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IDENTIFICATION OF BENZOTHIAZOLE DERIVATIVES AND POLYCYCLIC AROMATIC HYDROCARBONS AS ARYL HYDROCARBON RECEPTOR AGONISTS PRESENT IN TIRE EXTRACTS

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Abstract—Leachate from rubber tire material contains a complex mixture of chemicals previously shown to produce toxic and biological effects in aquatic organisms. The ability of these leachates to induce Ah receptor (AhR)-dependent cytochrome P4501A1 expression in fish indicated the presence of AhR active chemicals, but the responsible chemicals and their direct interaction with the AhR signaling pathway were not examined. Using a combination of AhR-based bioassays, we have demonstrated the ability of tire extract to stimulate both AhR DNA binding and AhR-dependent gene expression and confirmed that the responsible chemicals were metabolically labile. The application of CALUX (chemical-activated luciferase gene expression) cell bioassay-driven toxicant identification evaluation not only revealed that tire extract contained a variety of known AhR-active polycyclic aromatic hydrocarbons but also identified 2-methylthiobenzothiazole and 2-mercaptobenzothiazole as AhR agonists. Analysis of a structurally diverse series of benzothiazoles identified many that could directly stimulate AhR DNA binding and transiently activate the AhR signaling pathway and identified benzothiazoles as a new class of AhR agonists. In addition to these compounds, the relatively high AhR agonist activity of a large number of fractions strongly suggests that tire extract contains a large number of physiochemically diverse AhR agonists whose identities and toxicological/biological significances are unknown. Environ. Toxicol. Chem. 2011;30:1915–1925. © 2011 SETAC

Keywords—Ah receptor Tires Benzothiazoles CALUX Toxicant identification evaluation

INTRODUCTION

Vulcanized tire rubber represents a complex composition of hydrocarbons, minerals, metals, carbon blacks, process and extender oils, and other substances that have undergone an extensive and complex series of processing steps. In addition to the principal ingredient of rubber, tires contain a wide variety of chemicals that contribute to properties such as softness and resistance to skid, rolling, abrasion, aging, and others [1,2].

Chemical additives included in the processing of tires to obtain these characteristics include highly aromatic oils (known to contain high concentrations of polycyclic aromatic hydrocarbons [PAHs]), metals, peroxides, benzothiazole (BT) derivatives, phenols, phthalates, aromatic amines, and other chemicals as well as those that are formed during the tire vulcanization process [1–3]. Many of these chemicals are known to be toxic or carcinogenic. Given the number of tires currently used and those that are disposed of, the amount of tire particles on roadways released as a result of road wear and the increasing and extensive use of shredded or crumb tires in a wide variety of applications, a substantial amount of chemicals can be or are known to be released from vehicle tire rubber into the environment as a result of weathering and leaching [1–4]. Accordingly, significant concerns exist regarding the environmental and toxicological impact of chemicals that can be released (leached) from car tire rubber during weathering, and numerous studies have examined the toxicity of tire leachate (for review see Wik and Dave [2]).

Leachates or extracts of rubber tire have been shown to produce toxicity in a variety of aquatic organisms, including fish, amphibians, invertebrates, bacteria, and plants [1,2,5–9], and in human lung cell lines [10]. Although acute lethality was the most common effect, mutagenic, teratogenic, growth inhibition, oxidative stress, and alterations in estrogen receptor and progesterone receptor-dependent gene expression (endocrine-disrupting activity) have also been reported [11,12]. Water leachates of tires have been shown to induce expression of cytochrome P4501A1 (CYP1A1) in fathead minnows (*Pimephalies promelas*) [2] and rainbow trout (*Onchorhynchus mykiss*) [6]. Induction of CYP1A1 is mediated by the aryl hydrocarbon receptor (AhR), 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 chemicals [13,14]. Together, these results suggest that a chemical present in tire leachate is responsible for activating the AhR and AhR-dependent gene (CYP1A1) expression, although the specific chemical responsible for this activation was not identified.

The best studied and highest affinity ligands for the AhR are 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, but recent studies have demonstrated that the AhR can be bound and activated by structurally diverse chemicals with little similarity to the high-affinity HAH and PAH ligands [13,15–19]. Although the majority of these latter ligands are relatively weak agonists compared with the very potent HAHs

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and PAHs, their structural diversity clearly demonstrates the promiscuous ligand-binding activity of the AhR and indicates that the overall inducing potency of a given sample extract likely would result from the combined AhR stimulating activity of many different chemicals present in the extract. This is supported by recent studies by Misaki and coworkers [12], who demonstrated the presence of AhR agonist activity of organic extracts from road dust (of which tire wear particles are a significant component). Their initial toxicant identification evaluation (TIE) analysis using an AhR-based cell bioassay not only suggested that PAHs and hydroxy-PAHs likely were involved in the induction response but indicated that a major contribution to the activity derived from unidentified highly polar chemicals, a unique observation insofar as most AhR agonists are hydrophobic. However, it remains to be determined whether the AhR activity identified by these investigators as associated with the road dust originated from tire wear particles or materials from other sources, such as asphalt and combustion particulates.

Although the above-mentioned studies clearly demonstrate the presence of AhR-active chemicals in tire extracts and leachates, no responsible chemicals have yet been identified. In our previous studies, we have utilized a combination of AhR-based bioassay systems to identify and characterize novel AhR agonists and antagonists [16,20–22] and demonstrated the presence of AhR agonists in newspaper and inks [16,23]. We have expanded our studies to include screening of extracts of diverse biological, environmental, commercial, and consumer products with the goal of identifying and characterizing novel AhR activators or inhibitors and to determine their AhR-dependent toxic and biological potency. Here we describe the results of studies using both in vitro and cell-based AhR bioassays coupled with TIE approaches to isolate and identify AhR agonists present in organic solvent extracts of tire. These studies have led to the identification of various PAHs and several BT derivatives as contributors to the overall AhR agonist activity of tire extract.

MATERIALS AND METHODS

Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was obtained from Steve Safe (Texas A&M University); [γ^{32}P]ATP (>5,000 Ci/mmol) was purchased from Amersham; dimethylsulfoxide (DMSO), hexane, toluene, ethyl acetate (EA), and methanol (MeOH) were from Fisher Scientific; and poly-dI·dC was from Roche Molecular Biochemicals. Cell culture reagents and media were purchased from Gibco/BRL; G418 was from Gemini Bio-Products; and luciferase lysis and assays reagents from Promega. Truck tire tread was collected from a highway roadside. Benzothiazole derivatives were purchased from several sources: N-cyclohexyl-2-benzothiazolylsulfenamide, 2-(4-chlorophenyl)-benzothiazole, 2-benzothiazolyl diethyldithiocarbamate, 2-(4-morpholinyldithio)-benzothiazole, and (2-benzothiazolylthio)acetic acid from TCI; 2-morpholinobenzothiazole and 2-benzothiazolyl disulfide from Pfaltz and Bauer; *N*-*t*-butyl-2-benzothiazolesulfenamide and *N*,*N*-dicyclohexyl-benzothiazolesulfenamide from Wako; BT, 2-methylthiobenzothiazole (MTBT), 2-mercaptobenzothiazole (MBT), 2-hydroxybenzothiazole (OBT), 2-(2-benzothiazolylthio)-ethanol, 2-benzothiazolamine, and 2-chloro-benzothiazole from Aldrich Chemical; 4-(2-benzothiazolyl)-2-methyl-benzenamine, *N*-(2-benzothiazolylthio)-*N*-cyclohexyl-2-benzothiazolesulfenamide, and 2,2′-dithiobis (6-nitro-benzothiazole) from TimTec; and 2-(benzylsulfinyl)-1,3-benzothiazole from ChemBridge.

