

## Aryl hydrocarbon receptor response to indigoids in vitro and in vivo

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### Abstract

Indigo and indirubin have been reported to be present at low levels in human urine. The possibility that indigoids are physiological ligands of the aryl hydrocarbon receptor (AhR) has been suggested by initial studies in yeast, where indirubin was found to be 50 times more potent than 2,3,7,8-tetrachlorodibenzo[*p*]dioxin (TCDD), and indigo was found to be equipotent. To demonstrate that these indigoids are bona fide agonists in mammalian systems, we employed a number of in vitro and in vivo measures of AhR agonist potency. In a hepatoma cell reporter system, indigo yielded an EC<sub>50</sub> of  $\sim 5 \times 10^{-6}$  M (indirubin 3'-oxime EC<sub>50</sub>  $\sim 5 \times 10^{-7}$  M, indirubin EC<sub>50</sub>  $\sim 1 \times 10^{-7}$  M). A comparison of these EC<sub>50</sub> values with that of 2,3,7,8-tetrachlorodibenzofuran (TCDBF) ( $\sim 3 \times 10^{-9}$  M) indicated that these compounds are less potent than classic halogenated-dibenzofurans or -dibenzo-*p*-dioxins. Competitive binding assays for AhR occupancy showed similar IC<sub>50</sub> values for indirubin and TCDBF ( $\sim 2 \times 10^{-9}$  and  $5 \times 10^{-9}$  M), with the IC<sub>50</sub> values of indigo and indirubin 3'-oxime being  $\sim 10$ -fold higher. When rats were treated with these indigoids in the range of 1.5–50 mg/kg, induction of hepatic cytochrome P450 1A1 was detected. Differences in the rank-order of potency observed in vivo and in vitro could, in part, be explained by metabolism. Although their biological potencies are not as high as has been previously suggested, collectively the results show that these indole-derived pigments are agonists of AhR in vivo. The in vivo results suggest that solubility, distribution, and metabolism influence the response to the compounds.

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**Keywords:** Indigo; Indirubin; Aryl hydrocarbon receptor; Cytochrome P450 induction

The history of the aryl hydrocarbon receptor (AhR)<sup>1</sup> system began with the early observations in the 1950s that polycyclic hydrocarbons and dietary components can inhibit carcinogenesis [2,3]. In the 1960s, the focus of interest was the regulated induction of cytochrome P450 (P450), measured as benzo[*a*]pyrene 3-hydroxyl-

ation, also known as aryl hydrocarbon hydroxylase activity [4,5]. In the 1970s and 1980s, the AhR was first characterized, purified, and cloned [6–10]. Current interest in the AhR system relates to three main areas: (i) regulation of Phase I and Phase II detoxication systems, (ii) the toxic mechanisms of dioxins and polycyclic aromatic hydrocarbons, and (iii) the hunt for the physiological ligand of the receptor.

At the present time, the physiological role of the receptor is unclear. One view is that this receptor might function as part of an adaptive chemical response, leading to the synthesis of P450s and other enzymes capable of metabolizing certain structurally related xenobiotic agents [11]. However, studies with “knockout” mice indicate that the AhR plays an important role in vascular development [12]. Such observations suggest the possibility of an endogenous physiological ligand [13]. Several candidates as endogenous ligands have

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<sup>1</sup> Abbreviations used: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear transport protein;  $\beta$ NF,  $\beta$ -naphthoflavone (7,8-benzoflavone); DMSO, dimethyl sulfoxide; DRE, dioxin responsive element; EROD, 7-ethoxyresorufin *O*-deethylation; MS, mass spectrum (electrospray); P450, cytochrome P450 (also termed “heme-thiolate protein P450”) [1]; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; <sup>125</sup>IBr<sub>2</sub>N<sub>3</sub>DpD, 2-azido-3-[<sup>125</sup>I]iodo-7,8-dibromodibenzo-*p*-dioxin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

been considered, including indole-containing compounds, bilirubin, lipoxin A4, 7-ketocholesterol, flavones, and indole-thiazoles [14–22].

Recently a yeast-based system was employed to isolate two components of normal human urine with AhR-“stimulating” activity [23]. The compounds were identified as indigo and indirubin (Fig. 1). The characterization of the indigoid dyes as AhR inducers is of interest to us given that these compounds are produced by oxidation of indole by human P450s (Fig. 1) [24–26]. The activity of indigoids as AhR agonists is also of interest because of the potential use of these compounds as drugs. Indigo and indirubin are constituents of a Chinese traditional medicine (Danggui Longhui Wan), and indirubin has been studied as an inhibitor of cyclin-dependent kinases and glycogen synthase kinase [27–29]. Although Adachi et al. [23] showed very strong responses for indigo ( $EC_{50}$  5 nM) and indirubin ( $EC_{50}$  ~0.5 nM), the exclusive use of a yeast-based AhR reporter system (cf.  $EC_{50}$  = 5 nM for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) generates uncertainty as to the potency of these indigoids in mammalian cells [23]. Thus, indigo was equivalent with TCDD and indirubin was more potent in the yeast system. Based upon our previous work with yeast systems, we suspected that potency estimates of these compounds derived from yeast system may not be a valid measure of their biological potency in mammalian cells [30,31].

