URINE CONCENTRATIONS OF INDIRUBIN IN RATS AND HUMANS AND ITS POSSIBLE INTERACTION WITH OTHER ARYL HYDROCARBON RECEPTOR LIGANDS

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Abstract
Urine concentrations of indirubin, a potent aryl hydrocarbon receptor (AhR) ligand, were analyzed in rats and humans using indirubin-specific ELISA assay. Indirubin concentrations were higher in rats (406±54 nM, and 266±36 nM for male and female respectively) as compared to those in humans (76±12 nM, and 68±12 nM for male and female respectively). No significant differences for indirubin concentrations in urine were observed among four estrous-cycle stages in female rats. AhR-specific reporter-gene assay in yeast was used to test xenobiotic-response element (XRE) specific gene-induction activities of AhR ligands. Indirubin showed relatively low EC_{25} of 20 nM, whereas other natural AhR ligands, indigo, indole-3-carbinol, and genistein showed EC25 of from 460 to 4100 nM, and beta–naphthoflavone, a synthetic ligand showed EC25 of 40 nM. Combinations of indirubin with one of the other ligands showed higher gene induction activities than simple addition of activity of each compound in this assay. Possible physiological role of indirubin as a candidate of endogenous ligands for AhR is discussed.

Introduction
Aryl hydrocarbon receptor (AhR) was shown to be indispensable for dioxin induced deleterious effects in mice[1]. AhR is ubiquitous from nematodes to humans and is thought to play important functions in organisms, however still not much was known about its physiological roles and its endogenous ligands. Indirubin was found to be a potent ligand of AhR in sewage and was shown to be derived from human urine[2]. Since indirubin is a metabolite of tryptophan, we assumed it as one of potential endogenous ligands for AhR, and to be a clue to elucidate physiological roles of AhR. We developed a sensitive enzyme-linked immunosorbent assay (ELISA)[3] for indirubin and investigated concentrations of indirubin in urines from rats and humans. We also assayed AhR-mediated gene induction activities of several natural AhR ligands including indirubin, using yeast reporter gene assay, to examine whether they can be effective as AhR ligands at the concentrations naturally available to humans and other organisms.

Materials and Methods
A sensitive ELISA assay specific for indirubin[3] was developed to analyze indirubin concentrations in biological samples. Indirubin (purity 99%) and its antibody were prepared. Other chemicals (pure chemical grade) were purchased from test-chemical suppliers in Japan. Chemicals were dissolved either in distilled water or in dimethylsulfoxide and appropriately diluted by phosphate buffer (pH7.5, 50 mM) before use. Urine samples were appropriately diluted with the phosphate buffer. Urine was collected from 30 male and female rats (BrLHan:WIST@Jcl(GALAS), ages 10 to 11 week) using metabolic cages in a laboratory of the Ina Research Inc.and kept frozen until use. Human urine was collected from healthy young volunteers (10 males and 9 females aged from 21 to 36) with informed consent. Since indirubin can be easily metabolized by CYP1A1 enzyme which it induces, urine was treated with β– D-glucuronidase/sulfatase to hydrolyze conjugated-forms potentially present in urine.

AhR reporter-gene assay was conducted using *Saccharomyces cerevisiae* strain YCM3 carrying human AhR and XRE responsive β–D-galactosidase reporter genes originally developed by Miller[4], kindly given by Dr. Tomonari Matsuda of Kyoto University. Briefly, yeast cell was incubated with test chemicals overnight for gene induction, collected and assayed for β–D-galactosidase reporter gene induction on the next day. β–Naphthoflavone was

used as the positive control. In addition to indirubin, indigo, indole-3-carbinol, genistein, daidzein, and resveratrol which were reported as agonists or antagonists of AhR were used as samples for yeast gene induction assay.

