INDUCTION OF THE MULTIDRUG-RESISTANT *Mdr1b* MEDIATED BY ARYL HYDROCARBON RECEPTOR IN MOUSE HEPATOMA Hepa-1c1c7 CELLS

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Introduction

Multidrug resistance (MDR) is a phenomenon of acquired cross-resistance to a large number of structurally and functionally unrelated cytotoxic drugs[1]. MDR1 protein is encoded by a small family of genes: There are two members in humans (*MDR1* and *MDR2*) and three in rodents (*mdr1a, mdr1b*, and *mdr2*)[2,3]. *MDR1, mdr1a* and *mdr1b* are known as a transporter of anticancer drugs, whereas *MDR2* and *mdr2* are known as a transporter of phospholipids. It is reported that the transcription of *mdr1b* gene is induced in the liver cells in response to treatment with various polycyclic aromatic hydrocarbons (PAHs) including 3-methylcholanthrene (3MC)[4-6]. Moreover, some of PAHs metabolites damage DNA severely and activate p53[7,8]. Thereby, activated p53 was shown to induce *mdr1b* through the binding to its promoter region in mouse hepatoma Hepa-1c1c7 cells after treatment with 3MC[9]. These results suggested that *mdr1b* gene expression is p53-dependent and indirectly regulated by the AhR. On the other hand, the induction of *mdr1b* was not observed in the liver of mice treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[10]. However, we reported that the TCDD-activated AhR bound to a stretch of DNA corresponding to the promoter region of *mdr1b* gene[11]. This suggested that *mdr1b* is one of the possible transcriptional targets of the AhR. Thus, involvement of the AhR in the regulation of *mdr1b* has been a controversial issue to be clarified. In this study, we investigated whether the transcriptional regulation of *mdr1b* depends on the AhR in Hepa-1c1c7 cells.

Materials and methods

Chemicals. TCDD and 3MC as the AhR ligands were obtained from AccuStandard (New Haven, CT, USA) and Sigma Chemical (St. Louis, MO, USA), respectively.

Cell culture and treatment conditions. The mouse hepatoma Hepa-1c1c7 was maintained in Eagle's minimum essential medium (Nissui Pharmaceutical, Kyoto, Japan) supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in atmosphere containing 5% CO_2. The cells were treated with 3MC, 1 nM TCDD or dimethyl sulfoxide (DMSO) as a solvent control for 24 h.

WST-1 cell proliferation assay. The cells were seeded at 1.5×10^4 cells per well in a 96-well plate in the presence or absence of 0.5, 1, 2, 5, 10, 20, 30 μM 3MC. In the control and test groups, 10 μl of WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) was added per well, and the cells were incubated at 37 °C for 2 h. Absorbance at 450 nm was determined using a microplate reader (Wallac ARVO SX 1420 multilabel counter, Perkin-Elmer Life Sciences, Boston, MA, USA) .

EROD activity. The cells were seeded at 1.5×10^4 cells per well in a 96-well plate. The medium was changed to a fresh one containing 1 μM 7-ethoxyresorufin as a substrate and 10 μM dicumarol. After the cells were incubated for 1 h at 37 °C, an aliquot of 75 μL of the medium was withdrawn from each well and transferred to another 96-well plate, and the EROD activity was measured as previously described[12].

Quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR analyses were performed based on the SYBR green gene-expression technology[13,14]. Total RNA extracted from Hepa-1c1c7 cells stimulated with 10 μM 3MC, 1 nM TCDD or 0.1% DMSO as a vehicle control for 24 h was reverse transcribed into cDNA using the Primescript RT reagent kit (Takara Bio, Shiga, Japan) and subjected subsequently to quantitative real-time PCR (45 cycles of 95°C for 15 s, 56°C for 20 s and 72°C for 10s) in a Cycler Dice Real Time System TP860 (Takara Bio) using a SYBR Premix EX-Taq perfect real time kit (Takara Bio). The relative gene expression values were calculated by the comparative CT method[15] using expression of the β-actin gene (*actb*) as the internal control.

Transient transfection and luciferase assay. The cells were seeded at 7.5×10^5 per well in a 24-well plate. The cells were transiently transfected using lipofectamine and plus reagent (Invitrogen, Carlsbad,

California) with pGL4 luciferase reporter constructs. Briefly, the cells were incubated with lipofectamine regents and DNA mixtures for 24 h, and they were cultured in fresh medium. After the cells were treated with 3MC or TCDD for 24 h, luciferase activity in the cells was measured using a dual luciferase assay kit (Promega, Madison, WI, USA) and Wallac ARVO SX 1420 multilabel counter.