In this study, we re-examined the AhR-mediated responses to various indole derivatives in mammalian systems. We report the response to indigo, indirubin, and indirubin 3'-oxime in mammalian cell culture, in rat liver, and in a mouse AhR binding assay. Indirubin 3'-oxime was included because of its high solubility and its previous history as a protein kinase inhibitor [27–29]. These three indigoids were all found to be AhR ligands and inducers of the AhR-mediated response, although they appear not to be as potent as suggested by the earlier studies.

## Materials and methods

### Chemicals

Indigo was purchased from Aldrich Chemical (Milwaukee, WI) and used without further purification. Indole 3-acetate (Sigma Chemical, St. Louis, MO) and isatin (Aldrich) were used in the synthesis of indirubin, prepared (under Ar) as described [27,32] (88% yield): mass spectrum (electrospray) (MS)  $m/z$  285 ( $MNa^+$ );  $\lambda_{max}$  533 nm ( $C_2H_5OH$ );  $^1H$  NMR ( $d_6$ -dimethyl sulfoxide (DMSO))  $\delta$  6.90 (d, 1H, H-7), 6.95 (t, 1H, H-5'), 7.03 (t, 1H, H-5), 7.26 (t, 1H, H-6), 7.42 (d, 1H, H-7'), and .59 (t, 1H, H-6'), 7.65 (d, 1H, H-4'), 8.77 (d, 1H, H-4), 10.90 (s, 1H, H-1(NH)), 11.03 (s, 1H, H-1' (NH)) [25] (Fig. 1). Indirubin 3'-oxime was prepared from indirubin with  $NH_2OH$  as described [27] (quantitative yield) and recrystallized from  $C_2H_5OH/H_2O$ ; MS  $m/z$  300 ( $MNa^+$ );  $\lambda_{max}$  501 nm ( $C_2H_5OH$ ); ( $d_6$ -DMSO)  $\delta$  6.90 (d, 1H, H-7), 6.95 (t, 1H, H-5'), 7.14 (t, 1H, H-5), 7.40 (d, 1H, H-7'), 7.41 (1H, t, H-6'), 8.22 (d, 1H, H-4'), 8.65 (d, 1H, H-4), 10.73 (s, 1H, H-1(NH)), 11.74 (s, 1H, H-1' (NH)).

2,3,7,8-Tetrachlorodibenzofuran (TCDBF) was a generous gift of A. Poland (National Cancer Institute, Bethesda, MD). The radioligand 2-azido-3-[ $^{125}I$ ]iodo-7,8-dibromodibenzo-*p*-dioxin ( $^{125}IBr_2N_3DpD$ ) was synthesized as described [33]. The above dyes were prepared as DMSO solutions for use in assays with cell cultures and as suspensions in corn oil for (ip) injection into rats.

### Induction of AhR response in cell culture

The AhR reporter cell line employed in this study, 101L, is a derivative of the human hepatoma HepG2 cell line that has been stably transfected with a luciferase gene under control of the *CYP1A1* promoter and dioxin responsive elements (DREs). These cells were a generous gift from R.H. Tukey (University of California, San

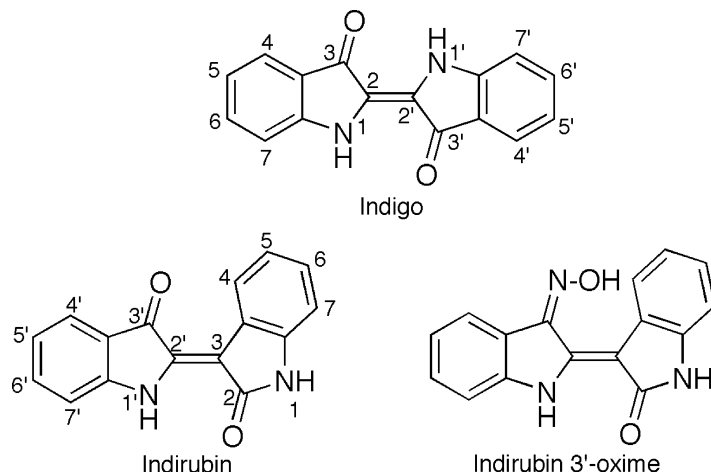


Fig. 1. Structures of indigoids used in this work.

