AH RECEPTOR AGONISTS PRESENT IN COMMERCIAL AND CONSUMER PRODUCTS: IDENTIFICATION AND CHARACTERIZATION OF NOVEL AGONISTS PRESENT IN TIRE EXTRACTS

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Abstract
The Ah receptor (AhR) is known to mediate the toxic and biological effects of a wide variety of structurally diverse chemicals. Application of the CALUX bioassay for the detection of AhR agonists in environmental, biological and many other matrices has led to the identification of novel chemicals and classes of chemicals that can bind to and activate the AhR. Since the presence of AhR agonists in extracts of these matrices can interfere with clear interpretation of CALUX assay results, their identification and characterization is necessary. Here we have demonstrated the presence of AhR agonists in solvent extracts of truck tire tread using both CALUX and AhR DNA binding bioassays. The application of bioassay-based fractionation coupled with instrumental analysis not only led to the isolation and identification of several known polycylic aromatic hydrocarbons which have been previously shown to activate the AhR signaling pathway, but it also identified 2-mercapto- and 2-hydroxybenzothiazole as AhR agonists.

Introduction
The Ah receptor (AhR) is a ligand-dependent transcription factor that mediates many of the toxic and biological effects of persistent organic pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and related dioxin-like chemicals. Following ligand (TCDD) binding, the cytosolic ligand:AhR complex translocates into the nucleus, dimerizes with the Ah receptor nuclear translocator (ARNT) protein, with the resulting conversion of the heteromeric complex into its high-affinity DNA binding form. The binding of the ligand:AhR:ARNT complex to its specific recognition site, the dioxin responsive element (DRE), stimulates transcription of adjacent downstream genes[1,2]. The best studied and highest affinity ligands for the AhR are primarily widespread synthetic environmental contaminants, which include both halogenated aromatic hydrocarbons (HAHs) such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene and dibenz[*a*,*h*]-anthracene. More recently, numerous other classes of natural and synthetic AhR ligands have been described, and these ligands differ dramatically from the strict structural requirements previously defined for HAH and PAH ligands[3,4]. Although the majority of these other classes of ligands are relatively weak agonists when compared to the more potent HAHs, such as TCDD, their structural diversity not only clearly demonstrates the promiscuous ligand-binding activity of the AhR, but it indicates that the overall ability of a sample extract to activate the AhR signaling pathway results from the combined activity of many different chemicals present in the extract.

While sample analysis using CALUX focuses upon dioxin-like HAHs, we have expanded our studies to include screening of extracts of diverse biological, environmental and commercial products with the goal of identifying and characterizing novel activators and/or inhibitors of the AhR pathway and to determine their AhR-dependent toxic and biological effects. In previous studies we identified AhR agonist activity in newspapers and newspaper ink and in various commercial and consumer products[5,6]. Recently, Stephensen and co-workers reported an increase in AhR-dependent induction of CYP1A1 mRNA and enzyme activity in rainbow trout maintained in tanks that contained immersed new tires, suggesting that some of the chemicals leaching from the tires were AhR agonists[7]. Given the widespread disposal of tires, their use as mulches in many applications and the fact that the areas adjacent to all highways will contain tire residue and fragments, we examined whether solvent extracts of tire contain AhR agonists that can bind to and activate AhR DNA binding and AhR-dependent gene expression and if so, to subject these extracts to CALUX bioassay directed fractionation in order to identify the responsible chemical(s).

Materials and Methods
Materials. TCDD was obtained from S. Safe (Texas A&M University, College Station, TX), luciferase

lysis and assays reagents were from Promega (Madison, WI). Benzothiazole (BT) and three 2-substituted BT derivatives: 2-methylthiobenzothiazole (MTBT), 2-mercaptobenzothiazole (MBT) and 2-hydroxybenzothiazole (OBT) were obtained from Aldrich (St. Louis, MO). A fragment of truck tire tread was rapidly collected from a highway roadside.

