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Induction of Cyp1a1 is a Non-Specific Biomarker of Aryl Hydrocarbon Receptor Activation: Results of Large Scale Screening of Pharmaceuticals and Toxicants *In Vivo* and *In Vitro*

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Running title: Biomarker specificity of Cyp1a1

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Abbreviations: AhR, aryl hydrocarbon receptor; DRE, dioxin response element; HAH, halogenated aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Cyp1a1, cytochrome P4501a1; Cyp1a2, cytochrome P4501a2; Ugt1a1, UDP-glucuronosyltransferase 1a1; Nqo1, NAD(P)H:quinone oxidoreductase 1; 3-MC, 3-methylcholanthrene; BNF, beta-naphthoflavone

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ABSTRACT

Expression of Cyp1a1 and its related enzyme activity have long been used as a biomarker for aryl hydrocarbon receptor (AhR) activation and a warning of dioxin-like toxicity. As a result, induction of Cyp1a1 by pharmaceutical drug candidates or environmental contaminants raises significant concern in risk assessment. The current study evaluates the specificity of Cyp1a1 induction as a marker for AhR affinity and activation, and provides context to assess the relevancy of AhR activation to risk assessment. *In vivo* experiments examined the expression of Cyp1a1 and other AhR-regulated genes in liver, kidney and heart in response to 596 compounds. From this dataset, a subset of 147 compounds were then evaluated for their ability to activate or bind to the AhR using a combination of gel shift, reporter gene, and competitive receptor binding assays. While *in vivo* Cyp1a1 mRNA expression is a sensitive marker for AhR activation, it lacks specificity, as 81 out of 137 (59%) compounds were found to significantly induce Cyp1a1 *in vivo* but were not verified to bind or activate the AhR *in vitro*. Combining *in vivo* and *in vitro* findings we identified 9 AhR agonists, 6 of which are FDA approved and marketed therapeutics, including leflunomide, flutamide and nimodipine. These drugs do not produce dioxin-like toxicity in rats or in humans. These data demonstrate that induction of Cyp1a1 is a non-specific biomarker of direct AhR affinity and activation, and lend further support to the hypothesis that Cyp1a1 induction and/or AhR activation is not synonymous with dioxin-like toxicity.

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INTRODUCTION

The aryl hydrocarbon receptor (AhR) regulates the expression of phase 1 and 2 metabolism genes including cytochrome P450s (CYP1A1, CYP1A2, and CYP1B1), UDP-glucuronosyltransferase 1a1 (Ugt1a1) and NAD(P)H:quinone oxidoreductase 1 (Nqo1), among others. Numerous experiments with AhR-null mice have demonstrated that the AhR mediates the toxicity of a number of environmentally persistent halogenated aromatic hydrocarbons (HAHs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin), the prototypical high affinity xenobiotic ligand). The toxic effects of TCDD exposure have been well-established and observed in a number of wildlife, domestic and laboratory species and include hepatotoxicity, hepatomegaly, severe weight loss, teratogenicity, reproductive and developmental toxicity, immunosuppression, thymic atrophy and tumorigenicity. In addition to the effects of P4501A1 on drug metabolism, including bioactivation of pro-mutagens, sustained activation of the AhR is associated with the potential for adverse effects in a number of organ systems due to its role in regulating development of hepatic, vascular, cardiac, immune and epidermal tissues (Gonzalez et al., 1996).

The induction of Cyp1a1 mRNA and resulting enzyme activity has long been used as a sensitive indicator of AhR activation in numerous *in vitro* and *in vivo* models to screen a variety of compounds, mixtures and environmental matrices (Behnisch *et al.*, 2001). As a result of the strong correlation observed between AhR binding affinity, Cyp1a1 induction and dioxin-like toxicity of structurally related HAHs, Cyp1a1 induction has

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been used as a biomarker for hazard identification and risk assessment of environmental pollutants, industrial chemicals and therapeutic compounds (Behnisch *et al.*, 2001, 2002). Such use assumes that induction of Cyp1a1 is specifically associated with AhR activation, and that activation of the AhR leads to dioxin-like toxicity. In contrast to this assumption, AhR-independent induction of Cyp1a1 has been documented (Delesculuse, *et al.*, 2000), and non-halogenated high affinity ligands of the AhR such as beta-napthoflavone, or high doses of weaker or labile endogenous ligands such as prostaglandins (Seidel *et al.*, 2001), heme degradation products (Phelan *et al.*, 1998) and tryptophan metabolites (Heath-Pagliuso, *et al.*, 1998), fail to induce dioxin-like toxicities in rodents. In addition, the AhR has been shown to bind and be activated by a diverse range of chemicals whose structures are dramatically different from the typical planar hydrophobic AhR agonists (Denison and Nagy, 2003; Denison et al., 1999, 2002). These findings raise questions about the validity of the use of Cyp1a1 and related enzyme activity as a specific biomarker of AhR activation, and the relevancy of HAH-induced effects to the safety assessment of non-persistent AhR agonists.

To evaluate the accuracy of *in vivo* Cyp1a1 induction as a biomarker of AhR agonist activity, we evaluated rat gene expression data in DrugMatrix®, a large toxicogenomic database of gene expression profiles for 596 compounds (Ganter *et al.*, 2005), and found that Cyp1a1 was induced by 239 compounds in a variety of tissues. The majority of the active compounds are marketed drugs with toxicity profiles unlike those produced by exposure to HAHs. To evaluate the sensitivity and specificity of *in vivo* Cyp1a1 induction to identify AhR agonists, a subset of 147 compounds were evaluated using a

combination of *in vitro* assays to assess their ability to stimulate AhR transformation and DNA binding, dioxin response element (DRE)-driven reporter gene expression and to compete with dioxin for binding to the AhR. The *in vivo* expression of other AhR-regulated genes, including Cyp1a2, Ugt1a1 and Nqo1, were also evaluated to determine if the expression of these DRE-driven genes could improve the accuracy for identifying AhR agonists. Although all AhR agonists induce Cyp1a1 gene expression, the induction of Cyp1a1 expression *in vivo* does not necessarily implicate that a chemical is a direct AhR agonist. Furthermore, 6 marketed drugs that activate and bind to the rat AhR were identified, as well as many treatments that induce Cyp1a1 in a tissue-specific manner and in a distinct pattern relative to other AhR-regulated genes. These results lend support to the hypothesis that AhR activation is not synonymous with AhR agonist activity and HAH-like toxicity for non-persistent compounds.

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MATERIALS AND METHODS

In Vivo Treatments

Animal and treatment details for the compounds discussed herein are as previously described (Ganter *et al.* 2005). This includes data on 596 compounds representing 3230 compound-dose-time point combinations. Briefly, *in vivo* short-term repeat dose rat studies have previously been conducted by Iconix Biosciences on reference compounds, including marketed, discontinued and withdrawn drugs, and toxicological and biochemical standards. For each compound, 6 to 8 week old male Sprague-Dawley rats (Crl:CD®(SD)(IGS)BR, Charles River Laboratories, Portage, MI) (three per group) were dosed daily at either a low (fully effective) or high (maximum tolerated) dose intended to reduce body weight gain or induce histopathological tissue injury. Animals were necropsied on days 0.25, 1, 3, and 5 or 7. Liver, kidney or heart tissues from treated rats were profiled for gene expression in biological triplicate on the CodeLink™ RU1 microarray platform (GE HealthCare Biosciences, Piscataway. NJ). Housing and treatment of the animals were in accordance with regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2 and 3)

Gene Expression Profiling

Gene expression profiling, data processing, quality control and statistical analysis were performed as previously described (Ganter *et al.* 2005). The microarray gene expression results reported herein are presented as $\log_{10}$ ratios for Cyp1a1 (X00469), Cyp1a2 (K02422), Ugt1a1 (J05132) and Nqo1 (NM_017000), where each experimental

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group is computed as the difference between the average of the logs of the normalized experimental signals and the average of the logs of the normalized control signals for each gene. Treatment related effects on gene expression were considered significant at $p<0.05$. All gene expression data presented herein for all 596 compounds, representing 3230 compound-dose-time point combinations in liver, kidney and heart, are provided in Supplementary Table S1.

Compound Selection for In Vitro AhR Screening

The 147 compounds analyzed *in vitro* were selected based on *in vivo* gene expression data to represent a diverse set of compounds that either induce, repress or do not significantly affect Cyp1a1 transcript levels in the liver, kidney or heart of treated rats. A number of these compounds that do not significantly induce Cyp1a1 *in vivo* were chosen in order to evaluate the potential for false negatives in the gene expression data. The compounds were obtained from a variety of different sources as previously described (Ganter *et al.*, 2005). A summary of all *in vitro* data presented herein for all 147 compounds are provided in Table 1.

Electrophoretic Mobility Shift Assay (EMSA)

All 147 compounds were tested for their ability to transform the rat hepatic cytosolic AhR into its DNA binding form using EMSA as previously described in detail (Denison *et al.*, 2002). Briefly, hepatic cytosol, prepared from 150 g male Sprague-Dawley rats (Charles River Laboratories) in HEDG buffer (25 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol (Denison *et al.,* 2002), was incubated with dimethyl

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sulfoxide (DMSO) (10 μl/ml), TCDD (20 nM), or the indicated test compound (10 μM) for 2 hr at room temperature. Ligand-dependent AhR binding to a [γ^{32}P]-labeled oligonucleotide probe containing the mouse DRE3 sequence (5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') was resolved by nondenaturing polyacrylamide gel electrophoresis and the amount of inducible protein:[^{32}P]DNA complex formation was determined by phosphorimager analysis (Molecular Dynamics). The difference between the amount of radioactivity in the induced protein:DNA complex in a treated sample lane minus that present in the identical position in a vehicle control (DMSO) lane represented the amount of specific protein:DNA complex and the results were expressed as a percentage of the amount of protein:DNA complex induced by 20 nM TCDD. The assay was performed six times for each test compound, and a compound was considered positive if it produced a visible band on the gel in at least three of the six replicate experiments.

