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Induction of Cyp1a1 Is a Nonspecific Biomarker of Aryl Hydrocarbon Receptor Activation: Results of Large Scale Screening of Pharmaceuticals and Toxicants in Vivo and in Vitro[S]

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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 data set, a subset of 147 compounds was 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. Whereas in vivo Cyp1a1 mRNA expression is a sensitive marker for AhR activation, it lacks specificity, because 81 (59%) of 137 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 nine AhR agonists, six of which are marketed therapeutics and have been approved by the U.S. Food and Drug Administration, 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 nonspecific 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.

The aryl hydrocarbon receptor (AhR) regulates the expression of phase 1 and 2 metabolism genes, including cytochromes P450 (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 promutagens, sustained activation of the AhR is associated with the potential for adverse effects in a number of organ systems because of its role in regulating the 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 matri-

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; DRE, dioxin-response element; HAH, halogenated aromatic hydrocarbon; 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, β-naphthoflavone; EMSA, electrophoretic mobility shift assay; HEDG buffer, HEPES/EDTA/dithiothreitol/glycerol; DMSO, dimethyl sulfoxide; FDA, Food and Drug Administration; PGC-1, peroxisome proliferator activated receptor-γ coactivator; PPAR, peroxisome proliferator-activated receptor; 5-HT2B, 5-hydroxytryptamine-2B; BW-723C86, 1-[5-(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl.

ces (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 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 nonhalogenated high-affinity ligands of the AhR such as β-naphthoflavone 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 et al., 1998, 2002; Denison and Nagy, 2003). 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 nonpersistent 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 was 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, was also evaluated to determine whether 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, six marketed drugs that activate and bind to the rat AhR were identified and 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 nonpersistent compounds.

Materials and Methods

In Vivo Treatments. Animal and treatment details for the compounds discussed herein are as described previously (Ganter et al., 2005). This includes data on 596 compounds representing 3230 compound-dose-time point combinations. In brief, in vivo short-term repeat-dose rat studies have been conducted previously 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, Chalfont St. Giles, Buckinghamshire, UK). Housing and treatment of the animals were in accordance with regulations outlined in the United States Department of Agriculture 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 described previously (Ganter et al., 2005). The microarray gene expression results reported herein are presented as $\log_{10}$ ratios for Cyp1a1 (GenBank accession no. X00469), Cyp1a2 (GenBank accession no. K02422), Ugt1a1 (GenBank accession no. J05132), and Nqo1 (GenBank accession no. NM_017000), in which each experimental 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 to evaluate the potential for false negatives in the gene expression data. The compounds were obtained from a variety of different sources as described previously (Ganter et al., 2005). A summary of all in vitro data presented herein for all 147 compounds is provided in Table 1.

Electrophoretic Mobility Shift Assay. All 147 compounds were tested for their ability to transform the rat hepatic cytosolic AhR into its DNA binding form using electrophoretic mobility shift assay (EMSA) as described previously in detail (Denison et al., 2002). In brief, 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 dithiothreitol, and 10% (v/v) glycerol] (Denison et al., 2002), was incubated with dimethyl sulfoxide (DMSO) (10 μl/ml), TCDD (20 nM), or the indicated test compound (10 μM) for 2 h 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 (GE Healthcare). 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 described previously

