METABOLISM OF INDIRUBIN AND INDIGO, ENDOGENOUS ARYL HYDROCARBON RECEPTOR LIGAND CANDIDATES, AND COMPETITIVE EFFECT WITH RESPECT TO 2,3,7,8-TETRA-CHLORODIBENZO-*p*-DIOXIN (TCDD)

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Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-binding transcription factor which was isolated as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) receptor in the cell, but remains an orphan receptor. Most of the effects of TCDD are mediated by cytosolic AhR. Binding of TCDD to AhR is considered to be the initial event leading to the manifestation of the biological and toxicological responses elicited by TCDD[1]. Indirubin and indigo were identified as AhR ligands in human urine and serum by means of a recombinant yeast assay[2]. The AhR ligand activity of indigo in this assay was about the same as that of TCDD, and the potency of indirubin was 50 times higher. The two compounds are plant products that have been used to dye cloth. Indirubin and its analogs have been used in traditional Chinese medicine to treat human chronic myelocytic leukemia, and they were reported to act by inhibiting cyclin-dependent kinases [3]. Pathways for the endogenous production of indirubin and indigo in the human body were postulated by Gillam *et al.*[4] In the previous report, we demonstrated that indirubin and indigo are potent inducers of cyp1a1/2 in mouse liver [5]. Since the inducing effect was abolished in AhR knock-out mice, the inducing effects should be mediated by AhR. Though indirubin has a higher AhR binding affinity than TCDD, its ability to induce drug-metabolizing enzymes *in vivo* is weaker than that of TCDD. Thus, these compounds might block the AhR-mediated toxicity of dioxins *in vivo.* However, their metabolism has not been examined. In this study, we examined the metabolism and excretion of indirubin, indigo and indigocarmine in rats and mice, and their competitive effect with respect to TCDD and MC *in vivo* and *in vitro*.

Materials and Methods

Animals, *in vivo* treatment and metabolism studies. Male C57BL/6JJcl mice (5-6 weeks old) from CLEA Japan, Inc. (Tokyo, Japan) were given indirubin (1-50 mg / kg body weight) dissolved in Panacete 810^{TM} (5 mL / kg) by gavage for three days. Vehicle control mice were given the same volume of Panacete 810^{TM}. For the competition experiment, indirubin was given for 4 days and TCDD or MC was given on the second day. Male Slc:SD rats (6-8 weeks old) from Japan SLC, Inc. (Shizuoka, Japan) were given indirubin (10-50 mg/kg) by gavage at 6, 12, 24 or 48 hours after TCDD or MC, and urine and feces were collected. Indirubin and its metabolites in urine and feces were extracted and measured with HPLC.

Preparation of liver preparations and enzyme assays. Mice were killed and the livers were quickly removed. Microsomes and cytosol were prepared according to usual methods. The ethoxyresorufin-*O*-dealkylase (EROD), methoxyresorufin-*O*-dealkylase (MROD) and pentoxyresorufin-*O*-dealkylase (PROD) activities in liver microsomes were assayed by a fluorophotometric method.

***In vitro* metabolism of indirubin and indigocarmine.** Indirubin and indigocarmine were incubated with liver microsomes or cytosol in 0.1 M Na-K phosphate buffer (pH 7.4), then metabolites and the substrate were measured by means of HPLC.

Hepatocyte preparation and incubation. Rat hepatocytes were isolated from male rats by collagenase perfusion as described previously. Hepatocytes ($2x10^6$ cells/ml) were suspended in Williams' E medium supplemented with 7 % fetal calf serum. After 7 hr of incubation at 37^oC under an atmosphere of 5 % CO_2 and 95 % air, the cells were harvested and the medium was changed. After an additional 17 hr of incubation, the enzyme inducers were added to the cell suspensions. Dimethyl sulfoxide was used for the solubilization of these inducers. After treatment of hepatocytes with the enzyme inducers, EROD activities were assayed and mRNA was measured by RT-PCR. Cell viability after isolation, estimated by means of the trypan blue exclusion test, was always greater than 90 %.

Enzyme assays in HepG2 cells. Human hepatocarcinoma HepG2 cells were grown to confluence at 37^oC under 5% CO_2 in MEM (Sigma Chemical Co.) with 5 % fetal bovine serum (Life Technologies, Rockville, MD). The cells were seeded in 24-well plates at $2.5x10^4$ cells/well and chemicals were added the next day. One hour after addition of the enzyme inducers, the EROD assay was performed. In this study, about 0.5 mg protein of HepG2 cells was used.

Results and Discussion

Time course of liver CYP activities after indirubin treatment in mice *in vivo*.

The microsomal alkyoxyresorufin-*O*-dealkylase activities, EROD, MROD and PROD, of male C57BL/6JJcl mice after treatment with indirubin (50 mg / kg body weight) by gavage for three days were examined. The EROD and MROD activities were induced to the extents of 1.9- and 2.7-fold at 24 hours after the last dose, then decreased time-dependently and returned to the original level after three days. In the case of TCDD the induction levels were still more than 70% of the peak after 4 weeks, whereas in the case of indirubin, they decreased promptly.

In vitro metabolism of indirubin by rat liver microsomes and cytosol.

In vitro metabolism of indirubin, indigo and indigocarmine was examined using rat liver preparations. When indirubin was incubated with rat liver microsomes, it was metabolized time-dependently. Liver microsomes from MC-treated rats were most effective compared with microsomes from untreated and phenobarbital-treated rats. The metabolic activities were inhibited by general CYP inhibitors, SKF-525A and carbon monoxide, and a specific inhibitor of CYP1A, α-naphthoflavone. Radical trapping agents, α-tocopherol, ascorbic acid and mannitol did not inhibit the activities. These facts indicate that indirubin is metabolized by CYP1A1/2 and that radical intermediates are not generated. Indigocarmine, indirubin and indigo were also reductively metabolized by liver microsomes and cytosol. Their leuco-form metabolites were detected under anaerobic conditions. The metabolites were auto-oxidized to the mother compounds by oxygen.