Luciferase Reporter Gene Assay

All 147 compounds were evaluated for their ability to induce AhR-mediated DRE-driven reporter gene expression using a recombinant H4IIE 1.1 rat hepatoma (H4L1.1c4) cell line stably transfected with DRE-driven firefly luciferase reporter gene directly under inducible control of the AhR. The cells were generated, grown, and maintained as previously described (Garrison *et al.*, 1996). DMSO (10 μl/ml), TCDD (1 nM), or test compound (10 μM) were added to the 96-well culture plate containing a monolayer of cells. After 4 hr incubation at 37°C, the cells were lysed and luciferase activity in an

aliquot (50 µl) was determined using an Anthos Lucy 2 microplate luminometer. Each compound was tested in triplicate in three independent experiments, and the results were expressed as percent of the luciferase activity induced by 1 nM TCDD. Statistical significance of the differences in luciferase activities between treatments and vehicle controls was determined with a Student's t-test ($p<0.01$). Additionally, only increases in luciferase activity greater than 10% of 1 nM TCDD were considered biologically relevant.

Ah Receptor-ligand Binding Assay

In order to confirm the ability of a compound to directly bind to the AhR, a competitive ligand binding assay was performed on compounds positive in both the reporter gene assay and the gel-shift assay using methods detailed elsewhere (Denison *et al.*, 2002) with minor modifications. Briefly, 500 µl aliquots of a rat cytosolic preparation (2 mg/ml total protein concentration) were pre-incubated at room temperature for 30 min with the compound of interest (10 µM), TCDF (200 nM), or with an equal volume of DMSO. [^{3}H]TCDD was then added to a final concentration of 20 nM. After two hours, 200 µl aliquots of the incubation mixture were added to tubes containing 250 µl HAP (0.5 mg/µl in HEGD buffer) and allowed to incubate for 30 min. Samples were centrifuged and pellets washed three times with HEGD buffer containing 0.05% (v/v) Tween 80. The radioactivity remaining in the HAP pellet was determined by liquid scintillation counting. Specific [^{3}H]TCDD binding was determined by subtracting the radioactivity measured in the TCDF samples (non-specific binding, NSB) from that measured in the samples that were incubated with [^{3}H]TCDD alone (Total binding). The assay was performed in

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triplicate for each compound and the results are presented as a mean percentage of the displacement of specific [^{3}H]TCDD binding.

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RESULTS

Cyp1a1 and AhR-regulated Genes are Frequently Induced in the Rat

Of the 596 compounds examined in the liver, heart and kidney of the rat in DrugMatrix, there were 600 (18.5%) treatment groups (compound-dose-time combinations) out of 3230 where Cyp1a1 transcript levels were significantly ($p<0.05$) increased relative to vehicle-treated controls. These included 123 compounds that induced Cyp1a1 mRNA expression in the liver, 79 in the heart, and 68 in the kidney (Table S1). Of these 239 total compounds (some compounds were profiled in more than one tissue), 158 (84%) are drugs approved for use by the Food and Drug Administration, while 37 are non-pharmaceuticals and are either prototypical toxicants, industrial chemicals or biochemical standards. The remaining 44 compounds are drugs registered outside of the US, withdrawn by the FDA or discontinued from development (Table S1). As expected, known AhR ligands such as beta-naphthoflavone (BNF; 1500 mg/kg/d) and 3-methylcholanthrene (3-MC; 300 mg/kg/d) significantly induced Cyp1a1, Cyp1a2, Ugt1a1 and Nqo1 in the liver at multiple time points, although the results for 3-MC were more variable for Ugt1a1 and Nqo1 (Figure 1A). In addition, there were many treatments, including albendazole, hydralazine, leflunomide, omeprazole and others that caused similar significant changes in gene expression across these AhR-regulated and 3-MC/BNF-inducible genes, suggesting these compounds are potential AhR agonists (Figure 1B). Other than omeprazole (Shih et al., 1999), these compounds have not been previously described as Cyp1a1 inducers or as AhR agonists. Cyp1a1 was induced more than 100-fold by leflunomide and phenothiazine. Consistent with

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previous findings, the benzimidazole drugs lansoprazole and rabeprazole had strong effects (>100-fold) on Cyp1a1 (Backlund *et al.*, 1999). Omeprazole also induced Cyp1a1 90-fold, which is consistent with published findings showing induction of Cyp1a1 in hepatocytes from a number of species (Shih *et al.*, 1999). By comparison, BNF and 3-MC maximally induced Cyp1a1 61- and 7-fold, respectively. Interestingly, the pineal gland hormone melatonin significantly induced Cyp1a1 over 32-fold, in addition to inducing Cyp1a2, Ugt1a1 and Nqo1 (Figure 1B).

There were many treatments that significantly induced Cyp1a1 and 1a2 in heart and kidney also (Figure 1C-D). To our knowledge the majority of these treatments have not been previously shown to induce the expression of Cyp1a1 or 1a2, or to bind to the AhR. In contrast to the results in liver, the expression of Ugt1a1 and Nqo1 did not appear to be co-regulated with Cyp1a1 and 1a2 in heart and kidney (Figure 1C-D). The most potent inducer of Cyp1a1 in heart was BW-723C86 (10-fold), a selective 5-HT2B receptor agonist (Figure 1C). Many other compounds evaluated in the kidney, including the HMG-CoA reductase inhibitors lovastatin and mevastatin also induced Cyp1a1 greater than 10-fold (Figure 1D). These results indicate that Cyp1a1 induction in liver, kidney and heart is very common among rats treated with marketed therapeutic drugs.

Co-regulation of Cyp1a1 and Other AhR-regulated Genes

There were a large number of treatments that significantly induced Cyp1a1, but not Cyp1a2, Ugt1a1 and Nqo1 concurrently. This included 73 treatments in liver, 134 in heart and 75 in kidney (Figure 2, Table S1). Many of these treatments slightly, but not

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significantly, increased the levels of these other AhR-regulated genes, thus suggesting a weak AhR agonist effect. However, there were a number of compounds that clearly had no effect on these genes or even repressed them, yet significantly induced Cyp1a1 (Figure 2). In liver, for example, a number of toxicants such as 1-naphthyl isothiocyanate, ethanol, N-nitrosodiethylamine, and valproic acid significantly induced Cyp1a1 while slightly repressing Cyp1a2 at both early and late time points (Figure 2A). A similar effect was particularly evident in heart where a number of compounds significantly induced Cyp1a1 while significantly repressing Cyp1a2, including bromisovalum (days 1 and 5), clofibric acid (days 3 and 5), isoprenaline (days 1 and 5) and vinorelbine (day 3) (Figure 2B). Similar effects in kidney were observed for bromisovalum, cadmium acetate and rifampin, although repression of Cyp1a2 was not as pronounced (Figure 2C). Dexfenfluramine, whose metabolite is a potent 5-HT2B receptor agonist, also significantly induced Cyp1a1 in heart (Figure 2B), but unlike the 5-HT2B receptor agonist BW-723C86, it did not induce Cyp1a2. This effect in heart was not evident in kidney where both Cyp1a1 and 1a2 were not significantly affected by dexfenfluramine (Table S1). These results indicate that Cyp1a1 may not be co-regulated with other AhR-regulated genes in heart and kidney. Furthermore, it suggests that Cyp1a1 is under regulatory control mechanisms distinct from the classic ligand binding and DRE-mediated transcription through the AhR, or that tissue-specific factors are needed to support induction of other DRE-regulated genes in these tissues.

Tissue-Specific Induction of Cyp1a1

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Due to the disparate induction pattern of Cyp1a1 compared to other AhR-responsive genes under certain treatment conditions, it was of interest to determine if similar effects on Cyp1a1 were observed across tissues. Of the 207 compound-dose-timepoint combinations that were profiled in more than one tissue and significantly induced Cyp1a1 in at least one of those tissues, only 41 (20%) did so in two out of the three tissues examined. For example, none of the 64 compound-dose-timepoint combinations that were profiled in all 3 tissues significantly induced Cyp1a1 consistently across all 3 tissues (Figure 3A). Interestingly, kidney-specific induction of Cyp1a1 was observed with the class of HMG-CoA reductase inhibitors. Consistent with the effects of mevastatin and lovastatin in kidney (Figure 1D), other HMG-CoA reductase inhibitors, including cerivastatin, atorvastatin, pravastatin, and simvastatin significantly induced Cyp1a1 in kidney but not liver (Figure 3B). The exception was cerivastatin on day 5, which significantly induced liver Cyp1a1 just over 2.5-fold. These results indicate that induction of Cyp1a1 can be tissue specific depending on the inducing agent.

Sensitivity of In Vivo Cyp1a1 Induction for Identifying AhR Agonists

In order to determine if the observed induction of Cyp1a1 *in vivo* is reflective of AhR binding and activation, 147 compounds were evaluated for their ability to transform the AhR into a DNA-binding complex *in vitro,* induce expression of a DRE-driven reporter gene in rat H4L1.1c4 cells, and bind to the rat AhR *in vitro*. Of the 147 compounds that were evaluated *in vitro*, only 9 compounds showed significant activity in all 3 *in vitro* assays and significantly induced Cyp1a1 *in vivo* (Table 1). This includes the known AhR ligands 3-MC, BNF and alpha-naphthoflavone, which have previously been shown

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to be active in these assays. The other 6 compounds are approved for use by the FDA for a variety of indications, including omeprazole (Prilosec®), nimodipine (Nimotop®), leflunomide (Arava®), flutamide (Eulexin®), mexiletine (Mexitil®), and atorvastatin (Lipitor®) (Figure 4). The most potent AhR agonist identified was leflunomide, a pyrimidine synthesis inhibitor indicated for rheumatoid arthritis, which induced luciferase activity as great as 1 nM TCDD, and completely displaced [^{3}H]TCDD from the AhR (Table 1). Nimodipine, a calcium channel blocker indicated for subarachnoid hemorrhage, and flutamide, an androgen receptor antagonist indicated for prostate cancer, also competitively displaced over 90% of [^{3}H]TCDD from the AhR. Omeprazole, previously thought to not bind the rat or human receptor (Backlund, *et al.*, 1997; Daujat *et al.*, 1992), was found to displace around 50% of TCDD from the rat AhR and induced AhR transformation as determined by EMSA. Atorvastatin and mexiletine had weaker effects on luciferase activity (<20% of TCDD) and displaced less than 33% of TCDD from the AhR (Table 1). By contrast, indomethacin was weakly positive in all 3 *in vitro* assays yet did not significantly induce Cyp1a1 *in vivo,* nor did it consistently induce Cyp1a2, Ugt1a1 or Nqo1 (Table S1). These results indicate that *in vivo* Cyp1a1 induction is a sensitive (9/10) indicator of AhR agonist activity, which is consistent with current understanding of AhR-mediated Cyp1a1 regulation (Figure 5).