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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 ($n = 9$), 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 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	EMSA	AhR Binding	In Vivo Cyp1a1 (-Fold-induction)
		mean ± S.D.		
Leflunomide	135.4 ± 27.1*	60.2 ± 10.3	99.3 ± 34.2	191.4
Phenothiazine	2.7 ± 2.0	N.D.	N.A.	173.0
Rabeprazole	3.1 ± 5.0	N.D.	N.A.	151.4
Fenbendazole	13.9 ± 21.5	N.D.	N.A.	93.5
Omeprazole	16.4 ± 4.8*	1.8 ± 1.8	47.6 ± 19.1	89.9
β-Naphthoflavone	51.2 ± 46.6*	62.2 ± 23.0	79.4 ± 96.1	72.9
Safrole	−1.1 ± 1.7	N.D.	N.A.	58.7
Melatonin	10.2 ± 11.2	N.D.	N.A.	32.8
Lovastatin	0.1 ± 2.1	N.D.	N.A.	31.0
1-Naphthyl isothiocyanate	3.5 ± 2.5	N.D.	N.A.	30.3
Pantoprazole	−1.1 ± 1.3	N.D.	N.A.	28.6
Albendazole	12.4 ± 4.3*	N.D.	N.A.	27.0
Sertraline	41.4 ± 3.8*	N.D.	25.2 ± 31.4	24.5
Bromhexine	−1.7 ± 3.7	N.D.	N.A.	23.2
Anastrozole	3.8 ± 1.9	11 ± 5.7	16.2 ± 25.6	18.0
Eperisone	0.7 ± 2.0	N.D.	N.A.	15.7
4,4′-Methylenedianiline	0.2 ± 0.5	N.D.	N.A.	15.7
Carvedilol	10 ± 15.9	N.D.	N.A.	12.8
α-Naphthoflavone	37.0 ± 7.6*	25.8 ± 13.6	13.8 ± 13.9	11.5
Zileuton	−0.4 ± 2.6	N.D.	N.A.	11.4
BW-723C86	0.8 ± 2.8	N.D.	N.A.	10.9
Clotrimazole	11.8 ± 14.3	N.D.	N.A.	10.8
Thioridazine	1.3 ± 3.7	N.D.	N.A.	9.0
Dipyrone	13.4 ± 18.3	12 ± 21.1	24.5 ± 35.1	8.7
Zomepirac	0.2 ± 0.5	N.D.	N.A.	8.6
Valproic acid	−0.7 ± 1.4	N.D.	N.A.	8.5
Cinnarizine	−1.0 ± 2.3	N.D.	N.A.	7.5
Doxazosin	3.6 ± 6.0	N.D.	N.A.	7.4
Diflunisal	7.5 ± 11.1	N.D.	N.A.	7.4
Troxipide	1.6 ± 6.3	N.D.	N.A.	7.3
Sulindac	30.0 ± 5.0*	N.D.	N.A.	7.0
3-Methylcholanthrene	168.7 ± 28.3*	58.2 ± 19.2	7.8 ± 11.5	7.0
Tenidap	43.5 ± 15.0*	N.D.	21.2 ± 36.6	6.7
Ampiroxicam	0.6 ± 2.8	N.D.	N.A.	6.5
Oxiconazole	−0.9 ± 0.6	N.D.	N.A.	6.4
N,*N*-Dimethylformamide	−0.1 ± 0.4	N.D.	N.A.	5.6
Sodium arsenite	1.4 ± 1.3	N.D.	N.A.	5.6
Tacrine	1.2 ± 2.0	N.D.	N.A.	5.5
N-Nitrosodiethylamine	−0.2 ± 0.5	N.D.	N.A.	5.4
Carbamazepine	−1.1 ± 1.5	N.D.	N.A.	5.2
Dexamethasone	41.8 ± 22.9*	N.D.	N.A.	5.2
Mexiletine	18.2 ± 16.3*	29.3 ± 14.6	32.2 ± 14.8	4.9
Simvastatin	0.1 ± 1.4	N.D.	N.A.	4.6
Benoxaprofen	17.8 ± 8.5*	N.D.	N.A.	4.3
Fludrocortisone acetate	43.6 ± 22.4*	N.D.	N.A.	4.3
Atorvastatin	17.1 ± 10.2*	11.5 ± 6.6	27.9 ± 35.2	4.2
Crotamiton	2.6 ± 2.8	N.D.	N.A.	4.1
Amitraz	−0.9 ± 1.1	N.D.	N.A.	4.0
Fenoprofen	10.2 ± 13.2	N.D.	N.A.	3.8
Bisphenol A	−0.3 ± 1.9	N.D.	N.A.	3.8
Timolol	−1.8 ± 4.2	N.D.	N.A.	3.7
Aceclofenac	1.0 ± 3.6	N.D.	N.A.	3.6
Sparteine	−1.2 ± 4.6	N.D.	N.A.	3.6
Dicyclomine	4.3 ± 6.6	N.D.	N.A.	3.5
Ethylene glycol	1.1 ± 1.0	N.D.	N.A.	3.3
Meloxicam	5.1 ± 7.6	N.D.	N.A.	3.3
Nimodipine	40.1 ± 12.8*	29.7 ± 14.6	111.4 ± 37.9	3.2
4-Methylpyrazole	6.3 ± 7.2	N.D.	N.A.	3.2
Prednisolone	40.2 ± 18.8*	N.D.	N.A.	3.1
Cadmium acetate	−4.8 ± 4.3	N.D.	N.A.	3.0
Rofecoxib	−5.4 ± 5.5	N.D.	N.A.	3.0
Amprenavir	5.5 ± 8.4	N.D.	N.A.	3.0
Famotidine	7.5 ± 10.5	N.D.	N.A.	2.8
Amoxapine	−1.9 ± 1.7	N.D.	N.A.	2.6
Rifabutin	18 ± 21.6	25 ± 13.9	55.6 ± 19.0	2.6
Fluvastatin	56.8 ± 9.9*	N.D.	1.6 ± 2.7	2.5
Nadolol	9.0 ± 13.1	N.D.	N.A.	2.4
Carboplatin	6.7 ± 8.5	N.D.	N.A.	2.3
Gliclazide	−1.5 ± 1.8	N.D.	N.A.	2.3
Valsartan	0.1 ± 1.8	8.4 ± 4.4	1.4 ± 2.5	2.3