Diego, CA) [34]. The cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (v/v), 1.0 mM L-glutamine, 1.0 mM sodium pyruvate, 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonate) (HEPES) (pH 7.5), minimum essential medium nonessential amino acids, and penicillin/streptomycin (Invitrogen). Cultures were maintained at 7.5% CO<sub>2</sub> (v/v) in a humidified atmosphere at 37 °C. Subconfluent cells were treated with a DMSO suspension of the indigoid compounds or TCDBF, at a final DMSO concentration of 1% (v/v), for 16–20 h. To assess the level of AhR activation, the treated cells were washed with phosphate-buffered saline (PBS) (Invitrogen) and incubated with Cell Culture Lysis Reagent (Promega, Madison, WI) for 5 min at room temperature. The luminescence obtained from reactions of the cell lysates and Luciferase Assay Substrate (Promega) was then measured in a Monolight 3010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

#### Competitive binding assay

A 1 nM concentration of the AhR ligand <sup>125</sup>IBr<sub>2</sub>N<sub>3</sub>DpD was incubated with increasing concentrations of competing ligand in 25 mM sodium morpholinopropane sulfonate buffer (pH 7.5) containing 0.025% NaN<sub>3</sub> (w/v), 1 mM EGTA, 10% glycerol (v/v), 15 mM NaCl, 1.0 mM dithiothreitol, 0.10% Nonidet NP-40 (v/v), and a liver cytosolic fraction obtained from C57BL/6J mice [35]. Incubation was performed at 20 °C for 30 min, followed by a step at 0 °C for 5 min to minimize the ligand off-rate. Charcoal/gelatin (final concentration 1%/0.1%, w/v) was added for 10 min at 0 °C to absorb the unbound ligand and then removed by centrifugation. The cytosolic fractions were UV-irradiated with four Photodyne 300 nm wavelength lamps at a distance of 4 cm for 1 min. The protein was precipitated by an overnight incubation in cold acetone at –20 °C, collected by centrifugation, and washed with cold acetone/water (9:1, v/v). The washed pellet was dissolved in sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on a 7.5% (w/v) polyacrylamide gel. Following staining and drying, the gel was exposed to film overnight at –80 °C with an intensifying screen. The 95-kDa AhR band was excised and the amount of <sup>125</sup>IBr<sub>2</sub>N<sub>3</sub>DpD covalently bound was quantified in a  $\gamma$  counter [33].

#### Animal experiments

Male Sprague–Dawley rats (125–150 g each) were purchased from Harlan Industries (Indianapolis, IN) and housed in the Vanderbilt facility with wood chip bedding and free access to a standard commercial chow and drinking water. All procedures were approved by

the Institutional Animal Care Committee. Rats indicated “untreated” received no injections. Other rats in the experiment received ip injections of 0.5 ml of corn oil or corn oil containing the indicated dose of chemical, once each day for 3 days. On the following day all animals were killed by CO<sub>2</sub> exsanguination and the livers were removed, weighed, and divided into three aliquots, each of which was frozen by immersion in liquid N<sub>2</sub> for storage at –80 °C. One piece of each liver was used to prepare microsomes using a standard protocol and the microsomes were stored frozen at –20 °C [36].

#### Assays with rat liver microsomes

Protein concentrations were estimated using a bicinchoninic acid (BCA) method according to manufacturer's recommendations (Pierce, Rockford, IL). SDS–polyacrylamide gel electrophoresis/immunoblotting (“Western blotting”) was done as described elsewhere [36,37] using an immunoglobulin G fraction (0.1 mg protein/ml) of rabbit antiserum raised against purified rat P450 1A1 [38] and cross-absorbed against rat liver microsomes prepared from untreated rats coupled to Sepharose to remove antibodies reacting with other P450s [39]. Blots were developed with 4-chloro-1-naphthol and imaging was done using a flatbed scanner and the program ImageQuant (Molecular Dynamics, Sunnyvale, CA; now available from Amersham Biosciences, Piscataway, NJ), with the same size of rectangle used for every band. Preliminary experiments were done with 0.1, 0.5, 2, and 10  $\mu$ g of microsomal protein from each rat. From this initial set of blots, a concentration of sample was selected to be in a linear range for analysis; duplicate aliquots of each sample were analyzed at the same time. The mean values (of the duplicate assays) were used for each set of rats (3–5) and the mean and SD values are presented in the figures.

7-Ethoxyresorufin O-deethylation (EROD) assays were done with the microsomes using a standard procedure [40] adapted for use in a microtiter plate fluorimeter. The microsomal protein concentration was in the range of 3–100  $\mu$ g/ml, depending upon the sample (adjusted following initial assays), with 5  $\mu$ M 7-ethoxyresorufin (37 °C, reaction time 10 min at 37 °C). The excitation wavelength was 544 nm and a filter was used to collect all emitted light >590 nm [40].