The agonist effects of leflunomide, nimodipine and flutamide were further tested using the reporter gene assay where H4L1.1c4 cells were treated with increasing concentrations of compounds up to 10μM (Figure 6A). Leflunomide was the most potent among the three compounds and induced luciferase activity to a significantly greater

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level than that of TCDD. Based on the dose response data, leflunomide had an EC_{50} of 0.17 μM, which was approximately 2700-fold higher than that of TCDD (EC_{50} = 6.2e-5 μM). Flutamide and nimodipine had EC_{50} values of 0.46 and 0.77 μM, respectively. Full dose response curves could not be generated for omeprazole, mexiletine and atorvastatin due to their relatively weak luciferase inducing potency.

To determine if leflunomide, flutamide and nimodipine were full or partial agonists in the luciferase assay, the compounds were co-treated with 1 nM TCDD. As shown in Figure 6B, both flutamide and nimodipine inhibited the response of 1nM TCDD by 40% indicating they are partial agonists. In contrast, co-treatment with leflunomide produced a synergistic increase in luciferase induction, increasing the maximum luciferase induction response of TCDD by 60%. These results establish the *in vivo* identification and *in vitro* validation of 6 novel AhR agonists in the rat.

Specificity of In Vivo Cyp1a1 Induction for Identifying AhR Agonists

Of the 137 parent compounds that were not consistently active in all 3 *in vitro* assays, 81 were found to significantly induce Cyp1a1 *in vivo*, thus indicating a high rate of false positives (59%) (Figure 5). Of the 81 false positives, a number of compounds significantly induced Cyp1a2, Ugt1a1 and Nqo1 gene expression concurrently with Cyp1a1, thus suggesting activation through the classic AhR signaling pathway. These compounds included albendazole (days 1 and 3), rabeprozole (days 1, 3, and 5), safrole (days 1 and 3), melatonin (days 1 and 3), phenothiazine (days 1, 3, and 5) and sulindac (day 1) (Figure 7A). While metabolic activation may be necessary for *in vivo* AhR

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agonist activity for these compounds, there were a number of compounds that induced Cyp1a1 over 10-fold, but did not significantly induce Cyp1a2, Ugt1a1, Nqo1, induce significant luciferase activity or transform the AhR into a DNA binding form. These compounds were not tested in the AhR binding assay and included lovastatin, ANIT, eperisone, carvedilol and zileuton (Figure 7B). Other compounds that significantly induced luciferase activity and Cyp1a1 over 10-fold, but failed to stimulate transformation of the AhR into a DNA binding form were also not tested in the binding assay. Notable compounds in this group include the corticosteroids dexamethasone and fludrocortisone in liver, prednisolone in heart, benoxaprofen and fenoprofen in liver, and cadmium chloride in kidney (Figure 7C).

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DISCUSSION

In the current study, a diverse set of drugs and industrial chemicals were examined in the rat across multiple organs to evaluate the concordance between induction of Cyp1a1 and other known AhR-responsive genes with AhR binding and activation. While the sensitivity of Cyp1a1 as an indicator of AhR binding and activation is not in question, the data reveal a surprisingly low specificity. Despite the common belief that the AhR is most often activated by polycyclic and planar aromatics, such as HAH's, a surprisingly large number (239 or 40%) of the 596 test compounds examined induced Cyp1a1 in at least one tissue. In the subset of compounds further examined for AhR agonist activity *in vitro*, 81 of the 137 (59%) compounds that induced Cyp1a1 were considered false positives, as they were not consistently active in all 3 *in vitro* assays. This may be an over-estimate since the discrepancy between the *in vivo* and *in vitro* findings may be due to a requirement for metabolic activation *in vivo* for agonist activity. Tissue-specific bioactivation may also explain the observed tissue-specific induction pattern of Cyp1a1 (Figure 3). Putative AhR pro-agonists identified in this study include albendazole, rabeprozole, safrole, melatonin, phenothiazine and sulindac (Figure 7A). The activity of albenzdazole and rabeprazole is consistent with results obtained with structurally related benzimidazoles that have been shown to activate the AhR (Backlund *et al.*, 1999). AhR agonist activity for the major metabolite of safrole, 4-allyl-1,2-dihydroxybenzene, or others have not been reported, although Cyp1a1 induction has been previously observed for safrole, isosafrole and related metabolites in mice (Cook and Hodgson, 1985; Lewandowski *et al.*, 1990). While melatonin is inactive as an AhR

agonist *in vitro* (Figure 7A; Heath-Pagliuso et al., 1998), potential active metabolites of melatonin include 6-hydroxymelatonin, which is produced in humans by CYP1A1, CYP1A2 and CYP1B1 (Ma *et al.*, 2005), thereby suggesting an auto-induction mechanism.

Numerous compounds have been reported to induce Cyp1a1 that do not appear to compete with TCDD for binding to the AhR, including thiazolium compounds, retinoids, carotenoids, benzimidazoles, carbamates and aminoquinoline (Daujat *et al.,* 1992; Aix *et al.,* 1994; Lesca *et al.,* 1995; Gradelet *et al.,* 1997; Ledirac *et al.,* 1997; Fontaine *et al.,* 1999). While the lack of *in vitro* AhR binding for the many Cyp1a1 inducers may result from technical limitations of the binding assays (Denison et al., 1998; Denison and Nagy, 2003), it has also been suggested that many of these compounds may induce Cyp1a1 through multiple modes of indirect AhR activation. For instance, a compound treatment may cause induction of endogenous metabolites or signaling molecules that regulate AhR. Aspartate aminotransferase has been shown to convert the proagonist L-tryptophan into a variety of AhR agonists (Bittinger *et al.*, 2003). Furthermore, Cyp1a1 is inducible in the absence of exogenous ligand under conditions of hyperoxia (Okamoto *et al.*, 1993), shear stress (Mufti and Shuler, 1996), and undefined serum factors (Guigal *et al.*, 2000). Compound-induced production of endogenous ligands such as tryptophan metabolites or prostaglandins and other bioactive lipids that have been identified as AhR agonists (Schaldach et al., 1999; Seidel et al., 2001) may also be involved, although these hypotheses have yet to be

confirmed. A more thorough understanding of these possible endogenous ligands and their levels in response to compound treatment may shed some light on this possibility.

There are data that support a role for numerous protein tyrosine kinases and mitogen activated protein kinases in modulating AhR activity (Chen and Tukey, 1996; Backlund et al., 1997), however, the evidence thus far suggests that these kinases facilitate and/or amplify the functionality of the AhR rather than modulate Cyp1a1 independent of the AhR. The cooperative effects of phosphorylation and ligand binding to the AhR may result in *in vivo* expression of AhR-regulated genes being more sensitive than reporter gene-based or cell-free assays for detecting weak or transient ligands. This is supported by evidence showing differential sensitivity of Cyp1a1 induction to tyrosine kinase inhibitors in response to the weak ligand omeprazole relative to a high affinity ligand like 3-MC (Lemaire *et al.*, 2004). Differences in the inducibility of the native Cyp1a1 promoter *in vivo* and the DRE-regulated reporter construct *in vitro* may exist, although we know of no examples of bona fide agonists that fail to activate the DRE-regulated construct. Assay conditions may also make it difficult for *in vitro* assays to detect the ability of weak affinity ligands to displace TCDD from the receptor given the strong affinity of TCDD (Kd in the pM range). Although other studies have reported that omeprazole is unable to displace TCDD from the receptor (Backlund *et al.*, 1997; Daujat *et al.*, 1992), we detected significant activity in all 3 *in vitro* assays for omeprazole (Table 1) suggesting the conditions used in our assays are more sensitive than those used by others. To this end, reports of AhR-independent induction of Cyp1a1 by

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chemicals have subsequently been reconsidered through the use of a more sensitive binding assay (Denison et al., 1998; Denison and Nagy, 2003).

Under certain treatment conditions, the expression of Cyp1a1 was induced while other DRE-regulated genes were not. Compounds with this profile deviate from the classic mechanism of AhR binding and transcriptional activation via DREs. Most notable among these compounds are the corticosteroids (Figure 7C). Dexamethasone has previously been shown to induce Cyp1a1 at high concentrations and potentiate TCDD-induced expression in a glucocorticoid receptor and protein synthesis-dependent manner (Lai *et al.*, 2004). Up-regulation or activation of certain transcription factors such as RXR, PGC-1 and HNF4-alpha, or calcium-dependent calpain may also contribute indirectly to Cyp1a1 induction (Gradelet et al., 1997; Dale and Eltom, 2006; Martinez-Jimenez *et al.*, 2006). PGC-1alpha was observed to be induced by most heart and kidney treatments concurrent with Cyp1a1 up-regulation (data not shown) where Cyp1a2, NQO1 and Ugt1a1 were not induced (Figure 2). Recently, two peroxisome proliferator-activated receptor (PPAR) response elements were found to mediate induction of human Cyp1a1 in response to PPAR-alpha agonists (Seree *et al*., 2006). PGC-1alpha positively regulates PPAR-alpha activity, thus suggesting that these transcription factors synergize to induce rat Cyp1a1 in a similar manner (Figure 2B). Interestingly, treatment of ischemic rats with a PPAR-alpha ligand has been shown to be cardioprotective as a result of nitric oxide production (Bulhak et al., 2006), which has also been shown to repress Cyp1a2 mRNA (Mulero-Navarro et al., 2003). Although definitive proof is still lacking, these findings and the observations in Figure 2 suggest a model whereby rat

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Cyp1a1 is specifically induced by PGC-1/PPAR-alpha in the heart with concomitant production of nitric oxide and a resulting down-regulation of Cyp1a2.

