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Novel Compound 2-Methyl-2*H*-pyrazole-3-carboxylic Acid (2-methyl-4-*o*-tolylazo-phenyl)-amide (CH-223191) Prevents 2,3,7,8-TCDD-Induced Toxicity by Antagonizing the Aryl Hydrocarbon Receptor

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental pollutant with many toxic effects, including endocrine disruption, reproductive dysfunction, immunotoxicity, liver damage, and cancer. These are mediated by TCDD binding to and activating the aryl hydrocarbon receptor (AhR), a basic helix-loop-helix transcription factor. In this regard, targeting the AhR using novel small molecule inhibitors is an attractive strategy for the development of potential preventive agents. In this study, by screening a chemical library composed of approximately 10,000 compounds, we identified a novel compound, 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide (CH-223191), that potently inhibits TCDD-induced AhR-dependent transcription. In addition, CH-223191 blocked the binding of TCDD to AhR and inhibited TCDD-mediated nuclear translocation and DNA binding of AhR. These inhibitory effects of CH-223191 prevented the expression of cytochrome P450 enzymes, target genes of the AhR. Unlike many known antagonists of AhR, CH-223191 did not have detectable AhR agonist-like activity or estrogenic potency, suggesting that CH-223191 is a specific antagonist of AhR. It is noteworthy that CH-223191 potently prevented TCDD-elicited cytochrome P450 induction, liver toxicity, and wasting syndrome in mice. Taken together, these results demonstrate that this novel compound, CH-223191, may be a useful agent for the study of AhR-mediated signal transduction and the prevention of TCDD-associated pathology.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a highly persistent environmental contaminant and one of the most potent man-made toxicants. The adverse effects of TCDD include a number of phenomena such as lethality (Blankenship et al., 2003), tumor promotion (Bertazzi et al., 1999; Cole et al., 2003), immunotoxicity (Kerkvliet, 1995; Ahmed et al., 2005), teratogenicity (Couture et al., 1990), and endocrine changes (Gregoraszczuk, 1995; Osteen and Sierra-Rivera, 1997). It is widely accepted that the majority of toxic effects of TCDD in higher organisms are mediated through activation of the cytosolic aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor of the basic helix-loop-helix family (Mimura and Fujii-Kuriyama, 2003; Mandal, 2005). Upon binding a ligand such as TCDD, AhR translocates to the nucleus, where it binds the aryl hydrocarbon receptor nuclear translocator (ARNT). This complex binds to enhancer sequences in target genes designated dioxin-responsive elements (DREs) that regulate expression of several genes, including cytochrome P450 enzymes (Mimura and Fujii-Kuriyama, 2003). Cytochrome P450 enzymes catalyze the epoxidation of certain classes of xenobiotics. The conse-

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; DRE, dioxin-responsive element; RT, reverse transcription; PCR, polymerase chain reaction; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ERE, estrogen responsive element.

quence of this can be the generation of highly reactive electrophilic metabolites and hydroxyl radicals, which may ultimately lead to cancer (Shimada and Fujii-Kuriyama, 2003; Chen et al., 2004).

AhR activation caused by the binding of TCDD is assumed to be one of the first and key steps in the development of TCDD toxicity. Therefore, the inhibition of AhR activation would be expected to provide protection against TCDD toxicity. Chemicals found in foods such as flavone and resveratrol and their derivatives have been reported to exert antagonistic activity on the binding of TCDD to AhR (Amakura et al., 2003; Zhang et al., 2003). However, at high concentrations, these compounds act as AhR agonists, causing an increase in cytochrome P450 expression (Zhang et al., 2003). In addition, some reported AhR antagonists have a high affinity for the estrogen receptor (Suetsugi et al., 2003; de Medina et al., 2005), which might cause estrogen-related effects. Therefore, a specific antagonist of AhR without agonistic potency would be potentially therapeutic for the prevention of human diseases that result from TCDD exposure.

In this study, we performed large-scale random screening of a synthetic chemical library in an effort to find compounds that can inhibit TCDD action. We identified a potent and specific antagonist for AhR, CH-223191, that can protect against TCDD-induced toxicity both ex vivo and in vivo.

Materials and Methods

Chemicals. A chemical library including CH-223191 was purchased from the Chembridge Corporation (San Diego, CA). 2,3,7,8-TCDD was obtained from Cambridge Isotope Laboratory (Andover, MA) and [^{3}H]TCDD was from ChemsynScience Labs (Lenexa, KS). Flavone, α-naphthoflavone, resveratrol, 7-ethoxyresorufin, resorufin, and 17β-estradiol were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Establishment of Stable Cell Line. HepG2 human hepatoma cells and MCF-7 human breast cancer cells were grown with Dulbecco's modified Eagle's medium supplemented 10% fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C. Hepa mouse hepatoma (Hepa) cells were grown in minimal essential medium, supplemented as above. To establish a stable reporter cell line, HepG2 cells were transfected with pMMTV-DRE-Luc plasmid, which encodes luciferase under the control of consensus sequences of DRE (dioxin-responsive element), and the MCF-7 cells were transfected with pMMTV-ERE-Luc plasmid that encodes luciferase under the control of consensus sequences of estrogen-responsive element (ERE) using electroporation. Positive clones were selected in the culture media containing 1 mg/ml G418, and their transcriptional responses were tested by luciferase activity assay after treatment with 1 nM TCDD or 17β-estradiol, respectively.

Luciferase Reporter Gene Assay. The cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.8, 1% Triton X-100, 150 mM NaCl, and 2 mM dithiothreitol). The cell lysate was mixed with luciferase activity assay reagent (Promega Corp., Madison, WI) and luminescence produced for 5 s was measured using a luminometer (Labsystems, Helsinki, Finland).

