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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. While the ability of these leachates to induce Ah receptor (AhR)-dependent cytochrome P4501A1 expression in fish indicated the presence of AhR active chemicals, the responsible chemical(s) 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 chemical(s) was 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.

Keywords

Ah receptor; Tire; 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].

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Many of these chemicals are known to be toxic and/or carcinogenic. Considering 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 tire in a wide variety of applications, a substantial amount of chemicals can and/or are known to be released from vehicle tire rubber into the environment as a result of weathering and leaching [1–4]. Accordingly, there are significant concerns 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 (reviewed in 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]. While acute lethality was the most common effect, mutagenic, teratogenic, growth inhibition, oxidative stress and alterations in estrogen receptor and progesterone receptor-dependent gene expression (i.e., 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(s) present in tire leachate is responsible for activating the AhR and AhR-dependent gene (CYP1A1) expression, although the specific chemical(s) responsible for this activation was not identified.

While 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, 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 when compared to the very potent HAHs 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 would likely 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 were likely involved in the induction response, but indicated that a major contribution to the activity derived from unidentified highly polar chemicals, a unique observation since 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 (i.e., asphalt, combustion particulates, etc.).

While the above 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 and commercial and consumer products with the goal of identifying and characterizing novel AhR activators and/or

inhibitors and to determine their AhR-dependent toxic and biological potency. Here we described 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 (TCDD, dioxin) was obtained from Steve Safe (Texas A&M University, TX, USA), [γ^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham (Piscataway, NJ, USA), dimethylsulfoxide (DMSO), hexane, toluene, ethyl acetate (EA), and methanol (MeOH) were from Fisher Scientific (PA, USA) and poly dI dC was from Roche Molecular Biochemicals (IN, USA). Cell culture reagents and media were purchased from Gibco/BRL (NY, USA), G418 was from Gemini Bio-Products (CA, USA), and luciferase lysis and assays reagents from Promega (WI, USA). 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 (OR, USA); 2-morpholinobenzothiazole and 2-benzothiazolyl disulfide from Pfaltz and Bauer (CT, USA); *N*-*t*-butyl-2-benzothiazolesulfenamide and *N*,*N*-dicyclohexyl-benzothiazolesulfenamide from Wako (VA, USA); BT, 2-methylthiobenzothiazole (MTBT), 2-mercaptobenzothiazole (MBT), 2-hydroxybenzothiazole (OBT), 2-(2-benzothiazolylthio)-ethanol, 2-benzothiazolamine and 2-chloro-benzothiazole from Aldrich Chemical (MO, USA); 4-(2-benzothiazolyl)-2-methyl-benzenamine, *N*-(2-benzothiazolylthio)-*N*-cyclohexyl-2-benzothiazolesulfenamide and 2,2'-dithiobis (6-nitro-benzothiazole) from TimTec (DE, USA); 2-(benzylsulfinyl)-1,3-benzothiazole from ChemBridge (CA, USA).

Tire sample preparation, fractionation and chemical analyses

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. The resulting supernatant (referred to as the original tire extract) was transferred into a fresh tube and an aliquot was serially diluted (10–10,000-fold range) in DMSO and 1 μl of each concentration analyzed for AhR agonist activity. For fractionation studies, 16 g of tire tread was 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, chromatographed through a hexane-washed silica gel column and the column sequentially eluted with 60 ml hexane, 60 ml hexane–toluene (1:1, v/v), 60 ml toluene and 60 ml MeOH to yield twenty-two primary fractions of approximately 10 ml each. Fractions were dried and resuspended in 1 ml of hexane and the AhR agonist activity in 1 μl of each fraction was determined using the mouse hepatoma H1L1.1c2 cells 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 twenty secondary fractions of approximately 5 ml each. Fractions were dried and resuspended in 1 ml of 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 auto-tune 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. 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.

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 to 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 repeated 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), 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 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 were 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 stored at −80°C [25]. Incubation of cytosol with TCDD or the indicated extracts or chemicals and subsequent gel retardation analysis was carried out as described 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') 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 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 non-denaturing 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 induces AhR-dependent gene expression

The presence of AhR agonists in leachates of tires have been suggested from several studies in aquatic organisms [2,6,12]. In order to systematically identify the responsible chemical(s), we first needed to confirm the presence of AhR agonists in tire extracts. The indicated concentration of tire extract was incubated for four hours 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 to 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 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/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. While the exact mechanism(s) responsible for the observed superinduction response is not known, we have previously described several possible mechanisms that may 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 and/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 since measurement of EGFP activity is carried out using intact cells in culture media [21,24]. While EGFP was induced in a concentration-dependent manner, the magnitude of gene induction by the tire extract initially increased over 48 hours 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, they also confirm that the responsible chemicals are not HAHs.

Tire extract stimulates AhR transformation and DNA binding *in vitro*

While the cell-based bioassays demonstrate induction of AhR-dependent reporter gene activity by the tire extract, 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 directly activate the AhR, 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.

TIE identification of AhR-active compounds in tire extracts

In order to identify the specific chemical(s) 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 determined using the recombinant mouse hepatoma (H1L1.1c2) cells. Interestingly, 19 of 22 fractions exhibited AhR agonist activity with a range in overall induction between 15 to 130% of that maximally induced by TCDD (Fig. 2B). The presence of AhR agonist activity in so many fractions suggests the presence of a large number of structurally different AhR active compounds. To further separate, analyze and identify responsible AhR agonists, the most active fraction (#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 AhR agonist activity of each determined using the H1L1.1c2 cells. Positive AhR agonist activity (defined as >10% of that 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 was shown to contain several BT derivatives (specifically BT, MTBT, MBT, and OBT (Fig.3 and Supplemental Data, Fig. S2); a number of 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 are AhR agonists

While instrumental analysis indicated the presence of several BT derivatives in active fraction 18, 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 (as compared to TCDD); BT and MTBT were inactive. While OBT was relatively efficacious and demonstrated a full concentration-dependent induction of luciferase activity (with an EC50 of ~4 μM), 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 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 as compared to 4 h, compare to that induced by 1 nM TCDD, indicates that these inducers are metabolically labile (Supplemental Data, Fig. S3), 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 to 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). While 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 and/or other factors.

Activation of the AhR by BT derivatives

The above analysis demonstrates the ability of the OBT and MBT to bind to and activate the AhR and AhR-dependent gene expression. While 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 raises the question as to whether AhR agonist activity is a common property of other BT derivatives. Accordingly, we examined the ability of sixteen 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 to 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 incubation using mouse hepatoma (H1L6.1c2) cells. Similar to the results with OBT and MBT, most of the BT derivatives induced significantly less luciferase activity at 24 h as compared to 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 and/or its metabolism/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) were relatively consistent with the mouse cell induction for compounds 1, 3, 7 to 10, 12 to 14 (Figs. 6A upper panel, 6C), indicating that they can directly activate the AhR; compound 11 was inactive in both assays. While compounds 15 and 16 induced reporter gene activity to 20 to 30 % of that of TCDD, they stimulated little or no AhR transformation or DNA binding (Fig. 6B,C). In contrast, compounds 2,4 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 the 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 they 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 results 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 current study has only 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 mechanism(s) 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 synergistically enhance the CALUX induction response to a level greater than that maximally induced by TCDD has been previously reported [26,32,33]. While 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 the level of a labile protein that represses AhR functionality and/or 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 a chemical(s) that affects 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 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 chemical(s) in the tire extract responsible for the superinduction response and the importance of this response on 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 represent 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]. While the reasons for these differences in response remain to be determined, they very likely result from species differences in the AhR in both assays (rodent versus guinea pig