The lack of specificity of Cyp1a1 as a biomarker of AhR activation raises significant concern over the use of Cyp1a1 and its related enzyme activities (i.e. ethoxyresorufin-O-deethylase (EROD) activity) to evaluate the potential of compounds or mixtures to activate the AhR. Given the lack of specificity, an overestimation of AhR activation potential and calculated toxicity equivalents may result from the strict reliance on Cyp1a1 mRNA, protein or enzyme activity alone without the use of more specific assays or a combination of functional or binding assays to confirm the dependence on AhR binding and transcriptional activation. With respect to estimates of dioxin-like toxicity, a rich body of literature indicates that metabolically persistent halogenated ligands of the AhR cause sustained activation of the receptor and result in a wide spectrum of toxic responses similar to TCDD, while metabolically labile, non-halogenated AhR ligands do not typically produce dioxin-like toxicities in animal studies. Recent studies in fish have demonstrated that inhibition of Cyp1a1-dependent metabolism of these labile AhR agonists can result in dioxin-like toxicity due to the increased persistence of the chemical (Wassenberg and Di Giulio, 2004). These results suggest that while binding and activation of the AhR are necessary prerequisite events for AhR-dependent dioxin-like toxicity, the actual occurrence of toxicity requires both continual presence of the AhR agonist and persistent activation of the AhR signaling pathway. In the current study, through a combination of *in vivo* and *in vitro* assays, a number of weak AhR ligands were identified, including nimodipine, leflunomide, flutamide, omeprazole,

mexiletine and atorvastatin. These compounds, which are approved for use by FDA, do not produce dioxin-like toxicities in rats and there is no evidence for chloracne, immunosuppression or other adverse dioxin-like effects in exposed humans. This could be due to both their reduced potency relative to TCDD and/or their rapid rate of clearance from the body relative to persistent halogenated ligands. It would appear that the toxicological consequences of transient or weak receptor activation are qualitatively and quantitatively distinct from persistent activation by metabolically stable and potent ligands.

Several lines of evidence presented in the current study are consistent with the conclusion that the induction of rat Cyp1a1 is a sensitive but not specific indicator of AhR binding and activation. Furthermore, the induction of Cyp1a1 and activation of AhR is not synonymous with dioxin-like toxicity in the rat for non-halogenated or metabolically labile compounds. Second tier hazard identification strategies such as the *in vitro* tests used herein should be considered for assessing exposure and toxicity related to AhR activation.

References

Aix L, Rey-Grobellet X, Larrieu G, Lesca P and Galtier P (1994) Thiabendazole is an inducer of cytochrome P450 1a1 in cultured rabbit Hepatocytes. *Biochem Biophys Res Commun* 202:1483-1489.

Backlund M, Johansson I, Mkrtchian S and Ingelman-Sundberg M (1997) Signal transduction-mediated activation of the aryl hydrocarbon receptor in rat hepatoma H4IIE cells. *J Biol Chem* 272:31755-31763.

Backlund M, Weidolf L and Ingelman-Sundberg M (1999) Structural and mechanistic aspects of transcriptional induction of cytochrome P450 1a1 by benzimidazole derivatives in rat hepatoma H4IIE cells. *Eur J Biochem* 261:66-71.

Behnisch PA, Hosoe K and Sakai S (2001) Bioanalytical screening methods for dioxins and dioxin-like compounds a review of bioassay/biomarker technology. *Environ Int* 27:413-439.

Behnisch PA, Hosoe K, Brouwer A and Sakai S (2002) Screening of dioxin-like toxicity equivalents for various matrices with wildtype and recombinant rat hepatoma H4IIE cells. *Toxicol Sci* 69:125-130.

Bittinger MA, Nguyen LP and Bradfield CA (2003) Aspartate aminotransferase generates proagonists of the aryl hydrocarbon receptor. *Mol Pharmacol* 64:550-556.

Bulhak AA, Sjoquist PO, Xu CB, Edvinsson L, Pernow J (2006) Protection against myocardial ischaemia/reperfusion injury by PPAR-alpha activation is related to production of nitric oxide and endothelin-1. *Basic Res Cardiol* 101:244-252.

Chen YH and Tukey RH (1996) Protein kinase C modulates regulation of the Cyp1a1 gene by aryl hydrocarbon receptor. *J Biol Chem* 271:26261-26266.

Cook JC and Hodgson E (1985) The induction of cytochrome P450 by isosafrole and related methylenedioxyphenyl compounds. *Chem Biol Interact* 54:299-315.

Dale Y and Eltom SE (2006) The induction of Cyp1a1 by oltipraz is mediated through calcium-dependent-calpain. *Toxicol Lett* 166:150-159.

Daujat M, Peryt B, Lesca P, Fourtanier G, Domergue J and Maurel P (1992) Omeprazole, an inducer of human Cyp1a1 and 1a2, is not a ligand for the Ah receptor. *Biochem Biophys Res Commun* 188:820-825.

Denison MS, Phelan D, Winter MG and Ziccardi MH (1998) Carbaryl, a carbamate insecticide, is a ligand for the hepatic Ah (dioxin) receptor. *Toxicol Appl Pharmacol* 152:406-414.

Denison MS and Heath-Pagliuso S (1998) The Ah receptor: A regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull Environ Contam Toxicol* 61:557-568.

Denison MS, Pandini A, Nagy SR, Baldwin EP and Bonati L (2002) Ligand binding and activation of the Ah receptor. *Chem Biol Interact* 141:3-24.

Denison MS and Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann Rev Pharmacol Toxicol* 43:309-334.

Fontaine F, Delescluse C, de Sousa G, Lesca P and Rahmani R (1999) Cytochrome 1a1 induction by primaquine in human hepatocytes and HepG2 cell: absence of binding to the aryl hydrocarbon receptor. *Biochem Pharmacol* 57:255-262.

Ganter B, Tugendreich S, Pearson CI, Ayanoglu E, Baumhueter S, Bostian KA, Brady L, Browne LJ, Calvin JT, Day GJ, Breckenridge N, Dunlea S, Eynon BP, Furness LM, Ferng J, Fielden MR, Fujimoto SY, Gong L, Hu C, Idury R, Judo MS, Kolaja KL, Lee MD, McSorley C, Minor JM, Nair RV, Natsoulis G, Nguyen P, Nicholson SM, Pham H, Roter AH, Sun D, Tan S, Thode S, Tolley AM, Vladimirova A, Yang J, Zhou Z and Jarnagin K (2005) Development of a large-scale chemogenomics database to improve drug candidate selection and to understand mechanisms of chemical toxicity and action. *J Biotechnol* 119:219-244.

Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP and Denison MS (1996) Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. *Fundam Appl Toxicol* 30:194-203.

Gonzalez FJ, Fernandez-Salguero P, and Ward JM (1996) The role of the aryl hydrocarbon receptor in animal development, physiological homeostasis and toxicity of TCDD. *J Toxicol Sci* 21:273-277.

Gradelet S, Astorg P, Pineau T, Canivenc MC, Siess MH, Leclerc J and d Lesca P (1997) Ah receptor-dependent Cyp1a induction by two carotenoids, canthaxanthin and carotenal, with no affinity for the TCDD binding site. *Biochem Pharmacol* 54:307-315.

Guigal N, Seree E, Bourgarel-Rey V and Barra Y (2000) Induction of Cyp1a1 by serum independent of AhR pathway. *Biochem Biophys Res Commun* 267:572-576.

Heath-Pagliuso S, Rogers W J, Tullis K, Seidel SD, Cenijn PH, Brouwer A and Denison MS (1998) Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochem* 37:11508-11515.

Lai KP, Wong MH and Wong CK (2004) Modulation of AhR-mediated CYP1A1 mRNA and EROD activities by 17beta-estradiol and dexamethasone in TCDD-induced H411E cells. *Toxicol Sci* 78:41-49.

Ledirac N, Delescluse C, de Sousa G, Pralavorio M, Lesca P, Amichot M, Berge JB and Rahmani R (1997) Carbaryl induces CYP1A1 gene expression in HepG2 and HaCaT cells but is not a ligand of the human hepatic Ah receptor. *Toxicol Appl Pharmacol* 144:177–182.

Lemaire G, Delescluse C, Pralavorio M, Ledirac N, Lesca P and Rahmani R (2004) The role of protein tyrosine kinases in Cyp1a1 induction by omeprazole and thiabendazole in rat hepatocytes. *Life Sci* 74:2265-2278.

Lesca P, Peryt B, Larrieu G, Alvinerie M, Galtier P, Daujat M, Maurel P and Hoogenboom L (1995) Evidence for the ligand-independent activation of the Ah receptor. *Biochem Biophys Res Commun* 209:474-482.

Lewandowski M, Chui YC, Levi PE and Hodgson E (1990) Differences in induction of hepatic cytochrome P450 isozymes by mice in eight methylenedioxyphenyl compounds. *J Biochem Toxicol* 5:47-55.

Ma XC, Idle JR, Krausz KW and Gonzalez FJ (2005) Metabolism of melatonin by human cytochrome P450. *Drug Metab Dispos* 33:489-494.

Martinez-Jimenez CP, Castell JV, Gomez-Lechon MJ and Jover R (2006) Transcriptional activation of Cyp2c9, Cyp1a1 and Cyp1a2 by HNF4 alpha requires coactivators PGC1 alpha and SRC1. *Mol Pharmacol* 101124/mol106025403.

Mufti NA and Shuler ML (1996) Possible role of arachidonic acid in stress-induced cytochrome P4501A1 activity. *Biotechnol Prog* 12:847-854.

Mulero-Navarro S, Santiago-Josefat B, Pozo-Guisado E, Merino JM, Fernandez-Salguero PM (2003) Down-regulation of CYP1A2 induction during the maturation of mouse cerebellar granule cells in culture: role of nitric oxide accumulation. *Eur J Neurosci* 18:2265-2272.

Okamoto T, Misuhashi M, Fujuta I, Shindhu RK and Kikkawa Y (1993) Induction of cytochrome P450 1A1 and 1A2 by hyperoxia. *Biochem Biophys Res Commun* 197:878–885.

Phelan D, Winter GM, Rogers WJ, Lam JC and Denison MS (1998) Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. *Arch Biochem Biophys* 357:155-163.

Schaldach CM, Riby J and Bjeldanes LF (1999) Lipoxin A4: a new class of ligand for the Ah receptor. Biochemistry 38:7594-7600.

Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B and Denison MS (2001) Activation of the Ah receptor signaling pathway by prostaglandins. *J Biochem Mol Toxicol* 15:187-196.

Seree E, Villard PH, Pascussi JM, Pineau T, Maurel P, Nguyen QB, Fallone F, Martin PM, Champion S, Lacarelle B, Savouret JF, Barra Y (2006) Evidence for a new human CYP1A1 regulation pathway involving PPAR-alpha and 2 PPRE sites. *Gastroenterology* 127:1436-1445.

