The Suggested Physiologic Aryl Hydrocarbon Receptor Activator and Cytochrome P4501 Substrate 6-Formylindolo[3,2-b]carbazole Is Present in Humans**

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Dioxins and other polycyclic aromatic compounds formed during the combustion of waste and fossil fuels represent a risk to human health, as well as to the well being of our environment. Compounds of this nature exert carcinogenic and endocrine-disrupting effects in experimental animals by binding to the orphan aryl hydrocarbon receptor (AhR). Understanding the mechanism of action of these pollutants, as well as the physiological role(s) of the AhR, requires identification of the endogenous ligand(s) of this receptor. We reported earlier that activation of AhR by ultraviolet radiation is mediated by the chromophoric amino acid tryptophan (Trp), and we suggested that a new class of compounds derived from Trp, in particular 6-formylindolo[3,2-b]carbazole (FICZ), acts as natural high affinity ligands for this receptor. Here we describe seven new FICZ-derived indolo[3,2-b]carbazole-6-carboxylic acid metabolites and two sulfoconjugates, and we demonstrate the following: (i) FICZ is formed efficiently by photolysis of Trp upon exposure to visible light. (ii) FICZ is an exceptionally good substrate for cytochromes P450 (CYP) 1A1, 1A2, and 1B1, and its hydroxylated metabolites are remarkably good substrates for the sulfotransferases (SULTs) 1A1, 1A2, 1B1, and 1E1. Finally, (iii) sulfoconjugates of phenolic metabolites of FICZ are present in human urine. Our findings indicate that formylindolo[3,2-b]carbazoles are the most potent naturally occurring activators of the AhR signaling pathway and may be the key substrates of the CYP1 and SULT1 families of enzymes. These conclusions contradict the widespread view that xenobiotic compounds are the major AhR ligands and CYP1 substrates.

The primary sequence and structure of AhR3 in different species have been highly conserved during the course of evolution, suggesting that this receptor, which belongs to the family of basic helix-loop-helix nuclear transcription factors, plays an important physiological role(s) in homeostatic processes (1, 2). However, despite intensive studies, this physiological role and the endogenous activators of the AhR remain to be elucidated (3). At the cellular level, activated AhR interacts with various signal transduction pathways, induces biotransformation enzymes; alters the cell cycle, cell adhesion, and migration; and causes apoptosis or aberrant cell growth (4–7). In vivo, the AhR plays significant roles in connection with development, immunological and reproductive functions, and adaptive responses to light and xenobiotics (8–11).

The AhR protein has been shown to bind the compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as similar highly lipophilic halogenated and non-halogenated hydrocarbons leading to cardiovascular, carcinogenic, and endocrine effects (12–16). Consequently, most studies designed to explore the functions of this receptor have been performed with TCDD. One remarkable feature of activation of AhR by dioxin-like compounds is sustained induction of both cytochromes P450 and other metabolizing enzymes, whereas other agonists cause only transient induction of these enzymes. It seems likely that for purposes of regulation, endogenous ligands of AhR are metabolized rapidly, so that the use of persistent xenobiotics such as dioxins to investigate this receptor might be inappropriate. A striking discrepancy between the effects of different types of AhR activators was made evident in two recent studies (17, 18). The authors showed that FICZ, the suggested physiologic AhR ligand, boosted T11,17-cell differentiation and worsened the experimentally induced autoimmune encephalomyelitis, whereas TCDD increased levels of Treg and suppressed the pathological effects in myelin-immunized mice.