Results and discussion

***Mdr1b* is suggested as a target gene of the AhR.** In some cases, AhR/Arnt is reported to bind to DNA without DRE consensus. For instance, the AhR is able to bind to a DRE-like sequence (GCGGG) in the *PON1* promoter[16] and to the CYP1A2 enhancer[17]. These findings suggest that the AhR sometimes binds to the DRE-like (or-related) sequence. It was also shown that the expression of *mdr1b* was induced by 3MC, and this induction depended upon the AhR[9]. These results implied that *mdr1b* might be an additional target gene of the AhR, and its transcriptional induction is mediated through binding of the AhR to the promoter region of *mdr1b*.

Determination of 3MC concentration. Cytotoxic effect of 3MC in Hepa-1c1c7 cells were determined by WST-1 assay. 3MC induced high cytotoxicity (<80%) when cells were treated with more over 5 μM (Figure 1), thereby the concentration of 3MC was determined to be up to 2 μM. 3MC is known to induce the expression of drug metabolizing enzymes such as CYP1Al through the AhR-dependent pathway. Therefore, EROD activity, namely the enzymatic activity of the CYP1A subfamily, was measured. 3MC increased EROD activity dose-dependently, and the strongest induction was observed at 0.25 μM (Figure 2).

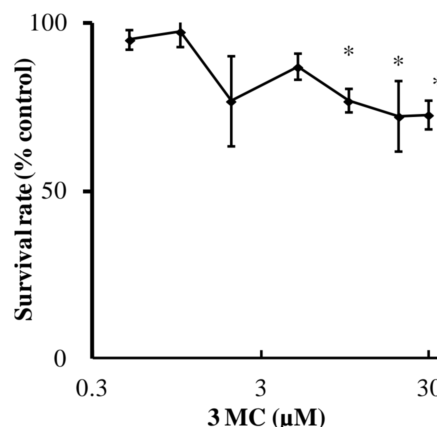

Figure 1. Cytotoxicity of 3MC.
Hepa-1c1c7 cells were treated with 0, 0.5, 1, 2, 5, 10, 20, 30 μM 3MC for 24h, and cytotoxicity was measured by WST-1 assay. Data represented as the means ± SD (n=3). Asterisks indicate statistical significance from the Hepa-1c1c7 cells treated with DMSO as a vehicle control (p < 0.01, Dunnett).

Transcriptional induction of *mdr1b*. The quantitative real-time RT-PCR technique was used to examine expression of *mdr1b* and *cyp1a1* in Hepa-1c1c7 after the treatment with either 1 nM TCDD or 0.25 μM 3MC. *Cyp1A1* expression increased after the treatment with both TCDD and 3MC in Hepa-1c1c7 cells (Figure 3A). In the case of *mdr1b* expression, the transcript level also increased after the treatment with 3MC, but did not with TCDD (Figure 3B). It is known that both *cyp1a1* and *mdr1b* were induced through AhR translocation[9]. However, *cyp1a1* was induced upon either 3MC- or TCDD-stimulation while the induction of *mdr1b* exclusively required 3MC, suggesting that differential activation of AhR by 3MC or TCDD.

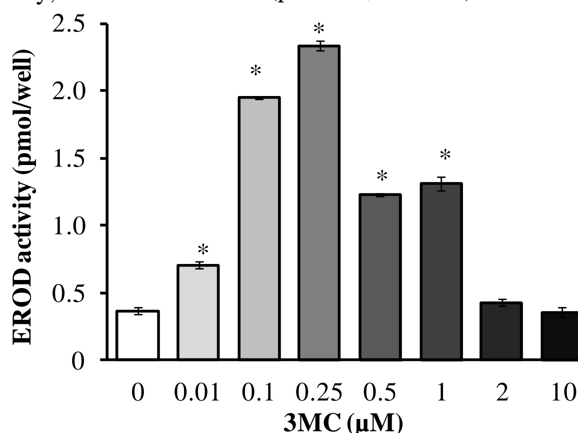

Figure 2. 3MC increased EROD activity.
Hepa-1c1c7 cells were treated with 0, 0.01, 0.25, 0.5, 1, 2, 10 μM 3MC for 24h, and EROD activity was measured. Data represented as the means ± SD (n=3). Asterisks indicate statistical significance from the Hepa-1c1c7 cells treated with DMSO as a vehicle control (p < 0.01, Dunnett).