Shih H, Pickwell GV, Guenette DK, Bilir B and Ouattrochi LC (1999) Species differences in hepatocyte induction of cyp1a1 and cyp1a2 by omeprazole. *Hum Exp Toxicol* 18:95-105.

Wassenberg DM, Di Giulio RT (2004) Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in Fundulus heteroclitus. *Environ Health Perspect* 112:1658-1664.

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Figure Legends

Figure 1. Consistent induction of AhR-regulated genes *in vivo* by putative AhR agonists. Gene expression results for known AhR-regulated genes, including cytochromes P4501a1 (Cyp1a1, X00469) and 1a2 (Cyp1a2, K02422), UDP-glucuronosyltransferase 1a1 (Ugt1a1, J05132) and NAD(P)H dehydrogenase, quinone 1 (Nqo1, NM_017000). Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to controls. Treatments are indicated by compound name, dose in mg/kg/d (mpk) and duration of treatment in days. A) Hepatic gene expression of AhR-regulated genes by known AhR agonists 3-methylcholanthrene (3-MC) and beta-naphthoflavone (BNF). B) Hepatic gene expression for treatments that significantly ($p<0.05$) induced Cyp1a1, Cyp1a2, Ugt1a1 and Nqo1. C) Cardiac gene expression for treatments that significantly ($p<0.05$) induced Cyp1a1 and Cyp1a2. D) Renal gene expression for treatments that significantly ($p<0.05$) induced Cyp1a1 and Cyp1a2.

Figure 2. Atypical induction of Cyp1a1 and AhR-regulated target genes *in vivo*. Gene expression results for known AhR-regulated genes, including cytochromes P4501a1 (Cyp1a1, X00469) and 1a2 (Cyp1a2, K02422), UDP-glucuronosyltransferase 1a1 (Ugt1a1, J05132) and NAD(P)H dehydrogenase, quinone 1 (Nqo1, NM_017000). Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to controls. Treatments are indicated by compound name, dose in mg/kg/d (mpk) and duration of treatment in days. Gene expression

results for treatments that significantly ($p<0.05$) and consistently induced Cyp1a1, but did not significantly induce Cyp1a2, Ugt1a1 or Nqo1 in A) liver, B) heart or C) kidney.

Figure 3. Tissue-specific regulation of Cyp1a1 expression *in vivo*. Gene expression results for Cyp1a1 (X00469) in liver, kidney and heart. Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to controls. Treatments are indicated by compound name, dose in mg/kg/d (mpk) and duration of treatment in days. A) Discordant gene expression results for Cyp1a1 for compound-dose-timepoints measured in both liver, heart and kidney. B) Gene expression results for Cyp1a1 for HMG-CoA reductase inhibitor treatments measured in both liver and kidney.

Figure 4. Structures of AhR agonists identified in this study. Compounds that were not previously reported as AhR agonists are shown. These compounds were found to significantly induce Cyp1a1 *in vivo*, positively induce DRE-driven luciferase activity in rat H4L1.1c4 cells, stimulate transformation of the AhR *in vitro*, and competitively bind to the AhR *in vitro*.

Figure 5. Concordance for *in vivo* Cyp1a1 induction and *in vitro* AhR activation. A compound was considered positive for Cyp1a1 induction if the compound significantly ($p<0.05$) increased Cyp1a1 (X00469) expression in liver, heart or kidney after 1, 3 or 5 days of repeated dosing at a maximum tolerated dose. A compound was considered positive for *in vitro* AhR activation if there was significant activity in the electromobility

shift assay, the reporter gene assay, and AhR binding, as described in materials and methods.

Figure 6. Dose response for luciferase induction. Three AhR agonists that had more than 50% TCDD activity in the reporter gene assay, including flutamide, leflunomide and nimodipine, were tested to determine the EC_{50} for luciferase induction. Results are expressed as the percentage of the luciferase activity induced by 1nM TCDD. A) dose-dependent effects of three AhR agonists on luciferase activity in H4L1.1c4 cells. B) Effects of co-treatment with AhR agonists on 1 nM TCDD-induced luciferase activity in H4L1.1c4 cells. Error bars represent the standard deviation of 3 replicates.

Figure 7. Discrepancy between *in vivo* Cyp1a1 induction and *in vitro* AhR activity. A) Putative pro-agonists of the AhR, which are active *in vivo* but not *in vitro*. B) Cyp1a1 inducers via non-AhR or -DRE-mediated mechanisms. C) Cyp1a1 inducers independent of ligand binding and/or DRE-mediated mechanisms. Gene expression results for known AhR-regulated genes, including cytochromes P4501a1 (Cyp1a1, X00469) and 1a2 (Cyp1a2, K02422), UDP-glucuronosyltransferase 1a1 (Ugt1a1, J05132) and NAD(P)H dehydrogenase, quinone 1 (Nqo1, NM_017000). Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to controls. Treatments are indicated by compound name, dose in mg/kg/d (mpk), duration of treatment in days and tissue. Results for DRE-driven luciferase activity, AhR transformation and DNA binding, AhR binding are shown for each compound. Values are the mean and standard deviation for the luciferase reporter gene

assay (n=9), electromobility shift assay (EMSA) (n=6) and the AhR binding assay (n=3). The results of the reporter gene and EMSA assays are expressed as a percentage of the maximum response observed for TCDD. The results of the receptor binding assay are presented as a percentage of the displacement of specific TCDD binding. Statistically significant ($p<0.05$) differences in luciferase activity compared to DMSO-treated controls is indicated by the asterisk (*). ND = not detected. NA = not determined.

Table 1. Summary of *in vitro* results for AhR agonist activity.

All 147 compounds evaluated *in vitro* are shown. Values are the mean and standard deviation for the luciferase reporter gene assay (n=9), electromobility shift assay (EMSA) (n=6) and the AhR binding assay (n=3). The results of the reporter gene assays and EMSA are expressed as a percentage of the response induced by TCDD (1 nM and 20 nM for the gene reporter assay and EMSA respectively). The results of the receptor binding assay are presented as a percentage of the displacement of specific TCDD binding. The results for Cyp1a1 gene expression *in vivo* are expressed as the maximum significant ($p<0.05$) fold-induction observed in liver, heart or kidney.

Compound Name	Luciferase Activity (mean ±_SD)	EMSA (mean ± SD)	AhR Binding (mean ± SD)	In Vivo Cyp1a1 (Fold-induction)
LEFLUNOMIDE	135.4 ± 27.1*	60.2 ± 10.3	99.3 ± 34.2	191.4
PHENOTHIAZINE	2.7 ± 2.0	ND	NA	173.0
RABEPRAZOLE	3.1 ± 5.0	ND	NA	151.4
FENBENDAZOLE	13.9 ± 21.5	ND	NA	93.5
OMEPRAZOLE	16.4 ± 4.8*	1.8 ± 1.8	47.6 ± 19.1	89.9
BETA-NAPHTHOFLAVONE	51.2 ± 46.6*	62.2 ± 23.0	79.4 ± 96.1	72.9
SAFROLE	-1.1 ± 1.7	ND	NA	58.7
MELATONIN	10.2 ± 11.2	ND	NA	32.8
LOVASTATIN	0.1 ± 2.1	ND	NA	31.0
1-NAPHTHYL ISOTHIOCYANATE	3.5 ± 2.5	ND	NA	30.3
PANTOPRAZOLE	-1.1 ± 1.3	ND	NA	28.6
ALBENDAZOLE	12.4 ± 4.3*	ND	NA	27.0
SERTRALINE	41.4 ± 3.8*	ND	25.2 ± 31.4	24.5
BROMHEXINE	-1.7 ± 3.7	ND	NA	23.2
ANASTROZOLE	3.8 ± 1.9	11 ± 5.7	16.2 ± 25.6	18.0
EPERISONE	0.7 ± 2.0	ND	NA	15.7

4,4'-METHYLENEDIANILINE	0.2 ± 0.5	ND	NA	15.7
CARVEDILOL	10 ± 15.9	ND	NA	12.8
ALPHA-NAPHTHOFLAVONE	37.0 ± 7.6*	25.8 ± 13.6	13.8 ± 13.9	11.5
ZILEUTON	-0.4 ± 2.6	ND	NA	11.4
BW-723C86	0.8 ± 2.8	ND	NA	10.9
CLOTRIMAZOLE	11.8 ± 14.3	ND	NA	10.8
THIORIDAZINE	1.3 ± 3.7	ND	NA	9.0
DIPYRONE	13.4 ± 18.3	12 ± 21.1	24.5 ± 35.1	8.7
ZOMEPIRAC	0.2 ± 0.5	ND	NA	8.6
VALPROIC ACID	-0.7 ± 1.4	ND	NA	8.5
CINNARIZINE	-1.0 ± 2.3	ND	NA	7.5
DOXAZOSIN	3.6 ± 6.0	ND	NA	7.4
DIFLUNISAL	7.5 ± 11.1	ND	NA	7.4
TROXIPIDE	1.6 ± 6.3	ND	NA	7.3
SULINDAC	30.0 ± 5.0*	ND	NA	7.0
3-METHYLCHOLANTHRENE	168.7 ± 28.3*	58.2 ± 19.2	7.8 ± 11.5	7.0
TENIDAP	43.5 ± 15.0*	ND	21.2 ± 36.6	6.7
AMPIROXICAM	0.6 ± 2.8	ND	NA	6.5
OXICONAZOLE	-0.9 ± 0.6	ND	NA	6.4
N,N-DIMETHYLFORMAMIDE	-0.1 ± 0.4	ND	NA	5.6
SODIUM ARSENITE	1.4 ± 1.3	ND	NA	5.6
TACRINE	1.2 ± 2.0	ND	NA	5.5
N-NITROSODIETHYLAMINE	-0.2 ± 0.5	ND	NA	5.4
CARBAMAZEPINE	-1.1 ± 1.5	ND	NA	5.2
DEXAMETHASONE	41.8 ± 22.9*	ND	NA	5.2
MEXILETINE	18.2 ± 16.3*	29.3 ± 14.6	32.2 ± 14.8	4.9
SIMVASTATIN	0.1 ± 1.4	ND	NA	4.6
BENOXAPROFEN	17.8 ± 8.5*	ND	NA	4.3
FLUDROCORTISONE ACETATE	43.6 ± 22.4*	ND	NA	4.3
ATORVASTATIN	17.1 ± 10.2*	11.5 ± 6.6	27.9 ± 35.2	4.2
CROTAMITON	2.6 ± 2.8	ND	NA	4.1
AMITRAZ	-0.9 ± 1.1	ND	NA	4.0