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3 The abbreviations used are: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; SULT, sulfotransferase; HPLC, high pressure liquid chromatography; CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ICIC, indolo[3,2-b]carbazole-6-carboxylic acid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MS, mass spectrometry; αNF, α-naphthoflavone; W, watt; EROD, 7-ethoxyresorufin-O-deethylase; XRE, xenobiotic-response element; dFICZ, 6,12-diformylindolo[3,2-b]carbazole.
Many endogenous compounds, including various indoles, heme, and arachidonic acid metabolites, have also been found to be AhR agonists, and Trp is the precursor for many of the most active ligands (3). Indeed, FICZ, which is formed upon exposure of solutions of Trp (19, 20), cell culture medium (21), or cells (9) to ultraviolet radiation, binds to AhR with greater affinity than TCDD (19), as well as all other substances tested previously (3). The $K_D$ value for FICZ is 0.07 nm, which is 2 or more orders of magnitude lower than for prostaglandin and lipoxin derivatives also suggested as physiological AhR ligands (22). FICZ up-regulates the expression of AhR-responsive genes efficiently and transiently and is rapidly metabolized in a feedback manner (23). This sequence of events typical for auto-regulatory loops in biological signaling indicates that this compound may be a physiological ligand for AhR. The purpose of this work was to characterize the light-dependent formation of FICZ, its metabolism by human enzymes, and to document the presence of stable metabolites of FICZ in humans.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Of the five new compounds used as standards, compound 8-SO$_3$FICZ was enzymatically produced (this study); the chemical synthesis of 2-SO$_3$FICZ is unpublished, and the synthesis of indolo[3,2-b]carbazole-6-carboxylic acid (CICZ), 2-OH-CICZ, and 2,8-dOH-CICZ (purity >98%) has been described in detail elsewhere (24).

**Irradiation of Trp**—For irradiation of Trp the following settings were utilized: visible light, a normal 100-watt bulb for 90 min at a distance of 18 cm (1.4 J/s m$^2$); UVA1-Osram UltraMed with a normal 13-watt bulb for 90 min (0.54 J/s m$^2$); and UVB-70 W UV240 DT, IP20, 2 Philips PL 36-W tubes for 15 min at a distance of 28 cm (1 J/s m$^2$). 100-ml aliquots of a Trp suspension were irradiated. Following irradiation, the solutes were concentrated by solid-phase extraction (SPE; SepPak$^{	ext{®}}$) and analyzed for the presence of FICZ by HPLC.

**Metabolic Systems**—Pooled human liver S9 fractions were purchased from MOLTOX™ (Molecular Toxicology Inc.) and used to obtain cytosolic and microsomal fractions by centrifugation at 105,000 × g for 60 min. Metabolism studies were performed according to Ref. 25. Three bicistronic plasmids encoding human NADPH-P450 reductase together with CYP enzymes 1A1, 1A2, or 1B1 were a generous gift from Dr. Fred Guengerich (Vanderbilt University School of Medicine, Nashville). The CYP1 enzymes were expressed in *Escherichia coli* DH5α, and bacterial membranes containing the recombinant enzymes were prepared according to the procedure described by Guengerich and co-workers (26). The levels of CYP1 proteins in the preparations were calculated on the basis of the carbon monoxide (CO) difference spectra (27). 7-Ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-demethylase activities were determined by the standard procedures described by Burke and Mayer (28). The kinetic properties of CYP1A1, -1A2, and -1B1 with FICZ as a substrate were determined on the basis of the decrease in fluorescence ($\lambda_{ex} = 460$ nm, $\lambda_{em} = 520$ nm) with time. Membrane preparations containing the different CYP1 enzymes were incubated separately with different concentrations of FICZ (ranging from 0.125 to 52.0 μM) at 37 °C in a mixture containing 25 nM CYP, 0.5 mM NADPH, 48 mM Tris-Cl (pH 7.4), and 0.96 mM EDTA (final concentrations). Antibodies used to investigate the importance of CYP1 enzymes in FICZ metabolism in human liver microsomal fractions were CYP1A1/1A2 inhibitory antibody (anti-rat CYP1A2 number 458131) and CYP1B1 antiserum (WB-rat number 458511) from BD Biosciences. Human SULTs were expressed in *Salmo-nella typhimurium* TA1538, and cytosolic preparations obtained from these bacteria were prepared according to Meinel and co-workers (29). The levels of SULTs in the bacterial cyto-solic fractions were determined based on immunoblots (supplemental Fig. S1) with homogeneous enzyme proteins as standards (29). Cytosolic preparations containing the different SULT enzymes were incubated separately with different concentrations of substrate (ranging from 0.0125 to 40.4 μM) in a mixture containing 0.02–0.1 μM SULT and 0.1 mM 3’-phospho-adenosino-5’-phosphosulfate in 100 mM sodium phosphate (pH 7.4) (final concentrations). The kinetic properties with 2- and 8-OH-FICZ as substrates were determined on the basis of the increase in fluorescence ($\lambda_{ex} = 390$ nm; $\lambda_{em} = 525$ nm) with time. For the kinetic studies the total cytosolic fraction, rather than the purified SULT enzymes, was employed because these enzymes are more stable in the presence of this fraction. Furthermore, other compounds in the cytosolic fraction rapidly degrade the de-sulfonated form, 3’-phospho-adenosino-5’-phosphate, of the cofactor, which is a potent inhibitor of SULTs.