Tire sample preparation and fractionation. For analysis of total tire extract, 1 gram of washed inner tire tread was cut into small pieces and immersed into 2 ml of DMSO overnight in a Teflon-lined screw-cap tube, followed by centrifugation for 15 min at 1500 rpm to remove the particulate material. The resulting supernatant was transferred into a fresh tube to obtain the final concentration of 0.5 g tire extract equivalent/ml DMSO. This original tire extract was then further diluted in DMSO and 1 μl of each dilution analyzed using the mouse H1L1.1c2 AhR luciferase cell bioassay. For fractionation studies, 16 g of tire tread were incubated in 100 ml of toluene overnight, the supernatant collected and the tire fragments extracted twice more with 100 ml of toluene. The combined toluene extract was evaporated to dryness and chromatographed through a silica gel column and eluted with 60 ml hexane, 60 ml hexane–toluene (1:1, v/v), 60 ml toluene and 60 ml methanol to give twenty two fractions of ~10 ml each. The AhR agonist activity in each fraction was determined using the H1L1.1c2 cells bioassay, with fractions 4–22 showing agonist activity. Fraction 20 was further chromatographed through a silica gel column eluted with 20 ml hexane–toluene (1:1, v/v), 20 ml toluene, 20 ml toluene–ethyl acetate (1:1, v/v), 20 ml ethyl acetate and 20 ml methanol to obtain twenty subfractions of ~5 ml each. Agonist activity was identified in fractions 8–12 and 18–20. Active fraction 18 was further analyzed by GC/MS in full scan mode using an EI-MS on a HP5973 mass spectral detector tuned with perfluorotributylamine using the system auto tune parameters. The mass spectrometer was interfaced with a HP 6890 gas chromatograph equipped with a 30 m x 0.25 m, 0.25 μM DB-5ms phase column. Optimized oven program used inlet, transfer line and quadrapole temperatures of 250°C, 280°C and 160°C, respectively. The oven program was an initial temperature of 50°C held for 1 min, ramped at 10°C/min to 320°C and held 10 min. Helium was used as the carrier gas at a constant flow of 0.8 ml/min. Analyses were executed in the splitless mode. These analyses resulted in the identification of four benzothiazoles (Figure 2) by comparing the mass spectra and retention times to authentic standards.

Cell culture and induction of luciferase activity. Recombinant mouse hepatoma cells H1L1.1c2 were grown and maintained as previously described[8-10]. These cells contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid whose transcriptional activation occurs in a ligand- and AhR-dependent manner. H1L1.1c2 cells, grown in 96-well microplates, were incubated with carrier solvent DMSO (10 μl/ml), TCDD (1 nM), or the indicated volume or concentration of total tire extract, MBT or OBT for 4 h at 37°C and luciferase activity measured as we have previously described[8-10].

Preparation of cytosol and gel retardation analysis. Hepatic cytosol was prepared from male Hartley guinea pigs (250-300 g) in HEDG buffer [25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol] as previously described[11] and was stored at −80°C. Gel retardation analysis was carried out as described in detail.[11] Briefly, a complementary pair of synthetic oligonucleotides containing the DRE3 binding site for the transformed AhR:Arnt complex (5'-GATCTGGCTCTTCTCAC-GCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') were synthesized, purified, annealed, and radiolabeled with [γ-^{32}P]ATP. An aliquot (125 μl) of liver cytosol (8 mg of protein/ml) was incubated with DMSO (2.5 μl), TCDD in DMSO (20 nM final concentration), 1.25 μl of the original tire extract in DMSO (equivalent to 0.625 ml of original tire extract), MBT or OBT (200 μM each) for 2 h at 20°C. A 10 μl aliquot of each incubation was analyzed by gel retardation analysis, and protein-DNA complexes were resolved by polyarylamide gel electrophoresis and autoradiography of the dried gel, with the amount of ^{32}P-labeled DRE present in the induced protein-DNA complex was determined using a Molecular Dynamics phosphoimager.

Results and Discussion

Crude DMSO tire extract induces DRE-dependent gene expression. We previously demonstrated the existence of AhR agonists in extracts of diverse commercial and consumer products, such as plastic, rubber and paper products[5,6] and have extended these analyses to examine the presence of AhR agonists in extracts of tires. Initial analyses involved determining the ability of a total DMSO tire extract to stimulate AhR-