Ethoxyresorufin-*O*-deethylase Assay. The ability of chemicals to affect cytochrome P450 enzyme activity was evaluated in intact cells by measurement of ethoxyresorufin-*O*-deethylase activity (Ciolino et al., 1998). In 96-well plates, HepG2 cells were incubated with increasing concentrations of compounds in the presence of 1 nM TCDD for 24 h. Cells were washed once with PBS. Medium containing 5 μM ethoxyresorufin was added for 30 min. Increasing fluorescence as a result of the conversion of ethoxyresorufin to resorufin by cytochrome P450 enzymes was measured using a fluorescence Spectrophotometer (Varian, Inc., Palo Alto, CA) with an excitation of 530 nm and emission at 590 nm.

Western Blot Analysis. For Western blot analysis, whole-cell lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF). Twenty micrograms of protein was separated on a denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Triton X-100. For detection of cytochrome P450 1A1 and actin, monoclonal anti-cytochrome P450 1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin antibody (ICN Biomedicals, Inc., Aurora, OH) were incubated with the membranes overnight at 4°C, respectively. Secondary antibody linked to horseradish peroxidase was used at 1:10,000, and signals were visualized by chemiluminescence.

RT-PCR. cDNA (1 μg) was reverse-transcribed from 5 μg of total cellular RNA prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's method using oligo(dT) primers and murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI). cDNA was amplified for 35 cycles using the following human cytochrome P450 1A1 gene-specific primers: 5′-TGGATGGAGAACGCCAATGTC-3′ (sense) and 5′-TGGGTTGACCCATAGCTTCT-3′ (antisense). The cycling parameters were as follows: 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 0.5 min at 72°C for polymerization. Meanwhile, the same amount of cDNA was amplified for 25 cycles using specific β-actin primers: 5′-TCACCAACTGGGACGACATG-3′ (sense) and 5′-GTACAGGGATAGCACAGCCT-3′ (antisense). The products were visualized after electrophoresis on a 2% agarose gel containing ethidium bromide.

Ligand-Binding Assay. The ability of CH-223191 to compete with TCDD for binding to the AhR was assessed by incubating aliquots of cytosol prepared from untreated Hepa cells (2.1–2.5 mg protein/ml) with CH-223191 or flavone and [^{3}H]TCDD at 3 nM for 2 h at room temperature. This concentration of TCDD is nonsaturating at these protein concentrations. Specific binding of [^{3}H]TCDD was determined in duplicate aliquots by the hydroxylapatite assay (Gasiewicz and Neal, 1982), with correction for nonspecific binding measured in the presence of 150-fold excess of unlabeled 2,3,7,8-tetrachlorodibenzofuran. Data were expressed for each antagonist concentration as a percentage of the specific binding of [^{3}H]TCDD in the absence of competitor.

Immunocytochemistry and Nuclear Staining. Cells were treated with compounds and/or TCDD, washed with phosphate-buffered saline, and fixed with 4% paraformaldehyde for 30 min at room temperature. After incubation with 100 μg/ml RNase A and subsequent blocking with phosphate-buffered saline containing 1% BSA and 0.1% TX-100 for 30 min at room temperature. They were incubated with 2 μg/ml anti-AhR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature, followed by 2 μg/ml biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) for 40 min, then fluorescein streptavidin (Vector Laboratories) for 30 min, and then 2.5 μg/ml propidium iodide for 10 min to visualize nuclei. Cells were examined under a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) to determine localization of AhR.

Electrophoretic Mobility Shift Assay. Aliquots of Hepa cell cytosol (21–25 μg of protein) from the above incubations with [^{3}H]TCDD, with or without compounds, were mixed with nonspecific DNA (herring sperm), 0.08 M NaCl, and 25,000 to 45,000 cpm of ^{32}P-endlabeled oligonucleotide. The annealed oligonucleotide contained a single consensus DRE that is recognized by the transformed AhR (for complete sequence, see Gasiewicz et al., 1996). Samples were subjected to nondenaturing electrophoresis (4% acrylamide).

Treatment of Animals. Male ICR mice (6 weeks old) were given oral vehicle (corn oil) or CH-223191 (10 mg/kg in corn oil) once a day for 25 days and treated i.p. with TCDD (100 μg/kg in corn oil) once