All kinetic measurements were performed utilizing a GENios PRO plate reader (Tecan), and the GraphPad Prism 5.0 software was employed to calculate the kinetic constants ($K_m$, $k_{cat}$, and $K_I$) in accordance with the substrate inhibition model.

**Cell Culture Conditions and Treatments**—HepG2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C in a 5% CO$_2$ atmosphere. The HepG2-derived cell line HepG2-XRE-Luc containing the stable pTX.DIR luciferase reporter with two rat XRE1 elements (30) was cultured in the same medium with addition of 800 μg/ml geneticin. All media and supplements were from Invitrogen. 1.4 × 10$^6$ HepG2 cells were seeded into 60-mm dishes and treated with FICZ (5 μM), α-naphthoflavone (αNF, 5 μM), and a combination of FICZ and αNF. The cells were briefly washed with phosphate-buffered saline, harvested in 850 μl of distilled H$_2$O, and sonicated on ice for FICZ determinations by HPLC. For studies of AhR activation of the HepG2-XRE-Luc, cells were plated into 12-well plates and grown to 70–80% confluence before treatment. The medium was replaced with fresh medium, containing the AhR ligands dissolved in DMSO (final concentration 0.1% (v/v)). At the indicated time points, the cells were lysed, and luciferase activity was measured in 30 μl of cell extract according to the manufacturer’s protocol (BioThema, Handen, Sweden). Luciferase activity was normalized to protein values determined with the DC protein assay (Bio-Rad).

**AhR Activating Capacities**—The AhR activating capacities of the different indolo[3,2-b]carbazoles were compared with TCDD using the enzyme-linked immunosorbent assay-based Ah-IMMUNOASSAY® (Biosense Laboratories, Nor-
TABLE 1
Formation of FICZ by light

| Light source | FICZ formed | − Riboflavin | + Riboflavin |
|--------------|-------------|--------------|-------------|
| Visible light | pmol/μmol | 9.5 ± 0.6 | 13.6 ± 1.7 |
| UVA | pmol | 2.5 ± 0.3 | 11.3 ± 1.4 |
| UVB | pmol | 13.6 ± 1.7 | 512 ± 15 |

way) according to the manufacturers protocol. The same range of concentrations was used for all compounds (10−2500 pm).

Chemical Analyses—Some samples were concentrated prior to analysis by HPLC using external or on-line SPE (SepPak® or restricted access materials columns), whereas others were injected directly. The HPLC separation was achieved on a 250-mm long, 5-μm particle size reverse phase C18 column using a 40-min linear mobile phase gradient from 45% B to 100% B (A, 1.5 mM formic acid in H2O; B, 1.5 mM formic acid in acetonitrile) at a flow rate of 0.8 ml/min. Both CICZ and FICZ were analyzed using fluorescence detection. Urine samples were sterilized by filtration and extracted with SPE. The LC/MS/MS separation of the sulfonated compounds was achieved on a 150-mm long, 1.8-μm particle size reverse phase C18 column. The temperature was elevated to 80 °C to reduce the viscosity of the mobile phase, which allowed the use of a conventional HPLC pump to obtain an acceptable flow rate. A gradient of 5–60% methanol was applied over a period of 30 min at a flow rate of 0.7 ml/min, and the separation was followed by negative electrospray ionization. Formic acid at a final concentration of 20 mM was added to the mobile phase to eliminate fluctuations in pH and variation in the retention times of the acidic sulfates. The MS detection was performed in the enhanced product ion mode, where the selected precursor ions (monosulfated metabolites, m/z 379) were isolated and fragmented at particular collision energy. For negative electrospray ionization, the following settings were utilized: curtain gas 40 p.s.i., gas1 30 p.s.i., gas2 40 p.s.i., ion spray voltage −4500 V, temperature 450 °C, declustering potential −40 V, entrance potential −10 V, and collision energy −70 V.