Deletion analysis of the *mdr1b* promoter region. To investigate the *mdr1b* promoter function, we performed a series of deletion analysis of the *mdr1b* promoter region leading expression of a reporter luciferase gene. Without any inducer compounds, P*mdr1b*, whose construct (-245 to +423) carrying the full-length promoter region, could lead the luciferase activity, suggesting that its basal promoter activity. When Hepa-1c1c7 cells were transfected with P*mdr1b*, 3MC induced the luciferase activity while TCDD did not (Figure 4), and this result was consistent with the results from the RT-PCR analysis (Figure 3). The significantly increase in the luciferase activity induced by 3MC was abolished by

deletion of the region -245 to +43 as in the P+44+423 construct (Figure 4), indicating that this region was indispensable for the induction in response to 3MC. This construct lacking the promoter region, containing the +1 position, still retained the promoter activity. When the luciferase activity of the P-245+43 construct was compared with that of the full-length construct, the 3MC-induced activity was reduced by deletion of position +44 to +423 but still higher than that of the P+44+423 construct (Figure 4). In addition, DMSO-treated control activity was also reduced by deletion of position +44 to +423. These results suggest that this region -245 to +44 containing the p53 site is involved in the basal transcriptional activity as well as the induction with 3MC of *mdr1b* promoter. On the other hand, the region +44 to +423 was required for the maximum levels of induction by 3MC, suggesting that AhR might be partially involved in the *mdr1b* induction with 3MC. From these results, it is implied that both p53- and AhR-binding to the *mdr1b* promoter region might cooperatively enhance the *mdr1b* expression. 3MC activates the AhR and induces the downstream genes for drug-metabolizing enzymes including *cyp1a1*, and the induced the enzymes produce the suicidal metabolites of 3MC to cause DNA-damage accompanied by the activation of p53[18,19]. The activated p53 and AhR bind to the cognate binding sites within the *mdr1b* promoter region and can induce *mdr1b* cooperatively. Further studies are needed to investigate the physiological consequences of AhR- and p53-mediated *mdr1b* induction.

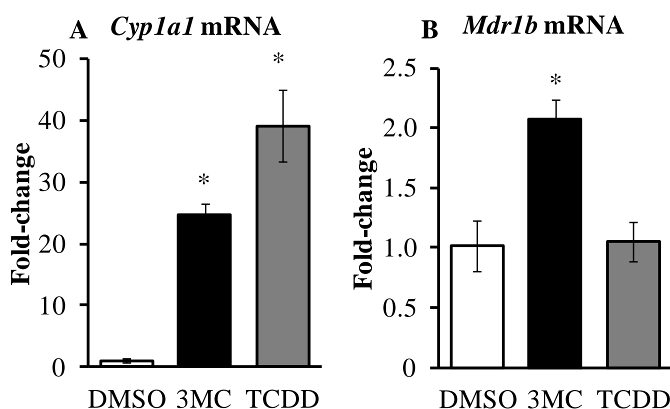

Figure 3. The expression of *mdr1b* was induced by 3MC.
Total RNA was prepared from Hepa-1c1c7 treated for 24 h with 1 nM TCDD (black bars), 0.25 μM 3MC (gray bars), or DMSO (white bars) as a vehicle control and subjected to quantitative real-time RT-PCR analysis. A, *cyp1a1*; and B, *mdr1b* expression. Data represented as the means ± SD (n=3). Asterisks indicate statistical significance from the Hepa-1c1c7 cells treated with DMSO ($p < 0.01$, Tukey-Kramer).

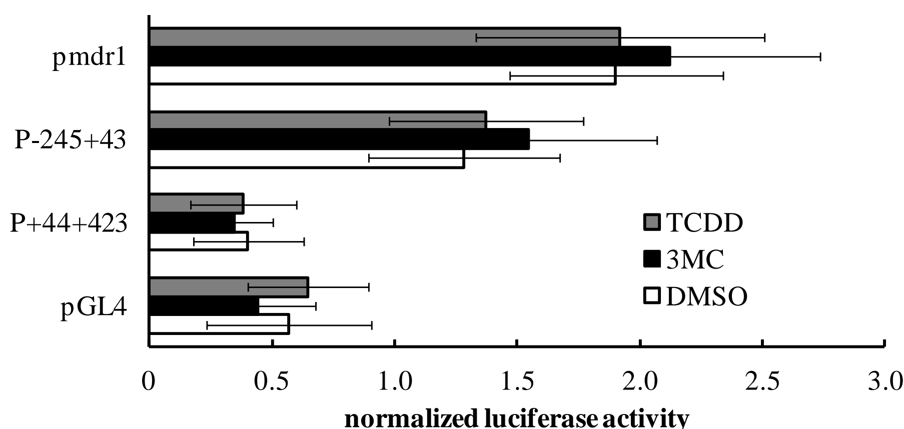

Figure 4. Deletion analysis of the *mdr1b* promoter region.
Hepa-1c1c7 cells were transfected with the plasmid constructs: The full length *mdr1b* promoter region (Pmdr1b); p53 binding region (P-245+43); DRE-like region (P+44+423); and the promoter less pGL-basic vector (pGL4). The transfected cells were treated with 1 nM TCDD (black bars), 0.25 μM 3MC (gray bars), or DMSO (white bars) as a vehicle control for 24 h and the luciferase activity was measured.

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