Incubations of indigoids with rat liver microsomes were done by incubating microsomal protein (2 mg protein/ml) with 20  $\mu$ M indigoid (added in DMSO, <1% (v/v)), 100 mM potassium phosphate buffer, and an NADPH-generating system [36] for 0–40 min at 37 °C. Reactions were quenched by the addition of two volumes of CH<sub>2</sub>Cl<sub>2</sub> and, following mixing with a vortex device and separation of the phases by centrifugation at 3  $\times$  10<sup>4</sup>g for 10 min, evaporation of an aliquot of the CH<sub>2</sub>Cl<sub>2</sub> layer to dryness in a 2.0 ml Reacti-vial (Pierce

Chemical, Rockford, IL). The residues were dissolved in a small volume of acetone (or *N,N*-dimethylformamide in the case of indigo) and aliquots were injected onto a reversed-phase HPLC column ( $6.2 \times 80$  mm Zorbax octadecylsilane,  $3 \mu\text{m}$ , Mac-Mod, Chadds Ford, PA). Elution was at a flow rate of 2.0 ml/min and the gradient was from 50 to 90%  $\text{CH}_3\text{OH}$  (in  $\text{H}_2\text{O}$ ) over 15 min, with the effluent directed into a ThermoSeparations UV3000HR rapid-scanning detector (ThermoSeparations, Piscataway, NJ), scanning from 400 to 720 nm.

## Results

### AhR response in mammalian cell culture

We used a mammalian cell culture system to demonstrate the relative potency and efficacy of the indigoids as AhR agonists. In this assay, receptor activation is measured directly with a luciferase reporter driven by DREs from the *CYP1A1* promoter [34]. We observed that indigo was a relatively weak inducer, with an  $\text{EC}_{50}$  value of  $\sim 5 \times 10^{-6}$  M (Fig. 2). Greater potency was observed with indirubin 3'-oxime and indirubin, with  $\text{EC}_{50}$  values of  $\sim 5 \times 10^{-7}$  and  $\sim 1 \times 10^{-7}$  M, respectively (Fig. 2). The  $\text{EC}_{50}$  value for the reference compound, TCDBF, was  $\sim 3 \times 10^{-9}$  M, 30-fold less than that of indirubin.

### Competitive binding of indigoids

Competition of the indigoids with  $^{125}\text{IBr}_2\text{N}_3\text{DpD}$  for AhR occupancy was used to quantify the relative affinity for the receptor. In the modified competitive binding assay employed herein, we measured the amount of

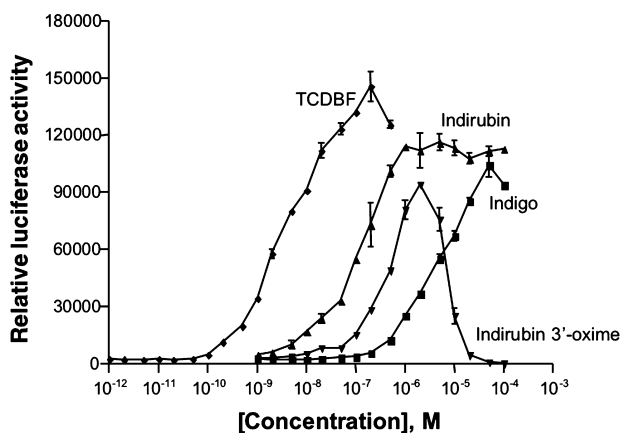


Fig. 2. DRE-driven luciferase activity of indigoids. 101L cells (0.5 ml) were treated with the indicated concentration of each compound (added in  $5 \mu\text{l}$  DMSO) for 16 h at  $37^\circ\text{C}$  prior to the luciferase assay. Each point represents the mean of two cell culture samples, and the error bar indicates the range. Indigo (■); indirubin (▲), indirubin 3'-oxime (▼); and TCDBF (◆).

$^{125}\text{IBr}_2\text{N}_3\text{DpD}$  bound to the AhR through the assessment of covalently linked  $^{125}\text{I}$  counts. Although efficiency of cross-linking is only approximately 1%, we assume the measurement is an accurate representation of the proportion of total radioligand bound [33]. The  $\text{IC}_{50}$  values are used as measurements of relative affinity (not actual  $K_D$ ). A mouse liver cytosol model was used, in which  $^{125}\text{IBr}_2\text{N}_3\text{DpD}$  is mixed with test compounds and the bound radioligand is subsequently covalently attached by photochemical crosslinking [33]. The potent agonist TCDBF was used as a positive control (Fig. 3). Indirubin was nearly as strong a ligand as TCDBF in this assay with  $\text{IC}_{50}$  values of  $2 \times 10^{-9}$  and  $5 \times 10^{-9}$  M, respectively. The  $\text{IC}_{50}$  values of indigo and indirubin 3'-oxime were approximately  $2 \times 10^{-8}$  M.