RESULTS AND DISCUSSION

Formation of Photoproducts—Our earlier findings (21), as well as those of others (31), indicate that exposure of Trp to visible light results in the formation of FICZ. In those studies the contribution of UV light could not be unequivocally excluded. Here we quantitatively compared the formation of this compound upon irradiation with visible light with the amounts formed following UVA and UVB irradiation (Table 1). Exposure of 80 μmol aqueous solution of Trp to light from a regular 100-W bulb for 20 min yielded 9.5 pmol of FICZ. Per unit of energy (joule), UVB was the most efficient in generating FICZ. 40−400-fold increases in FICZ formation were observed in the presence of the photosensitizer riboflavin (vitamin B12), especially in the case of UVA and visible light because, in contrast to Trp, riboflavin absorbs light efficiently at these longer wavelengths. This relatively easy conversion of Trp to FICZ in the presence of riboflavin and light suggests that the same process could occur, e.g., in the skin, and that the formation of FICZ could probably explain the reported UV-dependent activation of CYP1 enzymes in human skin (32).

Metabolism—Subsequently, we examined the major pathways of FICZ metabolism and determined the principal enzymes involved in order, among other things, to know which metabolites to look for in human urine. An S9 fraction prepared from human liver catalyzed mono- and di-hydroxylation of the ring moieties, as well as oxidation of the aldehyde group. Seven previously unknown metabolites (5, 6a−c, and 7a−c) were detected employing LC/MS/MS and their structures determined by LC/MS/MS (Fig. 1, A and B). Metabolites 5, 6a, and 7a (designated h1, h3, and h5 in Ref. 25) showed the same mass fragmentation pattern as the authentic standards CICZ, 2-OH-CICZ, and 2,8-dihydroxy-CICZ, respectively (see supplemental Fig. S2). Although 2, 6c, 7b, and 7c (designated MA, h2, h4, and h6 in Ref. 25) did not match any of the standards available, analysis by LC/MS/MS together with their UV spectra, molecular weight, fragmentation patterns, and comparison with the product-precursor relationships revealed the molecular structures shown in Fig. 1B. 2 exhibited the same molecular
Tryptophan Photoproduct FICZ Is Present in Humans

FIGURE 2. CYP1-mediated disappearance of FICZ and FICZ-dependent AhR activation. A, human liver microsomal fraction (2 nmol of cytochrome P450/ml) was preincubated with CYP1A1/1A2- and CYP1B1-blocking antibodies (Ab) for 20 min followed by addition of FICZ (10 nM) and NADPH (0.5 mM). Incubations with FICZ without addition of NADPH were performed to control for non-P450-catalyzed FICZ metabolism. The amount of FICZ remaining after 5 and 10 min was measured using HPLC. B, HepG2 cells were treated for 0–24 h with FICZ (5 nM) either alone or in combination with αNF (5 μM), or a combination of FICZ and αNF, and the effects on AhR-dependent transcriptional activity were estimated using a luciferase reporter assay. All data are from triplicate incubations and show means ± S.D.

FIGURE 3. Kinetic properties of recombinant human CYP1A1, -1A2, and -1B1. Dependence of the rate of hydroxylation of FICZ (E) and dealkylation of 7-ethoxyresorufin (EOR, a) and 7-methoxyresorufin (MOR, b) by CYP1A1, -1A2, and -1B1 on substrate concentration.

TABLE 2
Kinetic properties of recombinant human CYP1A1, -1A2, and -1B1

| Enzyme | Substrate | \( k_{cat} \) | \( K_m \) | \( K_i \) | \( k_{cat}/K_m \) |
|--------|-----------|-------------|---------|---------|-----------------|
| CYP1A1 | FICZ      | 257 ± 12    | 0.053 ± 0.012 | 15.4 ± 3.70 | 8.10 ± 1.10 |
|        | EOR       | 22.7 ± 3.4  | 0.24 ± 0.017  | 1.80 ± 0.52 | 0.16 ± 0.03 |
| CYP1A2 | FICZ      | 367 ± 44    | 0.89 ± 0.17   | 3.90 ± 1.00 | 0.69 ± 0.09 |
|        | EOR       | 33.2 ± 5.0  | 0.31 ± 0.08   | 0.98 ± 0.23 | 0.18 ± 0.03 |
| CYP1B1 | FICZ      | 174 ± 10    | 0.21 ± 0.04   | 13.3 ± 3.00 | 1.40 ± 0.16 |
|        | EOR       | 36.4 ± 5.2  | 0.23 ± 0.06   | 0.98 ± 0.23 | 0.27 ± 0.04 |

weight (300) and MS/MS fragments as the other monohydrated FICZ metabolites but was less stable, suggesting hydroxylation on the nitrogen atom. 6b co-eluted with 4b (2,10-dOH-FICZ). On the basis of its 