Tire sample preparation, fractionation, chemical analyses

For analysis of total tire extract, 1 g of washed inner tire tread was cut into small pieces and immersed into 2 ml DMSO overnight in a Teflon®-lined screw-cap tube, followed by centrifugation for 15 min at 1,500 rpm. The resulting supernatant (referred to as the ''original tire extract'') was transferred to a fresh tube, and an aliquot was serially diluted (10–10,000-fold range) in DMSO, and 1 μl of each concentration was analyzed for AhR agonist activity. For fractionation studies, 16 g of tire tread was incubated in 100 ml toluene overnight, the supernatant was collected, and the tire fragments were extracted twice more with 100 ml toluene. The combined toluene extract was evaporated to dryness and chromatographed through a hexane-washed silica gel column, and the column was sequentially eluted with 60 ml hexane, 60 ml hexane–toluene (1:1, v/v), 60 ml toluene, and 60 ml MeOH to yield 22 primary fractions of approximately 10 ml each. Fractions were dried and resuspended in 1 ml hexane, and the AhR agonist activity in 1 μl of each fraction was determined using the mouse hepatoma H1L1.1c2 cell bioassay as described below, with fractions 4 to 22 showing agonist activity. This concentration of hexane had no adverse effect on the cells (data not shown). The most active fraction (number 20) was subjected to a secondary fractionation by silica gel column chromatography and eluted with 20 ml hexane–toluene (1:1, v/v), 20 ml toluene, 20 ml toluene–EA (1:1, v/v), 20 ml EA, and 20 ml MeOH to obtain 20 secondary fractions of approximately 5 ml each. Fractions were dried and resuspended in 1 ml hexane (fractions 1–17) or methanol (fractions 18–20), and agonist activity in 1 μl of each fraction was determined, with positive AhR activity identified in fractions 8 to 12 and 18 to 20. Hexane solvent blanks were also run before sample fractionation to check for contaminants released from the chromatographic matrix. Analysis of these solvent blanks by gas chromatography–mass spectrometry (GC-MS) and gel retardation analysis revealed no chemical contamination or AhR activity. Active fractions were further analyzed by GC-MS in full scan mode using an electron impact ionization mode on a HP5973 mass spectral detector tuned with perfluorotributylamine using the system autotune parameters. The mass spectrometer was interfaced with a HP 6890 gas chromatograph equipped with a 30 m × 0.25 m, 0.25 μM DB-5ms phase column. The optimized oven program used inlet, transfer line, and quadrapole temperatures of 250, 280, 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.

Cell culture and induction of luciferase activity

Analysis of chemicals, extracts, and chromatographic fractions for their ability to stimulate AhR-dependent gene expression was carried out using recombinant mouse hepatoma (Hepa1c1c7) cell-based CALUX (H1L1.1c2 and H1L6.1c2) and CAFLUX (H1G1.1c3) clonal cell lines. The CALUX clonal cell lines are essentially identical and contain a stably integrated AhR/dioxin-responsive element (DRE)-driven firefly luciferase plasmid (pGudLuc1.1 or pGudLuc6.1, respectively) whose time course of luciferase induction varies as a result of differences in intracellular localization and stability of the luciferase gene product (with H1L1.1c2 cells inducing rapidly and maximally

by 4–6 h after agonist treatment and H1L6.1 cells inducing more slowly, with little activity at 4 to 6 h but with maximal induction observed by 24 h after agonist treatment [20,21]). The CAFLUX clonal cell line contains a stably integrated AhR/DRE-driven enhanced green fluorescent protein (EGFP) reporter gene plasmid whose activity can be repeatedly measured in intact cells for time course studies [21,24]. Transcriptional activation in the CALUX and CAFLUX cell lines occurs in a ligand-, dose-, and AhR-dependent manner [20,21,24]. For CALUX analysis, cells grown in white, clear-bottomed 96-well microplates were incubated with carrier solvent DMSO (10 μl/ml); TCDD (1 nM); and the indicated concentration of total tire extract, chromatographic fraction, or the indicated compound for 4 h (in H1L1.1c2 cells) or 24 h (in H1L6.1c2 cells) at 37°C, and luciferase activity was measured in a Berthold microplate luminometer as previously described [19–22]. For CAFLUX analysis, cells grown in black, clear-bottomed 96-well microplates were incubated with carrier solvent DMSO (10 μl/ml), TCDD (1 nM), or the indicated concentration of total tire extract at 33°C for the indicated time. Concentrations of DMSO up to 2% produced no visual signs of cytotoxicity in CALUX or CAFLUX cell lines. EGFP was measured in intact cells without removal of media, using a Fluostar microtiter plate fluorometer (Phenix Research Products) with an excitation wavelength of 485 nm (25 nm bandwidth) and an emission wavelength of 515 nm (10 nm bandwidth) as previously described [24]. Normalization between experiments involved adjusting the instrument fluorescence gain setting so that the level of EGFP fluorescence induced by 1 nM TCDD resulted in 9,000 relative fluorescence units. Samples were run in triplicate, and the fluorescent activity present in wells containing media only was subtracted from the fluorescence in all samples.

Preparation of cytosol and gel retardation analysis

Hepatic cytosol was prepared from male Hartley guinea pigs (250–300 g) in buffer containing 25 mM Hepes (pH 7.5), 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, and 10% (v/v) glycerol as previously described, and aliquots were stored at −80°C [25]. Incubation of cytosol with TCDD or the indicated extracts or chemicals and subsequent gel retardation analysis were carried out as described elsewhere in detail [25]. 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′) was synthesized, purified, annealed, and radiolabeled with [γ^{32}P]ATP. An aliquot (125 μl) of liver cytosol (8 mg protein/ml) was incubated with DMSO (2.5 μl), TCDD in DMSO (20 nM final concentration), and 1.25 μl of the original tire extract in DMSO or the indicated compound (200 μM each in DMSO) 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 nondenaturing polyarylamide gel electrophoresis and autoradiography of the dried gel. The amount of ^{32}P-labeled DRE present in the induced protein–DNA complex was determined using a molecular dynamics phosphoimager.