### In vivo results: effects of indigoids on liver weight

Male Sprague–Dawley rats were administered ip injections of indigo, indirubin, or indirubin 3'-oxime. In the preliminary phase of the work, we noted that administration of indigo or indirubin 3'-oxime (results not shown) had no effects on rat liver weight when given at doses up to 50 mg/kg body weight daily for 3 days. However, following administration of 50 mg/kg of indirubin the liver weights were reduced by 36% (statistically significant,  $p < 0.05$ , results not presented). Although indigoids are highly colored, there was no appearance of excess residual dyes in the peritoneal cavities of any of the rats after the 3-day administration.

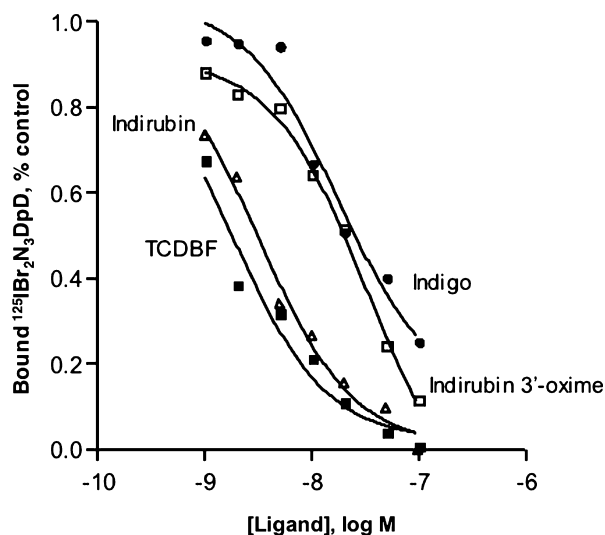


Fig. 3. Competitive binding to mouse cytosol AhR. The ligand was  $^{125}\text{IBr}_2\text{N}_3\text{DpD}$ , which was competed with the indicated concentrations of TCDBF (■) or the indigoids (indigo, ●; indirubin, ▲; and indirubin 3'-oxime, □); the undisplaced radiolabel was then photochemically linked and the bound radioactivity was determined following separation of the proteins using SDS–polyacrylamide gel electrophoresis [33]. The plots shown are for specific binding.

*In vivo results: induction of P450 1A1*

Two established methods were used to evaluate enzyme induction related to the AhR response. Liver microsomes were prepared from treated rats and analyzed for the induction of P450 1A1 using an immunoblotting method (Fig. 4A). The initial blots suggested weak induction of P450 1A1 by indigo and indirubin and a higher level of induction by indirubin 3'-oxime. The response was defined more quantitatively in the analyses reported in Figs. 4B–D. Although a positive response was clearly seen with 50 mg indirubin/kg body weight (Fig. 4C), the response to indigo and indirubin was significant at only some doses because of the variability among animals. A stronger response was seen with indirubin 3'-oxime, although the level of P450 1A1 was only  $\sim 1/3$  that produced with the same dose (50 mg/kg) of  $\beta$ -naphthoflavone ( $\beta$ NF) (Fig. 4D). The enzyme ac-

tivity EROD is also a well-established marker of P450 1A1 activity [40,41]. The pattern of this response was very similar to that observed with P450 1A1 immunoblotting (Fig. 5), with some response observed in the case of indirubin 3'-oxime (although less than seen with  $\beta$ NF) (Fig. 5).

*Oxidation of indigoids by rat liver microsomes*

One consideration in the evaluation of the biological response to these indigoids is their potential for metabolism, and a question is whether enzymes induced by the indigoids can oxidize them. To examine this possibility, the three indigoids of interest were incubated with liver microsomes prepared from rats that had been treated with corn oil or  $\beta$ NF (and fortified with NADPH to support P450 reactions) (Figs. 4 and 5). Studies with indigo were hampered by variable recovery