versus human) and/or differences in the overall rate of metabolism/degradation between these experimental systems. In fact, although HAHs show similar rank order potency with regards to AhR ligand binding and AhR activation between species, dramatic differences in ligand specificity and potency of nonHAH ligands between species (particularly between 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 to 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 (as compared to 4 h) suggested that this compound was either metabolically stable (leading to increased AhR-dependent gene expression for a longer period of time), metabolized into a more potent AhR agonist and/or it exerted additional effects that enhanced luciferase reporter gene expression. This compound has been previously shown to be a potent and selective antitumor agent and independently identified as an AhR agonist [36]. *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. While 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 since 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 previously that have found many AhR agonists to be significantly more potent in the in vitro, cell-free AhR bioassays as compared to 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 upon a variety of conditions, factors and the 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 that are 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 anti-algal agents and as 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 atmospheric deposition [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, that AhR agonist activity was identified in almost all fractions of the tire solvent extract and 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 provide 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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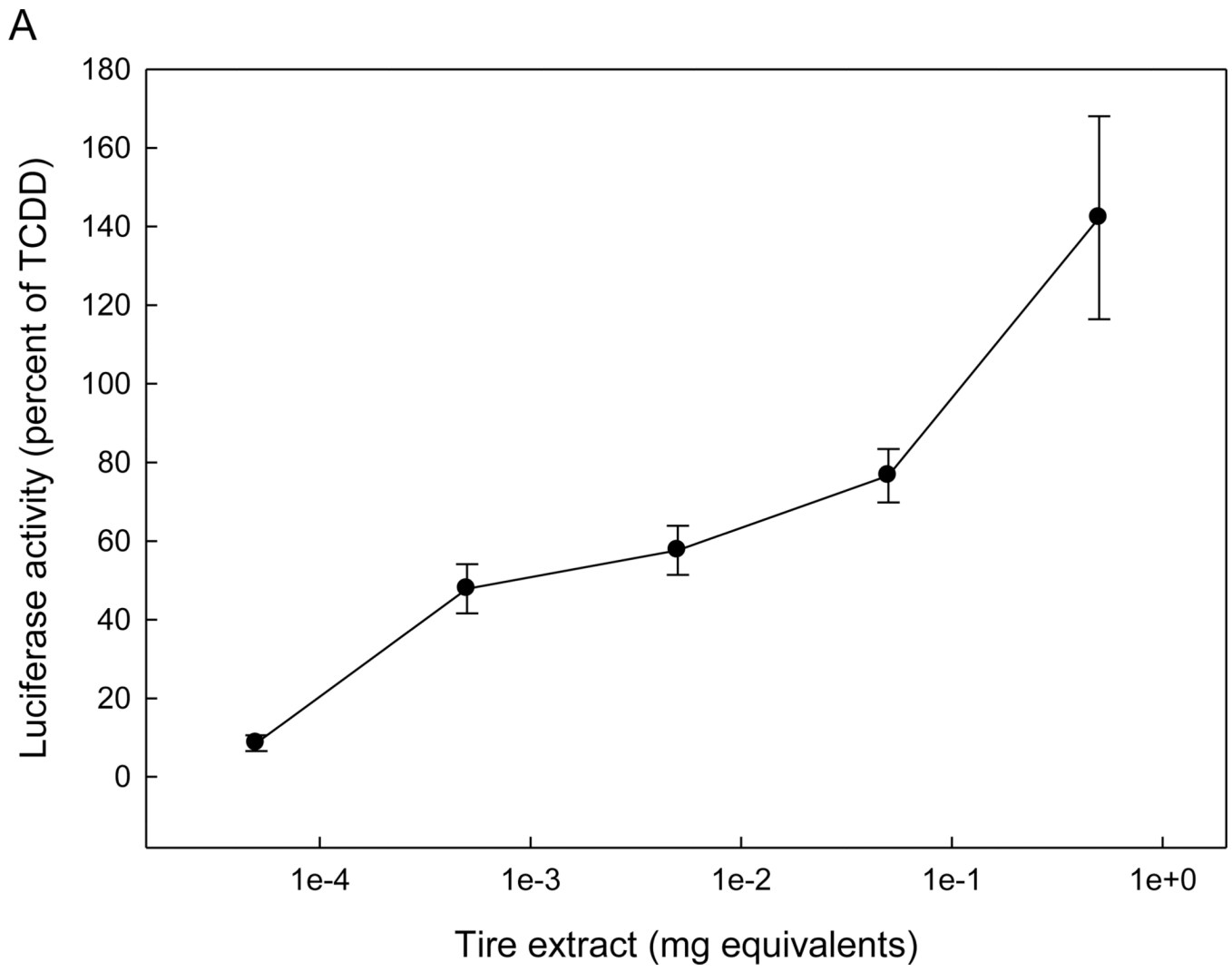
REFERENCES

1. Wik A. Toxic compounds leaching from tire rubber. Bull Environ Contam Toxicol. 2007; 79:114–119. [PubMed: 17476445]
2. Wik A, Dave G. Occurrence and effects of tire wear particles in the environment – A critical review and an initial risk assessment. Environ Pollut. 2009; 157:1–11. [PubMed: 18990476]
3. Evans JJ. Rubber tire leachates in the aquatic environment. Rev Environ Contam Toxicol. 1997; 151:67–115. [PubMed: 9216257]
4. Sheehan PJ, Warmerdam JM, Ogle S, Humphrey DN, Patenaude SM. Evaluating the risk to aquatic ecosystems posed by leachate from tire shred fill in roads using toxicity tests, toxicity identification evaluations, and groundwater modeling. Environ Toxicol Chem. 2006; 25:400–411. [PubMed: 16519300]
5. Day KE, Holtze KE, Metcalfe-Smith JL, Bishop CT, Dutka BJ. Toxicity of leachate from automobile tires to aquatic biota. Chemosphere. 1993; 27:665–675.
6. Stephensen E, Adolfsson-Erici M, Celander M, Hulander M, Parkkonen J, Sturve J, Hasselberg L, Bengtsson M, Forlin L. Biomarker responses and chemical analyses in fish indicate leakage of polycyclic aromatic hydrocarbons and other compounds from car tire rubber. Environ Toxicol Chem. 2003; 22:2926–2931. [PubMed: 14713032]
7. Stephensen E, Adolfsson-Erici M, Hulander M, Parkkonen J, Förlin L. Rubber additives induce oxidative stress in rainbow trout. Aqua Toxicol. 2005; 75:136–143.
8. Mantecca P, Gualtieri M, Andrioletti M, Bacchetta R, Vismara C, Vailati G, Camatini M. Tire debris organic extract affects Xenopus development. Environ Int. 2007; 33:642–648. [PubMed: 17328953]
9. Turner A, Rice L. Toxicity of tire wear particle leachate to the marine macroalga, *Ulva lactuca*. Environ Pollut. 2010; 158:3650–3654. [PubMed: 20828903]

10. Gualtieri M, Mantecca P, Cetta F, Camatini M. Organic compounds in tire particle induce reactive oxygen species and heat-shock proteins in the human alveolar cell line A549. Environ Int. 2008; 34:437–442. [PubMed: 17976723]
11. Zhang QH, Xu Y, Schramm K-W, Jiang GB, Kettrup A. Antiestrogenic and antiprogesteronic activity of tire extracts with yeast-based steroid hormone receptor gene transcription assay. Bull Environ Contam Toxicol. 2002; 69:863–868. [PubMed: 12428164]
12. Misaki K, Suzuki M, Nakamura M, Handa H. Aryl hydrocarbon receptor and estrogen receptor ligand activity of organic extracts from road dust and diesel exhaust particulates. Arch Environ Contam Toxicol. 2008; 55:199–209. [PubMed: 18180859]
13. Safe S. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit Rev Toxicol. 1990; 21:51–88. [PubMed: 2124811]
14. Denison, MS.; Phelen, D.; Elferink, CJ. The Ah receptor signal transduction pathway. In: Denison, MS.; Helferich, WG., editors. Xenobiotics, Receptors and Gene Expression. Philadelphia, PA, USA: Taylor and Francis; 1998. p. 3-33.
15. Denison, MS.; Seidel, SD.; Rogers, WJ.; Ziccardi, M.; Winter, GM.; Heath-Pagliuso, S. Natural and synthetic ligands for the Ah receptor. In: Puga, A.; Wallace, KB., editors. Molecular Biology Approaches to Toxicology. Philadelphia, PA, USA: Taylor & Francis; 1999. p. 393-410.
16. Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS. Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonists. Toxicol Sci. 2000; 55:107–115. [PubMed: 10788565]
17. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol. 2003; 43:309–334. [PubMed: 12540743]
18. Nagy SR, Liu G, Lam K, Denison MS. Identification of novel Ah receptor agonists using a high-throughput green fluorescent protein-based recombinant cell bioassay. Biochemistry. 2002; 41:861–868. [PubMed: 11790108]
19. Knockaert M, Blondel M, Bach S, Leost M, Elbi C, Hager GL, Nagy SR, Han D, Denison M, French M, Ryan XP, Magiatis P, Polychronopoulos P, Greengard P, Skaltsounis L, Meijer L. Independent actions on cyclin-dependent kinases and aryl hydrocarbon receptor mediate the antiproliferative effects of indirubins. Oncogene. 2004; 23:4400–4412. [PubMed: 15077192]
20. Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, Denison MS. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachloridobenzo-*p*-dioxin-like chemicals. Fund Appl Toxicol. 1996; 30:194–203.
21. Han D-H, Nagy SR, Denison MS. Comparison of recombinant cell bioassays for the detection of Ah receptor agonists. Biofactors. 2004; 20:11–22. [PubMed: 15096657]
22. Zhao B, DeGroot D, Hayashi A, He G, Denison MS. CH223191 is a ligand-selective antagonist of the Ah (dioxin) Receptor. Toxicol Sci. 2010; 117:393–403. [PubMed: 20634293]
23. Bohonowych JE, Zhao B, Timme-Laragy A, Jung D, Di Giulio RT, Denison MS. Newspapers and newspaper ink contain agonists for the Ah receptor. Toxicol Sci. 2008; 98:99–110. [PubMed: 17431010]
24. Nagy SR, Sanborn JR, Hammock BD, Denison MS. Development of a green fluorescent protein based cell bioassay for the rapid and inexpensive detection and characterization of AhR agonists. Toxicol Sci. 2002; 65:200–210. [PubMed: 11812924]
25. Denison, MS.; Rogers, JM.; Rushing, SR.; Jones, CL.; Tetangco, SC.; Heath-Pagliuso, S. Analysis of the Ah receptor signal transduction pathway. In: Maines, M.; Costa, LG.; Reed, DJ.; Sassa, S.; Sipes, IG., editors. Current Protocols in Toxicology. New York, NY, USA: John Wiley & Sons; 2002. p. 4.8.1-4.8.45.
26. Baston DS, Denison MS. Considerations for potency equivalent calculations in the Ah receptor-based CALUX bioassay: Normalization of superinduction results for improved sample potency estimation. Talanta. 2011; 83:1415–1421. [PubMed: 21238730]