Results and Discussion

(1) Indirubin concentrations in urine

Indirubin concentrations in rat urine are shown in Table 1. Male rats (analysis of remaining samples underway) showed higher indirubin concentrations in urine than female rats. Female rats showed not significant differences in indirubin concentrations during their estrous cycle. Significant increase (166 and 144% for male and female, respectively) of indirubin detection after β–D-glucuronidase/sulfatase treatment suggests part of indirubin was metabolized and conjugated before excretion in urine. The data corresponds to 26 pmole/mg creatinine, and 31 pmole/mg creatinine for male and female respectively

Table 1 Indirubin concentrations in rat urine (nM)

	Average ± S.D.		Samples (n)
	+ Enzyme treatment	- Enzyme treatment	
Male	406 ± 54	244 ± 22	7
Female	266 ± 36	185 ± 29	120
Female P-E	259 ± 45	183 ± 33	30
Female E-M	266 ± 42	185 ± 34	30
Female M-D	272 ± 30	189 ± 26	30
Female D-P	265 ± 28	183 ± 25	30

P: proestrus, E: estrus, M: metestrus D: diestrus, Enzyme: β–D-glucuronidase/sulfatase

Human urine concentrations of indirubin are shown in Table 2. Although the number of samples was limited, it is apparent that human urine concentrations were much lower than those in rats. β–D-Glucuronidase/sulfatase treatment increased detection of indirubin to threefold (i.e., higher ratio of conjugated form in urine as compared to in rats) suggested that indirubin metabolism/conjugation reactions are more efficient in humans than in rats.

Table 2 Indirubin concentrations in human urine (nM)

	Average ± S.D.		Samples (n)
	+ Enzyme treatment	- Enzyme treatment	
Male	76 ± 12	26 ± 8	10
Female	68 ± 12	25 ± 5	9

Enzyme: β–D-glucuronidase/sulfatase

Isatin and oxindole, precursors of indirubin, did not show cross-reactivity, but several hydroxyl-derivatives of indirubin showed various levels (50 to 90% compared to indirubin) of reactions. Currently, we are trying to determine indirubin and its metabolites quantitatively using GC-MS, and LC tandem MS technique.

(2) Reporter-gene assay of AhR-mediated activity

Fig.1 shows chemical structures of test chemicals for reporter-gene assay. AhR has been known to interact with many polycyclic aromatic hydrocarbons, however other chemicals containing indole or flavone structure are found to react with AhR to express XRE-specific gene in CALUX (chemical activated luciferase gene expression) assay using mouse hepatoma cell line[5]. Resveratol was shown to be an antagonist to this reaction[6]. β–Naphthoflavone which is a synthetic chemical, was used as a positive control.

In our assay, indirubin showed the highest AhR-ligand activity among test chemicals with lowest 12.5% (of the highest activity in the test) effective concentration ($EC_{12.5}$) of 12 nM (Table 3). Other natural ligands, indigo,

indole-3-carbinol, genistein were much weaker in XRE-specific gene expression activity with $EC_{12.5}$ of 210-2300 nM. Daidzein and resveratrol did not show agonist activity. We examined the activity in combination exposure to these chemicals using indirubin as one partner existing endogenously in the body to others which may be

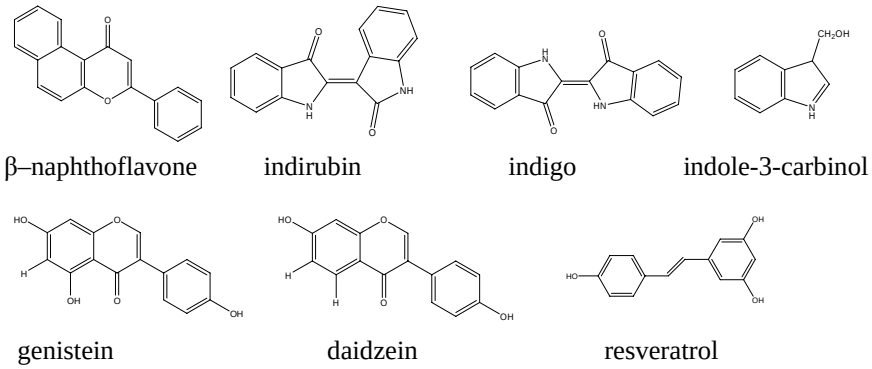

Fig. 1 Chemical structures of the gene induction assay samples

Table 3 Effective concentrations of various chemicals for gene induction assay (nM)