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TABLE 1
Continued

Compound Name	Luciferase Activity	EMSA	AhR Binding	In Vivo Cyp1a1 (-Fold-induction)
		mean ± S.D.		
Flutamide	41.8 ± 14.6*	11 ± 17.0	97.4 ± 68.4	2.3
Quinapril	−2.9 ± 2.8	N.D.	N.A.	2.1
Metoprolol	7.6 ± 7.8	6.2 ± 6.1	38.1 ± 33.2	2.1
Methotrexate	0.4 ± 2.0	N.D.	N.A.	2.1
Cadmium chloride	19.5 ± 12.6*	N.D.	N.A.	2.1
Tiapride	−0.2 ± 3.4	N.D.	N.A.	2.1
Fluoxetine	0.4 ± 6.4	N.D.	N.A.	2.0
Bithionol	−0.7 ± 0.2	N.D.	N.A.	2.0
Propranolol	−1.8 ± 4.7	N.D.	N.A.	2.0
Epinephrine	5.0 ± 2.4	N.D.	N.A.	1.9
Captopril	−1.1 ± 3.5	N.D.	N.A.	1.9
Digitonin	−0.5 ± 0.4	N.D.	N.A.	1.9
Losartan	9.1 ± 11.6	N.D.	N.A.	1.9
Aconitine	8.7 ± 2.3	40.7 ± 24.0	29.8 ± 25.9	1.9
Fluocinolone acetonide	7.4 ± 4.4	N.D.	N.A.	1.8
Diphenidol	0.6 ± 2.3	N.D.	N.A.	1.8
Pyrilamine	−2.5 ± 2.0	27 ± 23.0	22.8 ± 30.6	1.8
Cilostazol	−2.7 ± 1.0	N.D.	N.A.	1.7
Buflomedil	2.2 ± 1.8	N.D.	N.A.	1.7
Esmolol	6.1 ± 9.3	N.D.	N.A.	1.7
Fluphenazine	13.1 ± 8.2*	N.D.	N.A.	N.S.
Indomethacin	13.0 ± 11.0*	22.6 ± 7.8	14.4 ± 18.7	N.S.
Citalopram	11.8 ± 17.3	N.D.	N.A.	N.S.
Nevirapine	10.7 ± 8.9*	N.D.	N.A.	N.S.
Azithromycin	9.4 ± 11.9	N.D.	N.A.	N.S.
Gemfibrozil	8.4 ± 11.1	N.D.	N.A.	N.S.
Nimesulide	7.4 ± 9.2	N.D.	N.A.	N.S.
Cyproheptadine	7.0 ± 6.5	N.D.	N.A.	N.S.
Cimetidine	7.0 ± 8.0	N.D.	N.A.	N.S.
Prednisone	6.8 ± 5.0	N.D.	N.A.	N.S.
Pioglitazone	6.2 ± 6.5	5.6 ± 7.0	14.4 ± 12.8	N.S.
Acetazolamide	5.0 ± 4.3	N.D.	N.A.	N.S.
Tacrolimus	4.8 ± 5.2	14.4 ± 9.0	N.A.	N.S.
Ifosfamide	4.7 ± 4.9	N.D.	N.A.	N.S.
Doxapram	4.5 ± 6.3	N.D.	N.A.	N.S.
Digoxin	4.0 ± 1.4	22.6 ± 2.0	51.6 ± 7.8	N.S.
Diclofenac	3.9 ± 3.5	N.D.	N.A.	N.S.
Acetaminophen	3.8 ± 4.5	N.D.	N.A.	N.S.
Celecoxib	3.6 ± 4.2	N.D.	N.A.	N.S.
Naproxen	3.5 ± 5.3	N.D.	N.A.	N.S.
Vinblastine	2.9 ± 4.5	N.D.	N.A.	N.S.
Azathioprine	2.6 ± 3.8	N.D.	N.A.	N.S.
Amiodarone	2.6 ± 4.9	N.D.	N.A.	N.S.
Zidovudine	1.6 ± 10.0	N.D.	N.A.	N.S.
Stavudine	1.4 ± 3.0	N.D.	N.A.	N.S.
Temafloxacin	1.2 ± 2.9	N.D.	N.A.	N.S.
Methyldopa	1.1 ± 1.2	N.D.	N.A.	N.S.
Sotalol	0.8 ± 1.2	N.D.	N.A.	N.S.
Digitoxin	0.4 ± 0.5	N.D.	N.A.	N.S.
Nystatin	0.3 ± 1.5	N.D.	N.A.	N.S.
Olanzapine	0.1 ± 3.4	N.D.	N.A.	N.S.
Lead (II) acetate	0.0 ± 0.2	N.D.	N.A.	N.S.
Gentamicin	−0.1 ± 0.6	19 ± 10.0	N.A.	N.S.
Cisplatin	−0.3 ± 0.4	N.D.	N.A.	N.S.
Diethylstilbestrol	−0.4 ± 0.5	N.D.	N.A.	N.S.
Clemastine	−0.8 ± 0.3	N.D.	N.A.	N.S.
Vecuronium bromide	−1.0 ± 3.2	N.D.	N.A.	N.S.
Idarubicin	−1.3 ± 5.0	N.D.	N.A.	N.S.
Venlafaxine	−1.8 ± 4.0	N.D.	N.A.	N.S.
Spironolactone	−1.8 ± 3.8	N.D.	N.A.	N.S.
Pralidoxime chloride	−2.1 ± 4.3	N.D.	N.A.	N.S.
Doxorubicin	−2.8 ± 1.0	N.D.	N.A.	N.S.
Rapamycin	−3.3 ± 7.2	4.9 ± 7.0	14.9 ± 25.8	N.S.
Daunorubicin	−4.2 ± 1.8	N.D.	N.A.	N.S.
Cyclosporin A	−5 ± 4.5	N.D.	N.A.	N.S.
Rosiglitazone	−6.3 ± 5.3	N.D.	N.A.	N.S.
Epirubicin	−7.3 ± 3.2	N.D.	N.A.	N.S.
Nizatidine	−1.1 ± 2.4	N.D.	N.A.	0.7
Dexchlorpheniramine	1.6 ± 2.1	N.D.	N.A.	0.6
Clofibrate	1.9 ± 2.4	N.D.	N.A.	0.5
Neomycin	0.0 ± 0.5	N.D.	N.A.	0.5
Lead (IV) acetate	−0.2 ± 0.3	N.D.	N.A.	0.5
Ibuprofen	4.3 ± 3.3	N.D.	N.A.	0.5
Benzethonium chloride	3.1 ± 6.1	5.9 ± 5.3	59.5 ± 38.7	0.5

TABLE 1
Continued

Compound Name	Luciferase Activity	EMSA	AhR Binding	In Vivo Cyp1a1 (-Fold-induction)
		mean ± S.D.		
Fenofibrate	6.9 ± 4.1	N.D.	N.A.	0.5
Bezafibrate	4.7 ± 4.1	8.8 ± 10.2	16.9 ± 16.5	0.4
Aspirin	−0.1 ± 0.6	N.D.	N.A.	0.4

N.D., not detected; N.S., nonsignificant; N.A., not determined.
* Statistically significant ($p < 0.05$) luciferase induction relative to DMSO controls.

(Garrison et al., 1996). DMSO (10 μl/ml), TCDD (1 nM), or test compound (10 μM) was added to the 96-well culture plate containing a monolayer of cells. After 4 h of 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 a percentage 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 ($p < 0.01$). In addition, only increases in luciferase activity greater than 10% of 1 nM TCDD were considered biologically relevant.

