CH223191 IS A LIGAND-SPECIFIC ANTAGONIST OF THE AH RECEPTOR

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Abstract
The Ah receptor (AhR) is a ligand-dependent nuclear receptor that regulates gene expression in a wide variety of species and tissues. While the best-characterized high affinity AhR ligands include halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachloridibenzo-p-dioxin (TCDD, dioxin) and polycyclic aromatic hydrocarbons (PAHs), the AhR can bind and be activated by a structurally diverse range of chemicals. While differences in the binding of ligands within the AhR ligand binding domain (LBD) could account for some of the observed diversity of AhR ligand structure, strong support for the hypothesis is lacking. Utilizing a combination of AhR ligand and DNA binding and gene expression assays we have identified a novel antagonist (CH223191) that can specifically inhibit the ability of HAHs, but not PAHs or PAH-like chemicals, to bind to and/or stimulate AhR DNA binding or AhR-dependent gene expression. These results not only demonstrate that HAHs and PAHs (and PAH-like chemicals) differentially interact within the AhR LBD, but that CH223191 is an HAH-specific antagonist.

Introduction
The Ah receptor (AhR) is a ligand-dependent transcription factor that regulates the expression of a battery of genes and is responsible for mediating the toxicity of dioxin-like HAHs in a wide range of species and tissues.[1,2] The cytosolic AhR is a multi-protein complex that translocates into the nucleus following ligand (agonist) binding. Dimerization of the AhR with a closely related nuclear protein ARNT (Ah receptor nuclear translocator) stimulates release of the AhR from its associated protein subunits and the AhR:ARNT complex is converted into its high affinity DNA binding form. Binding of the heteromeric ligand:AhR:Arnt complex to its specific DNA recognition site, the dioxin response element (DRE), results in chromatin modification and stimulation of transcription of adjacent genes.[1,2] The best-characterized high affinity ligands for the AhR include a variety of HAHs, such as the polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls, and numerous PAHs and PAH-like chemicals, such as benzo(a)pyrene, 3-methylcholanthrene and beta-naphthoflavone (BNF).[3] The relatively recent identification of a large number of natural and synthetic AhR agonists whose structure and physicochemical characteristics are dramatically different from the prototypical HAH and PAH AhR ligands, demonstrate that the AhR has an extremely promiscuous LBD.[3,4] While the spectrum of biochemical and toxicological responses mediated by the AhR are species- and tissue-specific, ligand-specific differences have also been observed.[1,2,5,6] These studies demonstrate that HAHs, but not PAHs (or PAH-like agonists), can produce a common spectrum of TCDD-like and AhR-dependent toxic and biological effects. Although differential responsiveness to HAHs and PAHs can result from a variety of biochemical, physiological and pharmacological characteristics in target cells, the greater potency of HAHs has been suggested to be due to their significantly higher AhR binding affinity and resistance to metabolism. However, recent studies have also suggested that some of the differences in the potency and biological responses produced by PAHs and HAHs are directly related to ligand-dependent differences in the structure of the AhR protein and/or AhR protein complex that alters its functionality and relative affinity/specificity for DNA/chromatin as well as for coactivators and other nuclear factors and thus the overall biological/toxic response to a given ligand.

The structural activity relationship (SAR) has been established for many HAHs, however, numerous attempts to establish a SAR with all known AhR ligands has not been successful and this is likely due to the established promiscuity of AhR ligand structure and the lack of any 3-D protein structural information about the AhR LBD. Numerous studies have examined differences in biological and toxicological responses to HAH and PAH AhR agonists, however, only one has demonstrated a difference in the interaction of structurally diverse ligands to bind to and/or activate the AhR. The site-directed mutagenesis studies of Backlund and Ingelman-Sundberg

identified a specific mutation within the human AhR LBD that negatively affected the ability of the relatively weak benzothiazole AhR agonist omeprazole, but not TCDD to activate the AhR.[7] These results could suggest that the binding of omeprazole and TCDD to the AhR LBD may involve distinct amino acids within the LBD. Taking this concept further it could be hypothesized that, the observed promiscuity of AhR ligands could be explained, at least in part, by the ability of structurally diverse ligands (or classes of ligands) to differentially interact with amino acids within the AhR LBD. In fact, the differential binding of ligands (agonists) to different residues within a receptor binding pocket of the steroid hormone receptor PXR has been previously demonstrated.[8] Unlike that of PXR, there exists no 3-dimensional crystal or NMR structure of the AhR LBD bound by different ligands to test this hypothesis. However, we propose that if ligands can actually differentially interact within the AhR LBD, then it should be possible to identify an antagonist that would differentially affect two structurally distinct AhR agonists.