27. Gillner M, Bergman J, Cambillau C, Alexandersson M, Fernstrom B, Gustafsson J-A. Interactions of indolo[3,2-b]carbazoles and related polycyclic aromatic hydrocarbons with specific binding sites for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rat liver. Mol Pharm. 1993; 44:336–345.
28. Behnisch PA, Hosoe K, Sakai S-I. Brominated dioxin-like compounds: in vitro assessment in comparison to classical dioxin-like compounds and other polyaromatic compounds. Environ Int. 2003; 29:861–877. [PubMed: 12850102]
29. Petko PI, Rowlands JC, Budinsky R, Zhao B, Denison MS, Mekenyan O. Mechanism based common reactivity pattern (COREPA) modeling of AhR binding affinity. SAR QSAR Environ Res. 2010; 21:187–214. [PubMed: 20373220]
30. Noguerol T, Boronat S, Casado M, Raldua D, Barcelo D, Pina B. Evaluating the interactions of vertebrate receptors with persistent pollutants and antifouling pesticides using recombinant yeast assays. Anal Bioanal Chem. 2006; 385:1012–1019. [PubMed: 16705413]
31. Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L. Ligand binding and activation of the Ah receptor. Chemico-Biol Interact. 2002; 141:3–24.
32. Long WP, Pray-Grant M, Tsai JC, Perdew GH. Protein kinase C activity is required for aryl hydrocarbon receptor pathway-mediated signal transduction. Mol Pharm. 1998; 53:691–700.
33. Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B, Denison MS. Activation of the Ah receptor signaling pathway by prostaglandins. J Biochem Mol Toxicol. 2001; 15:187–196. [PubMed: 11673847]
34. Kikuchi H, Kato H, Mizuno M, Hossain A, Ikawa S, Miyazaki J, Watanabe M. Differences in inducibility of CYP1A1-mRNA by benzimidazole compounds between human and mouse cells: evidences of a human-specific signal transduction pathway for CYP1A1 induction. Arch Biochem Biophys. 1996; 334:235–240. [PubMed: 8900397]
35. Zhou J-G, Henry EC, Palermo CM, Dertinger SD, Gasiewicz TA. Species-specific transcriptional activity of synthetic flavonoids in guinea pig and mouse cells as a result of differential activation of the aryl hydrocarbon receptor to interact with dioxin-responsive elements. Mol Pharm. 2003; 63:915–924.
36. Loaiza-Pérez AI, Trapani V, Hose C, Singh SS, Trepel JB, Stevens MF, Bradshaw TD, Sausville EA. Aryl hydrocarbon receptor mediates sensitivity of MCF-7 breast cancer cells to antitumor agent 2-(4-aminophenyl)benzothiazole. Mol Pharm. 2002; 61:13–19.
37. Phelan D, Winter GM, Rogers WJ, Lam JC, Denison MS. Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. Arch Biochem Biophys. 1998; 357:155–163. [PubMed: 9721195]
38. Brownlee BG, Carey JH, Mclnnis GA, Pellizzari IT. Aquatic environmental chemistry of 2-(thiocyanomethylthio)benzothiazole and related benzothiazoles. Environ Toxicol Chem. 1992; 11:1153–1168.
39. Bujdakova H, Kralova K, Sidoova E. Anti- fungal activity of 3-(2-alkylthio-6-benzothiazolylamino-methyl)-2-benzothiazolinethiones in vitro. Pharmazie. 1994; 49:375–376. [PubMed: 8016186]
40. Reemtsma T, Fiehn O, Kalnowski G, Jekel M. Microbial transformations and biological effects of fungicide derived-benzothiazoles determined in industrial wastewater. Environ Sci Technol. 1995; 29:478–485. [PubMed: 22201395]



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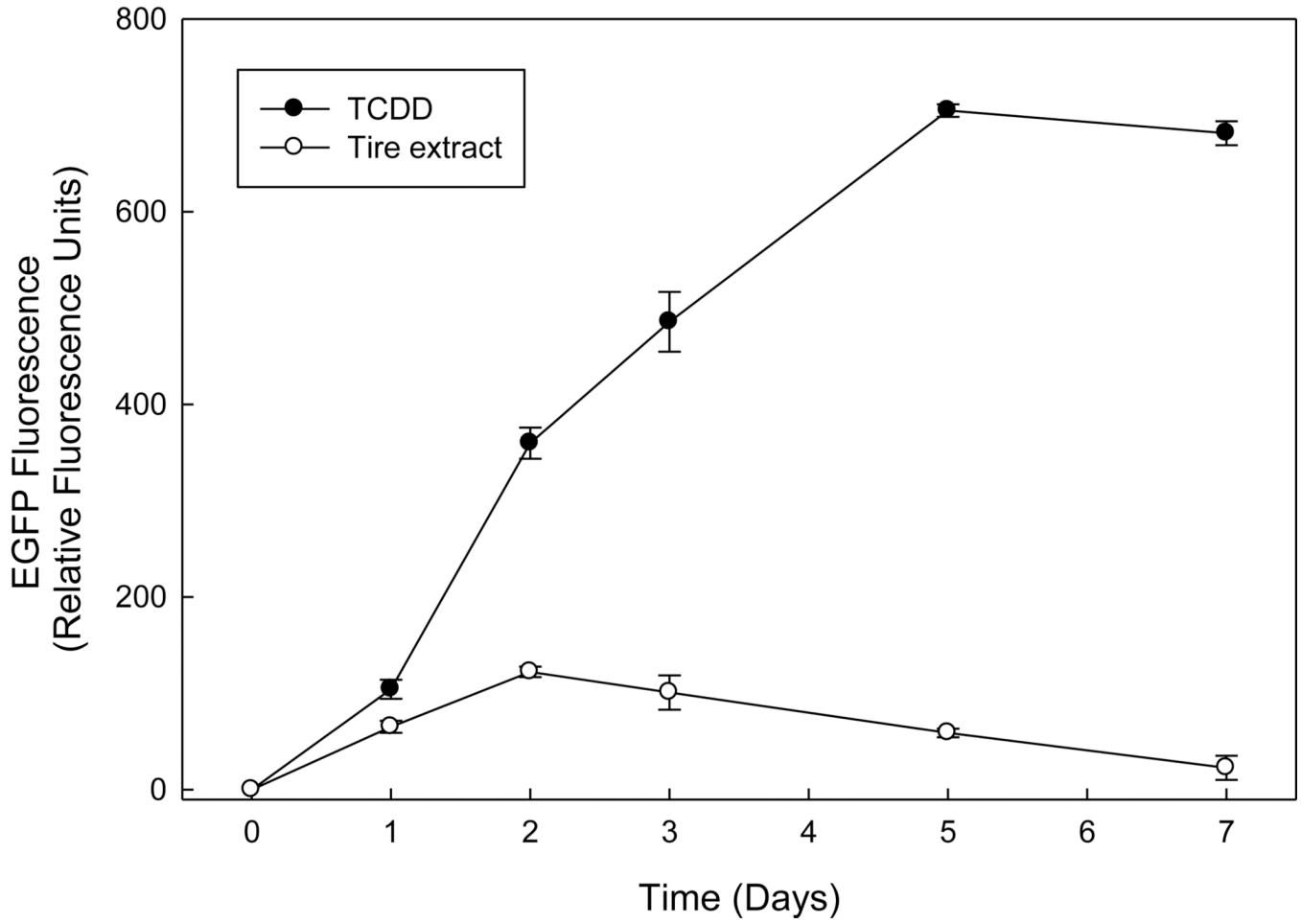
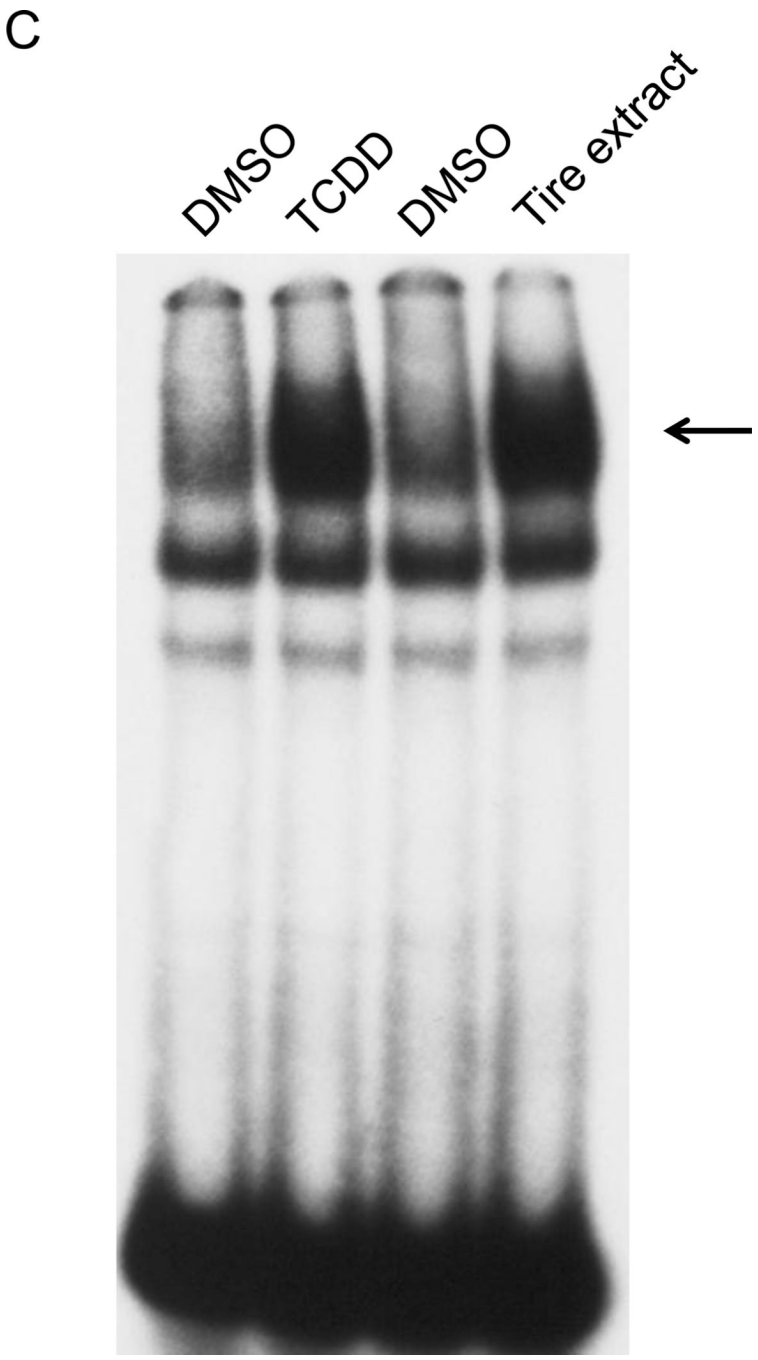