Ah Receptor-Ligand Binding Assay. 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. In brief, 500-μl aliquots of a rat cytosolic preparation (2 mg/ml total protein concentration) were preincubated 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 2 h, 200-μl aliquots of the incubation mixture were added to tubes containing 250 μl of HAP (0.5 mg/μl in HEDG buffer) and allowed to incubate for 30 min. Samples were centrifuged, and pellets were washed three times with HEDG 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 (nonspecific binding) from that measured in the samples that were incubated with [^{3}H]TCDD alone (total binding). The assay was performed in triplicate for each compound, and the results are presented as a mean percentage of the displacement of specific [^{3}H]TCDD binding.

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) of 3230 in which 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 (Supplementary 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 U.S. Food and Drug Administration, whereas 37 are nonpharmaceuticals and are either prototypical toxicants, industrial chemicals, or biochemical standards. The remaining 44 compounds are drugs registered outside of the United States, withdrawn by the FDA, or discontinued from development (Supplementary Table S1). As expected, known AhR ligands such as β-naphthoflavone (BNF; 1500 mg/kg/day) and 3-methylcholanthrene (3-MC; 300 mg/kg/day) 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 (Fig. 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 that these compounds are potential AhR agonists (Fig. 1B). Other than omeprazole (Shih et al., 1999), these compounds have not been described previously as Cyp1a1 inducers or as AhR agonists. Cyp1a1 was induced more than 100-fold by leflunomide and phenothiazine. Consistent with 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. It is interesting that the pineal gland hormone melatonin significantly induced Cyp1a1 more than 32-fold in addition to inducing Cyp1a2, Ugt1a1, and Nqo1 (Fig. 1B).

There were many treatments that significantly induced Cyp1a1 and 1a2 in heart and kidney also (Fig. 1, C and D). To our knowledge, the majority of these treatments have not been shown previously to induce the expression of Cyp1a1 or Cyp1a2 or to bind to the AhR. In contrast to the results in liver, the expression of Ugt1a1 and Nqo1 did not seem to be coregulated with Cyp1a1 and Cyp1a2 in heart and kidney (Fig. 1, C and D). The most potent inducer of Cyp1a1 in heart was BW-723C86 (10-fold), a selective 5-HT2B receptor agonist (Fig. 1C). Many other compounds evaluated in the kidney, including the HMG-CoA reductase inhibitors lovastatin and mevastatin also induced Cyp1a1 greater than 10-fold (Fig. 1D). These results indicate that Cyp1a1 induction in liver, kidney, and heart is very common among rats treated with marketed therapeutic drugs.

Coregulation 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 (Fig. 2 and Supplementary Table S1). Many of these treatments slightly but not 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 (Fig. 2). In liver, for example, a number of toxicants such as 1-naphthyl isothiocyanate, ethanol, *N*-nitrosodiethylamine, and valproic acid significantly induced Cyp1a1 but slightly repressed Cyp1a2 at both early and late time points (Fig. 2A). A similar effect was particularly evident in heart, in which a number of compounds significantly induced

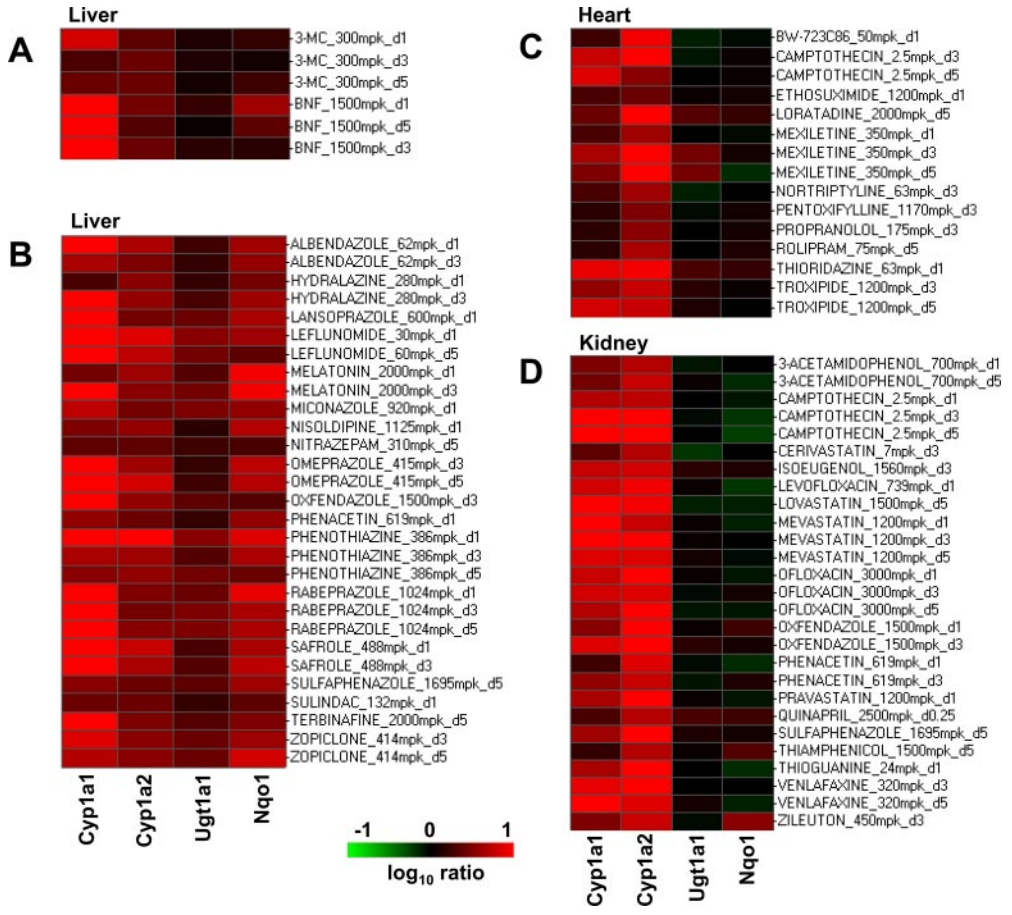

Fig. 1. Consistent induction of AhR-regulated genes in vivo by putative AhR agonists. Gene expression results for known AhR-regulated genes, including Cyp1a1 (X00469) and Cyp1a2 (K02422), Ugt1a1 (J05132), and Nqo1 (NM_017000). Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to controls. Treatments are indicated by compound name, dose in milligrams per kilogram per day (mpk) and duration of treatment in days. A, hepatic gene expression of AhR-regulated genes by known AhR agonists 3-MC and 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.

