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Archives of Biochemistry and Biophysics 470 (2008) 187–199
ABB
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# Antagonistic and agonistic effects of indigoids on the transformation of an aryl hydrocarbon receptor

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Received 12 September 2007, and in revised form 6 November 2007
Available online 18 December 2007

**Abstract**

Halogenated and polycyclic aromatic hydrocarbons, exogenous ligands of the aryl hydrocarbon receptor (AhR), cause various toxicological effects through the transformation of the AhR. In this study, we investigated the antagonistic effects of indigoids on the transformation in addition to their agonistic ones. In a cell-free system, indigoids induced the transformation dose-dependently, but suppressed the transformation induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the binding of 3-methylcholanthrene to the AhR. In mouse hepatoma Hepa-1c1c7 cells, indigoids, especially indirubin, suppressed the transformation and expression of CYP1A1 by inhibiting the translocation of AhR into the nucleus. When orally administered to mice at 10 mg/kg BW/day for three successive days, indigoids did not induce AhR transformation and expression of the CYP1A subfamily in the liver, while indirubin and indigo upregulated quinone reductase activity. These results indicate that indigoids are able to bind to the AhR as ligands and exhibit antagonistic effects at lower concentrations in mammalian cells.


*Keywords:* Aryl hydrocarbon receptor; Indigoid; Indirubin; Indigo; TCDD; Transformation; CYP1A1; Quinone reductase

The aryl hydrocarbon receptor (AhR),[1] a basic helix–loop–helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family, is a ligand-activated transcription factor that is present in most cell and tissue types. A previous study using AhR-deficient mice suggested to be involved in the development of the liver and immune systems [1]. The AhR is an orphan receptor whose endogenous ligands and biochemical functions have not been fully elucidated at the moment, although it is known to be stable in the cytosol where it forms a complex with a dimer of the 90-kDa heat shock protein (hsp90) [2], an AhR-associated protein (ARA9, also termed XAP2 or AIP) [3] and p23 [4]. It has been found that halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs) including dioxins

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[1] *Abbreviations used:* AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; ARE, antioxidant response element; bHLH, basic helix–loop–helix; CYP, cytochrome P450; DMSO, dimethylsulfoxide; DRE, dioxin response element; $ED_{50}$, 50% effective dose; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; EROD, ethoxyresorufin *O*-deethylase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferease; HAH, halogenated aromatic hydrocarbon; HAP, hydroxyapatite; hsp90, 90-kDa heat shock protein; $IC_{50}$, 50% inhibitory concentration; MC, 3-methylcholanthrene; NF-E2, nuclear factor-erythroid 2p45; Nrf2, NF-E2-related factor; PAH, polycyclic aromatic hydrocarbon; PAS, Per-Arnt-Sim; QR, quinone reductase; SW-ELISA, southwestern ELISA; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UGT, UDP-glucuronosyltransferase.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.11.021

bind to the AhR as exogenous ligands [5]. Among these exogenous ligands, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and causes various toxicological effects through an AhR-dependent pathway. The binding of a ligand to the AhR results in its transformation to a DNA-binding form via the following process: A conformational change in the receptor protein, its translocation into the nucleus, the dissociation of hsp90, and heterodimerization with another bHLH/PAS family protein, an AhR nuclear translocator (Arnt) [6]. This heterodimer binds to a dioxin response element (DRE), a *cis*-acting element found in the 5′-regulatory regions of dioxin-responsive genes, and regulates the expression of a battery of genes, particularly genes encoding drug-metabolizing enzymes, such as cytochrome P450 (CYP) 1A subfamily [7], quinone reductase (QR) [8], glutathione *S*-transferase (GST), [9] and UDP-glucuronosyltransferase (UGT) [10].

Regarding the ligands of the AhR, indole derivatives [11], bilirubin [12], 7-ketocholesterol [13], flavonoids [14] and resveratrol [15] have been reported to interact with the AhR and induce or inhibit the transformation. Certain natural compounds are considered candidates for endogenous ligands of the AhR. Indirubin and indigo were detected in human urine and/or fetal bovine serum (FBS), and had AhR ligand activity in a yeast-based AhR reporter system [16] and caused AhR transformation in mammalian cells [17,18]. Indigo is produced by the fermentation of plant materials from *Polygonum tinctorium*, *Isatis tinctoria* and *Indigofera tinctoria*, and has been used as a dye for cloth of jeans and other fabrics. Indirubin, a pink pigment, is a by-product of indigo synthesis. These compounds are ingredients of the traditional Chinese medicine 'Dang gui Long hui wang' used against chronic myelogenous leukemia [19]. It has been also reported that they are formed from indole through oxidation by human CYPs and/or dimerization in the human body [20]. Therefore, we assumed that indigoids do not have the toxicity of HAHs and PAHs, although they were better inducers of AhR transformation than TCDD in a yeast-based AhR reporter system [16].