Materials and Methods

Chemicals

TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and [^{3}H]TCDD were generously provided by Dr. Steven Safe (Texas A&M University, College Station, TX) and β-naphthoflavone (BNF) and DMSO were purchased from Aldrich (St. Louis, MO). CH223191 was purchased from Chembridge Corporation (San Diego, CA) and its structure confirmed by NMR (Figure 1). Cell culture reagents and media were purchased from Gibco/BRL (Grand Island, NY) and fetal calf serum from Atlanta Biologicals (Norcross, GA).

Cell Culture, Chemical Treatment and AhR-Dependent Luciferase Reporter Gene Expression

Recombinant guinea pig intestinal adenocarcinoma (G16L1.1c8) cells were grown and maintained as previously described.[9] G16L1.1c8 cells contain the stably transfected DRE-driven firefly luciferase reporter plasmid pGudLuc1.1, whose transcription occurs in a ligand-, dose-, time- and AhR-dependent manner.[9] Cells were plated into white, clear-bottomed 96-well tissue culture plates (75,000 cells per well) and allowed to attach for 24 hrs. Cells were incubated with carrier solvent DMSO (1% final solvent concentration), or the indicated concentration of TCDD, BNF or a variety of other AhR agonists, or the indicated chemicals plus CH223191 for 4 hrs at 37ºC. Luciferase activity was measured as we have previously described using Promega stabilized luciferase reagent and activity was expressed relative to that induced by TCDD.

DNA Binding Analysis

Guinea pig hepatic cytosol was prepared as we have demonstrated previously and aliquots were stored frozen at -80ºC until use and gel retardation analysis carried out as previously described.[10] For this analysis, cytosol (8 mg of protein/ml) was incubated with indicated concentration of TCDD (2 nM), BNF (2 μM) or carrier solvent (DMSO) or co-incubation of these compounds with the indicated concentration of CH223191 for 2 hr at 20ºC, followed by gel retardation analysis.[10] The amount of [^{32}P]-labeled DRE present in the TCDD-inducible protein-DNA complex was measured using a Molecular Dynamics Phosphorimager, and the amount of radioactivity in the inducible protein-DNA complex minus that present in the same position in the DMSO sample lane allowed calculation of the amount of ligand-induced AhR:Arnt: DRE complex. The amount of induced protein-DNA complex formation was expressed relative to that produced by a maximal TCDD or BNF induction.

Results and Discussion

CH223191 Inhibits TCDD- but not BNF-induced AhR-dependent luciferase gene expression in cells

CH223191 has been previously shown to antagonize the ability of TCDD to stimulate AhR-dependent CYP1A1 gene expression in mice in vivo and in cells in culture.[11] To determine whether CH223191 can antagonize the induction of AhR-dependent gene expression by both TCDD and BNF, we examined its effect in guinea pig (G16L1.1c8) cells that contain a stably transfected DRE-luciferase reporter plasmid (figure 2). While CH223191 decreased induction of luciferase by 1 nM TCDD in a concentration-dependent manner, with 10 μM CH223191 completely inhibiting TCDD induced luciferase activity, induction of luciferase activity by 10 μM BNF was only inhibited by 20% at the highest concentration of CH223191 (100 μM). These results demonstrate that CH223191 preferentially affects the ability of TCDD to activate AhR-dependent gene expression. The ability of CH223191

to inhibit luciferase gene induction by TCDD but not BNF was also observed in rat, mouse and human cell lines (data not shown) demonstrating that this effect is not a species-specific. The relative inhibitory potency (IC_{50}) of CH223191 against TCDD-dependent gene induction in G16L1.1c8 cells was estimated from these studies to be ~1.1 μM, a value significantly higher than the IC_{50} of 0.03 μM previously reported for CH223191 inhibition of AhR-dependent gene expression in human hepatoma (HepG2) cells and this may reflect species specific differences between the guinea pig and human AhRs and/or cell lines.[11] Taken together, these results demonstrate that CH223191 is a preferential inhibitor of TCDD induced gene expression. In additional studies using AhR-defective mouse hepatoma cells (Tao-Hepa1c1c7) that contain a stably transfected fluorescently tagged AhR expression vector, we demonstrated that CH233191 could inhibit TCDD-, but not BNF-dependent nuclear translocation of the AhR (data not shown). These results demonstrated that the effect of CH233191 occurred prior to ligand-dependent nuclear localization of the AhR.