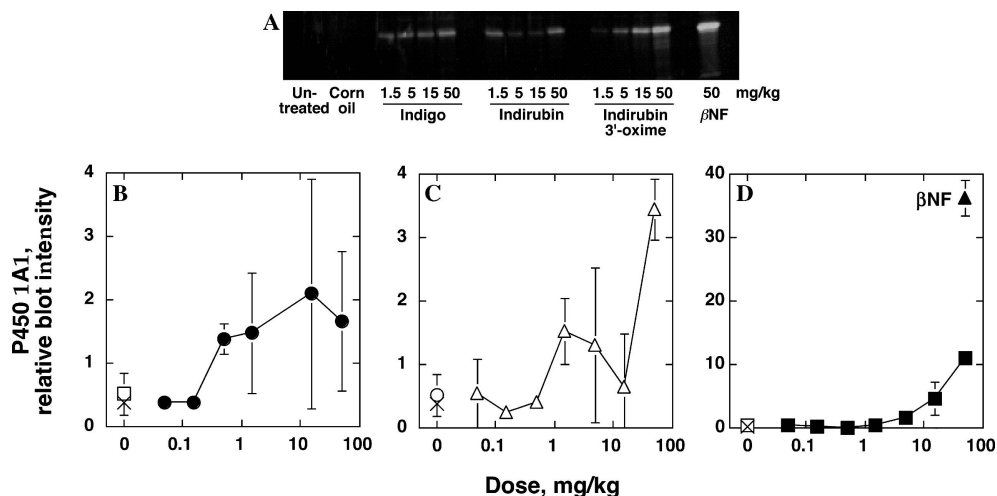


Fig. 4. (A) Effects of indigoids on P450 1A1 in rat liver. In each case, 2  $\mu$ g of microsomal protein was loaded and blotting was done with rabbit anti-P450 1A1. Untreated and corn oil-treated animals are indicated; doses (mg/kg) are presented in units of mg/kg body weight. (B–D) Quantitative results are presented from a more extensive series of blotting experiments for indigo (B), indirubin (C), and indirubin 3'-oxime (D). The value with  $\beta$ NF is shown only in part D ( $\blacktriangle$ ). Results are presented as means of results obtained with 3–4 animals ( $\pm$ SD).

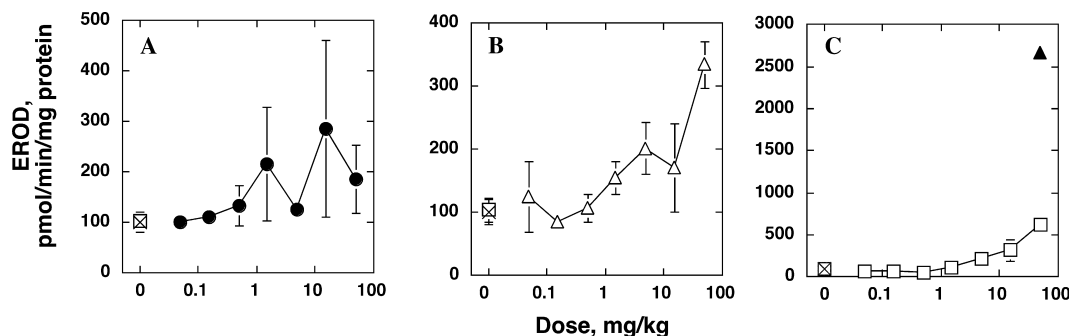


Fig. 5. Effects of indigoids on EROD activity in rat liver. Results are shown as means of duplicate assays with each microsomal sample and then presented as means  $\pm$  SD for each treatment group. Indigo (A); indirubin (B); and indirubin 3'-oxime (C). The value (mean  $\pm$  SD, less than width of symbol) is shown for the  $\beta$ NF-treated rats ( $\blacktriangle$ ) only in part C. Results are presented as means of results obtained with 3–4 animals ( $\pm$ SD).

of this rather insoluble compound, but the HPLC profiles did not show the presence of any products (absence or presence of NADPH) with spectra in the range 400–720 nm (data not presented). Under the same conditions there was limited disappearance of indirubin (Fig. 6A), but the appearance of several new products was detected. These products were NADPH-dependent, and the extent of formation was  $\sim 10\times$  faster in microsomes prepared from  $\beta$ NF-treated rats than from corn oil-treated rats. The three broadly eluting (groups of) products eluted at 5, 7, and 9 min had  $\lambda_{\max}$  values of 550, 570, and 550 nm, respectively (cf. 550 nm for indirubin). Incubation of indirubin 3'-oxime with rat liver microsomes led to the loss of the parent compound and the appearance of a leading peak eluted just prior to indirubin 3'-oxime (same  $\lambda_{\max}$  at 505 nm) and a set of broad peaks ( $t_R$  4–6 min, with complex spectra in the

region 465–480 nm) (Fig. 6B). The oxidation of the oxime was NADPH-dependent and about 2-fold faster with microsomes prepared from  $\beta$ NF-treated rats than from corn oil-treated rats. The disappearance of the parent indigoid appeared to be more extensive than for indirubin under the same conditions. Mass spectrometry of the products was attempted but was unsuccessful (our previous experience with indoles and indigoid dyes indicates that atmospheric pressure chemical ionization is generally the best technique [25] but even this method was not sensitive enough to use on-line with these products).