Without addition of the cofactor NADPH and/or when employing the cytosolic fraction from human liver S9, oxidation of the aldehyde moiety was the only metabolism that occurred. This reaction converted FICZ to CICZ (5) and the hydroxylated FICZ metabolites to the corresponding hydroxylated metabolites of CICZ (6a, c and 7a–c). The independence of NAD(P)H for formation of CICZ, the cytosolic localization, and the inhibition of FICZ oxidation by the aldehyde oxidase inhibitor menadione (supplemental Fig. S3) but not by the xanthine oxidase inhibitor allopurinol (data not shown) strongly suggest that oxidation of the aldehyde moiety is catalyzed by aldehyde oxidase.

By HPLC analyses we could demonstrate the primary role of the first family of cytochrome P450 enzymes, CYP1, in catalyzing FICZ catabolism both in human liver microsomes incubated with antibodies specific toward CYP1 and in human hepatoma HepG2 cells treated with the highly efficient chemical CYP1 inhibitor αNF (Fig. 2, A and B). A 20-min preincubation with a mixture of CYP1A1/1A2 and CYP1B1 antibodies inhibited almost completely (93%) the NADPH-dependent FICZ metabolism (Fig. 2A). The inhibiting capacity of the antibodies was confirmed by inhibition of the EROD activity by 98% (supplemental Fig. S4). The complete and rapid depletion of intracellular levels of FICZ seen in FICZ-treated HepG2 cells was attenuated when αNF was added (Fig. 2B). This CYP1 inhibitor gave rise to a prolonged AhR activation in HepG2 cells. It caused a sustained luciferase reporter response in contrast to the transient responses seen in control incubations or in incubations with FICZ alone (Fig. 2C). Thus, the inhibitor behaved as an AhR agonist in these experiments. In parallel, we showed that αNF behaved as an antagonist when added together with TCDD (supplemental Fig. S5).

The observed prolonged AhR response when the CYP1A1 enzyme was inhibited with αNF is in accordance with our earlier demonstration that mouse Hepa-1 cells (c37) lacking functional CYP1A1 enzyme exhibit a higher constitutive CYP1A1 expression and respond to FICZ treatment with sustained up-regulation of CYP1A1 expression (23). Higher constitutive CYP1A1 mRNA levels in CYP1A1-deficient mouse Hepa-1 cell clones and African green monkey kidney CV-1 cells had also been reported by others (33, 34) These investigators had shown that constitutive activity of the AhR-ARNT transcriptional complexes was regulated by the catalytic activity of the CYP1A1 enzyme. They pointed out the possibility that endogenous CYP1A1 substrates could be the AhR activators in the absence of exogenous ligands. Our results suggest that FICZ is such an endogenous ligand, which is efficiently metabolized by CYP1A1. This provides a likely mechanistic explanation for the earlier reported phenomena.

Enzyme Kinetics—To examine the efficiency with which the CYP1 enzymes metabolize FICZ, we compared the rates of hydroxylation of this compound to the standard EROD and methoxyresorufin-O-demethylase activities using human
TABLE 3  
Kinetic parameters of recombinant human SULT1A1, -1A2, -1B1, and -1E1  