CH223191 specifically inhibits TCDD- but not BNF-stimulated AhR transformation and DNA binding

Decreased TCDD-dependent nuclear translocation of the AhR by CH233191 could result from its ability to inhibit TCDD-dependent transformation of the AhR into its DNA binding form. To test this, we determined the effect of CH233191 on TCDD- and BNF-dependent transformation and DNA binding of the AhR in vitro using gel retardation analysis. Incubation of guinea pig hepatic cytosol with TCDD or BNF results in transformation and DNA binding of the AhR (Figure 3, first 3 lanes, ligand:AhR:Arnt:DRE complex indicated by the arrow). Similar to the gene expression results, CH223191 inhibited the ability of TCDD to stimulate AhR transformation/DNA binding; no effect on BNF-dependent AhR transformation/DNA binding was observed. (Figure 3, compare lanes 1-3 with 4-6). The lack of effect on preformed TCDD:AhR:Arnt:DRE complexes (data not shown) indicates that CH233191 inhibits DNA binding by the AhR rather than disrupting DNA bound AhR complexes and suggests an effect on AhR ligand (TCDD) binding. The ability of CH233191 to compete with [^{3}H]TCDD for specific binding to the AhR was demonstrated in subsequent ligand binding experiments (data not shown). These results demonstrate that although CH233191 is an antagonist of the AhR and its inability to produce any agonist activity even at relatively high concentrations (100 μM) and to compete with [^{3}H]TCDD specific binding to the AhR is consistent with it being a pure AhR antagonist. In addition, the ability of CH233191 to inhibit TCDD-dependent activation of the AhR and AhR signaling pathway, with little effect on BNF-dependent AhR activation, suggests that it is a ligand (TCDD) specific AhR antagonist. These results, combined with the previously published ability of CH233191 to antagonize TCDD-dependent gene induction and toxic effects in mice in vivo, supports it utility as a potential therapeutic agent to inhibit the adverse effects of toxic HAHs. Further studies are in progress to define the SAR aspects of CH233191 responsible for its ligand-selective activity and to examine its interactions within the current homology model of the AhR LBD using docking in order to gain insights into its ligand-specificity.

Acknowledgements

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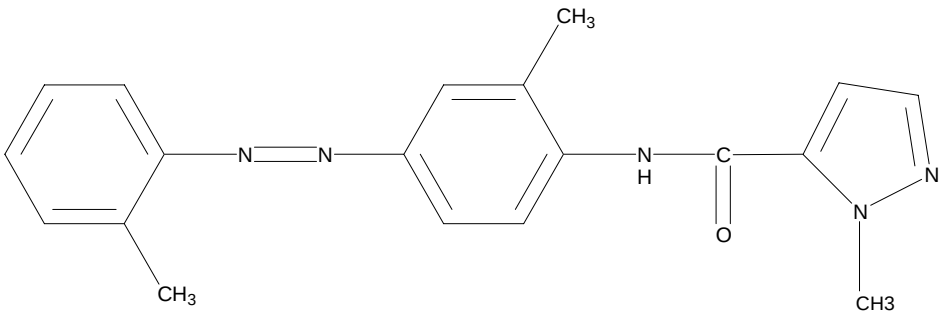