RESULTS

Tire extract induction of AhR-dependent gene expression

The presence of AhR agonists in leachates of tires has been suggested by several studies in aquatic organisms [2,6,12]. To identify the responsible chemicals systematically, we first had to confirm the presence of AhR agonists in tire extracts. The indicated concentration of tire extract was incubated for 4 h with recombinant mouse hepatoma (H1L1.1c2) cells, which contain a stably integrated AhR-responsive luciferase reporter gene [22,21]. These results demonstrated that tire extract induced luciferase in a concentration-dependent manner in the H1L1.1c2 cells (Fig. 1A), producing a response between 40 and 170% of that maximally induced by TCDD. This induction response was back-calculated to result from a range of extract that was equivalent to 0.5 μg to 0.5 mg of original tire. Superinduction of reporter gene activity by this extract was unexpected but was not surprising given our previous observation of the ability of other sample solvent extracts to produce a synergistically enhanced induction response [26]. Similar concentration-dependent induction or superinduction by this crude tire extract was also observed in recombinant AhR-responsive guinea pig, rat, and human cell lines containing a stably transfected AhR-responsive reporter gene (Supplemental Data, Fig. S1), demonstrating that this induction response was not unique to the mouse AhR and that this extract contained AhR agonists. Maximal induction in these cell lines was approximately 210, 170, and 230% of that induced by 1 nM TCDD, respectively. Although the exact mechanisms responsible for the observed superinduction response is not known, we have previously described several possible mechanisms that might contribute to synergistic increases in AhR-dependent gene expression [26].

To determine whether the induction response was by metabolically stable HAHs that might have been extracted from the tire or by other metabolically labile AhR ligands (such as PAHs and others), we examined the time course of induction of AhR-dependent gene expression in recombinant rat hepatoma (H1G1.1c3) cells that contain a stably transfected AhR-responsive EGFP reporter gene. A major advantage of these cells is that the same cells can be analyzed repeatedly in time course studies, because measurement of EGFP activity is carried out using intact cells in culture media [21,24]. Although EGFP was induced in a concentration-dependent manner, the magnitude of gene induction by the tire extract initially increased over 48 h and then progressively decreased over time (Fig. 1B); TCDD continued to induce EGFP to a stable maximal activity. These results not only indicate that the responsible AhR agonists extracted from tire are metabolically labile and produce only a transient induction response but they also confirm that the responsible chemicals are not HAHs.

Tire extract and AhR transformation, DNA binding in vitro

The cell-based bioassays demonstrate induction of AhR-dependent reporter gene activity by the tire extract, but they do not directly demonstrate the ability of chemicals in the tire extract to bind to and activate the AhR. To confirm the ability of the tire extract to activate the AhR directly, we examined its ability to stimulate AhR transformation and DNA binding in vitro. Incubation of guinea pig hepatic cytosol with crude tire extract and subsequent gel retardation analysis revealed that the tire extract did indeed induce AhR transformation and DNA binding (Fig. 1C). Phosphoimager quantitation revealed that the amount of AhR–ARNT–DRE complex formed by incubation with tire extract was comparable to that produced by a maximally stimulating concentration of TCDD (Fig. 1D). These results suggest that the ability of the tire extract to stimulate AhR-dependent gene expression is due to the ability of chemicals present in the extract to bind to and stimulate AhR transformation and DNA binding.

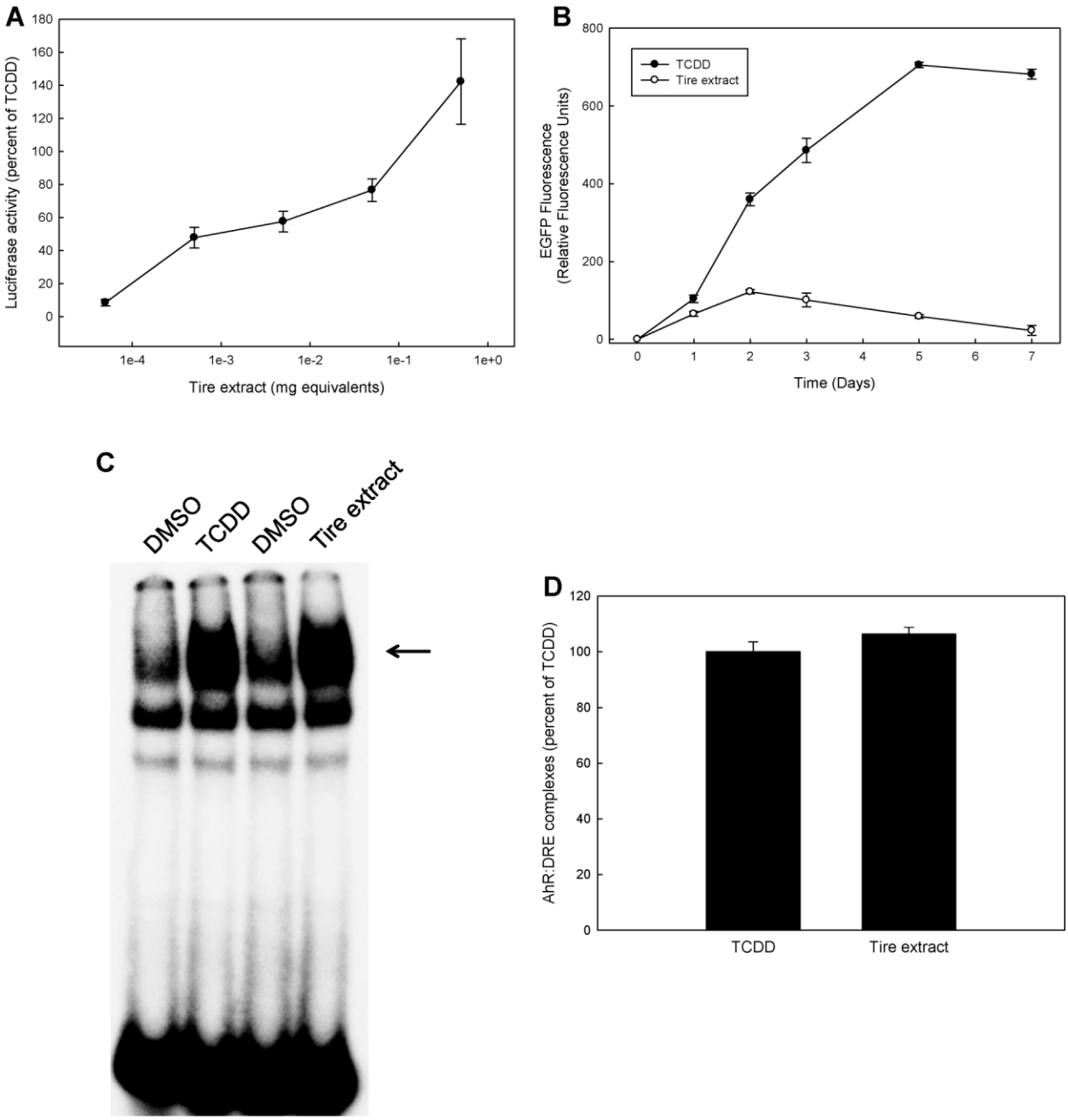

Fig. 1. Tire extract contains Ah receptor (AhR) agonist activity. (**A**) Concentration-dependent induction of AhR-dependent luciferase reporter gene expression by crude tire extract in H1L1.1c2 cells was determined after 4 h of exposure as described in *Materials and Methods*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percentage of the activity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). (**B**) Time course of induction of enhanced green fluorescent protein (EGFP) gene expression by crude tire extract. H1G1.1c3 cells were treated with TCDD (1 nM) or tire extract (equivalent to 0.005 mg of original tire) at 33°C, and EGFP fluorescence was measured at the indicated time points, as described in *Materials and Methods*. Values represent the mean ± SD of triplicate determinations. (**C,D**) Tire extract stimulates AhR transformation and DNA binding in vitro. Guinea pig hepatic cytosol was incubated with 2.5 μl dimethylsulfoxide (DMSO), 20 nM TCDD (in DMSO), or tire extract (equivalent to 0.625 mg of the original tire material) for 2 h at 20°C. The amount of AhR transformation and DNA binding was determined by gel retardation analysis (a representative gel is shown in C), and the amount of AhR–DNA complex was quantified by PhosphorImager analysis (D) as described in *Materials and Methods*. Values represent the mean ± SD of at least triplicate determinations and are expressed relative to the amount of complex induced by TCDD.