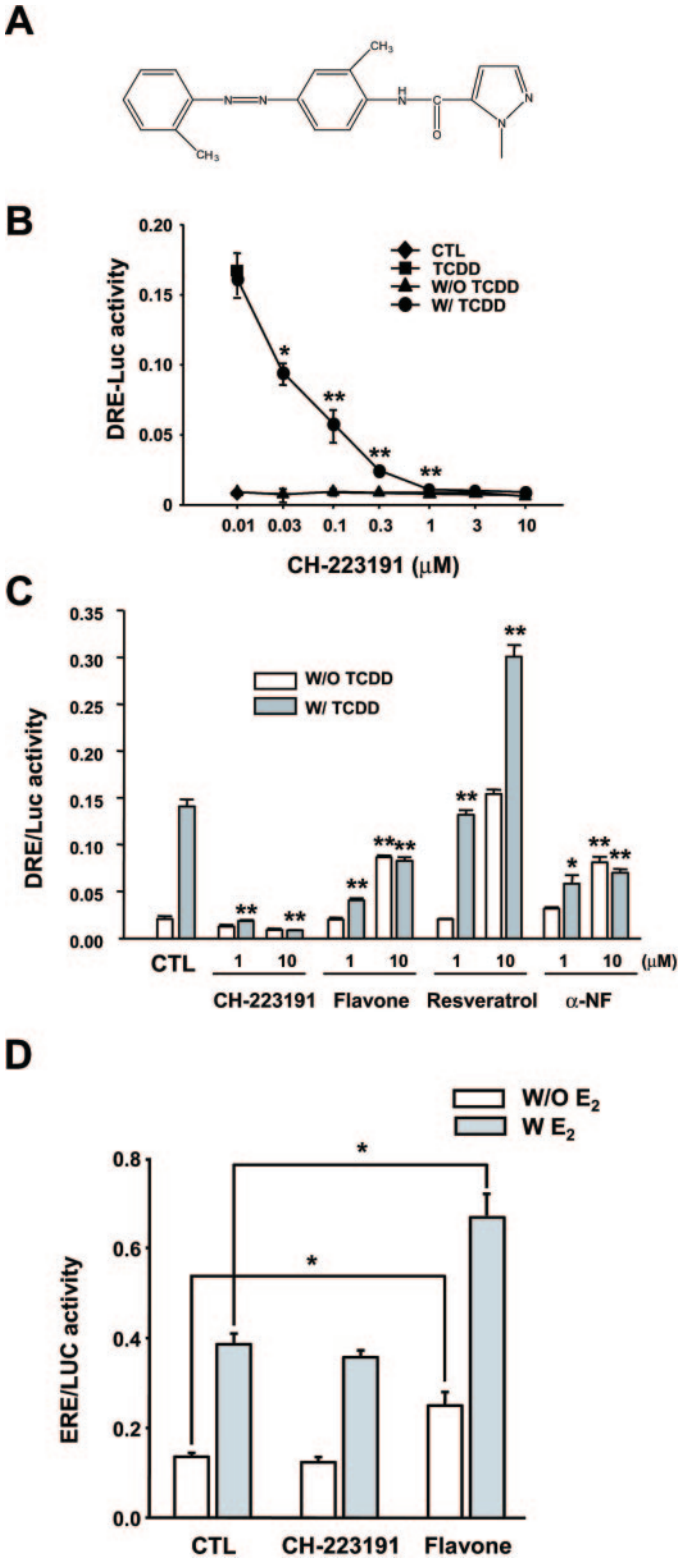


Fig. 1. CH-223191 inhibits TCDD-mediated AhR-dependent transcription. A, structure of CH-223191. B, HepG2 cells were stably transfected with DRE-controlled luciferase reporter gene (HepG2-DRE-luc-cells). Cells were pretreated with the indicated concentrations of CH-223191 or flavone for 1 h and then with 3 nM TCDD for 24 h, and then luciferase activity was assayed. Data represent the means ± S.E.M. of three separate experiments, each conducted in duplicate. C, HepG2-DRE-luc-cells were pretreated with 1 and 10 μM CH-223191, flavone, resveratrol and α-naphthoflavone (α-NF) for 1 h and then treated with 3 nM TCDD for 24 h, and then luciferase activity was assayed. Data represent the means ± S.E.M. of three separate experiments, each conducted in triplicate. *, $P < 0.05$; **, $P < 0.01$ versus vehicle treatment. D, MCF-7 cells were stably transfected with ERE-controlled luciferase reporter gene. Transfected cells were pretreated with 10 μM CH-223191 or flavone for 1 h and then treated with 1 nM 17β-estradiol for 24 h and then luciferase activity was assayed. Data represent the means ± S.E.M. of three separate experiments, each conducted in triplicate. *, $P < 0.05$.

after the first week of CH-223191 treatment. The body weights of all mice were measured before dosing and at termination.

AST and ALT Assay. We determined the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, in plasma, using kits purchased from Sigma-Aldrich.

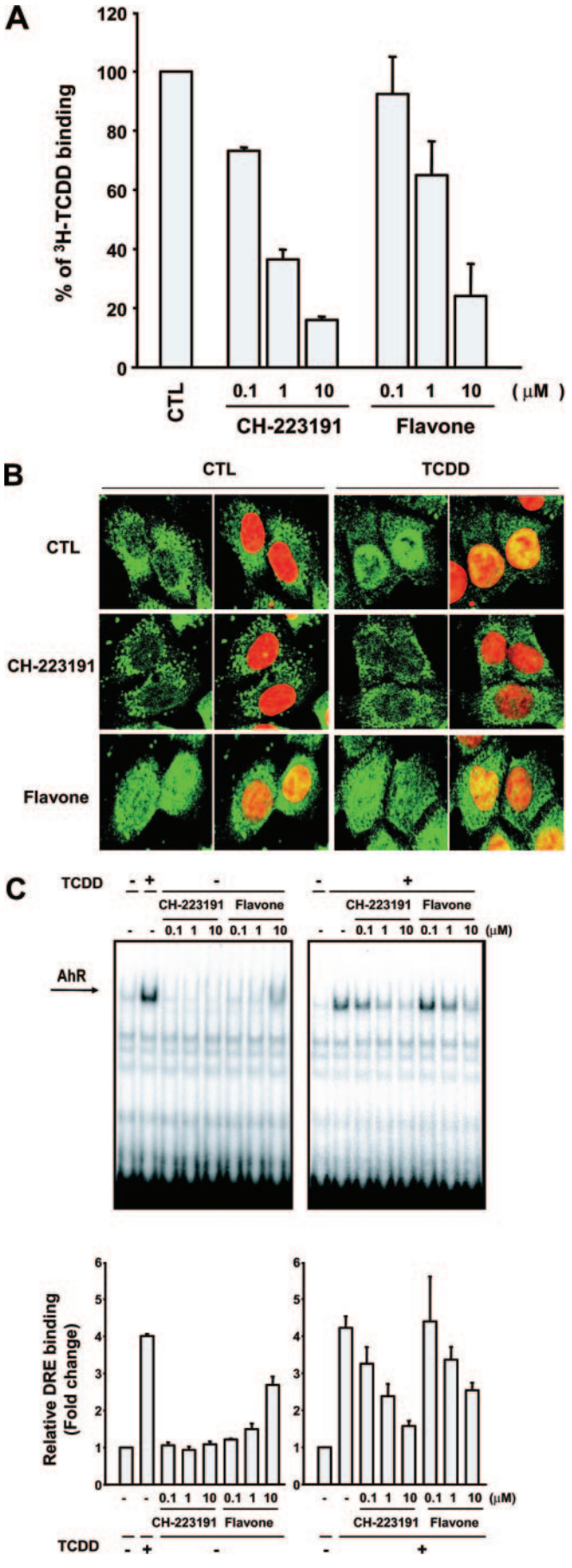
Histology of Liver. Livers were fixed in an iso-osmolar paraformaldehyde/glutaraldehyde-phosphate-buffered solution, postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanol solutions ranging from 30 to 100%, treated with propylene oxide, and embedded in Spurr's resin. Sections of liver (1 μm) were stained with hematoxylin and eosin, and the extent of toxicity was quantified morphometrically.

