ARYL HYDROCARBON RECEPTOR ENHANCES THE EXPRESSION OF MULTIDRUG-RESISTANT *mdr1b* THROUGH p53 IN MOUSE HEPATOMA 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 transpoter of phospholipids and overexpressed in drug-sensitive cells. It is reported that 3-methylcholanthrene (3MC) promote the transcription of *Mdr1b* gene[4-6]. It is also reported that aryl hydrocarbon receptor (AhR) enhances the expression of Cytochrome 1A1 (CYP1A1), and CYP1A1 metabolizes the 3MC. Certain metabolites of PAHs damage DNA severely and activate p53[7,8]. Thereby, activated p53 induces Mdr1b through the binding to its promoter region in mouse hepatoma Hepa-1c1c7 cells after treatment with 3MC[9]. On the other hand, 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD) did not affect the induction of Mdr1b in the liver of mice[10]. These results suggested that Mdr1b gene expression is a p53-dependnet manner indirectly regulated by the AhR. However, in some cases, AhR/ARNT is reported to bind to DNA without dioxin responsive element (DRE) consensus. For instance, the AhR is able to bind to a DRE-like sequence (GCGGG) in the PON1 promoter[11] and to the CYP1A2 enhancer[12], suggesting that the AhR binds to the DRE-like sequence. It was also shown that the expression of Mdr1b was induced by 3MC, and this induction dependent upon the AhR. Furthermore, we reported that the TCDD-activated AhR bound to stretch of DNA corresponding to the promoter region of Mdr1b gene[13]. 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. 3MC and TCDD were obtained from Sigma-Aldrich (St. Lous, MO, USA) and AccuStandard (New Haven, CT, USA), respectively.

Cell culture and treatment conditions. The mouse hepatoma Hepa-1c1c7 cells were obtained from the American Type Culture Collection, were maintained in Eagle's minimum essential medium (Nissui Pharmaceutival Kyoto, Japan) supplemented with 5% fetal bovine serum, 4 mM L-glutamine, 100U/ml penicillin, and 100 µg/ml streptomycin at 37ºC in atmosphere containing 5% CO_2. The cells were treated with various concentration of 3MC, 1nM TCDD, or dimethyl sulfoxide (DMSO) for 24 h.

Southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA). SW-ELISA was performed as described previously[14,15] . Briefly, FITC-labeled DNA probes used were a DRE sequence from the promoter region of *Cyp1a1* and DNA fragment corresponded to the promoter region of *Mdr1b*[14], whose genomic position of chromosome number was start-end as chr5:8798186-8798565. Each of the FITC-labeled probes (25 fmoles) was put into the respective wells of a 96-well microtiter plate, and captured by a rabbit anti-FITC antibody (Dako Cytomation, Glostrup, Denmark), which was previously fixed on the bottom. The nuclear extract was prepared from Hepa-1c1c7 cells after treatment with 1 nM TCDD for 2 h as previously described[13], and served as the source of the AhR-ARNT heterodimer. The nuclear extract (15 mg) was applied to the wells containing the probe and incubated at 20ºC for 2 h. After washing, goat anti-ARNT antibody (Santa Cruz Biotechnology, CA, USA) was added to form the immune complexes containing the AhR-ARNT heterodimer bound to the probe. After another washing step, the immune complex was further reacted with biotinylated rabbit anti-goat IgG antibody (Jackson Immuno Research Laboratory, West Grove, PA), which was visualized by the labeled-streptavidin biotin method with peroxidase-conjugated streptavidin and tetramethylbenzidine. After sulfuric acid was added to terminate the visualizing reaction, absorbance at 450 nm in the wells was

measured by using a Wallac ARVO sx multilabel counter (Perkin-Elmer Life Sciences, Boston, Massachusetts). The AhR-binding score of the probe, representing its specific affinity for AhR, was calculated by dividing the absorbance obtained in the presence of the probe by that in the absence of the probe.

Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR (qRT-PCR) analysis was performed based on the SYBR green gene-expression technology. Total RNAs were 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^{o}C for 10 s and 60^{o}C for 20 s) in a LightCycler detection system (Roche Molecular Biochemicals) using a SYBR Premix EX-Taq perfect real time kit (Takara Bio). The relative gene expression values were calculated by the comparative CT method, using expression of the *β-actin* as the internal control. Gene-specific primers used for *Mdr1b*, *Cyp1a1*, and *β-actin* were as follows: *Mdr1b* (forward 5'-AGTGGACCCAACAGTACTCTGAT-3', reverse 5'-GCACCAATCCCGGTGTAATA-3'), *Cyp1a1* (forward 5'-TTGGCCACTTTGACCCTTA-3', reverse 5'-TCAAGTCCTTGAAGGCATC-3'), and *β-actin* (forward 5'-AAGGCCAACCGTGAAAAGAT-3', reverse 5'-GTGGTACGACCAGAGGCATAC-3').

Western bolt

Hepa-1c1c7 cells were incubated in the presence or absence of 10 μM 3MC or 1 nM TCDD for 24 h. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin). Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting using rabbit polyclonal anti-β-actin and anti-phospho-p53 (Ser15) (Cell Signaling, Danvers, MA, USA) antibodies, and rabbit polyclonal anti-p53 (Santa Cruz Biotechnology) antibody. Primary antibodies were immunoreacted with horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected using the Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Transient transfection and luciferase assay

Hepa-1c1c7 cells were cultured on a 48-well plate for 24 h. The cells were transiently transfected using lipofectamine and plus reagent (Invitrogen, Carlsbad, California) with pGL4 luciferase reporter constructs for 6 h. After the medium was replaced with fresh medium, the cells were incubated in the presence or absence of 10 μM 3MC or 1 nM TCDD for 24 h. Transfection efficiency was normalized using pRL-SV40 (control reporter vector). The cells were lysed, and firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase reporter assay kit and the Wallac ARVO SX 1420 multilabel counter. Data are expressed as the relative light units (RLU, the activity of firefly luciferase divided by that of *Renilla* luciferase).

Results and discussion

Interaction of AhR with promoter region of *Mdr1b* gene. The southwestern chemistry-based enzyme-linked immunosorbent assay (SW-ELISA) technique was used to assess binding of AhR to the *Mdr1b* probe, which contains the promoter region of *Mdr1b*. Nuclear extracts were prepared from Hepa-1c1c7 cells treated with or without 1 nM TCDD and subjected to the SW-ELISA using the *Mdr1b* probe. Another probe for the *Cyp1a1* promoter region, which is known to contain a direct AhR-binding site, was also employed in the assay. In Hepa-1c1c7 cells the treatment with TCDD enhanced binding of AhR to either *Mdr1b* or *Cyp1a1* probe (Table 1). These results suggest that AhR is able to bind to the promoter region (+44 to +423) of *Mdr1b* even though the region does not contain the consensus sequence for DRE.

Table 1. AhR binds to the promoter region of *Mdr1b*.

	Vehicle	1 nM TCDD
Cyp1a1	1.85 ± 0.13	3.76 ± 0.39*
Mdr1b	1.48 ± 0.19	2.61 ± 0.14*

Binding of the AhR to *Mdr1b* probe was estimated by SW-ELISA as described in the Materials and Methods. The AhR-binding score of a probe, representing its specific affinity for AhR, was calculated by dividing the absorbance obtained in the presence of the probe by that in its absence. The data are presented as means ± S.E. (n=3). *$p < 0.05$ vs. vehicle

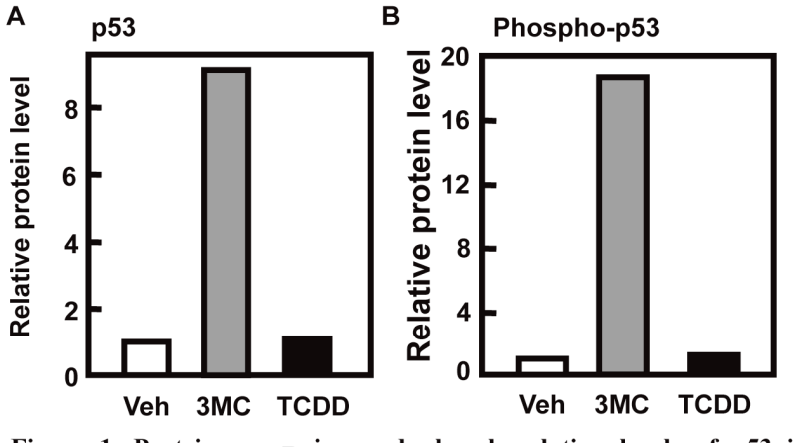
Effect of 3MC and TCDD on the expression of *Mdr1b gene* in Hepa-1c1c7 cells. The qRT-PCR technique was used to examine expression of *Mdr1b* and *Cyp1a1* in Hepa-1c1c7 after the treatment either with 3MC or TCDD as the AhR ligands. 3MC and TCDD increased the expression of Cyp1a1 (Table 2). On the other hands, the mRNA level of *Mdr1b* also increased by 3MC, but not by TCDD. From these data, it is indicated that both *Cyp1a1* and *Mdr1b* depend on AhR for their induction. However, *Cyp1a1* was induced upon either 3MC or TCDD stimulation, while the induction of *Mdr1b* exclusively required 3MC.