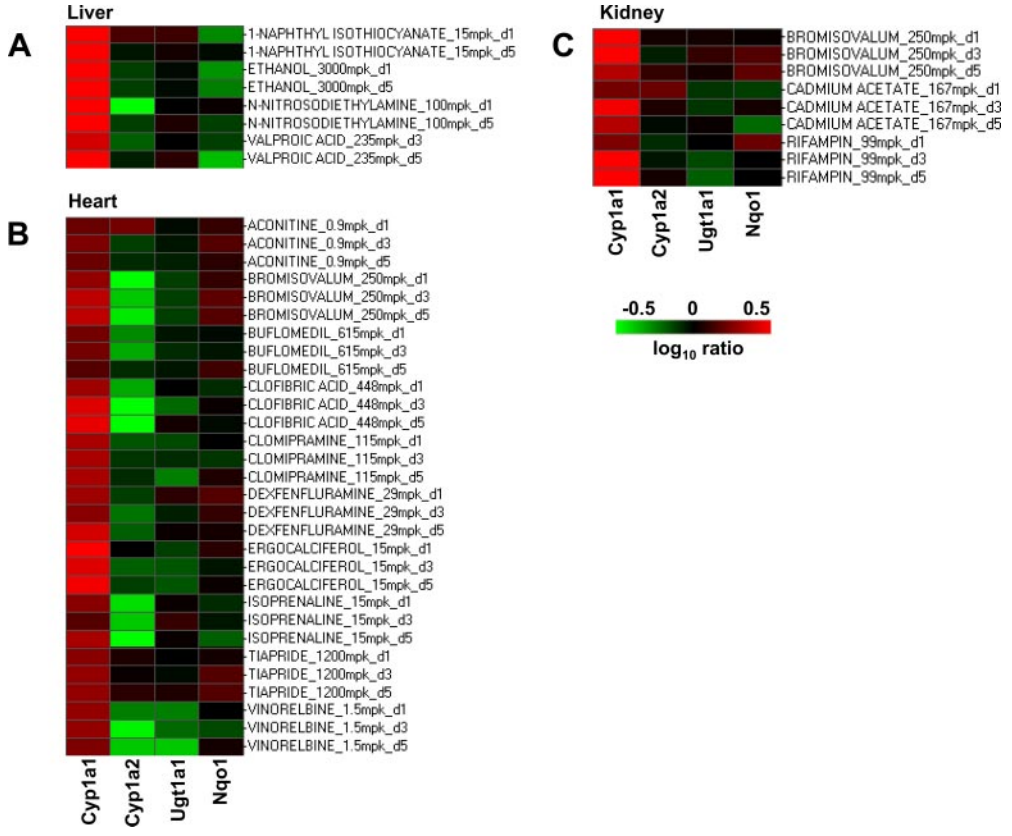

Fig. 2. Atypical induction of Cyp1a1 and AhR-regulated target genes in vivo. Gene expression results for known AhR-regulated genes, including Cyp1a1 (X00469) and Cyp1a2 (K02422), Ugt1a1 (J05132) and Nqo1 (NM_017000). Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to control rats. Treatments are indicated by compound name, dose in milligrams per kilogram per day (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 liver (A), heart (B), or kidney (C).

Cyp1a1 but significantly repressed Cyp1a2, including bromisovalum (days 1 and 5), clofibric acid (days 3 and 5), isoprenaline (days 1 and 5), and vinorelbine (day 3) (Fig. 2B). Similar effects in kidney were observed for bromisovalum, cadmium acetate, and rifampin, although repression of Cyp1a2 was not as pronounced (Fig. 2C). Dexfenfluramine, whose metabolite is a potent 5-HT2B receptor agonist, also significantly induced Cyp1a1 in heart (Fig. 2B), but unlike the 5-HT2B receptor agonist BW-723C86, it did not induce Cyp1a2. This effect in heart was not evident in kidney, in which both Cyp1a1 and 1a2 were not significantly affected by dexfenfluramine (Supplementary Table S1). These results indicate that Cyp1a1 may not be coregulated 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 the induction of other DRE-regulated genes in these tissues.

Tissue-Specific Induction of Cyp1a1. Because of the disparate induction pattern of Cyp1a1 compared with other AhR-responsive genes under certain treatment conditions, it was of interest to determine whether similar effects on Cyp1a1 were observed across tissues. Of the 207 compound-dose-time point 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 2 of the 3 tissues examined. For example, none of the 64 compound-dose-time point combinations that were profiled in all 3 tissues significantly induced Cyp1a1 consistently across all 3 tissues (Fig. 3A). It is interesting that 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 (Fig. 1D), other HMG-CoA reductase inhibitors, including cerivastatin, atorvastatin, pravastatin, and simvastatin significantly induced Cyp1a1 in kidney but not liver (Fig. 3B). The exception was cerivastatin on day 5, which significantly induced liver Cyp1a1 just more than 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. To determine whether 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 α-naphthoflavone, which have been shown previously to be active in these assays. The other six compounds are approved for use by the FDA for a variety of indications, including omeprazole