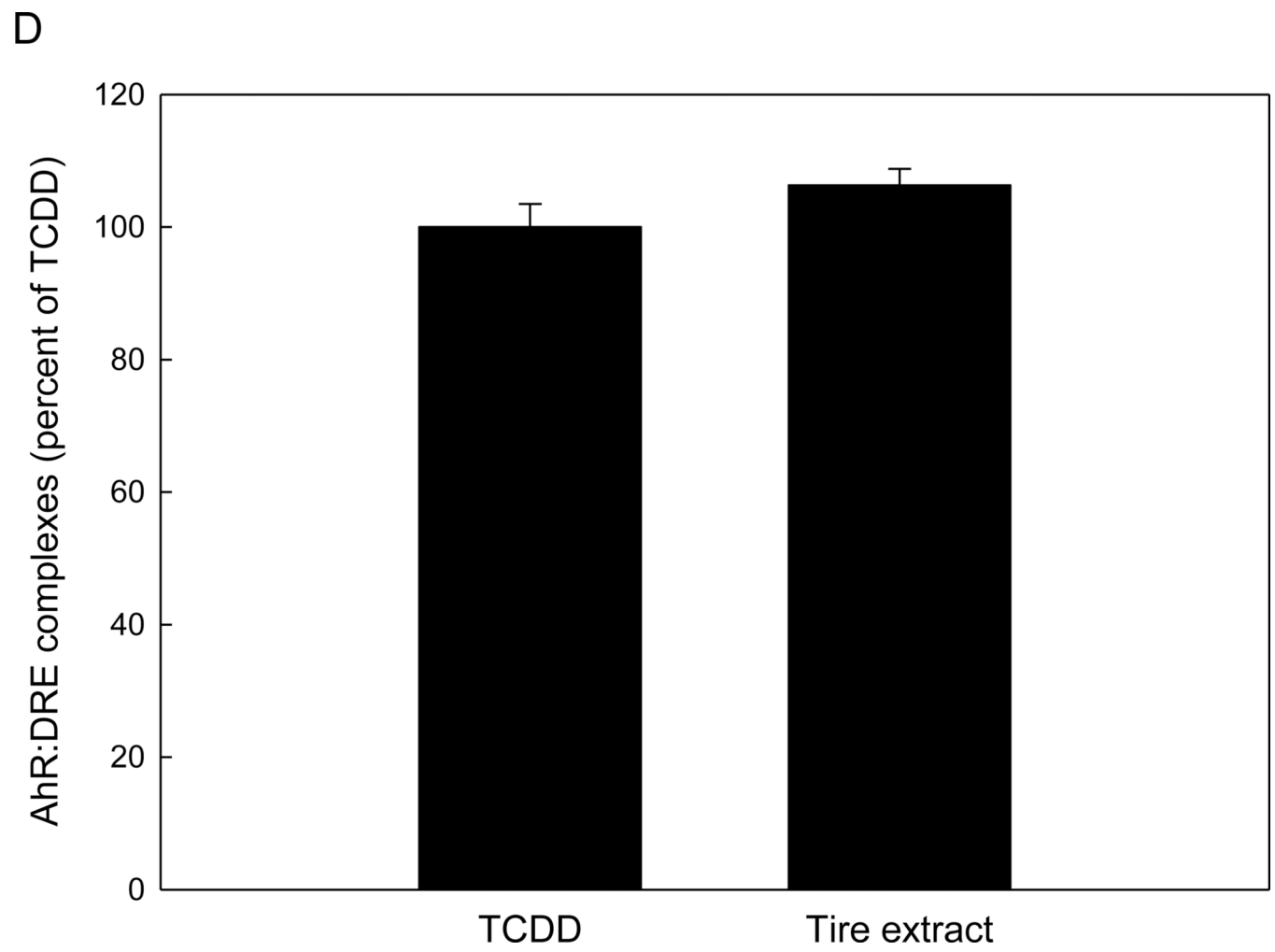
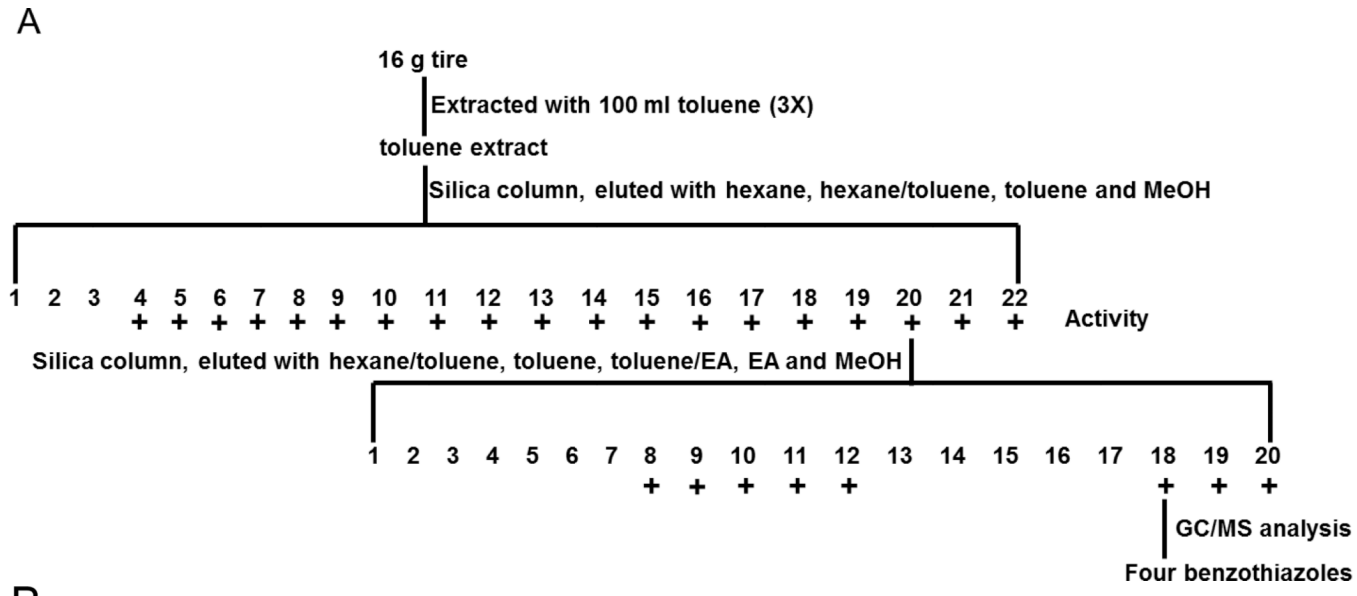
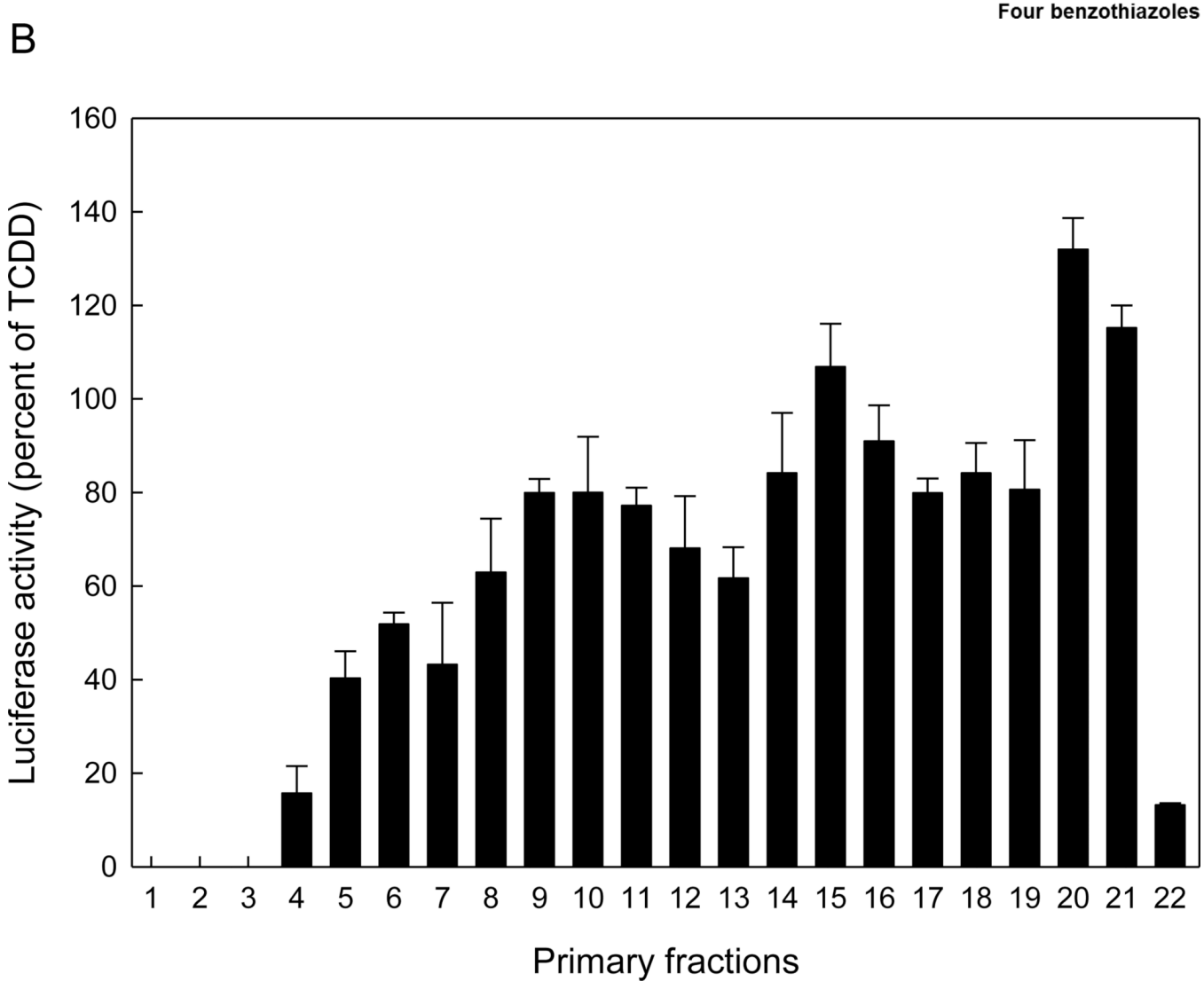