Table 2. The *Mdr1b* expression induced with 3MC depended on AhR.

	Fold induction		
Gene	Vehicle	10 μM 3MC	1 nM TCDD
Cyp1a1	1.00 ± 0.15	19.13 ± 1.32*	18.76 ± 1.89*
Mdr1b	1.00 ± 0.10	5.03 ± 0.05*	0.09 ± 0.09*

Total RNAs were prepared from Hepa-1c1c7 cells treated with 10 μM 3MC, 1 nM TCDD, or DMSO as a vehicle control and subjected to qRT-PCR analysis. Data represented as means ± S.D. from three independent experiments. *$p < 0.05$ vs. vehicle

Protein expression and phosphorylation levels of p53 by 3MC and TCDD. To determine whether 3MC and TCDD enhance the phosphorylation of p53, Hepa-1c1c7 cells were incubated in the presence or absence of 3MC or TCDD. The expression level of p53 was increased by 3MC (Figure 1A), and 3MC further enhanced the phosphorylation of p53 at serine 15 (Figure 1B). In contrast, TCDD did not affect the expression level and phosphorylation level of p53. These results indicate that 3MC, but not TCDD is involved in the induction of p53.



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 (Figure 2A). Hepa-1c1c7 cells were

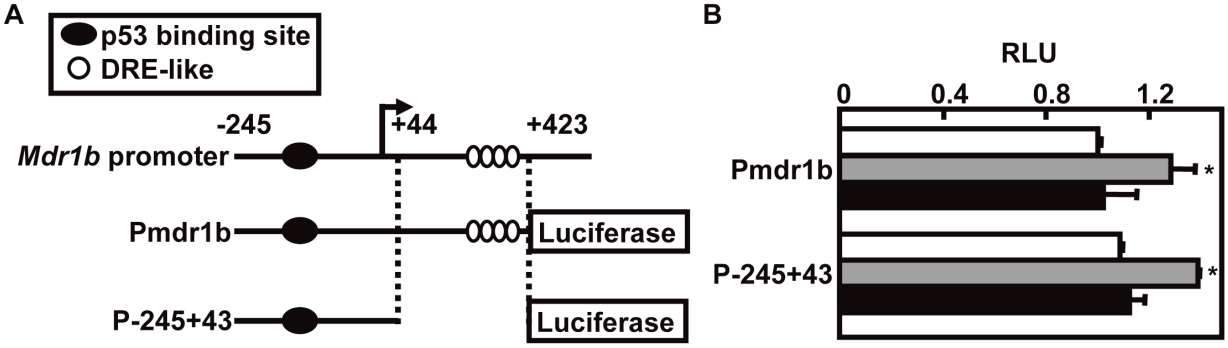


Figure 2. Deletion analysis of the *Mdr1b* promoter region.
(A) Luciferase reporter constructs of the mdr1b promoter. (B) Hepa-1c1c7 cells were transiently transfected with the reporter constructs shown in (A), followed by incubated with 10 μM 3MC (gray bars) or 1 nM TCDD (black bars), or DMSO (white bars) as a vehicle control. Luciferase activities were determined. The data are presented as means ± S.D. (n=3). Asterisks indicate statistical significance from control ($p < 0.05$, Student's t-test). The result is representative of three independent experiments.

transiently transfected with the Pmdr1b construct (-245 to +423) carrying the full-length promoter region.

Although 3MC induced the luciferase activity, TCDD did not induce the luciferase activity (Figure 2B), in agreement with the results from the previous RT-PCR analyses (Table 2). Furthermore, when the cells were transiently transfected with P-245+43 reporter construct, the luciferase activity was enhanced by 3MC, but not by TCDD. There results indicate that *Mdr1b* gene expression is indirectly regulated through AhR.

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