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Agonistic effects of diverse xenobiotics on the constitutive androstane receptor as detected in a recombinant yeast-cell assay

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ABSTRACT

The constitutive androstane receptor (CAR) is a nuclear receptor and transcription factor regulating proteins involved in xenobiotic metabolism. Agonist activation of the CAR can trigger metabolic activation and toxification as well as detoxification and clearance; accordingly, xenobiotic substances acting as CAR ligands may pose a threat to human and animal health. We used yeast cells transduced with the human CAR and the response pathway to measure the CAR-agonistic activities of 549 synthetic or natural compounds: 216 of the tested compounds exhibited CAR-agonistic effects. Eighty-four percent of CAR-activating compounds were aromatic compounds, and > 65% of these active compounds were aromatic hydrocarbons, bisphenols, monoalkyl phenols, phthalates, styrene dimers, diphenyl ethers, organochlorines, and organophosphates. The ten most potent compounds were 4-*tert*-octylphenol (4tOP; reference substance), 4-nonylphenol, diethylstilbestrol, benzyl *n*-butyl phthalate, 2-(4-hydroxyphenyl)-2,4,4-trimethylchroman, o,p′-DDT, methoxychlor, di-*n*-propyl phthalate, hexestrol, and octachlorostyrene. The activities of these nine non-reference compounds exceeded 10% of the 4tOP activity. Analysis of *para*-monoalkyl phenols suggests that branching of the alkyl group and chlorination at the *ortho* position raises potency. This study provides critical information for identifying the potential of CAR-mediated toxic hazards and for understanding the relevant mechanism.

1. Introduction

The constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR) are ligand-activated transcription factors involved in xenobiotic metabolism. These receptors modulate gene expression for proteins linked to the metabolism and excretion of the xenobiotics that activate them. In the case of an AhR bound by an agonist such as a halogenated polycyclic aromatic hydrocarbon, the receptor forms a heterodimer complex with the AhR nuclear translocator (ARNT), and this complex binds to a DNA sequence termed a xenobiotic responsive element (XRE), thereby stimulating transcription of various genes. In particular, the target genes may be those encoding phase I or phase II xenobiotic-metabolizing enzymes, including the cytochrome P450 (CYP) 1A subfamily (Nebert et al., 2000). The CAR and PXR are both members of the nuclear receptor subfamily. When they are bound by a xenobiotic agonist, the CAR remains as a monomer or forms a heterodimer complex with the retinoid X receptor (RXR), and PXR forms a heterodimer complex with the RXR. The CAR monomer or the CAR-RXR or PXR-RXR heterodimer complex binds to the relevant responsive elements on DNA, thereby stimulating gene transcription. The CAR and PXR are reported to share the following target genes: CYP2B, CYP2C, and CYP3A subfamilies as phase I oxidative enzymes; sulfotransferases, glutathione-S-transferases, and UDP-glucuronosyl-transferases as phase II conjugating enzymes; and multidrug-resistance protein 1, multidrug-resistance-associated protein 2, and organic anion transporter polypeptide 2 as phase III transport uptake and efflux proteins (Kliewer et al., 2002; Wada et al., 2009).

The CAR, PXR, and AhR promote xenobiotic detoxification and clearance; however, they can also trigger metabolic activation and toxification of ligand compounds or other xenobiotics by induction or inhibition of metabolizing enzymes or through target genes being expressed in an aberrant manner. After exposure to xenobiotic-metabolizing enzymes, polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (BaP) may form highly reactive, carcinogenic metabolites capable of attacking DNA. BaP is reportedly associated with the formation of highly reactive BaP-7,8-diol-9,10-epoxides after it is oxidized by the actions of CYP1A1 and CYP1B1 to intermediary BaP-7,8-oxides, followed by metabolization with microsomal epoxide hydrolase to BaP-7,8-diols and further activation by CYPs (Shimada and Fujii-Kuriyama, 2004). The CAR and PXR also induce expression of CYP3A4, one of the