Figure 1.

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 hours of exposure as described in the Materials and Methods *section*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percent 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 *section*. Values represent the mean ± SD of triplicate determinations. (**C** and **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/DNA binding was determined by gel retardation analysis (a representative gel is shown in **C**) and the amount of AhR:DNA complex quantified by PhosphorImager analysis (**D**) as described in the Materials and Methods *section*. Values represent the mean ± SD of at least triplicate determinations and are expressed relative to the amount of complex induced by TCDD.





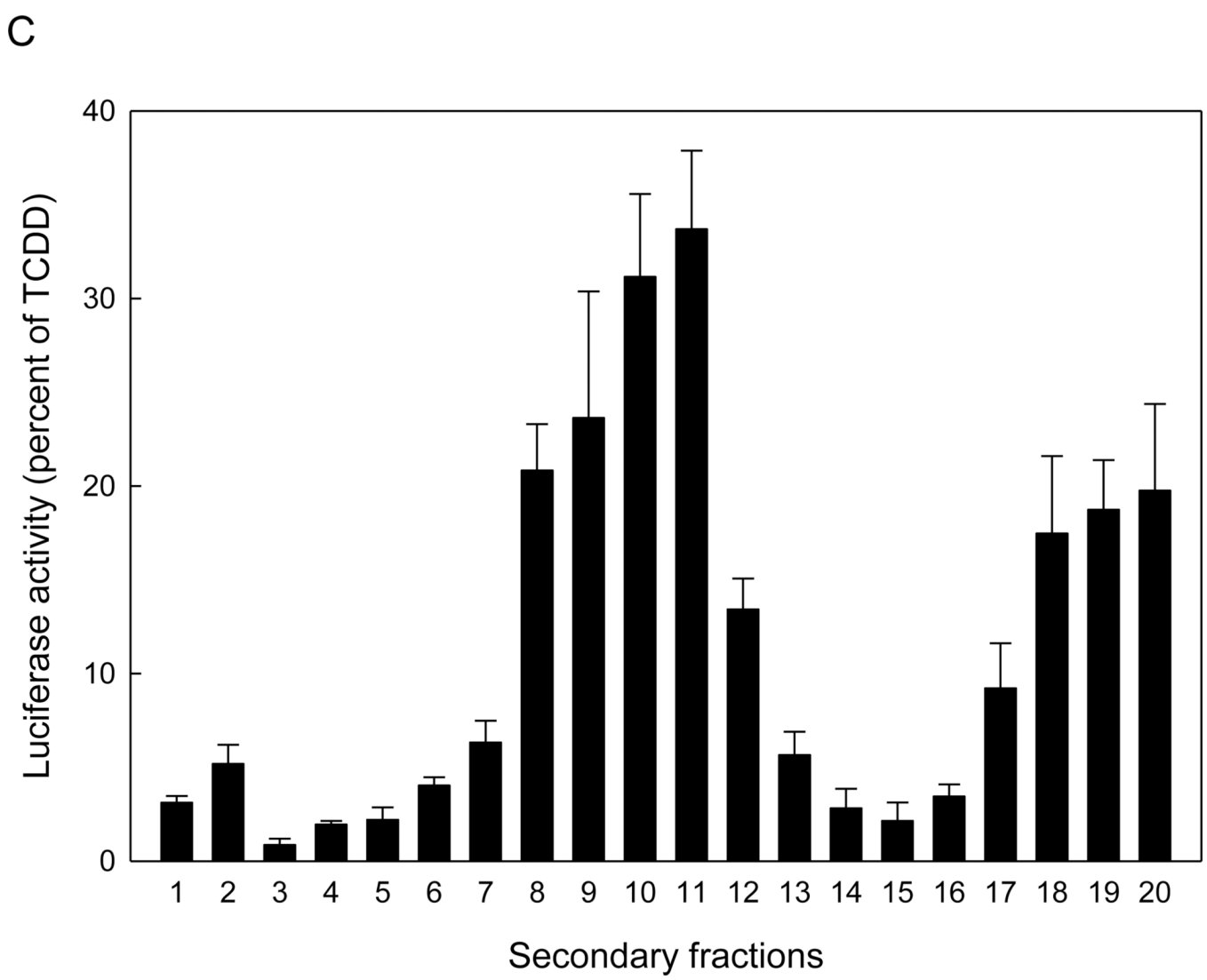


Figure 2.

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 twenty-two 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 twenty secondary fractions. (**B and C**) The AhR agonist activity in each primary fraction was determined using the mouse hepatoma H1L1.1c2 CALUX cell bioassay with (**B**) primary fractions 4 to 22 and (**C**) secondary fractions 8 to 12 and 18 to 20 containing AhR agonist activity.