Statistical Analysis. Statistical differences between group-mean values were determined by Student's pair-wise *t* tests. Analysis by nonlinear regression was used to calculate IC_{50} values, representing the concentration of CH-223191 at which TCDD-induced luciferase activity was reduced by 50%. The data are presented as means ± S.E.M.

Results

High-Throughput Screening of 10,000 Compounds. To screen for compounds that suppress TCDD-induced AhR activation, we established a human hepatoma cell line (HepG2) stably expressing a DRE fused to a luciferase reporter gene. Treatment of these cells with TCDD increased DRE-dependent luciferase in a dose-dependent manner (data not shown). After the validation of the HepG2-DRE-luc-cell-based assay, a pilot screen of ~10,000 compounds from Chembridge Corporation was done. We identified four hit compounds that, at 1 μM, inhibited more than 90% of the luciferase activity induced by TCDD. However, three of these compounds were excluded because they alone increased luciferase activity slightly (data not shown). Of the compounds examined, CH-223191 (Fig. 1A) was identified as the most potent compound that inhibited TCDD-induced luciferase activity (IC_{50} = 0.03 ± 0.005 μM) (Fig. 1B). In addition, CH-223191 had more inhibitory potency and no agonist-like effect, compared with flavone, resveratrol, and α-naphthoflavone, which are known AhR antagonists (Fig. 1C). In addition, CH-223191 had no effect on estrogen receptor-mediated luciferase activity, whereas flavone increased and potentiated the effect of 17β-estradiol on estrogen receptor-dependent transcription in MCF-7 breast cancer cells (Fig. 1D).

Effects of CH-223191 on the Binding of TCDD to AhR, DNA Binding, and Nuclear Translocation of AhR. Many compounds inhibiting TCDD action competitively inhibit the binding of TCDD to the AhR (Henry et al., 1999; Zhang et al., 2003). Therefore, to test CH-223191 for its ability to antagonize [^{3}H]TCDD binding to the AhR, competitive binding assays were performed using the cytosolic fraction from Hepa cells. Hepa cell cytosol was chosen for these assays because of the greater stability of the mouse AhR compared with the human AhR in cytosol obtained from HepG2 cells. It was first verified, however, that CH-223191 had the equivalent antagonist effect in Hepa cells as in HepG2 cells (data not shown). As shown in Fig. 2A, binding of [^{3}H]TCDD to the AhR was



inhibited by CH-223191 in a dose-dependent manner. In addition, immunocytochemistry and electrophoretic mobility shift assay confirmed that CH-223191 and flavone blocked TCDD-mediated nuclear translocation and DNA binding of AhR (Fig. 2, B and C), suggesting that, like flavone, CH-223191 acts as an antagonist of AhR. It is noteworthy that flavone demonstrated slightly increased nuclear translocation and DNA binding of AhR at higher concentrations in the absence of TCDD (Fig. 2, B and C). Other antagonists have also been reported to exhibit TCDD-like agonist activity (Henry et al., 1999; Zhang et al., 2003). However, CH-223191 elicited no AhR-activating activity up to a concentration of 10 μM. These results demonstrate that CH-223191 is an antagonist of AhR without agonistic effect.

Effects of CH-223191 on TCDD-Induced Cytochrome P450 1A1 Expression and Cytochrome P450 Enzymatic Activity in HepG2 Cells. We tested the ability of CH-223191 to block TCDD-mediated cytochrome P450 expression by using Western blot assay and RT-PCR in HepG2 cells. Treatment with TCDD caused an increase in the level of cytochrome P450 1A1 protein and mRNA compared with the DMSO control. Such cytochrome P450 1A1 induction was inhibited by CH-223191 in dose-dependent manner (Fig. 3, A and B). In addition, addition of CH-223191 caused a concentration-dependent inhibition of TCDD-induced cytochrome P450 enzyme activity (Fig. 3C). However, CH-223191 alone had no inductive effects at any concentration, whereas 10 μM flavone slightly increased cytochrome P450 1A1 expression and cytochrome P450 activity. Therefore, these results support the conclusion that CH-223191 has only AhR antagonist effects without agonist-like activity and unlike that observed for flavone.

Effects of CH-223191 on TCDD-Caused Toxicity in Vivo. Finally, we extended the results obtained ex vivo by assessing the antagonist effects of CH-223191 toward TCDD in vivo. Mice were treated as described under *Materials and Methods* with a combination of CH-223191 and TCDD. Cytochrome P450 1A1 induction was detectable after treatment with 100 μg/kg of TCDD. After treatment with CH-223191 or/and TCDD, the animals were sacrificed and cytochrome P450 1A1 protein was assayed in extracts of liver. As shown in Fig. 4A, TCDD elicited cytochrome P450 1A1 expression in

Fig. 2. CH-223191 inhibits TCDD-AhR binding and TCDD-induced nuclear translocation and DNA binding of AhR. A, the ability of CH-223191 or flavone to compete with [^{3}H]TCDD for binding to the receptor was assessed by incubating the cytosolic fraction from Hepa cells with CH-223191 or flavone as described under *Materials and Methods*. Values are presented as percentage of the specific binding of 3 nM [^{3}H]TCDD in the absence of the competitor. The data are means ± S.E.M. calculated from three separate experiments. B, Hepa cells were pretreated with 10 μM CH-223191 or flavone for 1 h. The localization of AhR was determined by immunofluorescence analysis. Then, cells treated with vehicle (CTL) or TCDD (3 nM, 1 h) in the absence or presence of CH223191 or flavone were stained with monoclonal antibody against AhR (green) and counterstained with propidium iodide (red). The left image in each pair shows only localization of AhR (green) and the right image shows the positions of nuclei (red) as well as AhR (green) by image merge. Immunofluorescence was detected by confocal laser microscopy (LAM510; Carl Zeiss GmbH, Jena, Germany) (400×). C, ^{32}P-end-labeled oligonucleotides were added to cytosol incubated with 3 nM [^{3}H]TCDD plus increasing concentrations of CH-223191 or flavone, and then DNA-protein complexes were separated by nondenaturing polyacrylamide gel electrophoresis then visualized and quantified by PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The AhR-and ligand-dependent bands are labeled by arrows. Each bar represents the average and S.E.M. of two independent experiments.