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CYPs that metabolize the mycotoxin aflatoxin B_1 (AFB_1) in the liver. CYP3A4 is reportedly the major contributor to both metabolic activation and detoxification of AFB_1, with the potent carcinogen AFB_1-8,9-epoxide and the metabolite aflatoxin QI (AFQ_1) as the respective products (Kamdem et al., 2006). Phenobarbital is a CAR activator and CYP2B inducer, and a known tumor promotor. In rodent studies, non-genotoxic and non-cytotoxic phenobarbital induced hepatic CYP2B and triggered the following key hepatic tumor-promoting events: increased cell proliferation, inhibition of apoptosis, hypertrophy, and formation of altered hepatic foci (International Agency for Research on Cancer (IARC), 2001; Whysner et al., 1996). Such tumor-promoting events are considered to be CAR dependent because phenobarbital-related hepatic tumors were induced in normal mice given the carcinogenic initiator diethylnitrosamine, but not in CAR knockout mice (Yamamoto et al., 2004).

AhR-interacting compounds represent a major concern for human and environmental health, in part because typical ligands for this receptor include chlorinated dibenzodioxins (dioxins) and polychlorinated biphenyls (PCBs), which are well-known pollutants; however, aside from some endogenous substances and medical products, CAR and PXR ligands and inhibitors have received less attention. Steroidal compounds include known ligands for CAR and PXR: for example, 5β-pregnane-3,20-dione and 17β-estradiol are agonists for both PXR and CAR while androstanol and progesterone are PXR agonists but CAR antagonists (Forman et al., 1998; Jones et al., 2000; Kawamoto et al., 2000; Moore et al., 2000). Among pharmaceutical compounds, the synthetic glucocorticoid dexamethasone, the glucocorticoid receptor antagonist mifepristone (RU486), and the mineralocorticoid receptor antagonist spironolactone all have agonistic effects on PXR (Jones et al., 2000; Moore et al., 2000). Reports on environmental chemicals in this category are rare, although researchers have investigated signal transductions initiated by CAR or PXR activation and the resultant responses. A small number of non-coplanar PCBs (rather than the coplanar PCBs, which are known to be highly toxic) are reported to directly activate CAR and PXR and increase transcription of genes for CYP2B and CYP3A (Al-Salman and Plant, 2012; Gährs et al., 2013). We have also demonstrated the agonistic effects of 21 of 34 PCBs and 72 of 91 mono-hydroxylated PCBs (OH-PCBs) on the CAR in a reporter gene assay (Kamata et al., 2015).

Accordingly, further research is needed to identify chemical substances that activate or inactivate receptors involved in xenobiotic metabolism: such research is very important for understanding potential metabolic aberrations and predicting possible health risks for humans and other animals. We previously screened potentially environmentally ubiquitous chemicals and elucidated a large number of chemical-cellular receptor interactions (Arulmozhiraja et al., 2005; Kamata et al., 2008, 2009; Morohoshi et al., 2005). In this study, we focused on CAR as a receptor involved in xenobiotic metabolism, and screened a wide range of xenobiotics and other compounds (total of 549 compounds) for CAR agonistic effects with a yeast-cell reporter gene assay. The scanned compounds fell into the following general categories: industrial chemicals, agrochemicals, natural compounds and related chemicals, and medicines and cosmetic chemicals. The yeast cells possessed a CAR signaling pathway transfected *via* a two-hybrid system with the human genes for CAR (NR1I3) and the nuclear receptor coactivator 2 (NCOA2). The assay employed chemiluminescence reagents for sensitive detection of a CAR response in high-throughput screening. As CAR agonistic effects were detected in various categories of chemicals, we provide the measured activities for the positively reacting compounds grouped according to chemical structure.