## Discussion

In a previous study, the suggestion was made that indigoids may act as endogenous ligands of the AhR [23]. This suggestion is based on the observations that the indigoids have been identified in human urine and that indirubin was found to be 50 times more potent than TCDD as a receptor agonist using a yeast-based system. Given that indigo and indirubin can be considered potential naturally occurring mammalian derivatives [23,25], we evaluated their potency as ligands of the mammalian AhR. Indirubin 3'-oxime is an unnatural derivative of indirubin and was included in this study to provide additional structure-activity information [27]. In support of their potential as physiological ligands, we observed that all three of the indigoids are ligands of the AhR and induce a biological response in mammalian cell culture and whole animals. In contrast to previous reports [23], we observed that these agonists are not as potent as halogenated-dioxins and -dibenzofurans.

In mammalian cell culture, the potency of each indigoid was measured using a reporter cell line derived from the human HepG2 cell line (Fig. 3). In this system, the most potent was indirubin, with an  $EC_{50}$  of  $1 \times 10^{-7}$  M. The rank-order of potency, indirubin > indirubin 3'-oxime > indigo, is similar to that reported from a yeast-based AhR system with a lac reporter [23]. Given that differences in reported  $EC_{50}$  values could be due to variables such as receptor number, kinetics, etc., we found it more useful to compare ligand potencies relative to a standard compound. In this regard, yeast studies suggest that indirubin is 50-fold more potent than TCDD. We observed that in mammalian cells indirubin is approximately 30-fold less potent than TCDBF. TCDBF was chosen in our study because its aqueous solubility is greater than that of TCDD and this yields more reproducible  $EC_{50}$  and  $IC_{50}$  values in vitro. TCDBF is a structurally related dibenzofuran and has similar binding affinity and potency for inducing aryl hydrocarbon hydroxylase activity as TCDD [6].

The strikingly different observations of the potency of indirubin relative to TCDD, made from the yeast-based

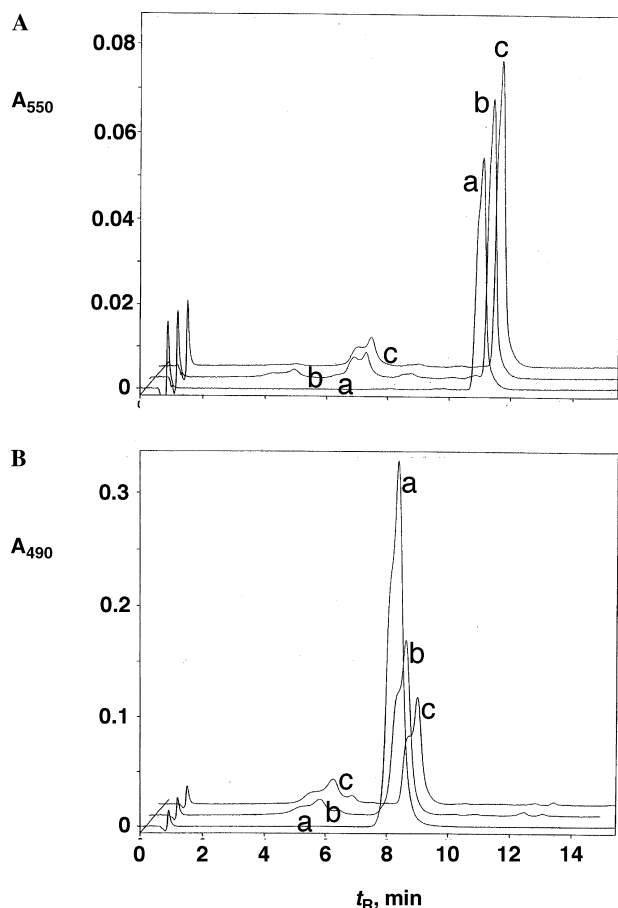


Fig. 6. Oxidation of indirubin (A) and indirubin 3'-oxime (B) by rat liver microsomes. In each case, the indigoid (20  $\mu$ M) was incubated with liver microsomes prepared from  $\beta$ NF-treated rats (2 mg protein/ml) in 0.10 M potassium phosphate buffer (pH 7.4) with an NADPH-generating system at 37  $^{\circ}$ C for 0 (A), 10 (B), or 40 (C) min. The extracts were analyzed by HPLC. Under these conditions indirubin eluted at  $t_R$  11 min (A) and indirubin 3'-oxime at  $t_R$  8.5 min (B); the (unidentified) metabolites were eluted in the  $t_R$  regions of 5–8 min (A) and 5–7 min (B).

system and our study in mammalian cells, highlight the difficulty of extending conclusions drawn from one organism to another. Although yeast has been widely employed in studies of AhR activation, a hyporesponsiveness of yeast to halogenated aromatics like TCDD has been documented. In this regard, several independent groups have reported that yeast-based systems tend to underestimate dioxin potency by at least 3 orders of magnitude [30,31,42]. This well-characterized attenuated response is the likely reason for the observed differences in relative potency of the indigoids and TCDD.