***In vivo* metabolism of indirubin**

In vivo metabolism of indirubin was examined using HPLC. Urine of rats dosed with indirubin (10 mg/kg) was treated with β-glucuronidase/arylsulfatase and extracted with ethyl acetate. Unchanged indirubin (about 0.1% of the dose) was detected in 24-hour urine.

Competitive effect of indirubin with respect to TCDD or MC for AhR in mice *in vivo*.

Indirubin (5 or 100 mg/kg) was administered to mice with TCDD (30 and 625 ng/kg) or MC (25 mg/kg). Indirubin was administered for 4 days, and TCDD or MC was given after indirubin on the second day. Liver CYP activities were then measured, but no significant difference of induction or mRNA levels were observed (Fig. 1).

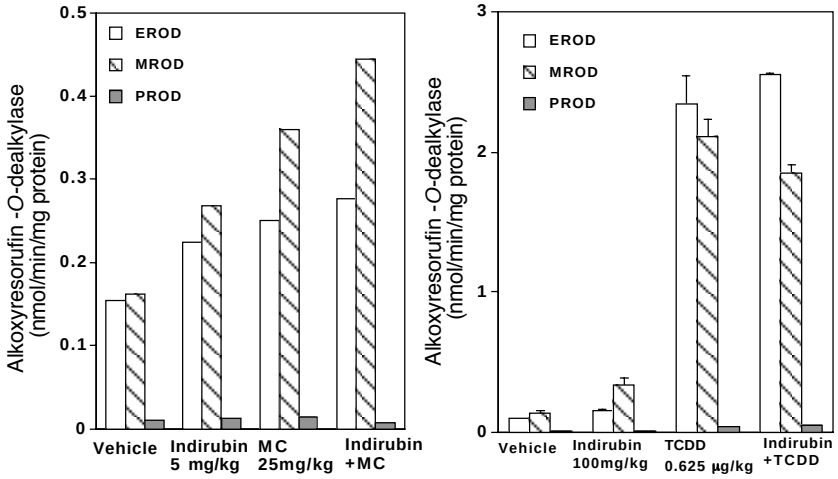

Fig. 1. Competitive effect with respect to MC (left), and TCDD (right) for AhR in mice *in vivo*

Effects of indirubin on rat hepatocytes and HepG2

The enhancing effect of indirubin and indigo on EROD activities in rat hepatocytes and HepG2 cells was examined. EROD activities in rat hepatocytes and HepG2 cells were increased by the addition of indirubin and indigo, as well as TCDD and 3-MC. The effect of indirubin after 10 hr was intermediate between those of TCDD and 3-MC in the concentration range of $1x10^{-9}$ - $1x10^{-5}$ M in HepG2 cells. When indirubin (10^{-6} M) was added with various concentrations of TCDD, a slight blocking effect on the action of TCDD was observed. However, a synergistic effect was observed with MC.

In this study, we observed a competitive effect of indirubin with respect to TCDD or MC *in vivo* and *in vitro*. The induction of liver CYP activities by indirubin was lower than that of TCDD and indirubin did not affect the inducing effect of TCDD. Indirubin was metabolized by liver microsomal cyp1a1/2, and reductive metabolism was catalyzed by cytosolic enzymes. The postulated metabolic pathways are illustrated in Fig. 2.

In conclusion, indirubin and indigo are easily metabolized and excreted *in vivo*. A blocking effect of indirubin on TCDD toxicity *in vivo* was not observed in this study.

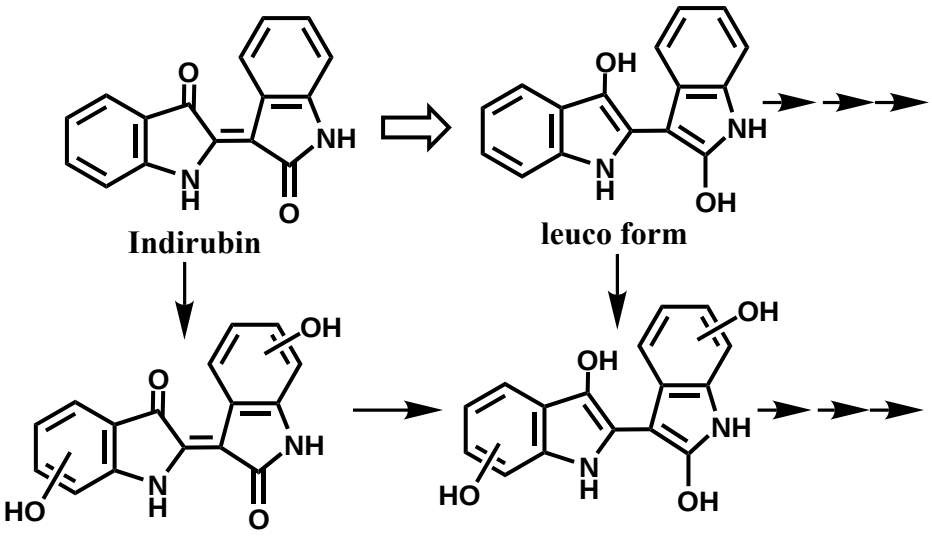

Fig. 2. Postulated metabolic pathways of indirubin in rats and mice

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