FENOPROFEN	10.2 ± 13.2	ND	NA	3.8
BISPHENOL A	-0.3 ± 1.9	ND	NA	3.8
TIMOLOL	-1.8 ± 4.2	ND	NA	3.7
ACECLOFENAC	1.0 ± 3.6	ND	NA	3.6
SPARTEINE	-1.2 ± 4.6	ND	NA	3.6
DICYCLOMINE	4.3 ± 6.6	ND	NA	3.5
ETHYLENE GLYCOL	1.1 ± 1.0	ND	NA	3.3
MELOXICAM	5.1 ± 7.6	ND	NA	3.3
NIMODIPINE	40.1 ± 12.8*	29.7 ± 14.6	111.4 ± 37.9	3.2
4-METHYLPYRAZOLE	6.3 ± 7.2	ND	NA	3.2
PREDNISOLONE	40.2 ± 18.8*	ND	NA	3.1
CADMIUM ACETATE	-4.8 ± 4.3	ND	NA	3.0
ROFECOXIB	-5.4 ± 5.5	ND	NA	3.0
AMPRENAVIR	5.5 ± 8.4	ND	NA	3.0
FAMOTIDINE	7.5 ± 10.5	ND	NA	2.8
AMOXAPINE	-1.9 ± 1.7	ND	NA	2.6
RIFABUTIN	18 ± 21.6	25 ± 13.9	55.6 ± 19.0	2.6
FLUVASTATIN	56.8 ± 9.9*	ND	1.6 ± 2.7	2.5
NADOLOL	9.0 ± 13.1	ND	NA	2.4
CARBOPLATIN	6.7 ± 8.5	ND	NA	2.3
GLICLAZIDE	-1.5 ± 1.8	ND	NA	2.3
VALSARTAN	0.1 ± 1.8	8.4 ± 4.4	1.4 ± 2.5	2.3
FLUTAMIDE	41.8 ± 14.6*	11 ± 17.0	97.4 ± 68.4	2.3
QUINAPRIL	-2.9 ± 2.8	ND	NA	2.1
METOPROLOL	7.6 ± 7.8	6.2 ± 6.1	38.1 ± 33.2	2.1
METHOTREXATE	0.4 ± 2.0	ND	NA	2.1
CADMIUM CHLORIDE	19.5 ± 12.6*	ND	NA	2.1
TIAPRIDE	-0.2 ± 3.4	ND	NA	2.1
FLUOXETINE	0.4 ± 6.4	ND	NA	2.0
BITHIONOL	-0.7 ± 0.2	ND	NA	2.0
PROPRANOLOL	-1.8 ± 4.7	ND	NA	2.0
EPINEPHRINE	5.0 ± 2.4	ND	NA	1.9

CAPTOPRIL	-1.1 ± 3.5	ND	NA	1.9
DIGITONIN	-0.5 ± 0.4	ND	NA	1.9
LOSARTAN	9.1 ± 11.6	ND	NA	1.9
ACONITINE	8.7 ± 2.3	40.7 ± 24.0	29.8 ± 25.9	1.9
FLUOCINOLONE ACETONIDE	7.4 ± 4.4	ND	NA	1.8
DIPHENIDOL	0.6 ± 2.3	ND	NA	1.8
PYRILAMINE	-2.5 ± 2.0	27 ± 23.0	22.8 ± 30.6	1.8
CILOSTAZOL	-2.7 ± 1.0	ND	NA	1.7
BUFLOMEDIL	2.2 ± 1.8	ND	NA	1.7
ESMOLOL	6.1 ± 9.3	ND	NA	1.7
FLUPHENAZINE	13.1 ± 8.2*	ND	NA	NS
INDOMETHACIN	13.0 ± 11.0*	22.6 ± 7.8	14.4 ± 18.7	NS
CITALOPRAM	11.8 ± 17.3	ND	NA	NS
NEVIRAPINE	10.7 ± 8.9*	ND	NA	NS
AZITHROMYCIN	9.4 ± 11.9	ND	NA	NS
GEMFIBROZIL	8.4 ± 11.1	ND	NA	NS
NIMESULIDE	7.4 ± 9.2	ND	NA	NS
CYPROHEPTADINE	7.0 ± 6.5	ND	NA	NS
CIMETIDINE	7.0 ± 8.0	ND	NA	NS
PREDNISONE	6.8 ± 5.0	ND	NA	NS
PIOGLITAZONE	6.2 ± 6.5	5.6 ± 7.0	14.4 ± 12.8	NS
ACETAZOLAMIDE	5.0 ± 4.3	ND	NA	NS
TACROLIMUS	4.8 ± 5.2	14.4 ± 9.0	NA	NS
IFOSFAMIDE	4.7 ± 4.9	ND	NA	NS
DOXAPRAM	4.5 ± 6.3	ND	NA	NS
DIGOXIN	4.0 ± 1.4	22.6 ± 2.0	51.6 ± 7.8	NS
DICLOFENAC	3.9 ± 3.5	ND	NA	NS
ACETAMINOPHEN	3.8 ± 4.5	ND	NA	NS
CELECOXIB	3.6 ± 4.2	ND	NA	NS
NAPROXEN	3.5 ± 5.3	ND	NA	NS
VINBLASTINE	2.9 ± 4.5	ND	NA	NS
AZATHIOPRINE	2.6 ± 3.8	ND	NA	NS

AMIODARONE	2.6 ± 4.9	ND	NA	NS
ZIDOVUDINE	1.6 ± 10.0	ND	NA	NS
STAVUDINE	1.4 ± 3.0	ND	NA	NS
TEMAFLOXACIN	1.2 ± 2.9	ND	NA	NS
METHYLDOPA	1.1 ± 1.2	ND	NA	NS
SOTALOL	0.8 ± 1.2	ND	NA	NS
DIGITOXIN	0.4 ± 0.5	ND	NA	NS
NYSTATIN	0.3 ± 1.5	ND	NA	NS
OLANZAPINE	0.1 ± 3.4	ND	NA	NS
LEAD (II) ACETATE	0.0 ± 0.2	ND	NA	NS
GENTAMICIN	-0.1 ± 0.6	19 ± 10.0	NA	NS
CISPLATIN	-0.3 ± 0.4	ND	NA	NS
DIETHYLSTILBESTROL	-0.4 ± 0.5	ND	NA	NS
CLEMASTINE	-0.8 ± 0.3	ND	NA	NS
VECURONIUM BROMIDE	-1.0 ± 3.2	ND	NA	NS
IDARUBICIN	-1.3 ± 5.0	ND	NA	NS
VENLAFAXINE	-1.8 ± 4.0	ND	NA	NS
SPIRONOLACTONE	-1.8 ± 3.8	ND	NA	NS
PRALIDOXIME CHLORIDE	-2.1 ± 4.3	ND	NA	NS
DOXORUBICIN	-2.8 ± 1.0	ND	NA	NS
RAPAMYCIN	-3.3 ± 7.2	4.9 ± 7.0	14.9 ± 25.8	NS
DAUNORUBICIN	-4.2 ± 1.8	ND	NA	NS
CYCLOSPORIN A	-5 ± 4.5	ND	NA	NS
ROSIGLITAZONE	-6.3 ± 5.3	ND	NA	NS
EPIRUBICIN	-7.3 ± 3.2	ND	NA	NS
NIZATIDINE	-1.1 ± 2.4	ND	NA	0.7
DEXCHLORPHENIRAMINE	1.6 ± 2.1	ND	NA	0.6
CLOFIBRATE	1.9 ± 2.4	ND	NA	0.5
NEOMYCIN	0.0 ± 0.5	ND	NA	0.5
LEAD(IV) ACETATE	-0.2 ± 0.3	ND	NA	0.5
IBUPROFEN	4.3 ± 3.3	ND	NA	0.5
BENZETHONIUM CHLORIDE	3.1 ± 6.1	5.9 ± 5.3	59.5 ± 38.7	0.5

FENOFIBRATE	6.9 ± 4.1	ND	NA	0.5
BEZAFIBRATE	4.7 ± 4.1	8.8 ± 10.2	16.9 ± 16.5	0.4
ASPIRIN	-0.1 ± 0.6	ND	NA	0.4

(*) Statistically significant ($p<0.05$) luciferase induction relative to DMSO controls.

ND = not detected. NS = non-significant. NA = not determined.