| Enzyme   | Substrate     | $k_{cat}$ | $K_m$ | $K_i$ | $k_{cat}/K_m$ | $K_m$ (m$^{-1}$s$^{-1}$) |
|----------|---------------|-----------|-------|-------|--------------|----------------------------|
| SULT1A1  | 2-OH-FICZ     | 99.3      | 0.25  | 3.1   | 6.62         | 0.29                       |
|          | 8-OH-FICZ     | 123       | 0.35  | 2.6   | 0.59         | 0.07                       |
|          | 4-Nitrophenol | 18        | 1.1   | 0.3   | 0.3          | 0.4                        |
| SULT1A2  | 2-OH-FICZ     | 91.6      | 0.14  | 0.36  | 10.9         | 0.91                       |
|          | 8-OH-FICZ     | 48.5      | 0.70  | 7.0   | 1.15         | 0.11                       |
|          | 4-Nitrophenol | 18        | 1.1   | 0.3   | 0.3          | 0.4                        |
| SULT1B1  | 2-OH-FICZ     | 7.90      | 0.03  | 2.10  | 4.39         | 0.37                       |
|          | 8-OH-FICZ     | 12.3      | 0.07  | 1.48  | 2.93         | 0.11                       |
|          | 4-Nitrophenol | 18        | 1.1   | 0.3   | 0.3          | 0.4                        |
| SULT1E1  | 2-OH-FICZ     | 12.2      | 0.51  | 3.2   | 0.40         | 0.03                       |
|          | 8-OH-FICZ     | 58.6      | 13.7  | 6.6   | 0.25         | 0.3                        |
|          | $17\beta$-estradiol | 1.3       | 0.05  | 0.02  | 1.03         | 0.04                      |

recombinant CYP1 enzymes expressed in E. coli. The catalytic efficiencies ($k_{cat}/K_m$) were found to be 5–50-fold higher for FICZ than for these standard substrates (Fig. 3 and Table 2) and at least 5000-fold higher compared with the hydroxylation of estrogens (35, 36). CYP1A1 catalyzed hydroxylation of FICZ extremely efficiently, with $k_{cat}/K_m$ of $8.1 \times 10^7$ M$^{-1}$ s$^{-1}$, which is similar to the corresponding value of $8.3 \times 10^7$ M$^{-1}$ s$^{-1}$ for the highly efficient carbonic anhydrase (37). The catalytic efficiency for FICZ as a CYP1A1 substrate is close to the limit of diffusion, and therefore FICZ is an excellent CYP1A1 substrate. FICZ is also a very good substrate for CYP1A2 and CYP1B1.

We then investigated whether the hydroxylated metabolites of FICZ are in turn good substrates for conjugating enzymes. Our earlier findings indicated that sulfoconjugation is an important pathway in this context (25). Such conjugation of exogenous and endogenous compounds generates water-soluble sulfuric acid esters that are excreted in the urine. Therefore, sulfation of the primary hydroxylated FICZ metabolites 3a and 3b by six human recombinant sulfotransferases (SULT1A1, -1A2, -1A3, -1B1, -1E1, and -2A1) was studied in detail.

Although SULT1A3 and -2A1 did not catalyze any conjugation (data not shown), the other four SULTs all exhibited high catalytic efficiencies. In all four cases the 2-hydroxylated metabolite 3b was more efficiently conjugated than the 8-hydroxylated metabolite 3a (Fig. 4 and Table 3). With a $k_{cat}/K_m$ of $1.1 \times 10^7$ M$^{-1}$ s$^{-1}$ with 3b as substrate, SULT1A2 exhibited the highest efficiency. This value is higher than for any other substrate and human SULT form reported in the literature (38, 39) and for other substrates in Table 3. In this connection it could be added that $k_{cat}/K_m$ for the analgesic and antipretic drug paracetamol for which sulfation is an important pathway in the elimination of the drug is 4 orders of magnitude lower.4 Furthermore, the dihydroxylated metabolites 4a–c were converted to disulfuric acid esters by SULT 1A1, -1A2, and -1B1, with intermediate formation of the monosulfonconjugates (not shown). Because sulfation of monohydroxylated metabolites of FICZ occurs rapidly and the second sulfation of dihydroxylated metabolites is less favorable, monosulfates would appear to be most likely to be excreted in the urine.