TIE identification of AhR-active compounds in tire extracts

To identify the specific chemicals responsible for activating the AhR and AhR signaling pathway, a toxicant identification evaluation approach was used. Tire extract was fractionated by silica gel column chromatography and progressively eluted with a series of solvents (hexane, hexane/toluene [1:1,v/v], toluene, and then methanol) into 22 fractions (Fig. 2A). Aliquots of each fraction were exchanged into hexane, and AhR agonist activity in each fraction was determined with the recombinant mouse hepatoma (H1L1.1c2) cells. Interestingly, 19 of 22 fractions exhibited AhR agonist activity with a range in overall induction between 15 and 130% of that maximally induced by TCDD (Fig. 2B). The presence of AhR agonist activity in so many

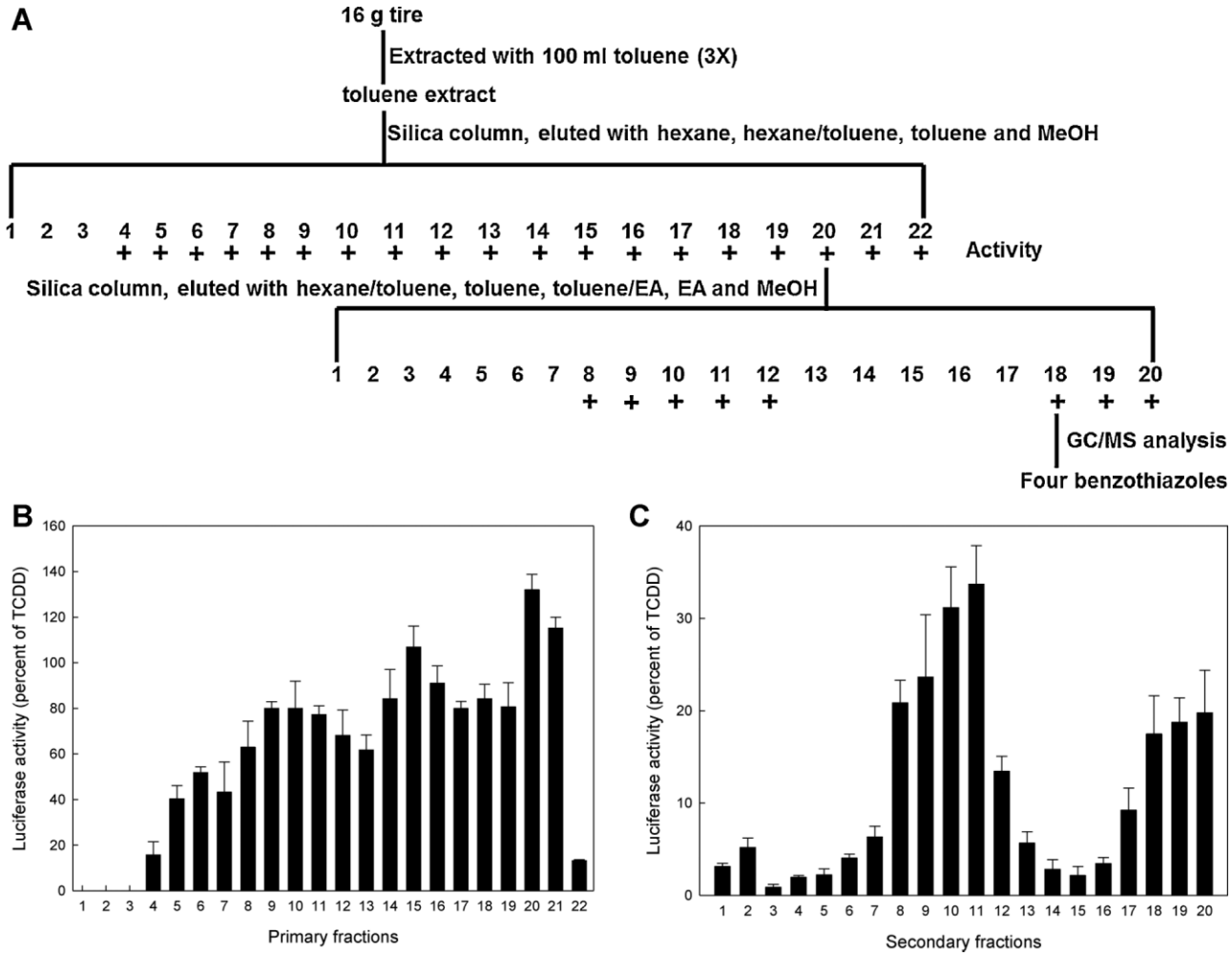

Fig. 2. Ah receptor (AhR)-based chemically activated luciferase expression (CALUX) cell bioassay-guided toxicant identification evaluation of tire extract. (**A**) Tire tread was cut into small pieces, extracted with toluene, and fractionated using silica gel chromatography by elution with the indicated solvents to obtain 22 primary fractions. The strongly inducing fraction 20 was subjected to a secondary fractionation using a silica gel column chromatography and eluted with the indicated solvents to obtain 20 secondary fractions. (**B,C**) The AhR agonist activity in each fraction was determined using the mouse hepatoma H1L1.1c2 CALUX cell bioassay with primary fractions 4 to 22 (B) and secondary fractions 8 to 12 and 18 to 20 (C) containing AhR agonist activity.

fractions suggests the presence of a large number of structurally different AhR active compounds. To separate further, analyze, and identify responsible AhR agonists, the most active fraction (no. 20) from the first fractionation scheme was subjected to additional silica gel chromatography and progressively eluted with a different series of solvents (hexane/toluene [1:1,v/v], toluene, toluene/ethyl acetate [1:1,v/v], ethyl acetate, and methanol) into 20 fractions (Fig. 2A). Aliquots of each fraction were exchanged into hexane (fractions 1–17) or methanol (fractions 18–20), and the AhR agonist activity of each was determined using the H1L1.1c2 cells. Positive AhR agonist activity (defined as >10% of that maximally induced by TCDD) was contained in fractions 8 to 12 and 18 to 20 (Fig. 2C). Instrumental analysis by GC/MS was carried out on each active fraction, and comparison of mass spectra and retention times to authentic standards revealed that active fraction 18 contained several BT derivatives (specifically BT, MTBT, MBT, and OBT; Fig. 3, and Supplemental Data, Fig. S2); several PAHs were also tentatively identified in fraction 9, although some of these PAHs were also present in other fractions (structures shown in Fig. 3). Many of these PAHs are known AhR agonists [15,27–29] or would be expected to have AhR agonist activity. The lack of chemical contaminants or AhR activity in the hexane solvent blanks run before extract fractionation confirmed that the AhR activity was derived from the tire extract.