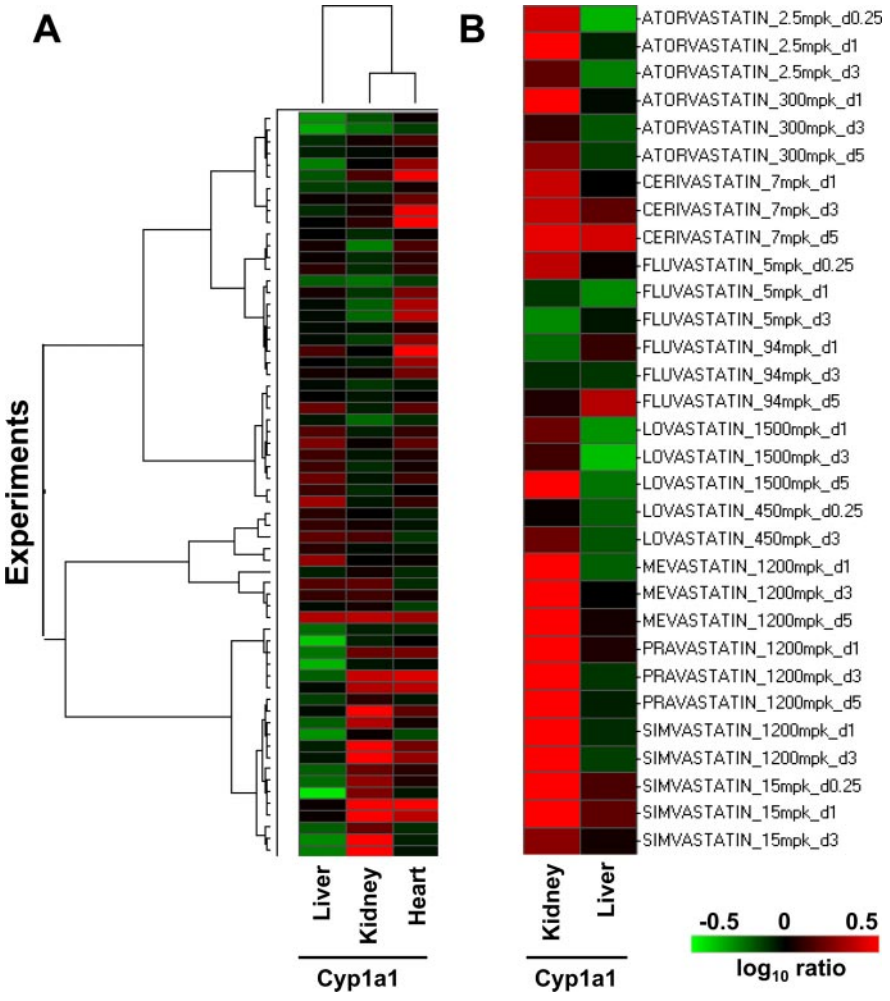

Fig. 3. Tissue-specific regulation of Cyp1a1 expression in vivo. Gene expression results for Cyp1a1 (X00469) in liver, kidney, and heart. Transcript levels were measured using microarrays and are represented as $\log_{10}$ ratios of expression in treated rats relative to control rats. Treatments are indicated by compound name, dose in milligrams per kilogram per day (mpk), and duration of treatment in days. A, discordant gene expression results for Cyp1a1 for compound-dose-time points measured in liver, heart, and kidney. B, gene expression results for Cyp1a1 for HMG-CoA reductase inhibitor treatments measured in both liver and kidney.

(Prilosec), nimodipine (Nimotop), leflunomide (Arava), flutamide (Eulexin), mexiletine (Mexitil), and atorvastatin (Lipitor) (Fig. 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 more than 90% of [^{3}H]TCDD from the AhR. Omeprazole, previously believed to not bind the rat or human receptor (Daujat et al., 1992; Backlund et al., 1997) was found to displace approximately 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). In contrast, indomethacin was weakly positive in all three in vitro assays yet did not significantly induce Cyp1a1 in vivo, nor did it consistently induce Cyp1a2, Ugt1a1, or Nqo1 (Supplementary Table S1). These results indicate that in vivo Cyp1a1 induction is a sensitive (9 of 10) indicator of AhR agonist activity, which is consistent with current understanding of AhR-mediated Cyp1a1 regulation (Fig. 5).

The agonist effects of leflunomide, nimodipine, and flutamide were further tested using the reporter gene assay, in which H4L1.1c4 cells were treated with increasing concentrations of compounds up to 10 μM (Fig. 6A). Leflunomide was the most potent among the three compounds and induced luciferase activity to a significantly greater level than that of TCDD. Based on the dose-response data, leflunomide had an EC_{50} value of 0.17 μM, which was approximately 2700-fold higher than that of TCDD ($EC_{50} = 6.2 \times 10^{-5}$ 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 because of their relatively weak luciferase inducing potency.

To determine whether leflunomide, flutamide, and nimodipine were full or partial agonists in the luciferase assay, the compounds were cotreated with 1 nM TCDD. As shown in Fig. 6B, both flutamide and nimodipine inhibited the response of 1 nM TCDD by 40%, indicating that they are partial agonists. In contrast, cotreatment 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 six 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%) (Fig. 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