AhR Activation—The key events involved in the processes regulated by AhR are activation of this receptor by ligand binding and subsequent binding of a complex consisting of the activated receptor and accessory proteins to specific responsive elements (XREs) in genes, thereby triggering their transcription. We examined whether the transient nature of the induction of CYP1A1 by FICZ (23) reflects rapid metabolism of this compound to derivatives with little or no affinity for AhR. When the capacities of FICZ and eight of its metabolites to activate AhR were compared with the AhR activating capacities of different indolo[3,2-b]carbazoles compared to TCDD (Table 4). With a $k_{cat}/K_m$ of $1.1 \times 10^7$ M$^{-1}$ s$^{-1}$ with 3b as substrate, SULT1A2 exhibited the highest efficiency. This value is higher than for any other substrate and human SULT form reported in the literature (38, 39) and for other substrates in Table 3. In this connection it could be added that $k_{cat}/K_m$ for the analgesic and antipretic drug paracetamol for which sulfation is an important pathway in the elimination of the drug is 4 orders of magnitude lower.4 Furthermore, the dihydroxylated metabolites 4a–c were converted to disulfuric acid esters by SULT 1A1, -1A2, and -1B1, with intermediate formation of the monosulfonconjugates (not shown). Because sulfation of monohydroxylated metabolites of FICZ occurs rapidly and the second sulfation of dihydroxylated metabolites is less favorable, monosulfates would appear to be most likely to be excreted in the urine.

AhR Activation—The key events involved in the processes regulated by AhR are activation of this receptor by ligand binding and subsequent binding of a complex consisting of the activated receptor and accessory proteins to specific responsive elements (XREs) in genes, thereby triggering their transcription. We examined whether the transient nature of the induction of CYP1A1 by FICZ (23) reflects rapid metabolism of this compound to derivatives with little or no affinity for AhR. When the capacities of FICZ and eight of its metabolites to activate this receptor were compared with those of the high affinity ligands 6,12-diformylindolo[3,2-b]carbazole (dFICZ), ICZ, and TCDD employing an enzyme-linked immunosorbent assay, the three indolo[3,2-b]carbazoles FICZ, ICZ, and dFICZ produced the greatest activation, as expected, with FICZ being almost twice as potent in this respect as TCDD (Table 4). Interestingly, one of the primary hydroxylated metabolites, 8-OH-FICZ, demonstrated approximately the same potency as TCDD, whereas all of the other metabolites were less potent, and the monosulfate conjugate (i.e. the end product) produced no detectable activation.

Presence of FICZ Metabolites in Humans—To demonstrate that FICZ actually occurs in humans, we analyzed seven 24-h

### TABLE 4  
The AhR activating capacities of different indolo[3,2-b]carbazoles compared to TCDD

| Compound | Relative capacity to activate AhR |
|----------|---------------------------------|
| TCDD     | 1                               |
| FICZ     | 1.89 ± 0.04                     |
| dFICZ    | 1.17 ± 0.09                     |
| ICZ      | 1.48 ± 0.07                     |
| 2-OH-FICZ| 0.43 ± 0.06                     |
| 8-OH-FICZ| 0.93 ± 0.05                     |
| 2,10-dOH-FICZ | 0.052 ± 0.003 |
| 4,8-dOH-FICZ | 0.035 ± 0.003 |
| 2,8-dOH-FICZ | ND                            |
| 2-SO$_2$-FICZ | ND                            |
| CICZ     | 0.044 ± 0.004                   |
| 2-OH-CICZ| 0.006 ± 0.001                   |

4 H. R. Glatt, unpublished results.

**FIGURE 4.** Kinetic properties of recombinant SULT1A1, -1A2, -1B1, and -1E1. The rates of conjugation of monohydroxylated metabolites of FICZ by different SULT1 enzymes as a function of the concentrations of 2-OH-FICZ (■) or 8-OH-FICZ (▲).
Tryptophan Photoproduct FICZ Is Present in Humans

**FIGURE 5.** Identification of monosulfate conjugates of FICZ (M, 380) in three human urine samples. In all the chromatograms shown, m/z 379 was selected and fragmented. Compounds with different retention times (boldface numerals) and slightly different fragmentation patterns (roman numerals) are shown. A overlay of LC/MS/MS chromatograms of unspiked (unfilled) and spiked (with 2- and 8-SO4-FICZ, filled) aliquots of one urine sample containing compounds 8–10. The retention times and fragmentation spectrum of the standards were compared with those of the unspiked urine, and 8-SO4-FICZ was definitively identified in this manner. B, LC/MS/MS chromatograms of two other urine samples containing compounds 11–20. Compound 13 is identical to 8-SO4-FICZ. The other compounds are most likely other sulfated forms of FICZ because they exhibited the same molecular weight and produced the same fragments as 2- and 8-SO4-FICZ, but they could not be definitely identified because appropriate standards are not available. C, fragmentation patterns of 2- and 8-SO4-FICZ standards (both giving pattern II) and four other potential sulfated forms of FICZ (III, IV, and V). The major ions m/z 299, 298, 283, 270, 269, 241, and 214 correspond to [M-SO3-H]+, [M-SO3-2H]+, [M-SO3-2CO-H]+, [M-SO3-2CO-3H]+, [M-SO3-2CO-3H]+, and [M-SO3-2CO-2CH-4H]+, respectively, where M is SO4-FICZ.