Figure 1. Structure of antagonist CH223191

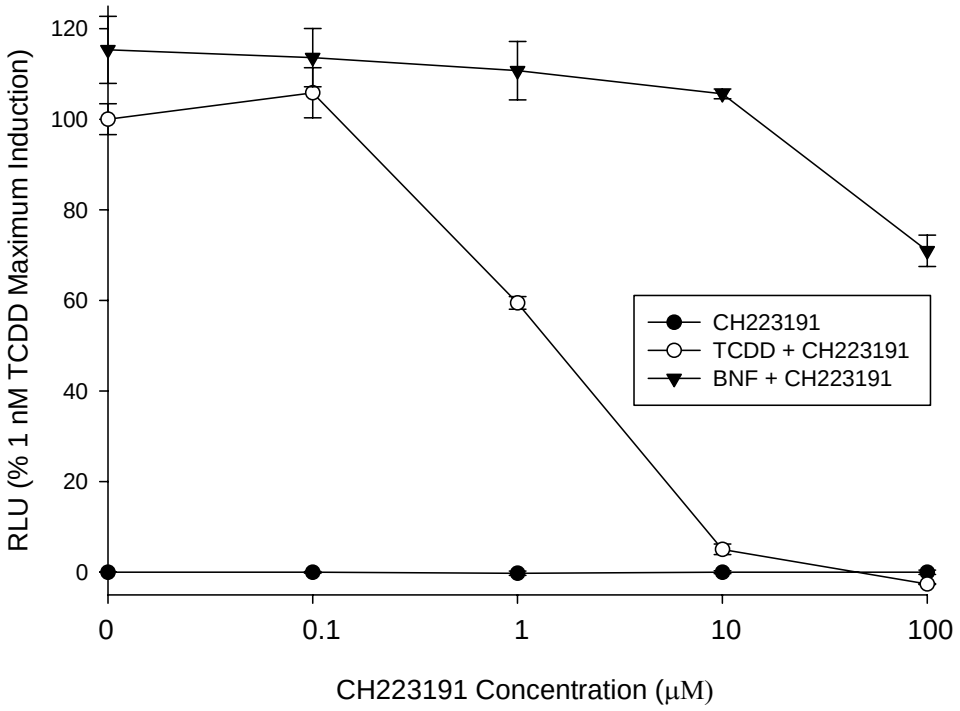

Figure 2. Effect of CH223191 to inhibit TCDD- or BNF-mediated AhR-dependent luciferase reporter gene expression in the guinea pig G16L1.1c8 cells.

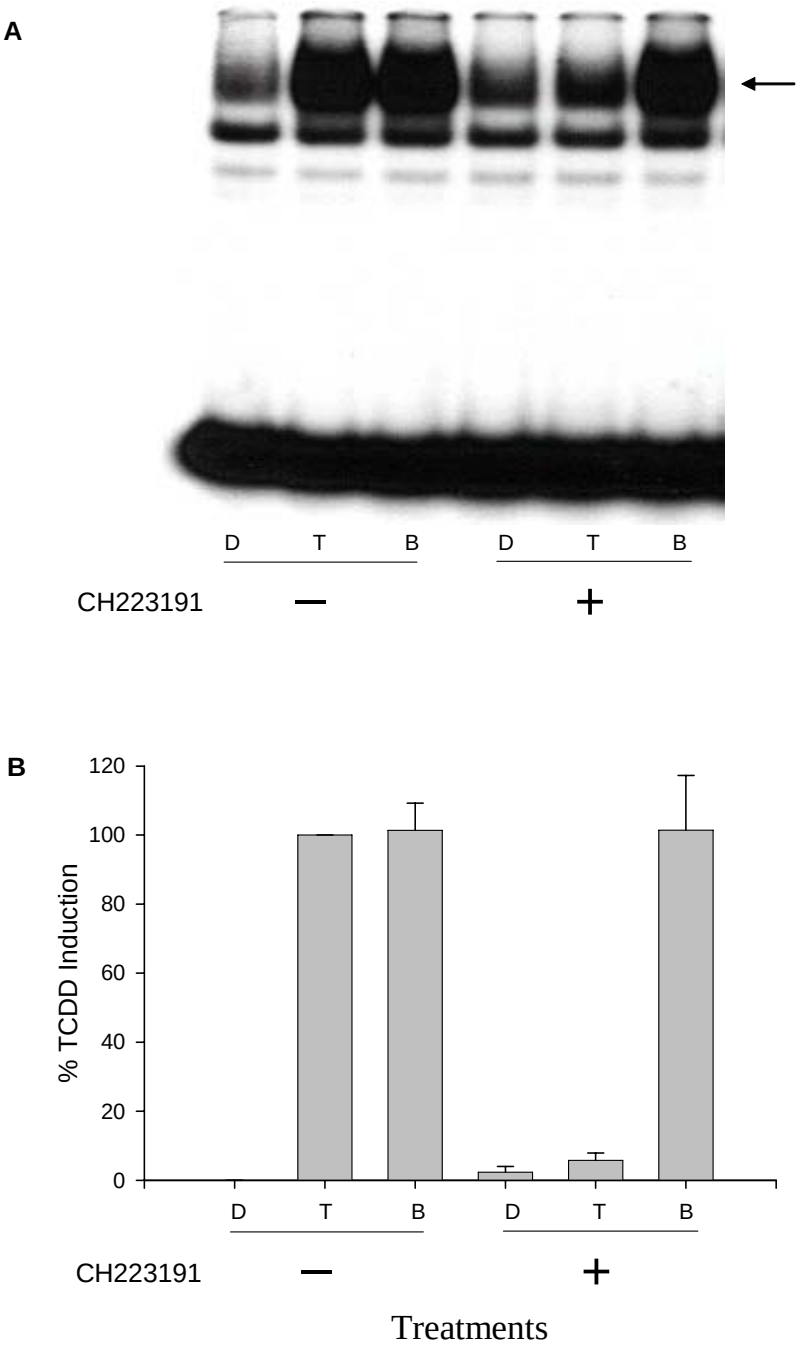

Figure 3. Effect of CH223191 to inhibit TCDD- or BNF-stimulated AhR transformation and DNA binding in vitro.