liver, and this induction was suppressed approximately 75% by administration of CH-223191. TCDD is known to cause fatty degeneration of the liver, which involves increased accumulation of lipids in the hepatocyte (Uno et al., 2004). As shown in Fig. 4B, the intrahepatocyte fat content increased by TCDD was significantly suppressed by CH-223191. In addition, we estimated the degree of TCDD-induced toxicity by measuring plasma AST and ALT levels. Activity of both AST and ALT in TCDD-treated mice was elevated, and these were reduced by CH-223191 (Fig. 4C). Furthermore, TCDD is known to cause a wasting syndrome, manifested as a failure to gain weight at normal rates and, in more severe instances, weight loss (Uno et al., 2004). Mice treated with TCDD showed the classic signs and symptoms of severe wasting, with significant weight loss rather than gain. This was significantly suppressed by CH-223191; mice exhibited weight gains that did not differ significantly from the untreated controls (Fig. 4D). Therefore, CH-223191 could antagonize TCDD both ex vivo and in vivo.

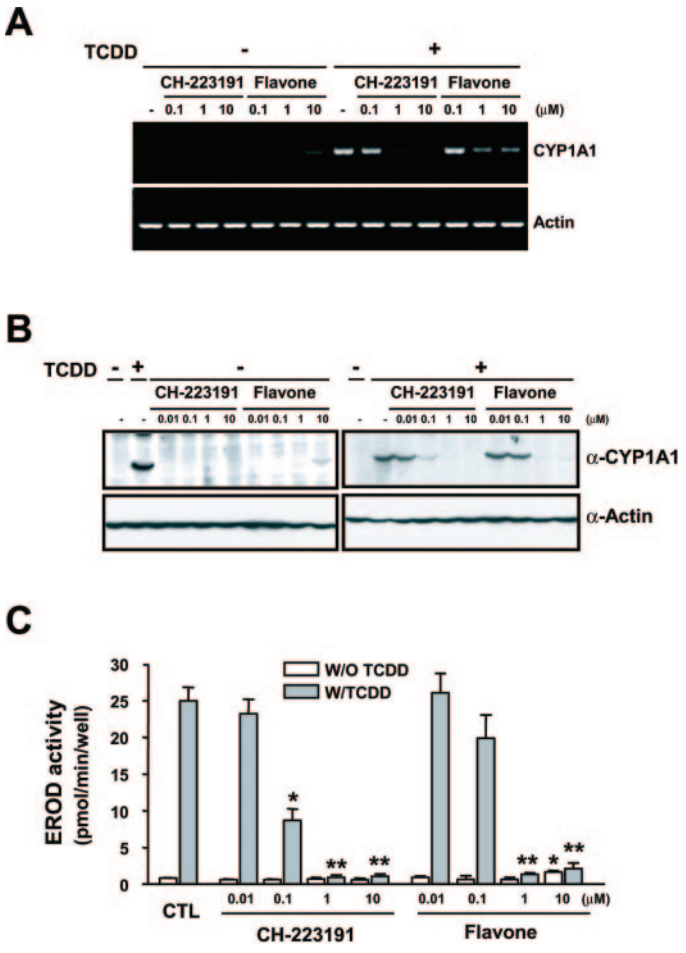


Fig. 3. CH-223191 inhibits TCDD-mediated cytochrome P450 1A1 induction. A, HepG2 cells were pretreated with the indicated concentrations of CH-223191 or flavone and then 3 nM TCDD for 12 h. RT-PCR with specific cytochrome P450 1A1 or β-actin primers was performed. B, HepG2 cells were pretreated with the indicated concentrations of CH-223191 or flavone for 1 h and then 3 nM TCDD for 24 h. Proteins were separated by SDS-PAGE, and then Western blot analysis with specific antibodies against cytochrome P450 1A1 or actin was performed. C, after pretreatment with 0.1, 1, or 10 μM compounds for 1 h, HepG2 cells were treated with 3 nM TCDD for 24 h. Cytochrome P450 activity was determined in intact cells by ethoxyresorufin-*O*-deethylase (EROD) assay. The results are expressed as means ± S.E.M. for three replicate determinations for each treatment group. *, $P < 0.05$; **, $P < 0.01$.

Discussion

These studies suggest that the novel synthetic compound CH-223191 is a specific and potent antagonist for AhR. So far, most, if not all, AhR antagonists also have some agonist activity, which might lead to TCDD-like effects at higher concentrations. In contrast, CH-223191 had no agonist effect on AhR, and prevented TCDD-induced toxicities both ex vivo and in vivo.

TCDD is a very potent agonist of AhR and its interaction with this basic helix-loop-helix transcription factor is essential for its toxicity. This has been clearly demonstrated in AhR-knockout mice, which are resistant to TCDD-induced biochemical and biological endpoints (Fernandez-Salguero et al., 1996). Therefore, the development of inhibitors of AhR-mediated signal transduction has been of great interest for the chemoprevention of effects elicited by dioxin-like xenobiotics. In this study, we developed a cell-based assay (HepG2-DRE-luc) to identify compounds inhibiting AhR-mediated signal transduction. This cell-based assay system can be used to perform high-throughout screening for inhibitors of AhR-dependent transcription and test for cytotoxic effects of the compounds simultaneously. Through a screen of ~10,000 compounds, we identified a novel compound, CH-223191 (Fig. 1A), that showed the greatest inhibition of TCDD-induced AhR-dependent transcription without cytotoxic effects (Fig. 1B and data not shown). In addition, the inhibitory effect of CH-223191 was more potent than several compounds, such as curcumin, indole-3-carbinol, and luteolin, that have been reported to possess AhR antagonist activity (Fig. 1C and data not shown).