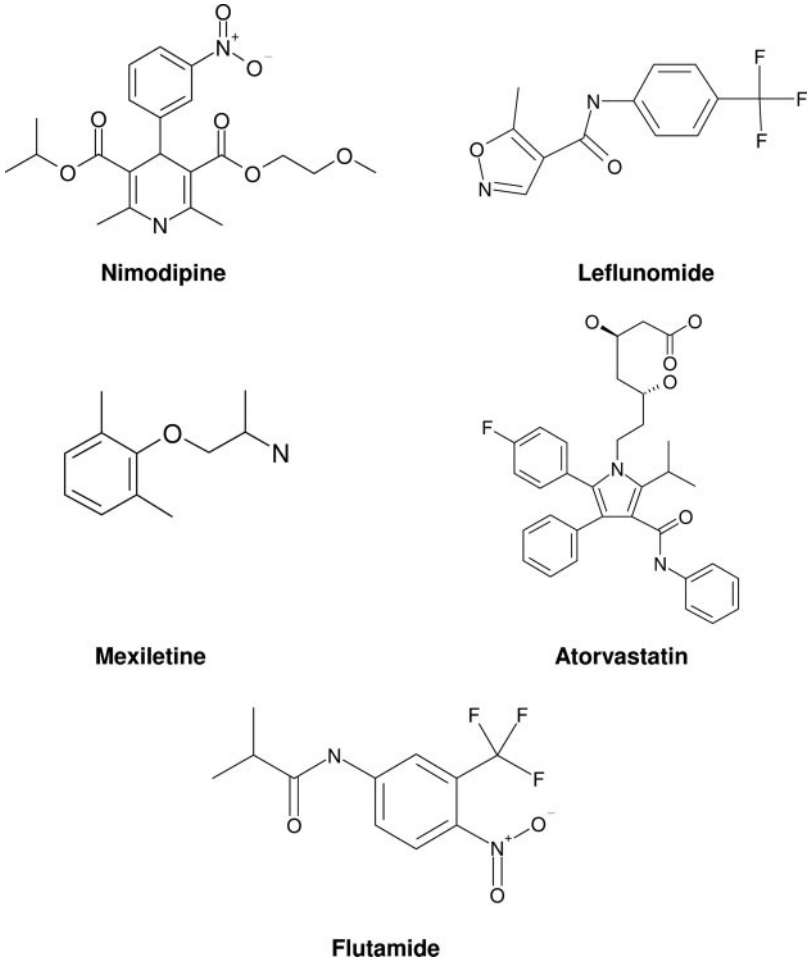

Fig. 4. Structures of AhR agonists identified in this study. Compounds that were not reported previously as AhR agonists are shown. These compounds were found to significantly induce Cyp1a1 in vivo, positively induce DRE-driven luciferase activity in rat H4L1.1c4 cells, stimulate transformation of the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

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) (Fig. 7A). Although metabolic activation may be necessary for in vivo AhR agonist activity for these compounds, there were a number of compounds that induced Cyp1a1 more than 10-fold but did not significantly induce Cyp1a2, Ugt1a1, or 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, 1-naphthylisothiocyanate, eperisone, carvedilol, and zileuton (Fig. 7B). Other compounds that significantly induced luciferase activity and Cyp1a1 more than 10-fold but failed to stimulate the 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 (Fig. 7C).

		In Vivo Cyp1a1 Induction +	In Vivo Cyp1a1 Induction -
In Vitro AhR Agonist	+	9	1
	-	81	56

Fig. 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 under *Materials and Methods*.

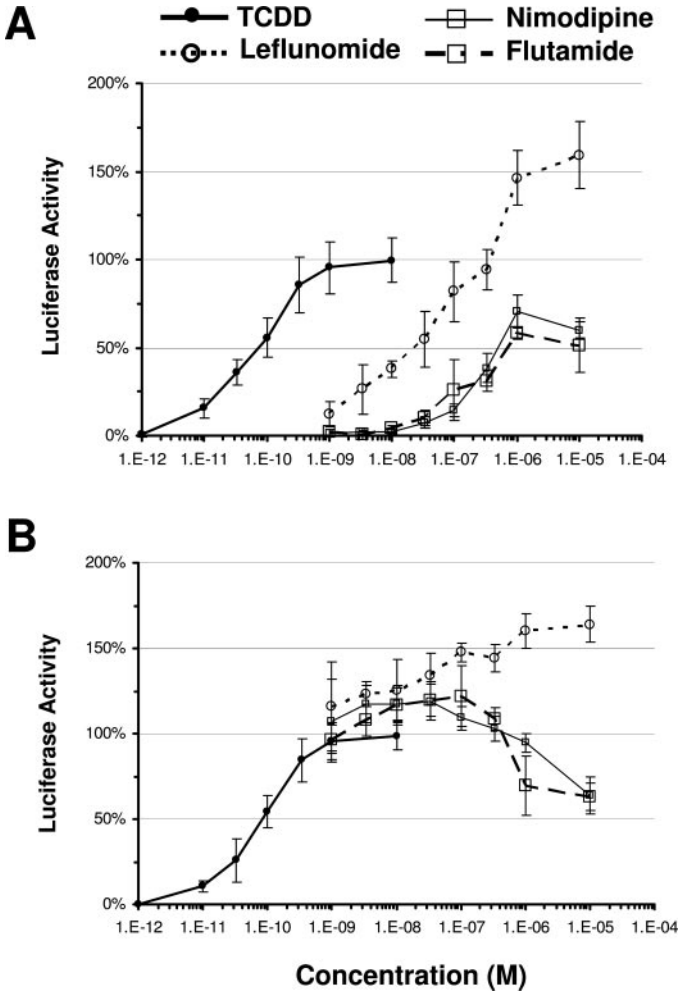

Fig. 6. Dose-response for luciferase induction. Three AhR agonists that had more than 50% TCDD activity in the reporter gene assay, including flutamide, leflunomide, and nimodipine, were tested to determine the EC_{50} value for luciferase induction. Results are expressed as the percentage of the luciferase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

Discussion

In the current study, a diverse set of drugs and industrial chemicals was 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. Although 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 HAHs, 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 (59%) of the 137 compounds that induced Cyp1a1 were considered false positives, because they were not consistently active in all 3 in vitro assays. This may be an overestimate, because 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 (Fig. 3). Putative AhR proagonists identified in this study include albendazole, rabeprazole, safrole, melatonin, phenothiazine, and sulindac (Fig. 7A). The activity of albendazole 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 has not been reported, although Cyp1a1 induction has been observed previously for safrole, isosafrole, and related metabolites in mice (Cook and Hodgson, 1985; Lewandowski et al., 1990). Although melatonin is inactive as an AhR agonist in vitro (Fig. 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 autoinduction mechanism.