In this study, we evaluated the antagonistic effects of indigoids on the transformation of the AhR and its downstream events, in addition to confirming their agonistic effects. In a cell-free system using rat liver cytosol, 1 nM indirubin and indigo significantly suppressed the binding of 0.25 nM [$^3$H] 3-methylcholanthrene (MC) to the AhR and the 1 nM TCDD-induced transformation, nevertheless indirubin and indigo induced the receptor's transformation dose-dependently consistent with previous reports [17,18]. In mouse hepatoma Hepa-1c1c7 cells, 100 nM indirubin and indigo suppressed the transformation and the expression of the CYP1A subfamily caused by 0.5 nM TCDD, although indigoids themselves prompted these events at a higher concentration range. Moreover, the oral administration of indigoids did not cause transformation of the AhR or expression of the CYP1A subfamily, but indirubin and indigo significantly upregulated QR activity in the liver. These results suggest that indigoids do not show HAH and PAH-like actions, but rather suppress the AhR-dependent toxicological actions of these environmental contaminants.

## Materials and methods

### *Materials*

Indirubin, indigo and isoindigo were synthesized according to a previous report [21] and TCDD was obtained from AccuStandard (New Haven, CT) and dissolved in dimethylsulfoxide (DMSO). Their structures are shown in Fig. 1. Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO). Corn oil and MC were obtained from Nacalai Tesque Inc. (Kyoto, Japan). For the electrophoretic mobility shift assay (EMSA), an oligonucleotide DRE probe containing an AhR-binding site, 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′ were synthesized at Hokkaido System Science (Sapporo, Japan). For the AhR ligand-binding assay, [$^3$H]MC (1.9 Ci/mmol) and hydroxyapatite (HAP) were obtained from Moravek Biochemicals Inc. (Brea, CA) and Bio-Rad Laboratories Inc. (Hercules, CA), respectively. For the Western blot analysis, anti-CYP1A1 goat IgG and anti-AhR mouse IgG antibodies were purchased from Daiichi Pure Chemicals (Tokyo, Japan) and Affinity BioReagents (Golden, CO), respectively, and anti-goat IgG and anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

### *A cell-free incubation system*

All animal treatments in this study were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. Male Sprague–Dawley rats (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Rat hepatic cytosol was prepared according to our previous report [14]. The cytosol (4 mg protein/mL) was incubated with various concentrations of indirubin, indigo and isoindigo with or without TCDD in HEDG buffer consisting of 25 mM Hepes, pH 7.4, 1.5 mM EDTA, 1.0 mM dithiothreitol (DTT) and 10% (v/v) glycerol at 20 °C for 2 h in the dark. The control treatment was carried out with the same volume of DMSO (10 μL/mL) alone as vehicle. After the incubation, the reaction mixture was subjected to EMSA or southwestern enzyme-linked immunosorbent assay (SW-ELISA) for determination of AhR transformation.

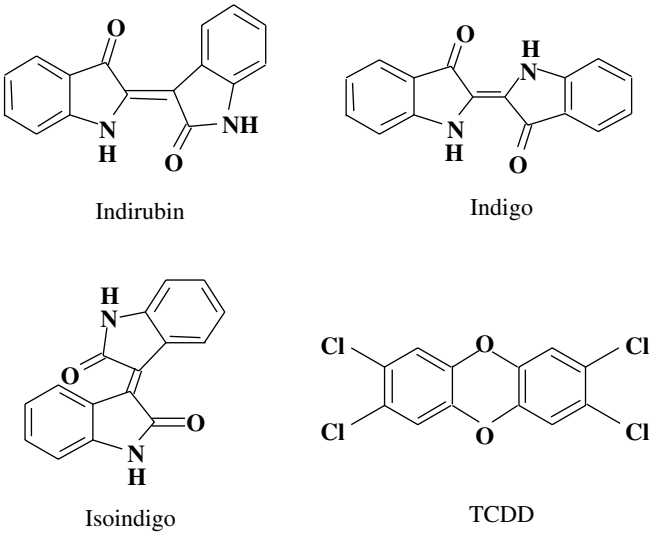


Fig. 1. Structure of indigoids and TCDD.