MBT and OBT as AhR agonists

Instrumental analyses indicated the presence of several BT derivatives in active fraction 18, but they do not confirm that these compounds are AhR active. Accordingly, we examined the ability of commercially obtained BT, MTBT, MBT, and OBT to stimulate AhR-dependent gene expression in the mouse H1L1.1c2 cell line (Fig. 4A). Although these four BTs have similar structures (Fig. 3), only MBT and OBT induced AhR-dependent gene expression, and they were relatively weak inducers (compared with TCDD); BT and MTBT were inactive. OBT was relatively efficacious and demonstrated a full concentration-dependent induction of luciferase activity (with an EC50 of ~4 μM), but MBT was only a partial AhR agonist, with a concentration of 100 μM MBT inducing luciferase to approximately 50% of that produced by a maximal inducing concentration of TCDD (Fig. 4A). To examine the metabolic stability of MBT and OBT as AhR agonists, we examined the ability of

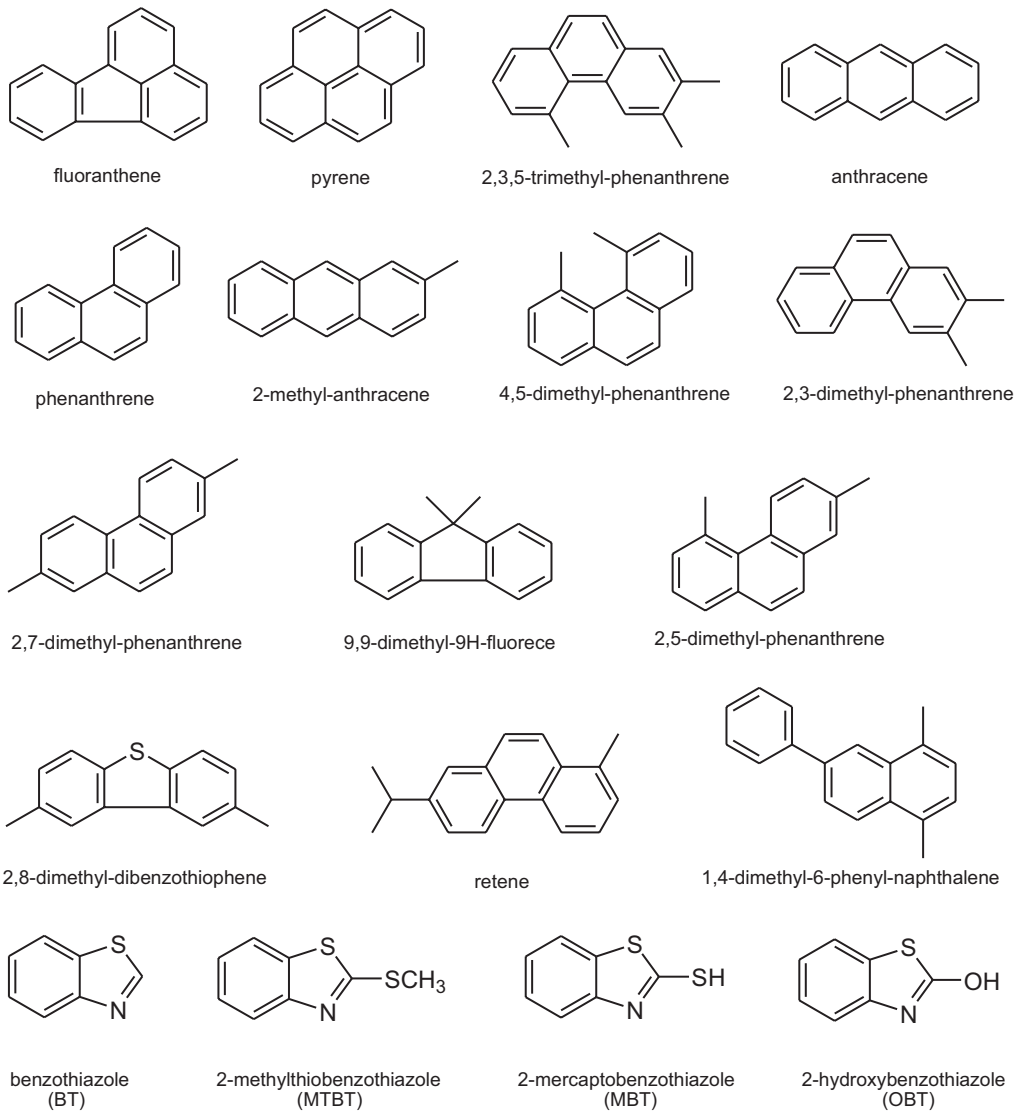

Fig. 3. Chemical structures of the benzothiazoles identified in fraction 18 and the polycyclic aromatic hydrocarbons tentatively identified in secondary fraction 9 of the tire extract.

10 μM of each compound to induce luciferase activity in two mouse hepatoma (Hepa1c1c7)-based CALUX clonal cell lines whose time course of maximal luciferase gene induction is relatively rapid (4–6 h for H1L1.1c2 cells) or slow (24 h for H1L6.1c3 cells) [21]. The lower relative magnitude of luciferase gene induction at 24 h compared with 4 h, compared with that induced by 1 nM TCDD, indicates that these inducers are metabolically labile (Supplemental Data, Fig. S3), which is consistent with the transient induction response by the crude tire extract (Fig. 1B). To demonstrate that the identified BTs can bind to and activate the AhR, we examined their ability to stimulate AhR transformation and DNA binding by gel retardation analysis. These analyses not only demonstrated that OBT and MBT could stimulate AhR transformation and DNA binding to between 40 and 50% of that of TCDD and confirmed them as AhR agonists but interestingly revealed that BT and MTBT also stimulated AhR transformation and DNA binding, albeit only to 15 to 20% of that produced by TCDD (Fig. 4B). Although these results would suggest that BT and MTBT are weak AhR agonists, their inability to induce AhR-dependent gene expression may result from lower potency/affinity, more rapid metabolism within the cell, or other factors.

Activation of the AhR by BT derivatives

The analysis described above demonstrates the ability of the OBT and MBT to bind to and activate AhR and AhR-dependent gene expression. Whereas a previous study identified a BT derivative as an AhR agonist [30], the widespread use of these chemicals in many materials and potential exposure to these compounds raise the question of whether AhR agonist activity is a common property of other BT derivatives. Accordingly, we examined the ability of 16 commercially available BT derivatives (Fig. 5) to stimulate AhR-dependent luciferase gene expression (Fig. 6A) as well as AhR transformation and DNA binding by gel retardation analysis (Fig. 6B). Although some compounds were inactive as activators of AhR-dependent gene expression in H1L1.1c2 cells after 4 h of incubation (compounds 2, 4–6, 11), several compounds (namely, compounds 3, 8–10, 13, 14) were moderately active, inducing luciferase reporter gene activity between 40 and 100% of that obtained with a maximally inducing concentration of TCDD. To examine the metabolic stability of these novel AhR agonists, we further examined their ability to induce luciferase gene expression after 24 h of incubation using mouse hepatoma