Numerous compounds have been reported to induce Cyp1a1 that do not seem 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). Although 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 com-

pounds 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 (K_d values in the picomolar range). Although other studies have reported that omeprazole is unable to displace TCDD from the receptor (Daujat et al., 1992; Backlund et al., 1997), we detected significant activity in all three in vitro assays for omeprazole (Table 1), suggesting that 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 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, whereas other DRE-regulated genes were not. Compounds with this profile deviate from the classic mechanism of AhR binding and transcriptional activation

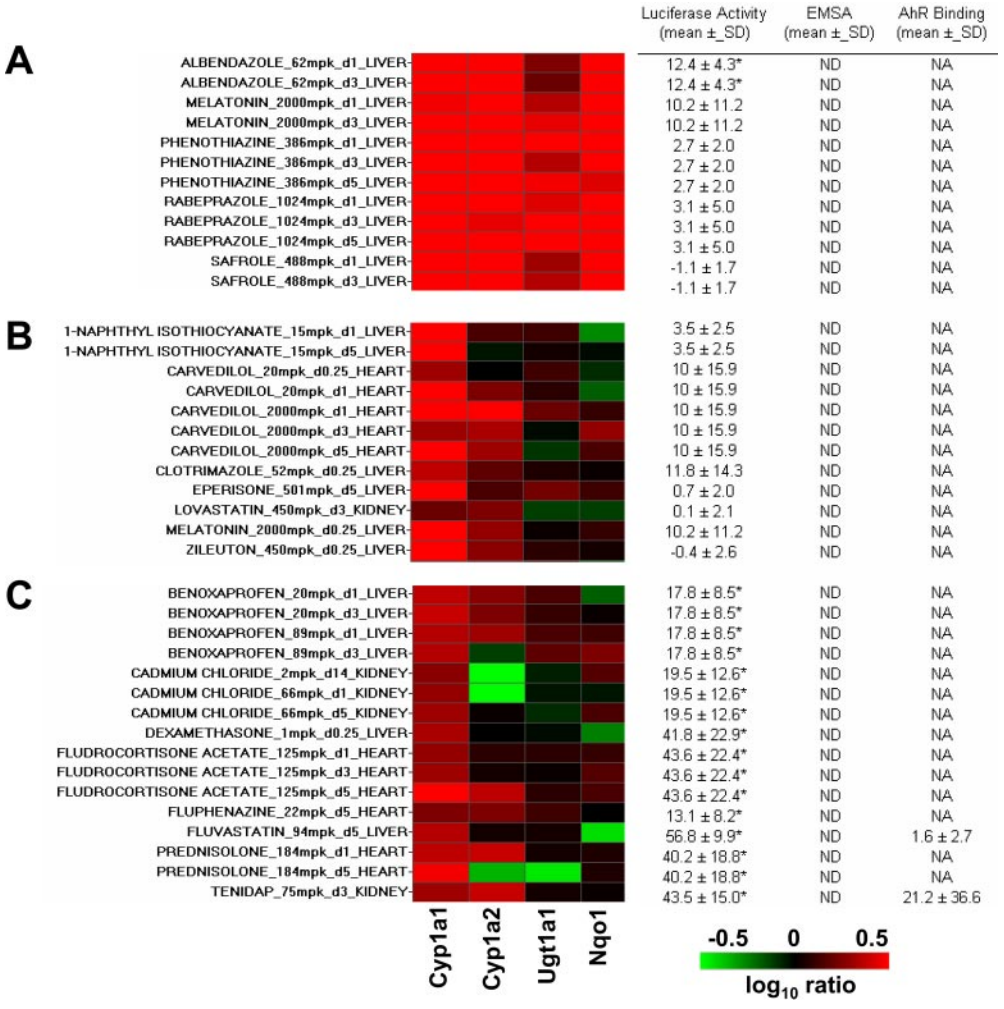

Fig. 7. Discrepancy between in vivo Cyp1a1 induction and in vitro AhR activity. A, putative proagonists of the AhR, which are active in vivo but not in vitro. B, Cyp1a1 inducers via non-AhR or non-DRE-mediated mechanisms. C, Cyp1a1 inducers independent of ligand binding and/or DRE-mediated mechanisms. Gene expression results for known AhR-regulated genes, including Cyp1a1 (X00469) and Cyp1a2 (K02422), Ugt1a1 (J05132), and 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 milligrams per kilogram per day (mpk), duration of treatment in days, and tissue. Results for DRE-driven luciferase activity, AhR transformation and DNA binding, and AhR binding are shown for each compound. Values are the mean and standard deviation for the luciferase reporter gene assay ($n = 9$), 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 with DMSO-treated controls is indicated by the asterisk (*). ND, not detected; NA, not determined.

via DREs. Most notable among these compounds are the corticosteroids (Fig. 7C). Dexamethasone has been shown previously 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 retinoid X receptor, PGC-1, and hepatic nuclear factor 4-α, 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-1α was observed to be induced by most heart and kidney treatments concurrent with Cyp1a1 up-regulation (data not shown) in which Cyp1a2, NQO1, and Ugt1a1 were not induced (Fig. 2). Two peroxisome proliferator-activated receptor (PPAR) response elements were found to mediate induction of human Cyp1a1 in response to PPAR-α agonists (Seree et al., 2006). PGC-1α positively regulates PPAR-α activity, thus suggesting that these transcription factors synergize to induce rat Cyp1a1 in a similar manner (Fig. 2B). It is interesting that treatment of ischemic rats with a PPAR-α 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 Fig. 2 suggest a model whereby rat Cyp1a1 is specifically induced by PGC-1/PPAR-α 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 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, whereas metabolically labile, nonhalogenated 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 because of the increased persistence of the chemical (Wassenberg and Di Giulio, 2004). These results suggest that whereas 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 the U.S. 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 seem 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 nonhalogenated 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.

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