dependent gene expression using recombinant mouse hepatoma (H1L1.1c2) cells which contain a stably integrated DRE-driven firefly luciferase reporter gene that responds to TCDD and related AhR agonists with the induction of luciferase activity in a time-, dose-, and chemical-specific manner[8,9]. The tire extracts induced luciferase activity in a concentration-dependent manner in the H1L1.1c2 cells (Fig. 1), producing a response between 40–170% of that maximally induced by TCDD. This induction response calculated out to result from extract that was equivalent to 5 ng–5 mg of original tire. We also demonstrated the ability of the DMSO tire extract to induce AhR-dependent luciferase gene expression in recombinant guinea pig, rat, and human cells lines in a concentration-dependent manner (data not shown). Together these results indicate that a crude DMSO extract contains relatively high AhR agonist activity in cell from a range of species. To determine whether the AhR agonists in the tire extract are metabolically stable (i.e. are HAHs) or not, we examined the magnitude of luciferase gene induction at 4 hours in H1L1.1c2 cells as compared to that at 24 hours using H1L6.1c2 cells, the identical cell line (hepa1c1c7) containing a slightly different reporter vector[9]. Relative luciferase expression was significantly reduced at 24 h as compared to 4 h (data not shown). We further examined induction of AhR-dependent gene expression by the DMSO tire extract using recombinant rat hepatoma (H1G1.1c3) cells containing a stably transfected AhR-responsive enhanced green fluorescent protein (EGFP) reporter gene[13]. These cells allow examination of AhR-reporter gene expression in the same cells for up to 10 days and thus evaluating the metabolic persistence of any AhR agonist[13]. Similar to the results with the luciferase cell lines, induction of EGFP decreased over time. Taken together, these results suggest that tire extract contains AhR agonists that are not metabolically stable, thus, the induction response is transient.

Tire extract stimulates AhR transformation and DNA binding in vitro. We have previously demonstrated that the AhR can be transformed in vitro into its high-affinity DNA-binding form by TCDD and a variety of structurally diverse chemicals[5,6,12]. Incubation of guinea pig hepatic cytosol with crude tire extract and subsequent gel retardation analysis revealed that tire extract induced formation of a protein-DNA complex, which migrated to the same position as that produced by TCDD and represents the ligand:AhR:Arnt:DRE complex (data not shown). Phosphoimager quantitation revealed that the tire extract resulted in an amount of inducible AhR:DRE complexes comparable to that maximally induced by TCDD (data not shown). These results confirm that the ability of the DMSO tire extract to stimulate AhR-dependent gene expression is due to the ability of AhR agonists in the extract to bind to and stimulate AhR transformation and DNA binding.

Identification of PAHs and benzothiazoles in AhR-active fractions of tire extract. We employed a CALUX bioassay-directed fractionation approach to isolate and identify the AhR agonists in tire extracts. Active fractions from one chromatography step were refractionated and tested and positive fractions were analyzed by GC/MS. These analyses resulted in the tentative identification of a variety of PAHs, some of which would be expected to be AhR agonists (data not shown). Interestingly, benzothiazole (BT) and three BT derivatives: 2-methylthiobenzothiazole (MTBT), 2-mercaptobenzothiazole (MBT) and 2-hydroxybenzothiazole (OBT) were also identified in an active fraction by comparing the mass spectra and retention times to authentic standards (Fig. 2).

Activation of the AhR and AhR signaling pathwaynby OBT and MBT. To test the AhR agonist activity of the identified compounds, we examined the ability of BT and the BT derivatives to stimulate AhR-dependent gene expression in the CALUX bioassay. Although these benzothiazoles have similar structures, only OBT and MBT induced AhR-dependent gene expression, and they were relatively weak inducers (as compared to TCDD). The EC_{50} values for CALUX luciferase induction were 4.0 and 1.8 μM for OBT and MBT, respectively, approximately 2×10^5 fold less potent than the most potent AhR agonist TCDD (Fig. 3). Gel retardation analysis confirmed the ability of OBT and MBT to stimulate AhR transformation and DNA binding in vitro to ~50% of that produced by TCDD (data not shown).

Our results demonstrate that the AhR agonist activity of tire extract is not simply the result of the release of AhR PAHs from the tire, but to other chemicals as well. While BT and OBT have been previously shown to be AhR agonists[14], albeit weak activators, we show for the first time that MBT is also an AhR agonist. We have subsequently identified a variety of other BT derivatives with the AhR agonist activity (data not shown). BTs are a class of polar and biological active industrial chemicals that have a considerable environmental half-life and thus could represent a potential source of AhR chemicals in relevant

environmental samples. They are widely used and are discharged from industrial processes that are not solely limited to tire manufacturing and they have been detected in various environmental compartments, including water, soils, sediments, groundwater, municipal wastewater effluent, industrial emission, and atmospheric deposition[15].