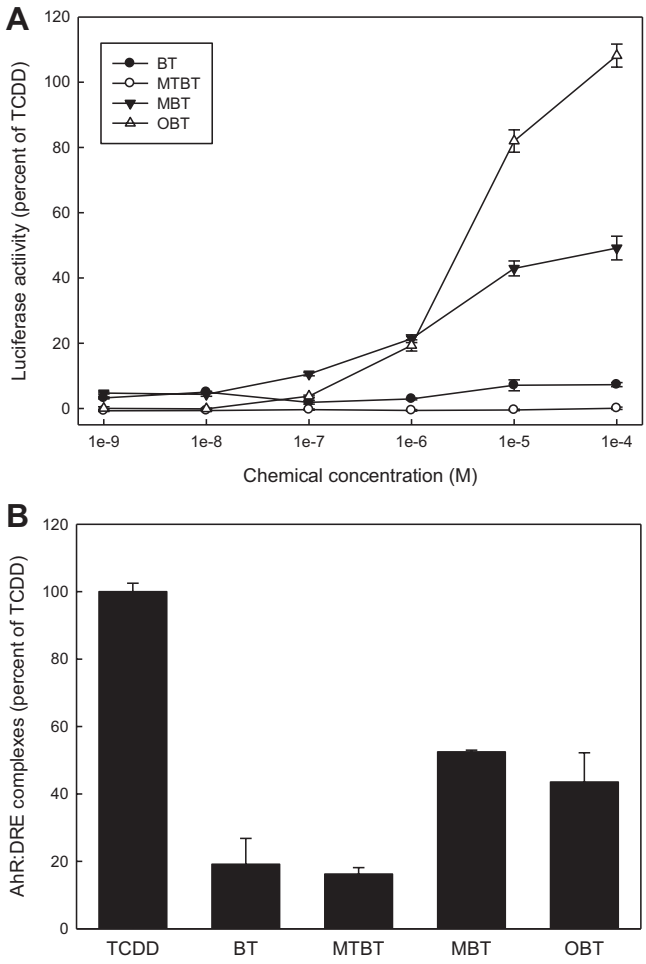

Fig. 4. Ability of benzothiazoles identified in tire extract to stimulate Ah receptor (AhR)-dependent gene expression and AhR DNA binding. (**A**) H1L1.1c2 cells were incubated with the indicated concentrations of benzothiazole (BT), 2-methylthiobenzothiazole (MTBT), 2-mercaptobenzothiazole (MBT), or 2-hydroxyenzothiazole (OBT) at 37°C for 4 h, and the luciferase activity was determined as described in *Materials and Methods*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percentage of maximal luciferase activity induced by 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). (**B**) Guinea pig hepatic cytosol was incubated with carrier solvent (2.5 μl dimethylsulfoxide [DMSO]), 20 nM TCDD (in DMSO), or 200 μM of the indicated benzothizole compound for 2 h at 20°C. Aliquots of each sample were analyzed by gel retardation analysis as described in the legend to Figure 1. The amount of induced AhR–DRE complex formed was expressed relative to that of TCDD, with values representing the mean ± SD of at least triplicate determinations. DRE = dioxin responsive element.

(H1L6.1c2) cells. Similarly to the results with OBT and MBT, most of the BT derivatives induced significantly less luciferase activity at 24 h compared with 4 h (Fig. 6A), indicating that they are metabolically labile AhR agonists. Interestingly, compound 14 induced significantly more luciferase activity at 24 h than at 4 h, suggesting a somewhat different mechanism of action for this compound or its metabolism or conversion into a more potent AhR agonist. The ability of these BT derivatives to stimulate guinea pig AhR transformation and DNA binding in vitro (Fig. 6B,C) was relatively consistent with the mouse cell induction for compounds 1, 3, 7 to 10, and 12 to 14 (Fig. 6A, upper panel; Fig. 6C), indicating that they can directly activate the AhR; compound 11 was inactive in both assays. Compounds 15 and 16 induced reporter gene activity to 20 to 30% of that of TCDD, but they stimulated little or no AhR transformation or DNA binding (Fig. 6B,C). In contrast, compounds 2 and 4 to 6 were similar to BT and MTBT in that they could stimulate AhR DNA binding (to 10–60% of that of TCDD; Fig. 6B,C), yet did not induce AhR-dependent gene expression (Fig. 6A). Previous studies have identified chemicals that can bind to and activate the AhR in vitro but fail to induce AhR-dependent gene expression in cells in culture [15,16,18], most likely as a result of rapid metabolic degradation of the chemicals within the cells.

DISCUSSION

Tire wear components are present throughout the environment, and numerous studies have not only catalogued the specific chemicals leached from these materials but have also reported on the toxic and biological consequences of exposure to leachates and extracts of these materials on the aquatic environment and organisms [1–3]. Human exposure to chemicals leached from tires, shredded tires, and tire wear material can occur by dermal exposure from the environmental sources and ingestion of contaminated materials as well as inhalation of airborne particulate matter derived from tire wear material [2]. Evaluating the exposure and possible toxicity of tire-derived materials becomes more complex considering that weathering and aging of tire materials as well as heating of the tires as a result of roadway friction likely result in the generation of other chemicals with significantly different biological and toxicological effects and potencies. Accordingly, tire rubber contains a wide variety of chemicals that can produce biological and toxicological effects through many distinct mechanisms [1–4]. The present study has focused on only one class of bioactive compounds, those that can interact with and active the AhR and AhR signaling pathway. The AhR bioassay-based TIE analysis of crude organic tire extract described here revealed that most fractions contained substantial AhR agonist activity. When we consider the range of elution solvents used to generate these fractions and the documented promiscuity of AhR ligand structure [15–19,31], it is very likely that the tire extract contains a large number of structurally and physiochemically diverse AhR agonists, only a few of which were identified in this report. Interestingly, superinduction of AhR-dependent luciferase reporter gene activity was observed with the crude tire extract in each of the CALUX cell lines from four different species (Fig. 1A, and Supplemental Data, Fig. S1), indicating a commonality in the mechanisms of this enhancement of the induction response among species and cell lines. The ability of chemicals and solvent extracts of environmental samples (e.g., soil and sediment) to enhance synergistically the CALUX induction response to a level greater than that maximally induced by TCDD has been previously reported [26,32,33]. Although the exact mechanisms of CALUX superinduction have not been defined, several mechanisms have been proposed, including chemical-dependent inhibition of protein synthesis that increases the stability of the ligand–AhR complex, decreases in the level of a labile protein that represses AhR functionality, and enhancement of AhR transcriptional activity via additional cell signaling pathways [26,32]. Given that the CALUX superinduction response was observed only with the crude tire extract and not with any of its fractions, it is more likely that the crude extract contains both AhR agonists and chemicals that affect another cellular signal transduction pathway that augments AhR-dependent luciferase expression from the reporter gene plasmid. Consistent with this possibility are previous observations that chemical-dependent activation of the