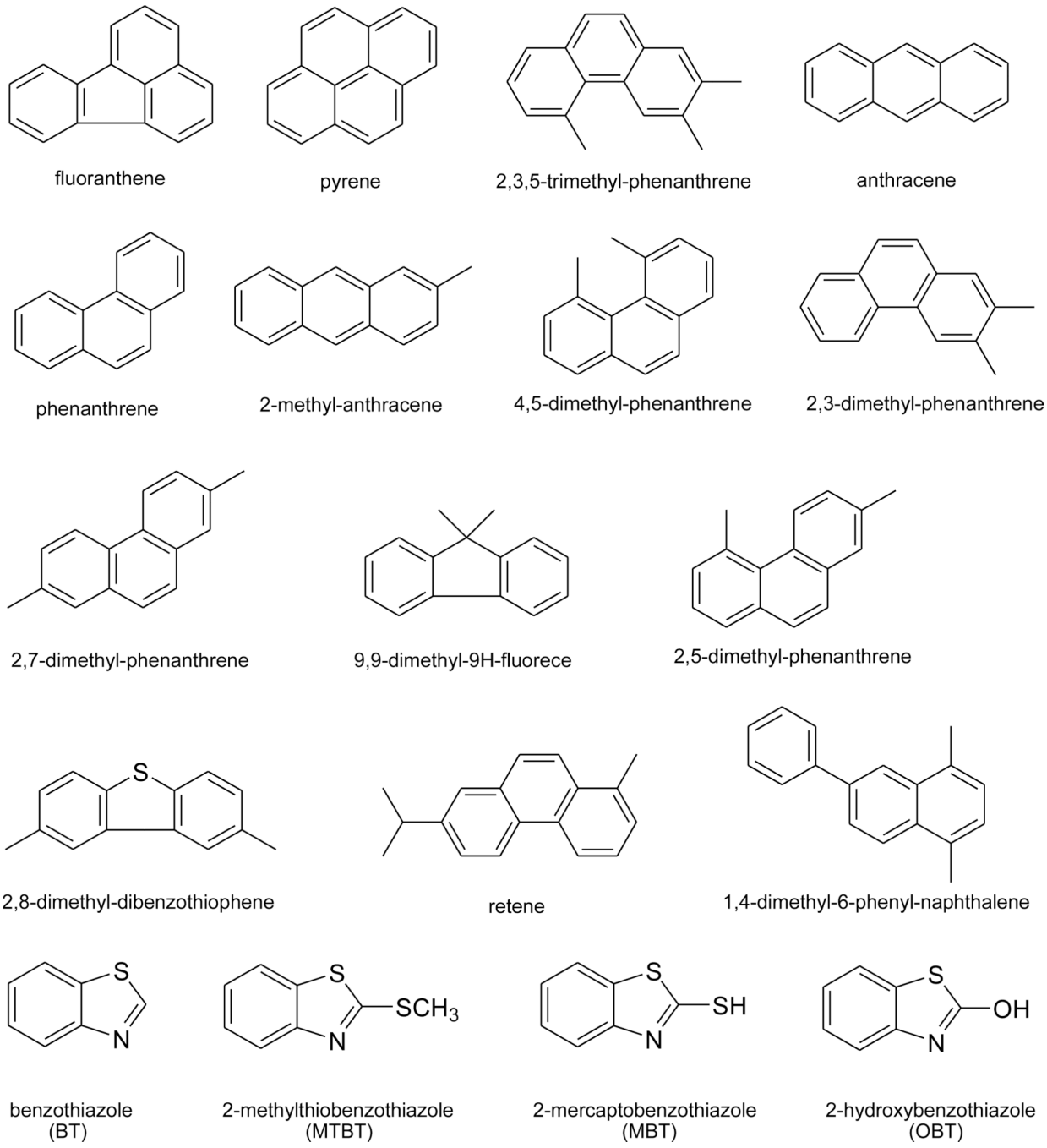
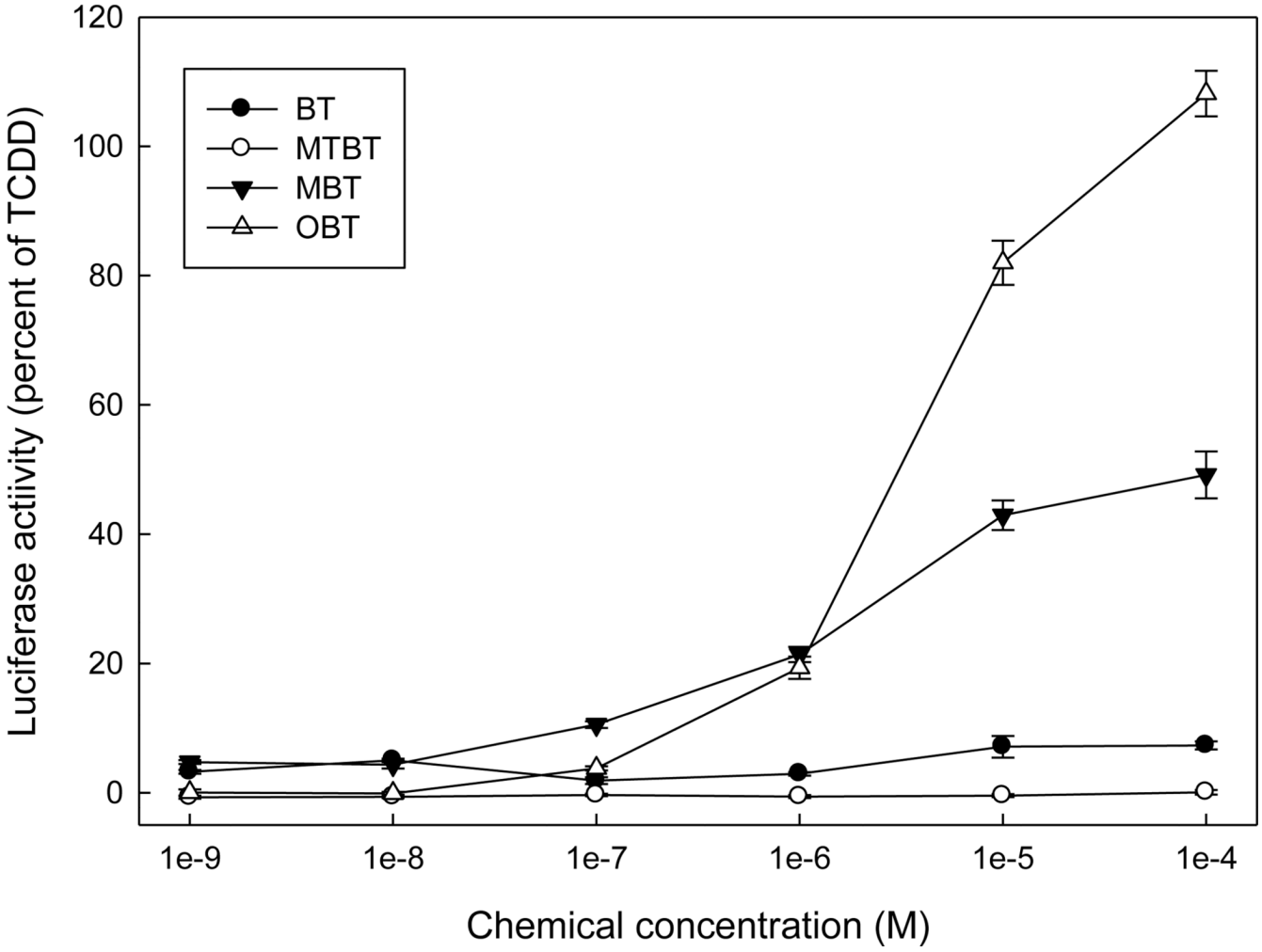


Figure 3.
Chemical structures of the benzothiazoles identified in fraction 18 and the polycyclic aromatic hydrocarbons tentatively identified in secondary fraction 9 of the tire extract.