It should be noted that other fractions also exhibited AhR agonist activity and the extraction conditions were such that these fractions would likely contain other classes of chemicals. Vulcanized tire rubber represents a complex composition of hydrocarbons, minerals, and metals that have undergone an extensive and complex series of processing steps. In addition to the principal ingredient rubber, tires contain various chemicals that confer desired properties such as softness and resistance to skid, rolling, abrasion and aging. Chemical additives included in the processing of tires to obtain the desired characteristics include as highly aromatic oils (known to contain high concentrations of PAHs), metals (such as zinc), peroxides, benzothiazoles, phenols, and aromatic amines[8] and others may be formed during the tire vulcanization process. As such, PAHs and BTs are only two of numerous additives used in the manufacture of tires and the overall AhR agonist activity of tire extracts very likely results from a combination of many distinctly different chemicals. Further investigations are needed to assess the contribution of other tire additives to the AhR ligand response in tire extract and their biological and toxicological activities.

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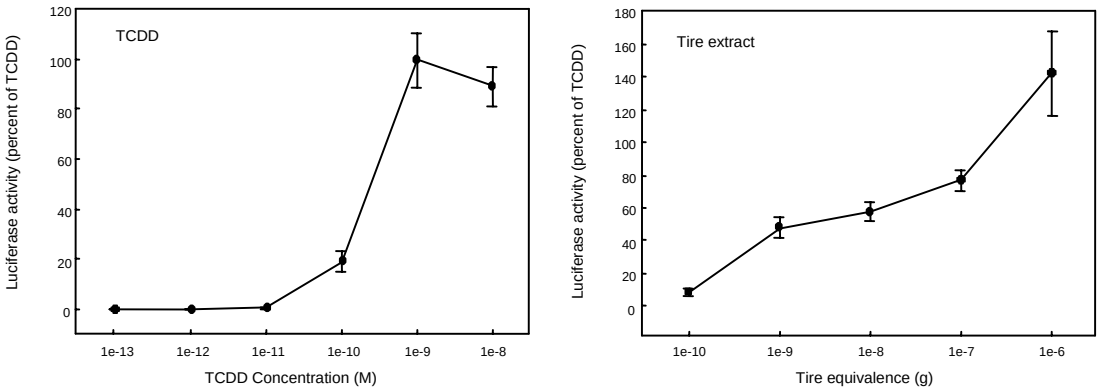

Fig. 1. Concentration-dependent induction of luciferase activity in recombinant mouse H1L1.1c2 cells by TCDD and total tire DMSO extract. Confluent plates of H1L1.1c2 cells were incubated with carrier solvent DMSO (1% final concentration) or increasing concentrations of TCDD or tire extract for 4 h at 37°C and luciferase activity in cell lysates determined as described in the Materials and Methods. Values represent the mean ± SD of triplicate determinations and are expressed as a percent of maximal TCDD induction.

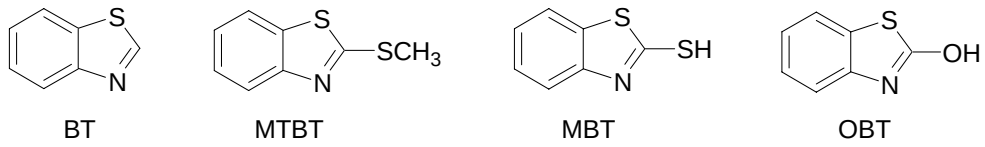

Fig. 2. Chemical structures of four benzothiazoles identified in fractionated tire extracts.

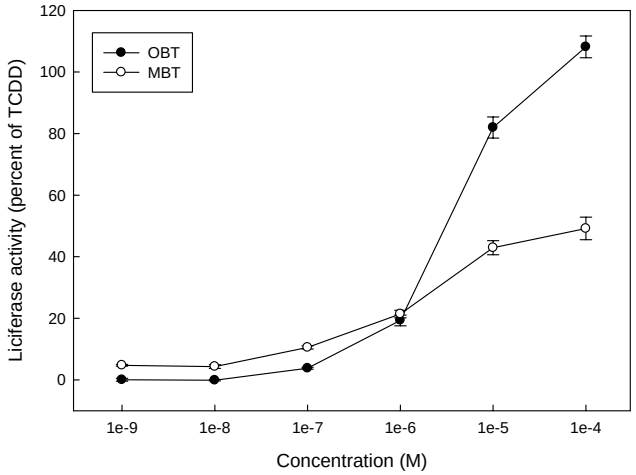

Fig. 3. Concentration-dependent induction of AhR-dependent luciferase reporter gene expression by 2-hydroxybenzothiazole (OBT) and 2-mercaptobenzothiazole (MBT). H1L1.1c2 cells were incubated with the indicated concentrations of OBT or MBT at 37°C for 4 hr, and the luciferase activity was determined as described in Materials and Methods. Values represent the mean ± SD of triplicate determinations.