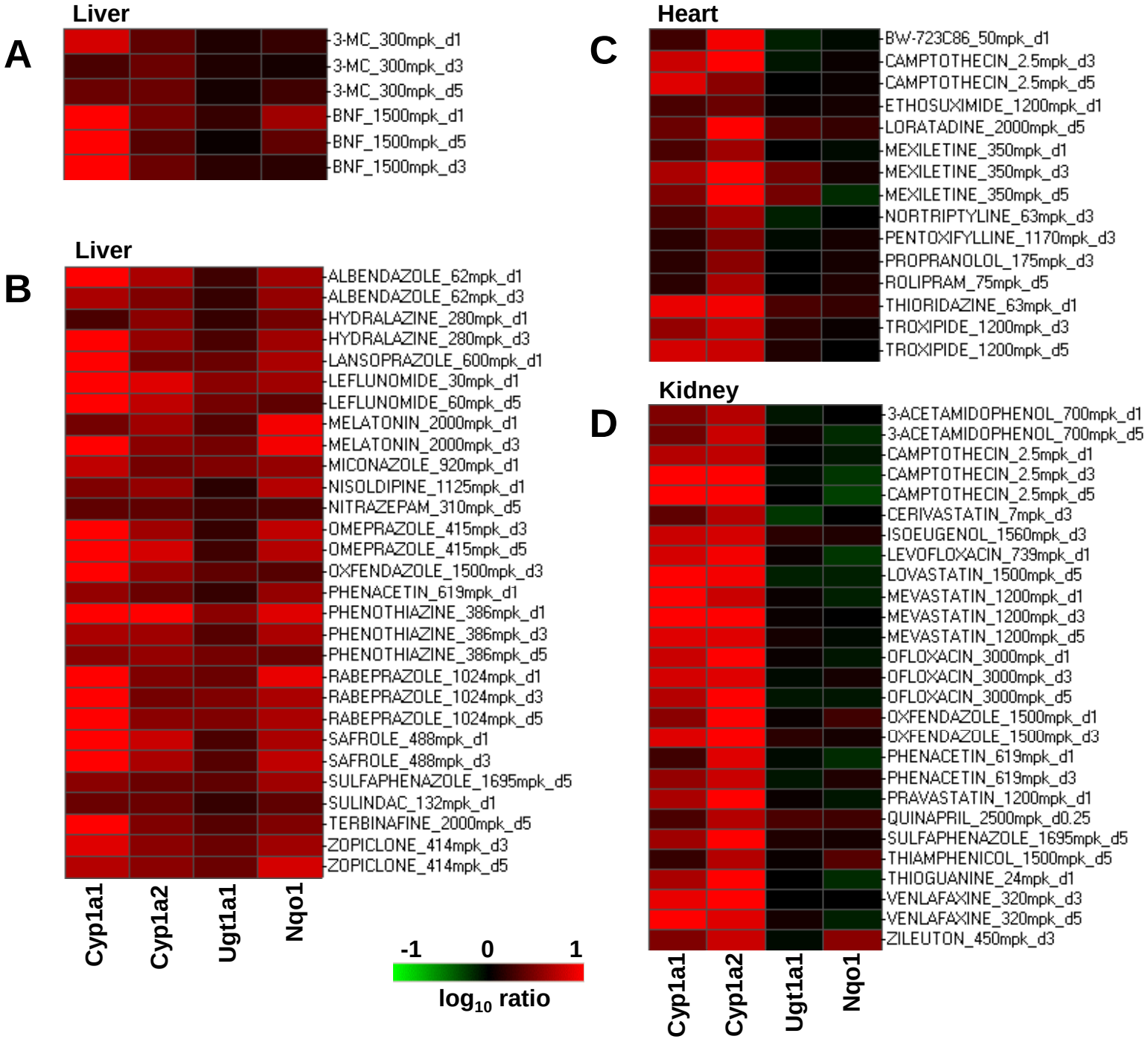


Figure 1

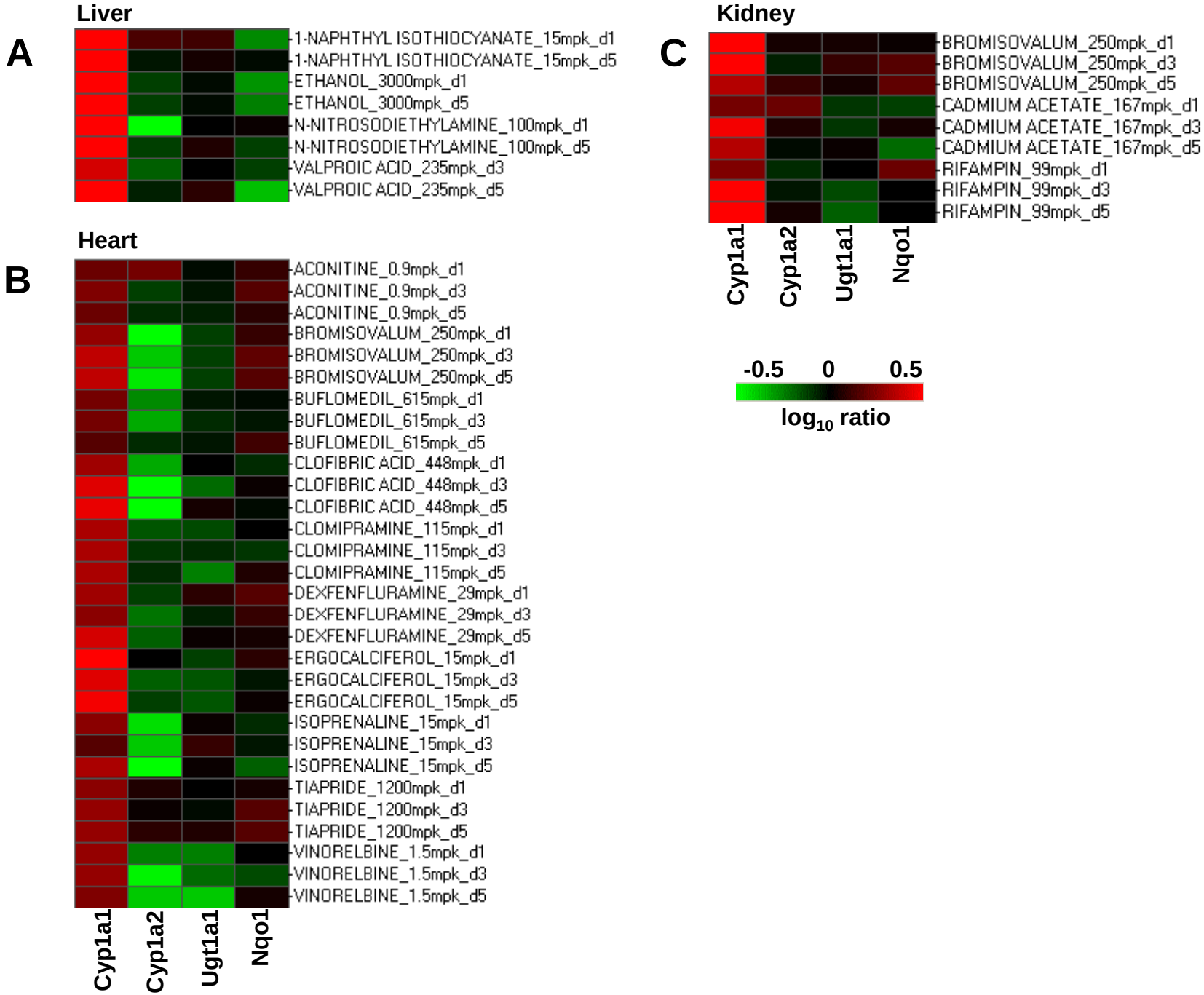


Figure 2

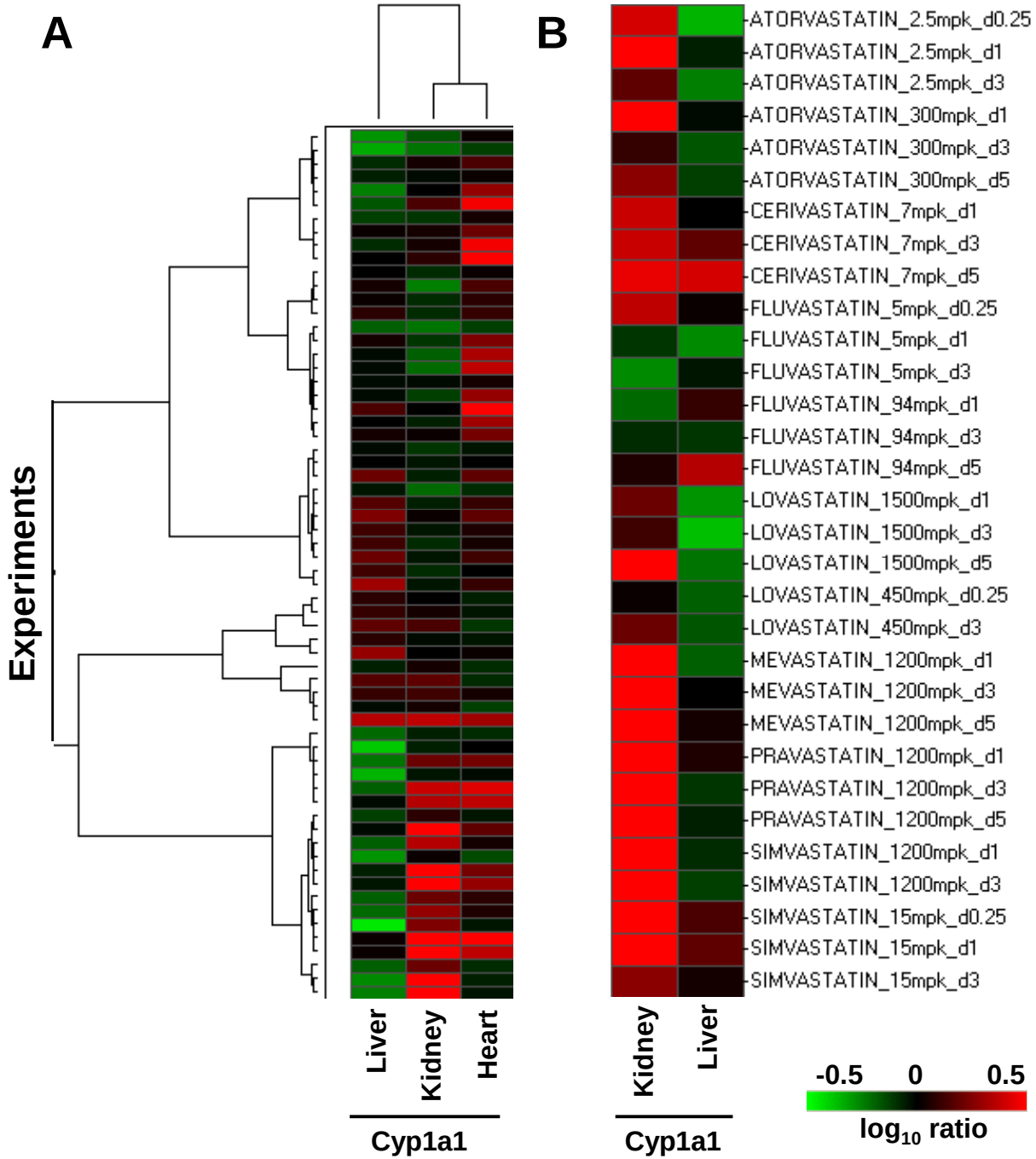
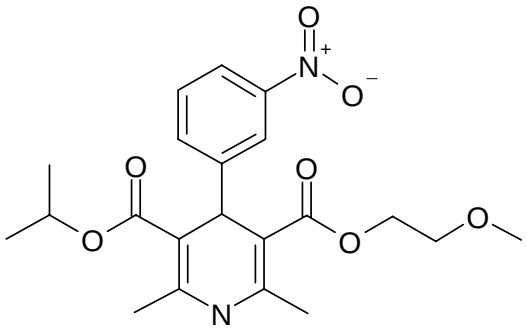
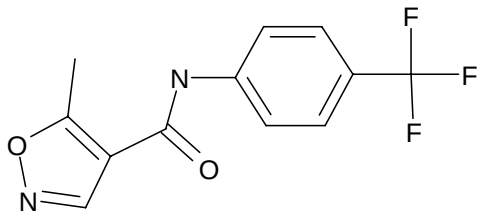