2. Materials and methods

2.1. Compounds

The 549 compounds investigated in this study are listed in Table 1, grouped according to their intended use and chemical structure. The compounds were purchased from Accu Standard, Inc. (New Haven, CT, USA), Acros Organics N.V. (Geel, Belgium), Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany), Cosmo Bio Co., Ltd. (Tokyo, Japan), Dr. Ehrenstorfer GmbH (Augsburg, Germany), GL Science, Inc. (Tokyo, Japan), Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Katayama Chemical Industries Co., Ltd. (Osaka Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Maruzen Petrochemical Co., Ltd. (Tokyo, Japan), MP Biochemicals (Solon, OH, USA), PerkinElmer, Inc. (Wellesley, MA, USA), Scientific Polymer Products, Inc. (Ontario, NY, USA), Sigma–Aldrich Corp. (St. Louis, MO, USA), Steraloids, Inc. (Newport, RI, USA), Tocris Bioscience (Ellisville, MO, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Toronto Research Chemicals Inc. (North York, ON, Canada), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or were donated by researchers who had synthesized them for other purposes. The purity of each compound was certified by the manufacturer or verified by the donor to be at least 90%, with the exception of anthrarobin (85% purity), 4-(2,4-dinitroanilino)phenol (70%), sodium omadine (40%), hexahydro-1,3,5-tris(hydroxyethyl)-*s*-triazine (80%), zineb (82.1%), hesperidin (80%), benzoyl peroxide (75%), and galaxolide (85%).

2.2. Yeast-cell assay

The agonistic effect of each compound on the CAR was evaluated with a reporter gene assay using yeast cells (*Saccharomyces cervisiae* Y190) carrying the β-galactosidase reporter gene. The cells were transfected by a two-hybrid system (Nishikawa et al., 1999) with the following two expression plasmids: pGBT9-CARLBD carrying the ligand-binding domain of the human CAR and the DNA-binding domain of the transcriptional activator GAL4, and pGAAD424-TIF-2 carrying the coactivator NCOA2 (otherwise known as TIF2) and the transcriptional activation domain of GAL4. The assay was performed using a chemiluminescent detection method (for β-galactosidase) employing a 96-well culture plate (Arulmozhiraja et al., 2005; Shiraishi et al., 2003).

In brief, yeast cells were pre-incubated for 24 h at 30 °C with shaking in modified SD medium lacking tryptophan and leucine (Kamata et al., 2015), and the cell density was adjusted to an absorbance of 1.65 to 1.80 at 595 nm. A dimethylsulfoxide (DMSO) solution of each test compound was stored at − 80 °C until immediately before the assay. The test solution diluted 25-fold with the medium was poured into two wells of a black 96-well culture plate, and a series of twofold dilutions was prepared with the medium, and the volume per well was adjusted to 60 μL. An aliquot of the yeast cell suspension (60 μL) was then added to the diluted solution on the plate, to prepare a solution containing 1% DMSO in each well. Each compound was tested at a minimum of seven concentrations (prepared by serial twofold dilutions) between 10 μM and 156 nM; for chemicals showing a strong CAR agonistic effect, additional lower concentrations were tested. The dissolved state of each test compound was visually confirmed; no insoluble material except yeast cells was visible in any final mixture up to at a test compound concentration of 10 μM. The plate was incubated at 30 °C under conditions of high humidity for 4 h. Reaction buffer was added to each well (80 μL). This mixture contained GalactLux substrate (AURORA GAL-XE, MP Biochemicals, Solon, OH, USA) for detection of chemiluminescent activity of released β-galactosidase, and zymolyase for enzymatic digestion (Kirin Brewery Co, Ltd., Tokyo, Japan). The plate was incubated at 37 °C for 1 h and then placed in a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan), and a light-emission-accelerator solution (AURORA GAL-XE, 50 μL) was added to each well. The chemiluminescence produced by released β-galactosidase was measured for each well. All test compounds were evaluated in duplicate. Test compounds showing agonistic activity in the initial duplicate assay were subjected to a minimum of two further separate assays (total of three experiments with independent biological replicates). A blank control containing all the components of the test