratio of -1 or +1 corresponds to a ±10 fold change relative to vehicle control. Similarly, a log ratio of -0.3 to +0.3 corresponds to a ±2 fold change relative to vehicle control.
Table 1

Global functions classifier from Ingenuity analysis of FLU responsive genes.

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<td>Cancer</td>
<td>6.15E-6 — 2.97E-2</td>
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<tr>
<td>Cellular Growth and Proliferation</td>
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<tr>
<td>Hematological Disease</td>
<td>9.86E-5 — 2.97E-2</td>
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<tr>
<td>Immunological Disease</td>
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<td>Cell Death</td>
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Table 2  Expression data for AhR ligand responsive genes

Gene expression changes relative to the vehicle are expressed as a log ratio, with a range of -2 to +2 (-100 to +100 fold). Thus a log ratio of -1 or +1 corresponds to a ±10 fold change relative to vehicle control. Similarly, a log ratio of -0.3 to +0.3 corresponds to a ±2 fold change relative to vehicle control. Up and down-regulated genes are color coded yellow and blue, respectively.

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<th>Accession Code</th>
<th>Gene Name</th>
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<th>BNF</th>
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<th>I3C</th>
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<td>Fos</td>
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Figure 4

Planar AhR ligands:
3 Compound Consensus Set (100 Genes)

Planar AhR Ligand Overlap with FLU and Atypical Inducers

Four Compound Consensus Set (BAP/3MC/BNF/I3C) Overlap with FLU and Atypical Inducers
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Figure 6