The identification of a nontoxic antagonist for AhR with specificity for this receptor is potentially of great medical interest. Some reports have shown that several antagonists of AhR, including flavone and resveratrol, have high affinity for the estrogen receptor (Suetsugi et al., 2003; de Medina et al., 2005). Flavone increased estrogen receptor-mediated transcription and potentiated the effect of 17β-estradiol. However, CH-223191 showed no effect on estrogen receptor-driven transcription (Fig. 1D). This property should minimize estrogen-related risks, such as the increased risk of breast cancer and genital cancers.

In our study, we found that CH-223191 blocked the binding of TCDD to the AhR as well as TCDD-mediated nuclear translocation and DNA binding activity of the AhR (Fig. 2). A number of chemicals found in foods or derived from naturally occurring compounds have been reported to act as AhR antagonists. However, most of these also have concentration-dependent AhR agonist activity (Chen et al., 1996; Rinaldi et al., 2002; Zhang et al., 2003; Zhou and Gasiewicz, 2003). This was supported by our observation that flavone functioned as an antagonist at low concentration, whereas high concentrations of flavone increased AhR agonist activity. In addition, as shown in Fig. 1C, a high concentration (10 μM) of flavone, resveratrol, or α-naphthoflavone stimulated AhR-dependent transcription in the absence of TCDD. In contrast, CH-223191 did not have any agonist actions up to 100 μM, strongly indicating that CH-223191 is a pure antagonist of AhR.

The best characterized molecular response to AhR activation is the transcriptional induction of cytochrome P450 1A1, cytochrome P450 1A2, and cytochrome P450 1B1 enzyme isoforms (Mimura and Fujii-Kuriyama, 2003). Several studies have suggested that chronically expressed cytochrome P450 contributes to TCDD-induced toxicity (Smith et al., 2001; Uno et al., 2004; Leung et al., 2005). This enzyme catalyzes the epoxidation of certain classes of xenobiotics, resulting in the generation of highly reactive electrophilic metabolites that may result in genotoxicity. This activity also generates NADH-dependent oxygen radicals, which may lead to indiscriminate damage to cellular macromolecules and ultimately in toxicity (Matsumura, 2003; Mandal, 2005). As expected, TCDD-induced cytochrome P450 1A1 mRNA, pro-