A



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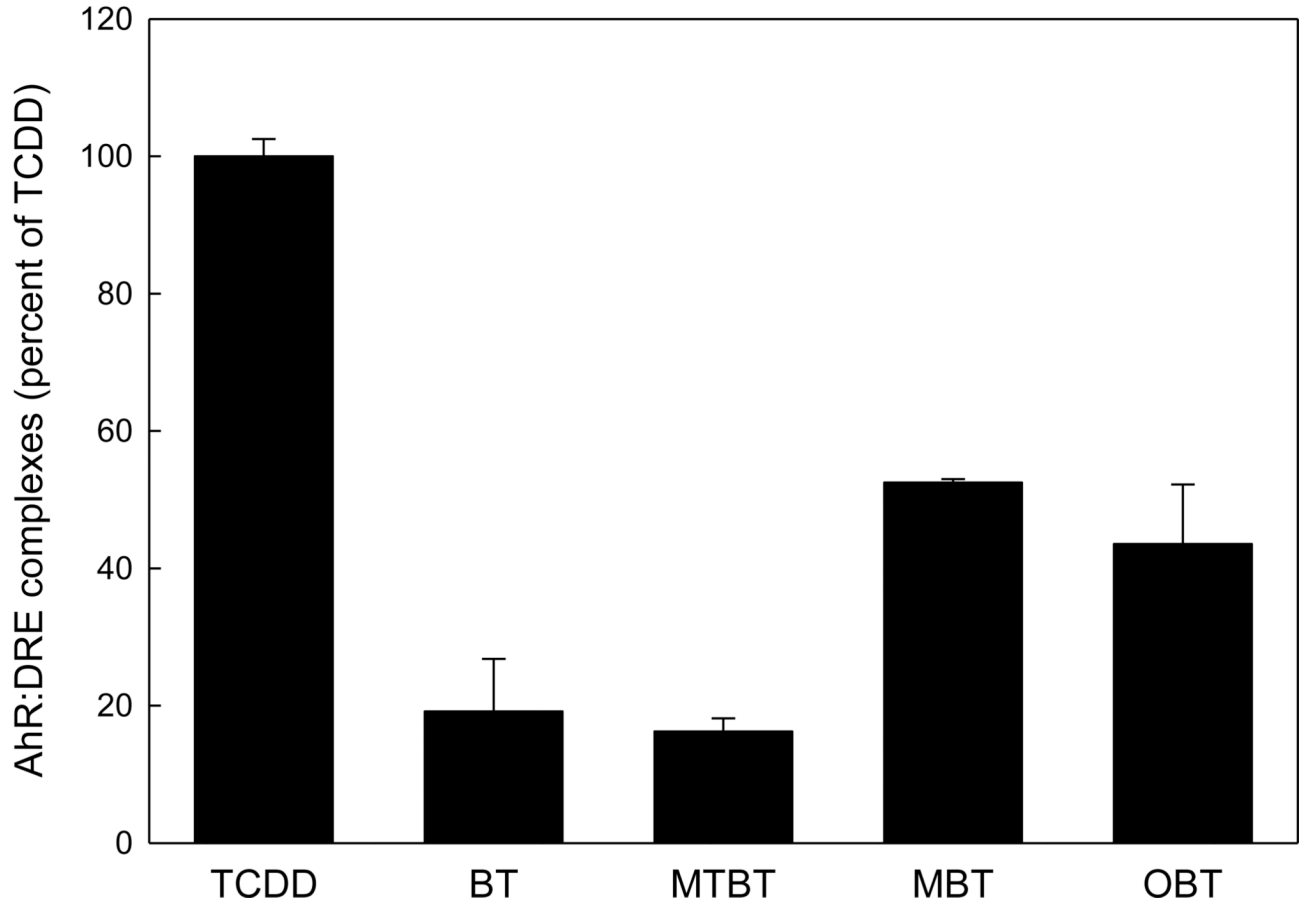


Figure 4.

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 determined as described in the Materials and Methods *section*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percent 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 of 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.

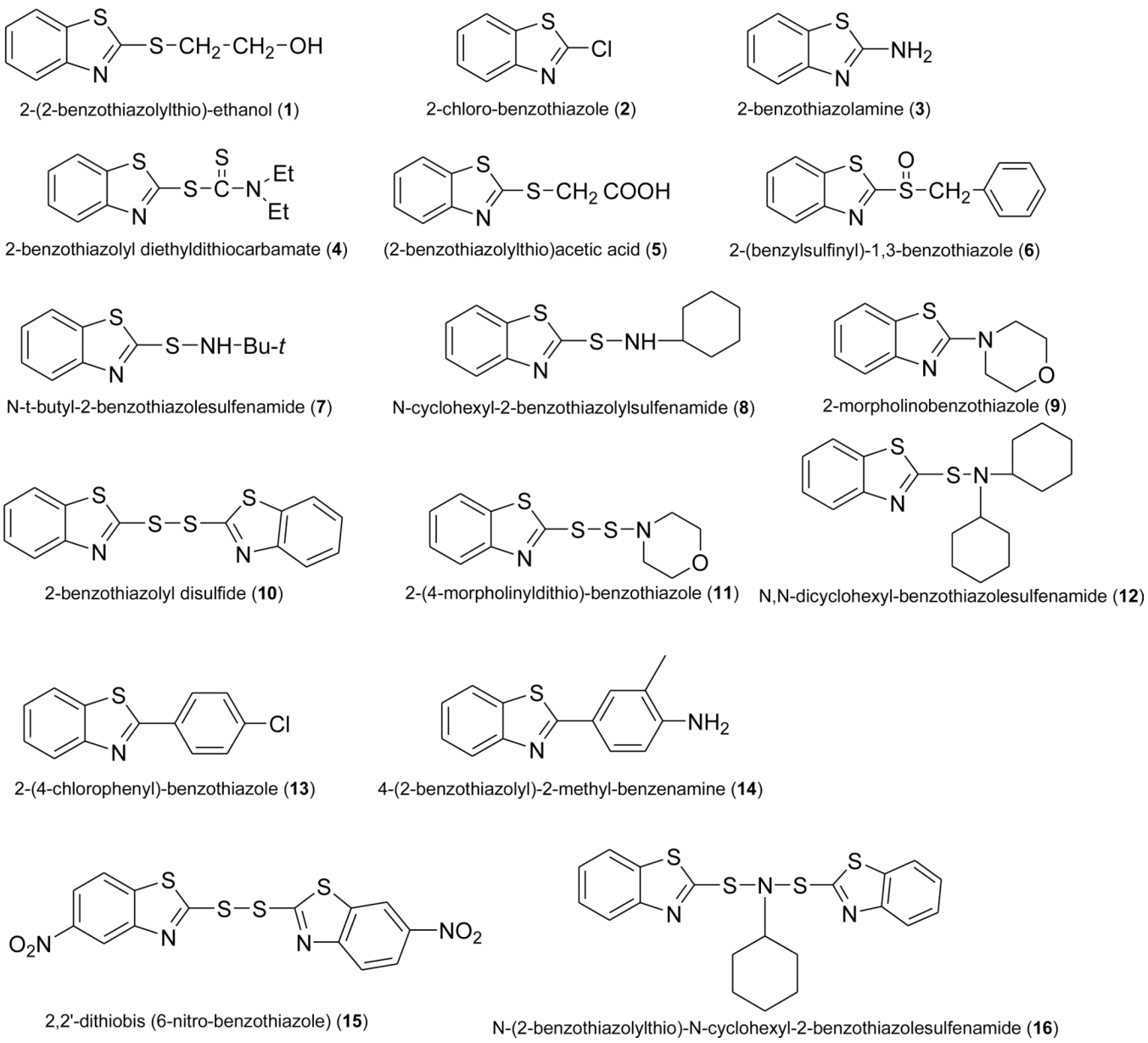
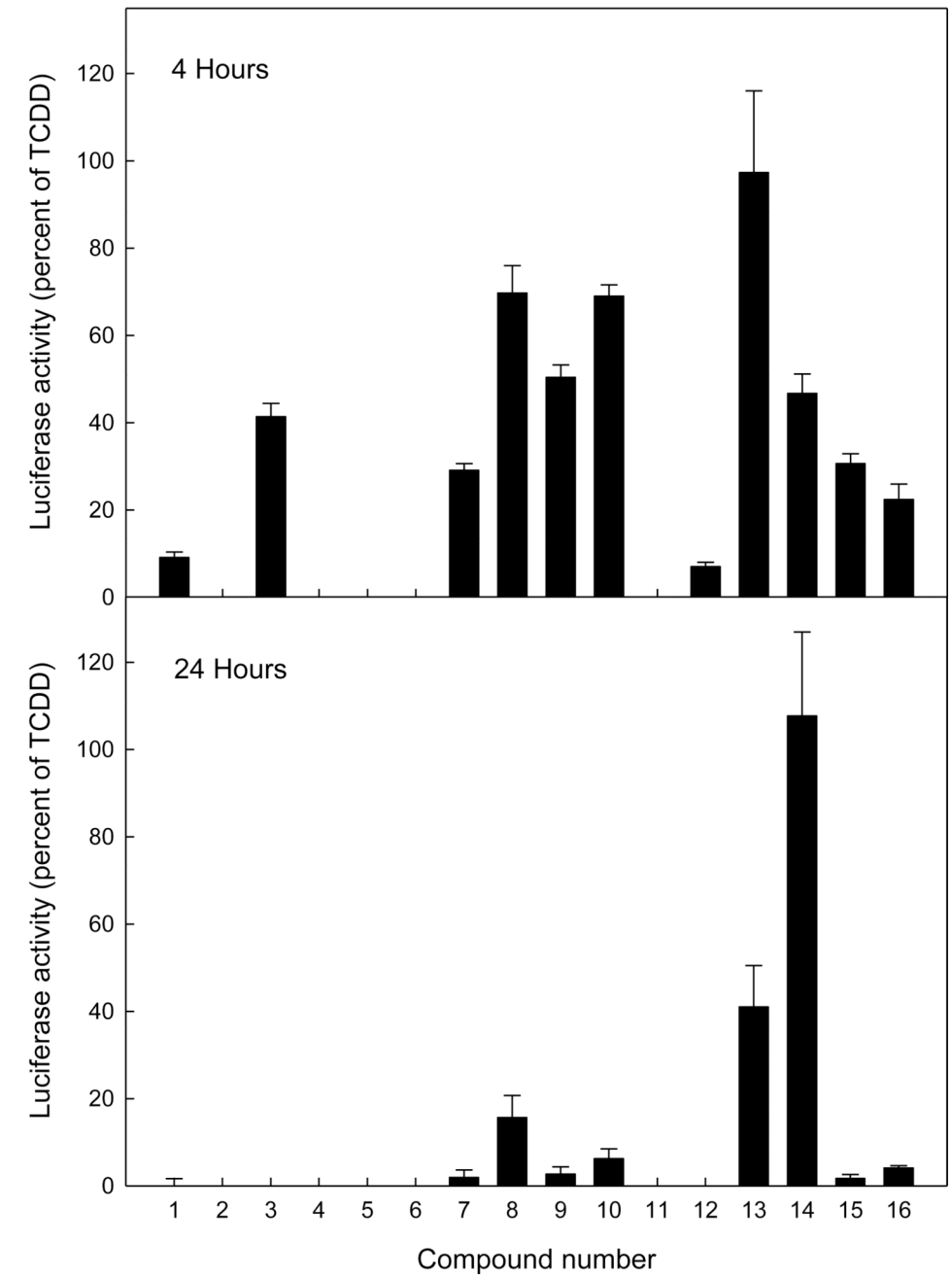


Figure 5.
Chemical structures of commercially available benzothiazole derivatives analyzed for Ah receptor (AhR) agonist activity.