FIGURE 6. Identification of monosulfate conjugates of FICZ (M, 380) in three human urine samples. In all the chromatograms shown, m/z 379 was selected and fragmented. Compounds with different retention times (boldface numerals) and slightly different fragmentation patterns (roman numerals) are shown. A overlay of LC/MS/MS chromatograms of unspiked (unfilled) and spiked (with 2- and 8-SO4-FICZ, filled) aliquots of one urine sample containing compounds 8–10. The retention times and fragmentation spectrum of the standards were compared with those of the unspiked urine, and 8-SO4-FICZ was definitively identified in this manner. B, LC/MS/MS chromatograms of two other urine samples containing compounds 11–20. Compound 13 is identical to 8-SO4-FICZ. The other compounds are most likely other sulfated forms of FICZ because they exhibited the same molecular weight and produced the same fragments as 2- and 8-SO4-FICZ, but they could not be definitely identified because appropriate standards are not available. C, fragmentation patterns of 2- and 8-SO4-FICZ standards (both giving pattern II) and four other potential sulfated forms of FICZ (III, IV, and V). The major ions m/z 299, 298, 283, 270, 269, 241, and 214 correspond to [M-SO3-H]+, [M-SO3-2H]+, [M-SO3-2CO-H]+, [M-SO3-2CO-3H]+, [M-SO3-2CO-3H]+, and [M-SO3-2CO-2CH-4H]+, respectively, where M is SO4-FICZ.

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enolamines, FICZ is also an excellent substrate for CYP1A1. Furthermore, the hydroxylated metabolites of FICZ are remarkably good substrates for the SULT1 enzymes. Our findings have important experimental implications, because FICZ is present in cells in culture (9, 21, 31) and probably also in other biological matrices containing Trp. Thus, conditions that influence formation or metabolism of FICZ may lead to misinterpretation of experimental data. This could be demonstrated by treatment of cells with the CYP1 inhibitor αNF, which caused a prolongation of the normally transient AhR activation induced by FICZ treatment. Similarly, αNF treatment alone increased the AhR activation induced by the medium change, i.e., in this case αNF behaved like an agonist, although mechanistically it is an inhibitor of FICZ metabolism. Together with earlier observations of high constitutive CYP1A1 expression in metabolically deficient cells (23, 34, 35), this illustrates the fact that AhR activation can be obtained in the absence of exogenously added AhR ligands or by compounds having low or insignificant AhR affinity if they inhibit the intracellular breakdown of FICZ. In summary, this study advances significantly our knowledge concerning the mechanisms of formation, biochemical properties, and metabolic fate of FICZ.

Our kinetic data demonstrate that, in addition to being a potent activator of AhR and, thereby, inducer of CYP1 enzymes, FICZ is also an excellent substrate for CYP1A1. Furthermore, the hydroxylated metabolites of FICZ are remarkably good substrates for the SULT1 enzymes. Our findings have important experimental implications, because FICZ is present in cells in culture (9, 21, 31) and probably also in other biological matrices containing Trp. Thus, conditions that influence formation or metabolism of FICZ may lead to misinterpretation of experimental data. This could be demonstrated by treatment of cells with the CYP1 inhibitor αNF, which caused a prolongation of the normally transient AhR activation induced by FICZ treatment. Similarly, αNF treatment alone increased the AhR activation induced by the medium change, i.e., in this case αNF behaved like an agonist, although mechanistically it is an inhibitor of FICZ metabolism. Together with earlier observations of high constitutive CYP1A1 expression in metabolically deficient cells (23, 34, 35), this illustrates the fact that AhR activation can be obtained in the absence of exogenously added AhR ligands or by compounds having low or insignificant AhR affinity if they inhibit the intracellular breakdown of FICZ. In summary, this study advances significantly our knowledge concerning the mechanisms of formation, biochemical properties, and metabolic fate of FICZ.
Tryptophan Photoproduct FICZ Is Present in Humans

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