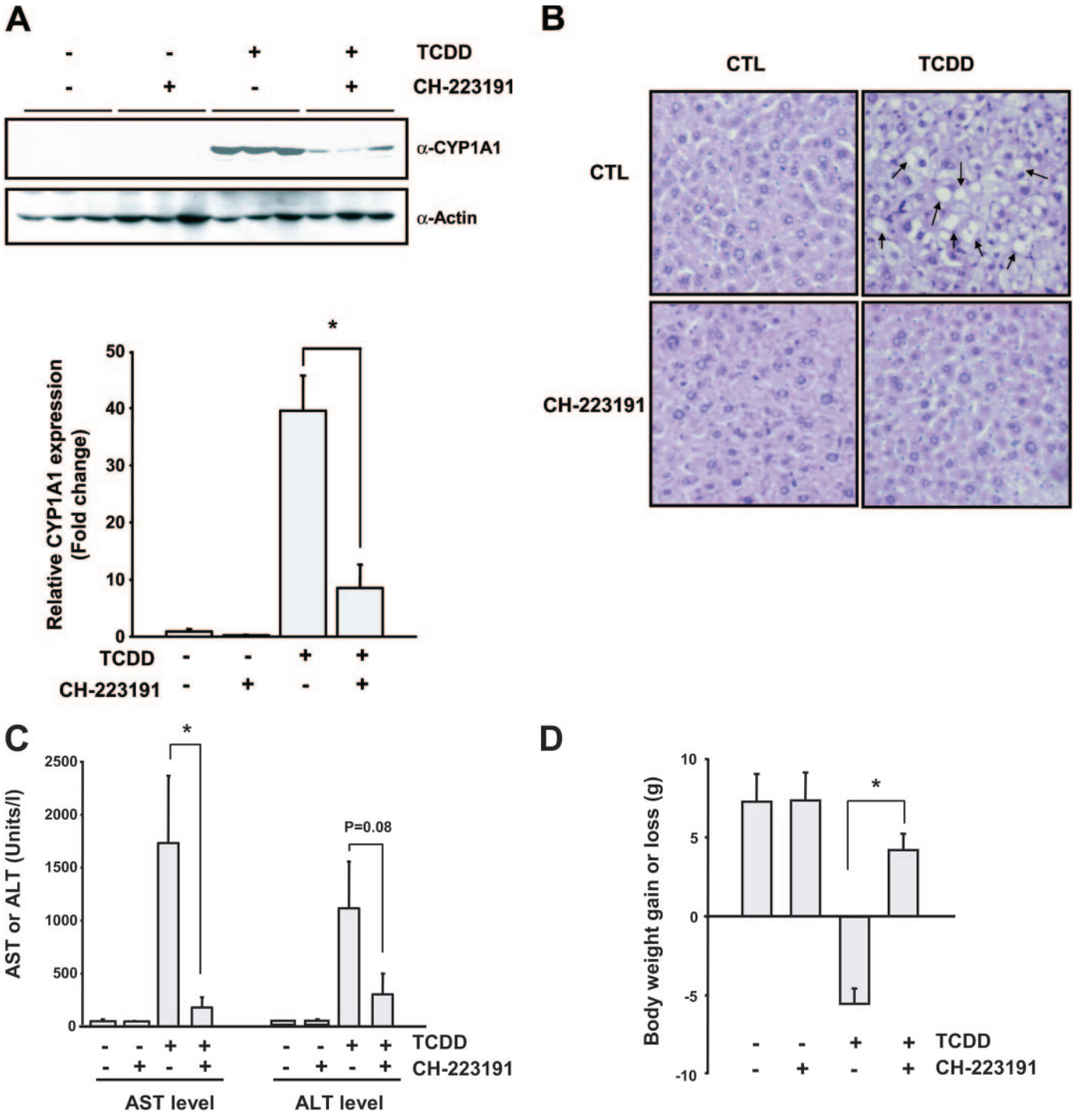


Fig. 4. CH-223191 prevents TCDD-induced toxicities in vivo. Male ICR mice (6 weeks old) were administered vehicle (corn oil) or CH-223191 (10 mg/kg in corn oil) orally once a day for 25 days and treated i.p. with TCDD (100 μg/kg in corn oil) once after the first week of CH-223191 treatment. A, Western blot was performed as for Fig. 3B for detection of cytochrome P450 1A1 in liver. Levels of cytochrome P450 1A1 protein were normalized to that of actin. $n = 3$ *, $P < 0.05$. B, histology of liver shows intrahepatocyte fat content (hematoxylin & eosin, 200×). The arrows point to lipid droplets. C, plasma AST and ALT levels. Each group was $n = 5$. The values represent the mean ± S.E.M. *, $P < 0.05$. D, change of body weight. Each group was $n = 5$. The values represent the mean ± S.E.M. *, $P < 0.05$.

tein and enzyme activity were suppressed by CH-223191 (Fig. 3). In addition, CH-223191 inhibited increased levels of cytochrome P450 1A2 and cytochrome P450 1B1 mRNA induced by TCDD (data not shown). Flavone and α-naphthoflavone directly inactivate cytochrome P450 enzymatic activity by competitively interacting with the substrate binding site (Lu et al., 1996; Ciolino et al., 1998). In contrast, we confirmed, using an in vitro microsomal cytochrome P450 activity assay (data not shown), that CH-223191 did not affect cytochrome P450 activity directly, which indicates that CH-223191 inhibits TCDD-mediated cytochrome P450 induction by transcriptional regulation.

Some flavonoids have not been examined extensively for potential therapeutic anti-dioxin activity because they often display adverse effects. These effects may be mediated by AhR agonistic actions at high concentrations or by other unknown mechanisms, as demonstrated for α-naphthoflavone or quercetin (Collman et al., 1986; Sahu and Washington, 1991). In this study, in the case of flavone, TCDD-induced AhR activation was significantly suppressed in cultured cells. However, flavone did not inhibit and rather potentiated TCDD-caused toxicity in mice (data not shown). Casper et al. (1994) showed that resveratrol is able to efficiently block the induction of cytochrome P450 1A1 by TCDD both ex vivo and in vivo. However, as mentioned above, resveratrol could bind to human estrogen receptor α and stimulate proliferation of human breast cancer cells (Schmitt et al., 2002). From the study of analogs of flavone and resveratrol, many specific and potent antagonists for AhR have been identified (Gasiewicz et al., 1996; Davis et al., 2003). However, their anti-dioxin activity has not always been confirmed in whole animals. In this study, we demonstrate that CH-223191 has antidioxin activity in vivo. CH-223191 potently suppressed cytochrome P450 1A1 expression in liver and suppressed AST and ALT levels that reflect toxicity, fatty liver and wasting syndrome caused by TCDD without adverse effects in mice (Fig. 4), suggesting that CH-223191 may be an important chemopreventive compound that targets the AhR for prevention of TCDD-caused damage in vivo. However, this preliminary evidence that CH-223191 is nontoxic is very limited and is restricted to short-term outcomes. For potential medical application of chemopreventive agents, their safety assessment through many toxicity studies (including genotoxicity assay, chronic toxicity studies, development toxicity studies, and life-time carcinogenicity assay) have to be performed, and a full understanding of pharmacokinetics must be achieved. The study about safety and pharmacokinetics of CH-223191 deserve further detailed investigation.

In conclusion, by screening a chemical library, we identified a small synthetic molecule, CH-223191, that acts as a potent and specific antagonist for AhR. CH-223191 significantly suppressed toxic responses caused by TCDD ex vivo and in vivo. Therefore, we believe that CH-223191 may be a potential preventive agent for the prevention of dioxin-associated toxicity.

References

Ahmed S, Shibazaki M, Takeuchi T, and Kikuchi H (2005) Protein kinase Ctheta activity is involved in the 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced signal transduction pathway leading to apoptosis in L-MAT, a human lymphoblastic T-cell line. *FEBS J* **272:**903–915.

Amakura Y, Tsutsumi T, Sasaki K, Yoshida T, and Maitani T (2003) Screening of the inhibitory effect of vegetable constituents on the aryl hydrocarbon receptor-mediated activity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biol Pharm Bull* **26:**1754–1760.

Bertazzi PA, Pesatori AC, Bernucci I, Landi MT, and Consonni D (1999) Dioxin exposure and human leukemias and lymphomas. Lessons from the Seveso accident and studies on industrial workers. *Leukemia* **1:**S72–4.

Blankenship AL, Hilscherova K, Nie M, Coady KK, Villalobos SA, Kannan K, Powell DC, Bursian SJ, and Giesy JP (2003) Mechanisms of TCDD-induced abnormalities and embryo lethality in white leghorn chickens. *Comp Biochem Physiol C Toxicol Pharmacol* **136:**47–62.

Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E, and Savouret JF (1994) Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. *Mol Pharmacol* **56:**784–790.

Chen I, Safe S, and Bjeldanes L (1996) Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem Pharmacol* **51:**1069–1076.