Since we finished these studies, some assays of indirubin potency have been reported [13,43,44]. All of these have involved only transcription reporter constructs in yeast and cultured mammalian cells. Kawanishi et al. [43] used a yeast-based system similar to those of Miller [31] and Adachi et al. [23] and compared mouse and human Ah receptors. The two systems were similar in their responses to TCDD but the human AhR system was  $>30\times$  more sensitive to the induction by indirubin than the mouse AhR-based system. The sensitivity of the mouse-based systems to indirubin was nearly identical to that of TCDD, but the human AhR-based system was at least 25-fold more sensitive to indirubin than TCDD, as reported by Adachi et al. [23]. Two reports involve mammalian cells. Spink et al. [44] reported that indirubin had a similar response to TCDD in inducing P450 1A1 and 1B1 in MCF-7 breast cancer cells, but the response was short-lived and metabolism of indirubin was involved (human P450 1A1 and 1B1, products not identified). Denison and Nagy [13] reported that indigo and indirubin were  $5 \times 10^4$  to  $10^5$  less potent compared to TCDD as AhR activators in mammalian cell culture.

The response of indirubin in these reporter systems seems to be better than TCDD in yeast systems but generally less in the mammalian cells [13,43,44]. None of these other studies either directly demonstrated that indigoids were Ah receptor ligands or if a response could be elicited *in vivo*.

The response to these indigoids in the rat further suggests that the compounds do activate the mammalian AhR but are not in the potency range of halogenated-dioxins and -dibenzofurans. The *in vivo* AhR activation was demonstrated by induction of the prototypic response, P450 1A1 (Fig. 4A). Upon treatment with the indigoids, both immunoreactive protein and associated EROD activity were elevated in rat liver (Figs. 4 and 5). The dose–response patterns were not as smooth for indigo and indirubin as observed for indirubin 3'-oxime (Figs. 4 and 5). The scatter of the response curves is likely the result of poor solubility, supported by the observation that indirubin 3'-oxime is more soluble than indigo and indirubin and that it also yields more reproducible responses (Figs. 4 and 5). The basis for the selective loss of liver weight by indirubin is unknown; it

could involve the action of indirubin as a protein kinase inhibitor [29]. The magnitude of the AhR activation elicited by any of the indigoids at higher doses was not as great as that of  $\beta$ NF (Figs. 4 and 5). Since  $\beta$ NF is a weaker inducer of P450 1A1 activity in murine liver than TCDD, requiring about  $10^3$ -fold higher concentration to achieve similar responses [45], these indigoids are probably not as potent activators of the AhR as TCDD *in vivo*.

The *in vivo* response to the indigoids also appears to be influenced by their differential metabolism. This conclusion is based on the observation that the rank order of potency differs between cell culture and animal studies (Figs. 2, 4, and 5). *In vitro* the rank order of  $EC_{50}$  values is indirubin  $>$  indirubin 3'-oxime  $>$  indigo, and *in vivo* the order is indirubin 3'-oxime  $>$  indirubin  $>$  indigo. Oxidative metabolism was directly demonstrated by the observation that incubation of the indigoids in microsomes produced a number of derivative compounds (Fig. 6). At low concentrations, the indigoids were oxidized in the order indirubin 3'-oxime  $>$  indirubin  $>>$  indigo. Although the metabolic products remain to be identified, they have been characterized by HPLC detection and visible spectra (Fig. 6). The NADPH-dependent oxidations were apparently enhanced by  $\beta$ NF-treatment of the rats, particularly in the case of indirubin. This observation supports a role for the AhR system in the regulation of indigoid levels *in vivo*. Indigoids might be conjugated in the body, but we have not examined this possibility.

We conclude that the indigoids examined can all activate the AhR in mammalian systems. Of the three, indirubin and indirubin 3'-oxime appear to be P450 substrates (Figs. 2–6). On the basis of our *in vivo* and *in vitro* findings, the indigoids are not as potent as TCDD in mammalian systems. Whether the indigoids are normally endogenous agonists of the AhR is still uncertain. Adachi et al. [23] measured levels of indirubin and indigo at  $\sim 2 \times 10^{-10}$  M. If such concentrations were found in cells or serum, contributing sources could be dietary, bacterial, or human P450s. It is premature to conclude that the measured levels of the indigoids reflect their effective concentrations, because we have not observed a biological response to these compounds at such concentrations in the systems used here. Finally, the work demonstrates that indirubins developed as drug candidates [27–29] might be expected to also be P450 1 family inducers when tested at high concentrations in biological assays.

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