A



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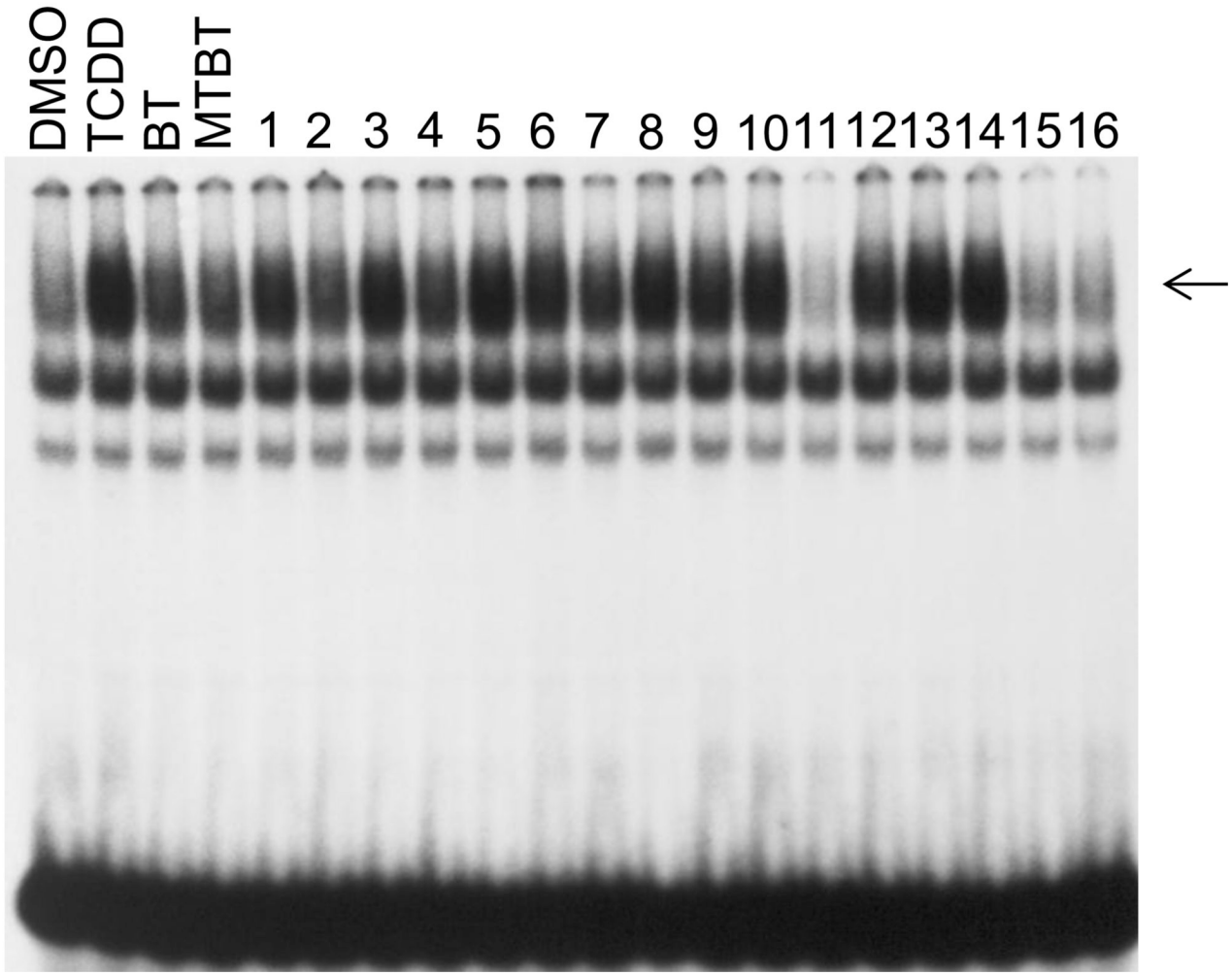

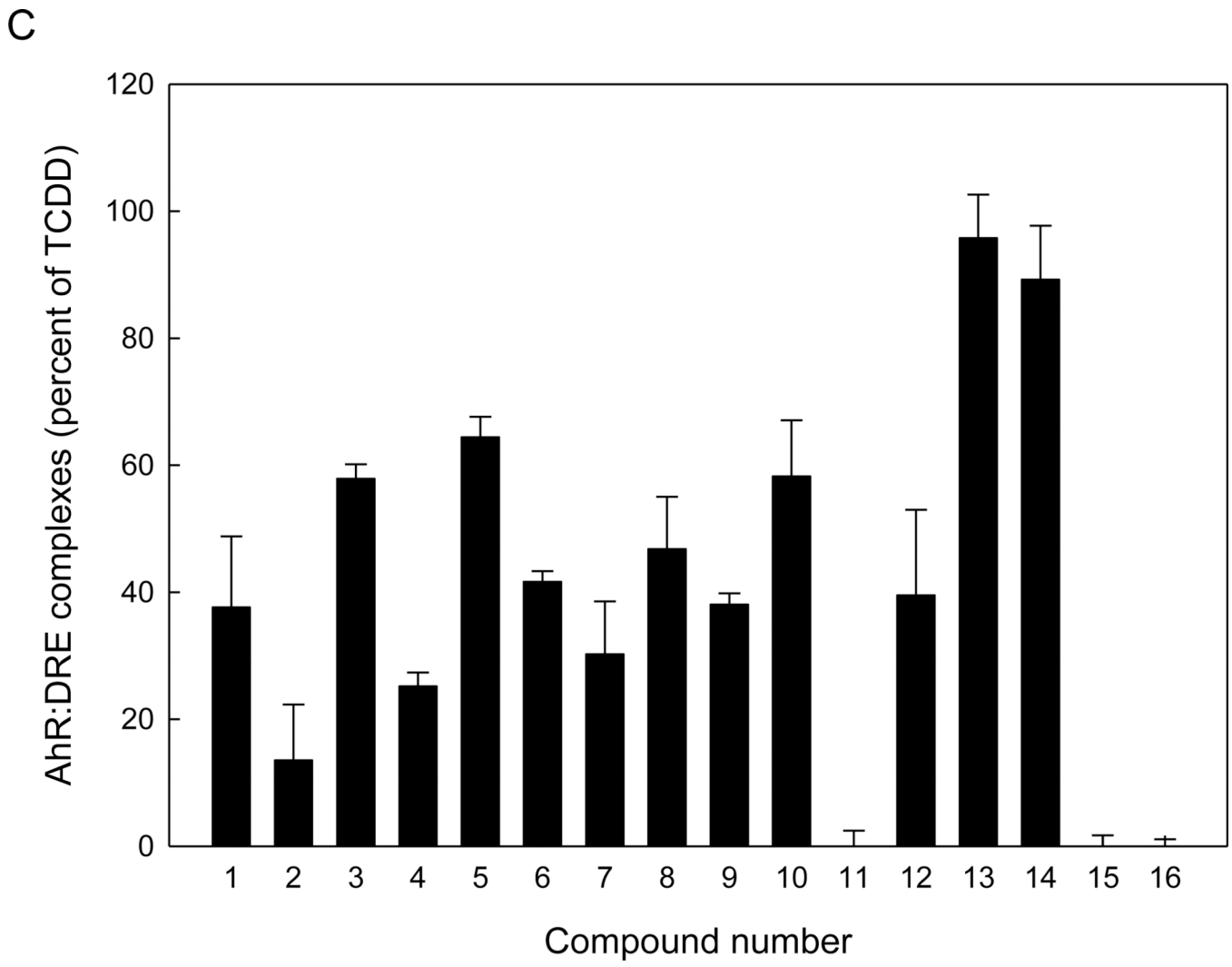

Figure 6.

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 the Materials and Methods *section*. Values represent the mean ± standard deviation (SD) of triplicate determinations and are expressed as a percent of TCDD. (**B** and **C**) Guinea pig hepatic cytosol was incubated with carrier solvent (2.5 μl of DMSO) or 20 nM TCDD (in DMSO), 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. 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.