Chen ZH, Hurh YJ, Na HK, Kim JH, Chun YJ, Kim DH, Kang KS, Cho MH, and Surh YJ (2004) Resveratrol inhibits TCDD-induced expression of cytochrome P4501A1 and cytochrome P4501B1 and catechol estrogen-mediated oxidative DNA damage in cultured human mammary epithelial cells. *Carcinogenesis* **25:**2005–2013.

Ciolino HP, Daschner PJ, Wang TT, and Yeh GC (1998) Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem Pharmacol* **56:**197–206.

Ciolino HP, Wang TT, and Yeh GC (1998) Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. *Cancer Res* **58:**2754–2760.

Cole P, Trichopoulos D, Pastides H, Starr T, and Mandel JS (2003) Dioxin and cancer: a critical review. *Regul Toxicol Pharmacol* **38:**378–388.

Collman GW, Lundgren K, Shore D, Thompson CL, and Lucier GW (1986) Effects of alpha-naphthoflavone on levels of sister chromatid exchanges in lymphocytes from active and passive cigarette smokers: dose-response relationships. *Cancer Res* **46:**6452–6455.

Couture LA, Abbott BD, and Birnbaum LS (1990) A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin: recent advances toward understanding the mechanism. *Teratology* **42:**619–627.

Davis JW, Burdick AD, Lauer FT, and Burchiel SW (2003) The aryl hydrocarbon receptor antagonist, 3′methoxy-4′nitroflavone, attenuates 2,3,7,8-tetrachlorodibenzo-p-dioxin-dependent regulation of growth factor signaling and apoptosis in the MCF-10A cell line. *Toxicol Appl Pharmacol* **188:**42–49.

de Medina P, Casper R, Savouret JF, and Poirot M (2005) Synthesis and biological properties of new stilbene derivatives of resveratrol as new selective aryl hydrocarbon modulators. *J Med Chem* **48:**287–291.

Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM, and Gonzalez FJ (1996) Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol Appl Pharmacol* **140:**173–179.

Gasiewicz TA, Kende AS, Rucci G, Whitney B, and Willey JJ (1996) Analysis of structural requirements for Ah receptor antagonist activity: ellipticines, flavones and related compounds. *Biochem Pharmacol* **52:**1787–1803.

Gasiewicz TA and Neal RA (1982) The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-p-dioxin using hydroxylapatite. *Anal Biochem* **124:**1–11.

Gregoraszczuk EL (1995) Dioxin exposure and porcine reproductive hormonal activity. *Cad Saude Publica* **18:**453–462.

Henry EC, Kende AS, Rucci G, Totleben MJ, Willey JJ, Dertinger SD, Pollenz RS, Jones JP, and Gasiewicz TA (1999) Flavone antagonists bind competitively with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the aryl hydrocarbon receptor but inhibit nuclear uptake and transformation. *Mol Pharmacol* **55:**716–725.

Kerkvliet NI (1995) Immunological effects of chlorinated dibenzo-p-dioxins. *Environ Health Perspect* **9:**47–53.

Leung YK, Lau KM, Mobley J, Jiang Z, and Ho SM (2005) Overexpression of cytochrome P450 1A1 and its novel spliced variant in ovarian cancer cells: alternative subcellular enzyme compartmentation may contribute to carcinogenesis. *Cancer Res* **65:**3726–3734.

Lu YF, Santostefano M, Cunningham BD, Threadgill MD, and Safe S (1996) Substituted flavones as aryl hydrocarbon (Ah) receptor agonists and antagonists. *Biochem Pharmacol* **51:**1077–1087.

Mandal PK (2005) Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. *J Comp Physiol B Biochem Syst Environ Physiol* **175:**221–230.

Matsumura F (2003) On the significance of the role of cellular stress response reactions in the toxic actions of dioxin. *Biochem Pharmacol* **66:**527–540.

Mimura J and Fujii-Kuriyama Y (2003) Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta* **1619:**263–268.

Osteen KG and Sierra-Rivera E (1997) Does disruption of immune and endocrine systems by environmental toxins contribute to development of endometriosis. *Semin Reprod Endocrinol* **15:**301–308.

Rinaldi AL, Morse MA, Fields HW, Rothas DA, Pei P, Rodrigo KA, Renner RJ, and Mallery SR (2002) Curcumin activates the aryl hydrocarbon receptor yet significantly inhibits (−)-benzo(a)pyrene-7R-trans-7,8-dihydrodiol bioactivation in oral squamous cell carcinoma cells and oral mucosa. *Cancer Res* **62:**5451–5456.

Sahu SC and Washington MC (1991) Quercetin-induced lipid peroxidation and DNA damage in isolated rat-liver nuclei. *Cancer Lett* **58:**75–79.

Schmitt E, Lehmann L, Metzler M, and Stopper H (2002) Hormonal and genotoxic activity of resveratrol. *Toxicol Lett* **136:**133–142.

Shimada T and Fujii-Kuriyama Y (2003) Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci* **95:**1–6.

Smith AG, Clothier B, Carthew P, Childs NL, Sinclair PR, Nebert DW, and Dalton TP (2001) Protection of the Cyp1a2(−/−) null mouse against uroporphyria and

hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Appl Pharmacol* **173:**89–98.
Suetsugi M, Su L, Karlsberg K, Yuan YC, and Chen S (2003) Flavone and isoflavone phytoestrogens are agonists of estrogen-related receptors. *Mol Cancer Res* **1:**981–991.
Uno S, Dalton TP, Sinclair PR, Gorman N, Wang B, Smith AG, Miller ML, Shertzer HG, Nebert DW (2004) Cyp1a1(−/−) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome and resistance to intrahepatocyte lipid accumulation and uroporphyria. *Toxicol Appl Pharmacol* **196:**410–421.
Zhang S, Qin C, and Safe SH (2003) Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environ Health Perspect* **111:**1877–1882.
Zhou J and Gasiewicz TA (2003) 3′-methoxy-4′-nitroflavone, a reported aryl hydrocarbon receptor antagonist, enhances Cyp1a1 transcription by a dioxin responsive element-dependent mechanism. *Arch Biochem Biophys* **416:**68–80.

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