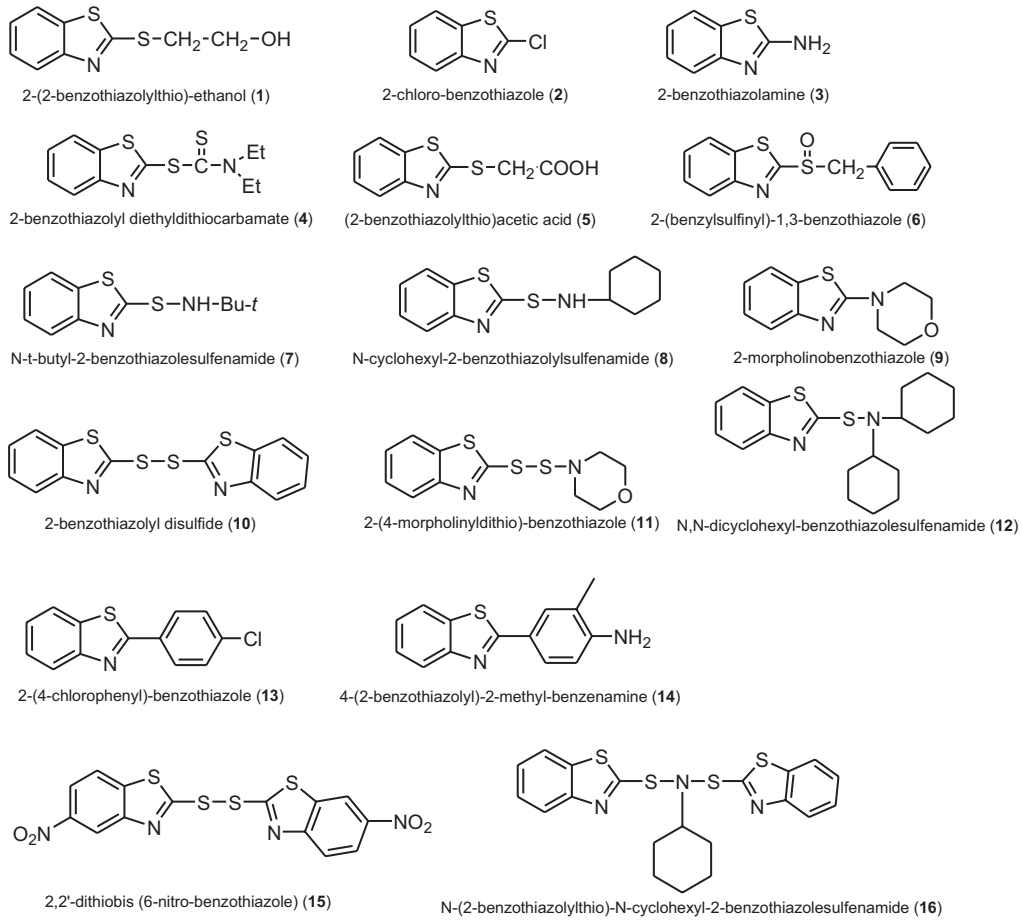

Fig. 5. Chemical structures of commercially available benzothiazole derivatives analyzed for Ah receptor (AhR) agonist activity.

protein kinase C signaling pathway or treatment of CALUX cells with selected prostaglandins, known signaling factors, can synergistically enhance AhR-dependent gene expression [26,32,33]. The mechanism and chemicals in the tire extract responsible for the superinduction response and the importance of this response in the overall toxicity of tire extracts remain to be determined.

Polycyclic aromatic hydrocarbons were found in numerous active fractions of tire extract, and many of the PAHs that were identified are either known to be AhR agonists or are similar in structure to known AhR-active PAHs [15,27–29]. Accordingly, the PAHs likely are a significant contributor to the overall AhR agonist activity of crude tire extract. Several BT derivatives were also specifically identified in tire extract, and two of the identified compounds (MBT and OBT) were confirmed to be AhR agonists by subsequent analysis in rodent cell line- and guinea pig cytosolic AhR-based bioassays. Interestingly, it was recently reported that injection of MBT into rainbow trout had no effect on AhR-inducible CYP1A-dependent ethyoxyresorufin *O*-deethylase activity, suggesting that MBT does not react with the AhR [7]. However, results of the present study clearly demonstrate that MBT is an AhR agonist in in vitro and cell-based AhR bioassays (Fig. 4). Similarly, although BT was only a very weak AhR agonist in our mammalian cell bioassays (i.e., 100 μM BT induced less than 10% of maximal AhR-dependent gene expression), a previous study identified BT as a relatively potent AhR agonist of the human AhR expressed in yeast [30]. The reasons for these differences in response remain to be determined, but they very likely result from species differences in the AhR in both assays (rodent vs. guinea pig vs. human) or differences in the overall rate of metabolism or degradation between these experimental systems. In fact, although HAHs show similar rank order potency with regard to AhR ligand binding and AhR activation between species, dramatic differences in ligand specificity and potency of non-HAH ligands between species (particularly among human, rodent, and fish AhRs) have been reported [31,34,35].

The AhR bioassay screening of commercially available BT derivatives identified many compounds with AhR agonist activity, and significant diversity in response was observed relative to the in vitro and cell-based bioassay systems. The ability of most of the BT derivatives to stimulate AhR-dependent luciferase gene expression correlated reasonably well with their ability to activate AhR transformation and DNA binding, consistent with them being direct AhR ligands. Additionally, the relative potency of these compounds to induce AhR-dependent gene expression was generally reduced with longer incubation times (compare 4 and 24 h of incubation in Fig. 6A), and this likely results from metabolic degradation of the BT inducer. Interestingly, the greater degree of induction by 4-(benzothiazolyl)-2-methyl-benzamine (compound 14) at 24 h (compared with 4 h) suggested that this compound was metabolically stable (leading to increased AhR-dependent gene expression for a longer period of time), metabolized into a more potent AhR agonist, or exerted additional effects that enhanced luciferase reporter gene expression. This compound has previously been shown to be a potent and selective antitumor agent and independently identified as an AhR agonist [36].