	$EC_{12.5}$	EC_{25}	EC_{50}
indirubin	12 ± 6	25 ± 9	70 ± 10
β–naphthoflavone	19 ± 12	39 ± 22	110 ± 50
indigo	210 ± 40	460 ± 60	1400 ± 100
indole-3-carbinol	330 ± 210	660 ± 320	1900 ± 700
genistein	2300 ± 800	4100 ± 1400	9200 ± 2600

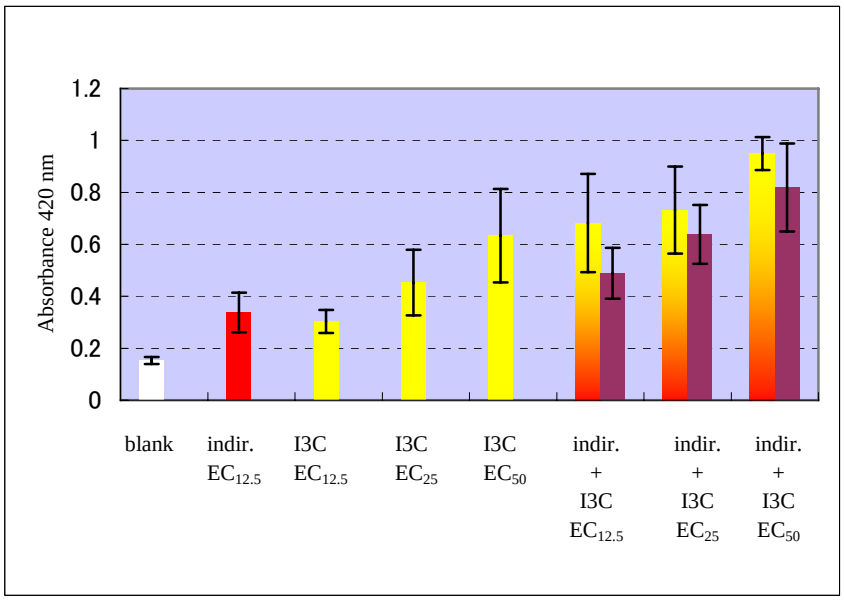

Fig.2 Example of combination assay: blank : buffer only, indir. : Indirubin, I3C: Indole-3-carbinol left bar of the two adjacent bars shows mixture activity, right bar of the two shows simple addition value

taken extraneously from foods. Fig.2 showed an example of the results in which combination exposure to indirubin and indole-3-carbinol expressed somewhat higher activity (142 ± 39% for indirubin $EC_{12.5}$ + indole-3-carbinol $EC_{12.5}$) than the simple addition of the activity of each component. Combination with indigo or genistein also showed higher (156 ± 50% or 189 ± 35%, respectively) than simple sum of each component activity.

(3) Possible role of indirubin in the human body

Relatively low $EC_{12.5}$ (12 nM) of indirubin in our reporter-gene assay and its concentrations in human urine (76 or 68 nM for male and female, respectively) suggest that indirubin may be present in enough amount to express its activity with AhR in human body. We are now measuring serum concentrations of indirubin in humans and rats.

Since indirubin was reported to be more sensitive in showing AhR activity than 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD)[2], and only trace amounts of dioxins are present in blood of Japanese (26 pg TEQ/g fat in the blood corresponding to ca 8 pg TEQ/L or 3 fM in blood) [7], it could be explained that exposure to extraordinarily excess amount of persistent dioxins can only interfere indirubin's physiological function by continuously expressing signals. True activity of indirubin in the human body needs to be examined, since originally yeast cells may not have CYP enzyme activity to metabolize indirubin.

In this regard, the significance of combination assay results needs further investigation. Vegetable constituents, such as flavones, flavonols, anthraquinones, resveratrol and coumestrol were shown to have inhibitory effects on the AhR activity induced by TCDD[6]. Indirubin is known to be a major component of herbal medicine effective to treat leukemia in China[8]. Indirubin is known to interact either directly or indirectly on cell-cycle regulation to arrest cell-cycle at G1-S stage[9]. Since complex cross-talk interactions between AhR and estrogen receptor were reported[10], it is interesting that some AhR reactive chemicals, such as genistein, daidzein and resveratrol show also estrogen receptor binding and gene induction activities.

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