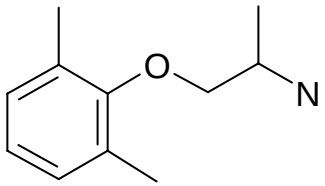
Figure 3



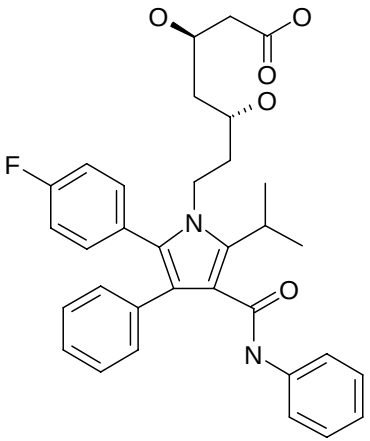
Nimodipine



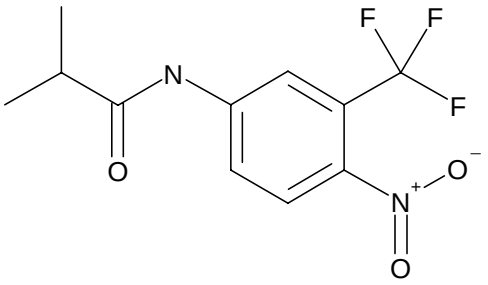
Leflunomide



Mexiletine



Atorvastatin



Flutamide

Figure 4

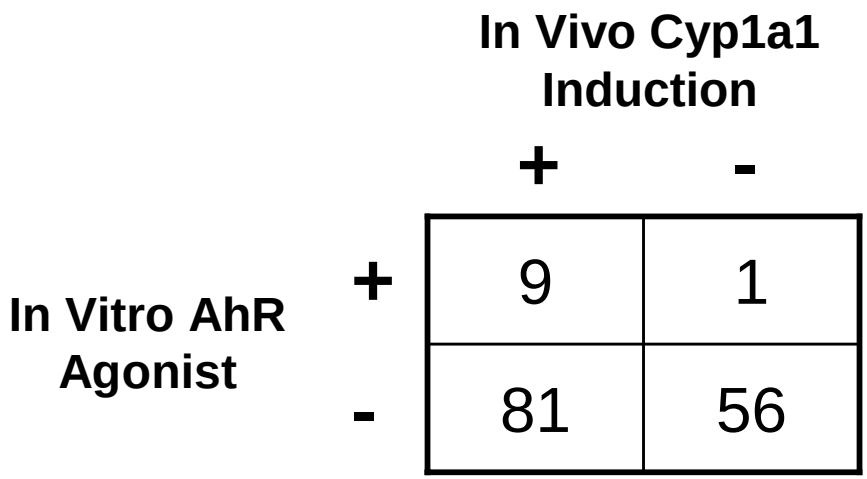


Figure 5

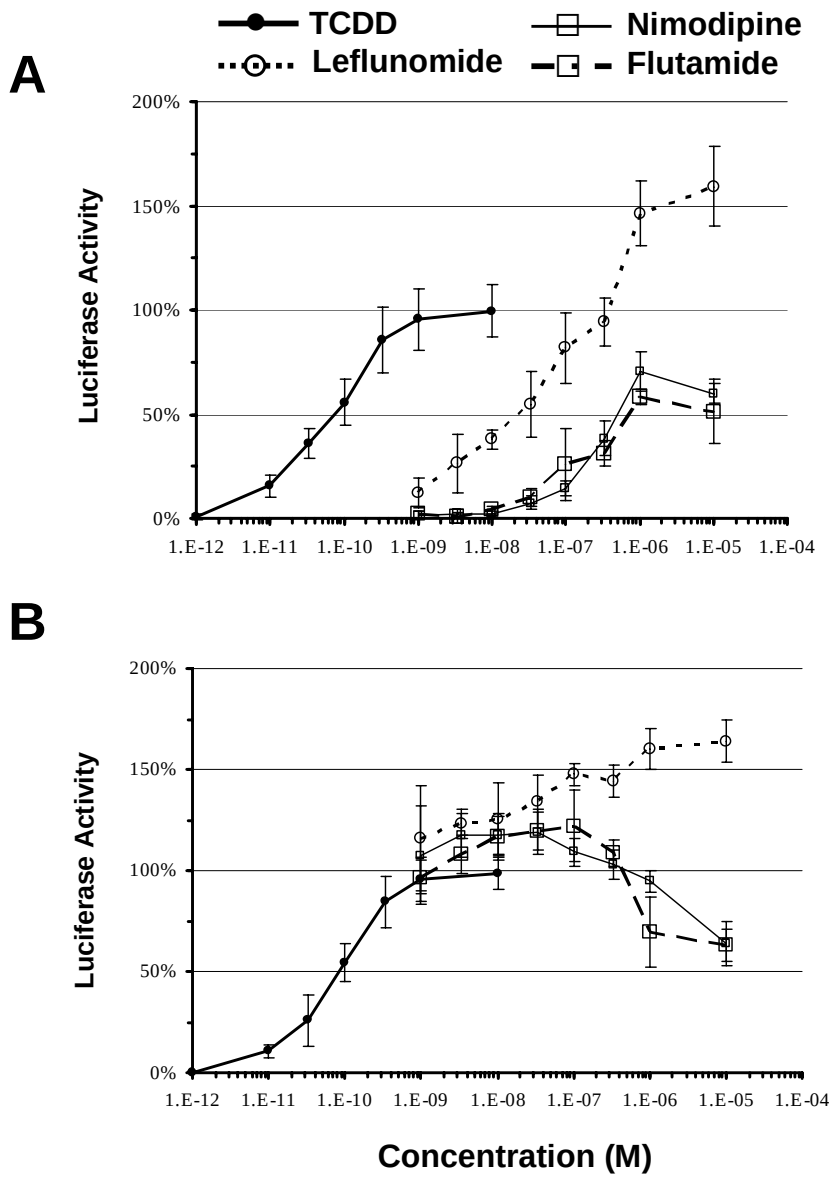


Figure 6

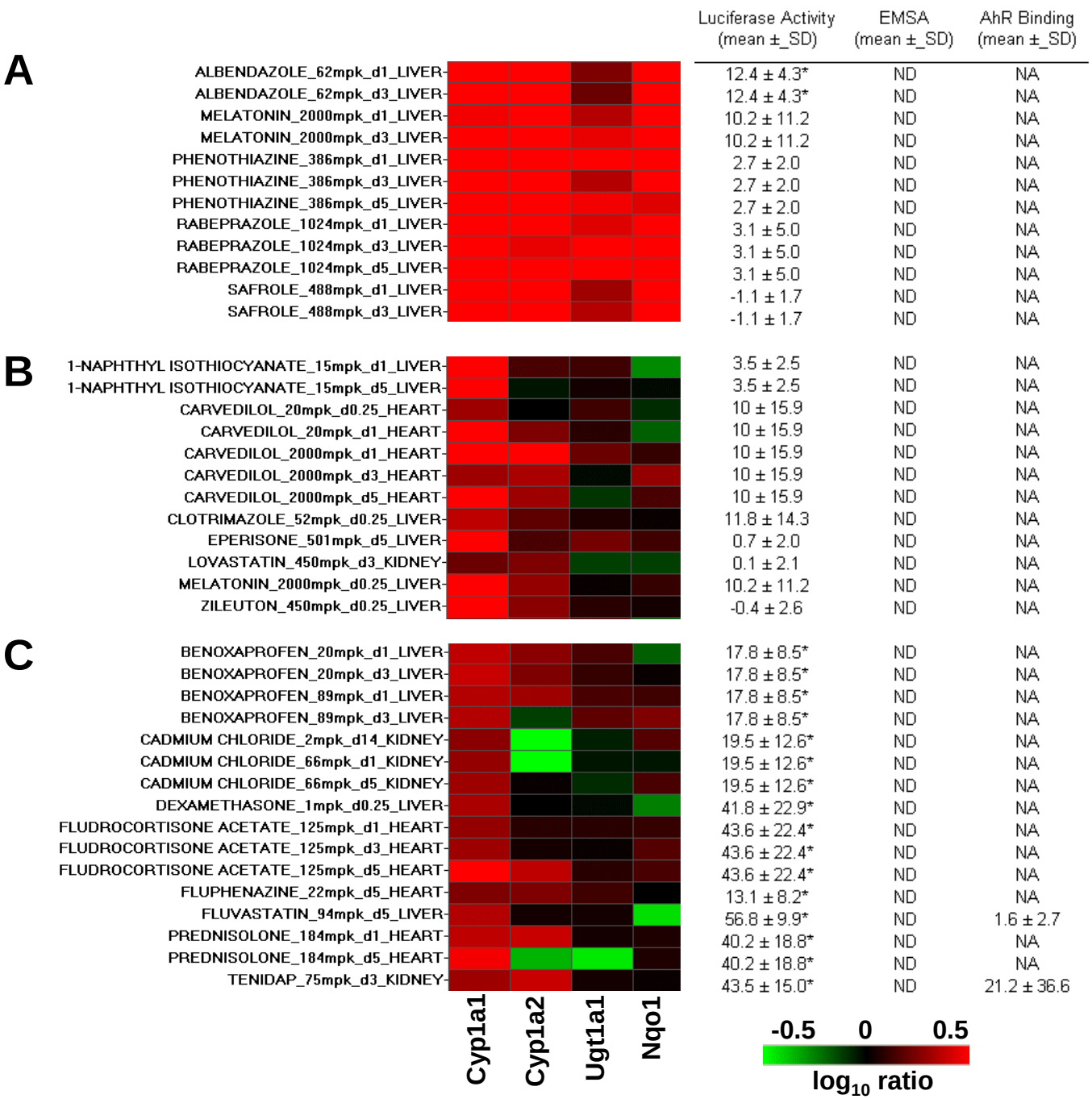


Figure 7