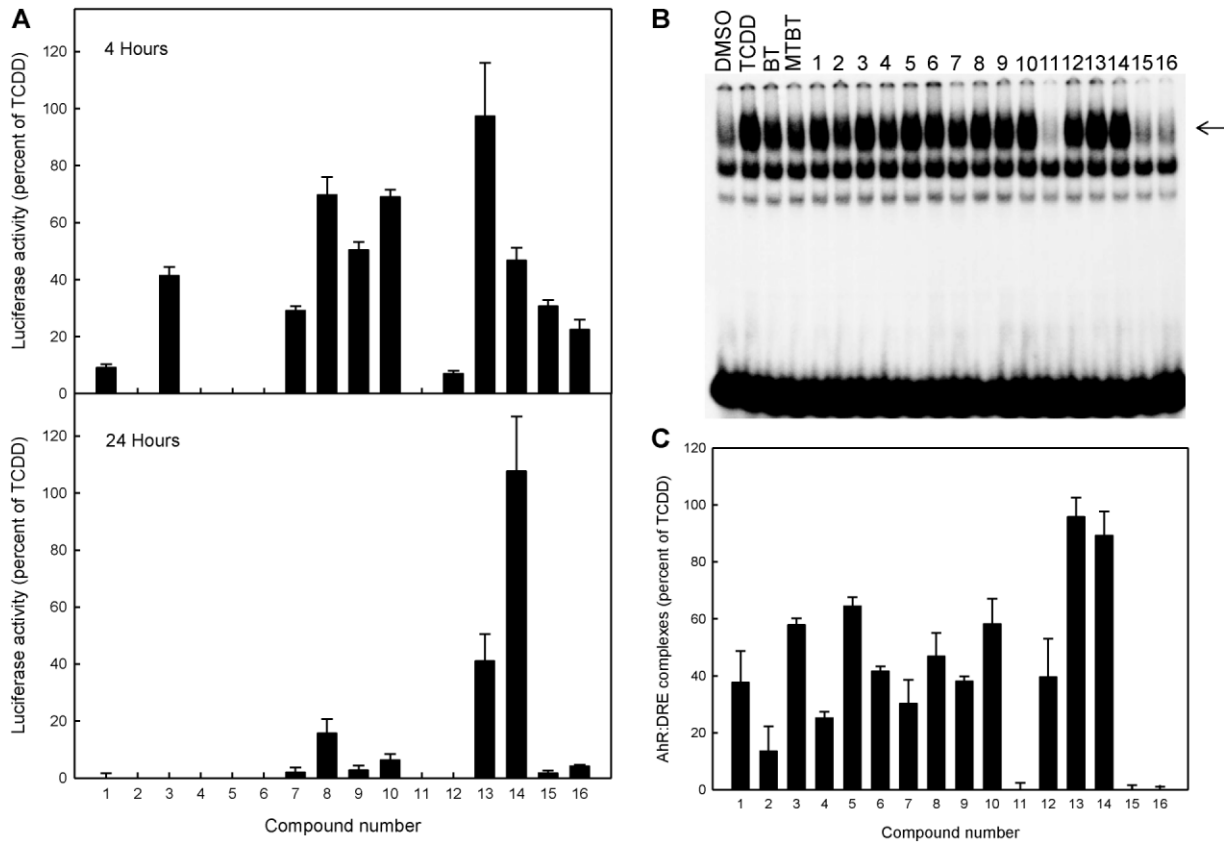

Fig. 6. Benzothiazole (BT) derivatives induce Ah receptor (AhR)-dependent luciferase reporter gene expression and stimulate AhR transformation and DNA binding. (**A**) Confluent plates containing the recombinant mouse hepatoma cells were incubated with the indicated benzothiazoles (10 μM) for 4 h (H1L1.1c2 cells) or 24 h (H1L6.1c2 cells) at 37°C. Dimethylsulfoxide (DMSO; 10 μl/ml final concentration) and 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were used as negative and positive controls. Luciferase activity in cell lysates was determined as described in *Materials and Methods*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percentage of TCDD. (**B,C**) Guinea pig hepatic cytosol was incubated with carrier solvent (2.5 μl of DMSO) or 20 nM TCDD (in DMSO) and the indicated benzothiazoles (200 μM) for 2 h at 20°C. Aliquots of each sample were analyzed by gel retardation analysis as described in the legend to Figure 1 (a representative gel is shown in B). The amount of induced AhR–DRE complex formation was expressed relative to that of TCDD (C), with values representing the mean ± SD of at least triplicate determinations. MTBT = 2-methylthiobenzothiazole.

N-(2-benzothiazolylthio)-*N*-cyclohexyl-2-benzothiazolesulfenamide (compound 16) and 2,2′-dithiobis (6-nitro-benzothiazole) (compound 15) were novel AhR agonists in that they were active in the cell-based luciferase assay yet inactive in stimulating AhR transformation and DNA binding in vitro. Although the exact mechanism for this differential effect is not known, it is possible that these compounds were metabolized into more potent AhR agonists in the intact cells (leading to AhR-dependent gene induction) and their inactivity in the DNA binding assay resulted from the lack of conversion of the compounds into AhR agonists, because this cytosolic-based assay lacks major xenobiotic metabolizing enzymes. This observation is comparable to our previously reported studies with bilirubin and biliverdin, which were very weak agonists in the AhR DNA binding assay yet were relatively potent inducers of AhR-dependent gene expression in intact cells [37]. The stimulation of AhR transformation and DNA binding and lack of induction of AhR-dependent gene expression by the compounds 2-chloro-benzothiazole (compound 2), 2-benzothiazolyl diethyldithiocarbamate (compound 4), 2-(benzylsulfinyl)-1,3-benzothiazole (compound 6), and (2-benzothiazolylthio)acetic acid (compound 5) are not surprising, given that many AhR agonists have been found to be significantly more potent in in vitro, cell-free AhR bioassays compared with cell-based gene induction assays [15,16,18]. In the cell-free AhR transformation and DNA binding bioassay, the agonist has direct access to the AhR in the cytosolic incubation, and, if it can bind to and activate the AhR, it will do so, resulting in a positive response. In contrast, for a compound to be positive in cell-based bioassays, it must enter the cell, avoiding sequestration (by membranes, lipids, proteins, and organelles) and metabolism (by degradation enzymes such as cytochrome P450s), and bind to the AhR, stimulating AhR nuclear localization, transformation, and DNA binding and induction of gene expression, all within the time frame of the bioassay. Thus, positive activity in the cell bioassay is dependent on a variety of conditions, factors, and physiochemical properties of the activating chemical, and those chemicals that do not induce AhR-dependent gene expression are negatively affected by one or more of these aspects. Overall, the results of these studies have identified BTs as a novel class of AhR agonists.

Benzothiazoles are a large class of widely used and globally synthesized industrial chemicals used in a wide variety of applications, including vulcanization accelerators in rubber production, as slimicides in the paper and pulp industry, fungicides, herbicides, antimicrobial and antialgal agents, and corrosion inhibitors in cooling water and in antifreeze for automobiles [2,38,39]. Considering that many of these uses result in release of these chemicals into the environment, coupled with the considerable environmental half-life of some BTs [38], it is not surprising that these compounds have been detected in water, soils, sediments, groundwater, municipal wastewater effluent, industrial emission, and the atmosphere [2,40]. Given the role of the AhR in mediating the toxic and

biological effects of structurally diverse chemicals, BTs could contribute to the overall impact and effect of environmental mixtures of AhR active compounds. Additionally, given that numerous additives are used in the manufacture of tires and that AhR agonist activity was identified in almost all fractions of the tire solvent extract along with the documented promiscuity of AhR ligand structure [15,17,29,31], the overall AhR agonist activity of tire extracts very likely results from a combination of many distinctly different chemicals. The identification of MBT, OBT, and several commercially obtained BTs as novel AhR agonists provides us with more insight into the structure–activity relationships that govern AhR agonist responses. Further studies are needed to assess the contribution of other tire additives to the overall AhR agonist activity of tire extract and to determine its potential for producing AhR-dependent toxic and biological effects.

SUPPLEMENTAL DATA

Fig. S1. Tire dimethylsulfoxide (DMSO) extract induces AhR-dependent luciferase reporter gene expression in stably transfected guinea pig intestinal adenocarcinoma (G16L1.1c8), rat hepatoma (H4L1.1c4), and human hepatoma(HG2L6.1c3) cells in a concentration-dependent manner.

Fig. S2. Mass chromatogram of fraction 18 from a tire extract and identification of four benzothiazoles.

Fig. S3. Transient induction of AhR-dependent gene expression by 2-mercaptobenzothiazole (MBT) and 2-hydroxyenzothiazole (OBT).(196 KB PDF).

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