

Regular article

Establishment of a Human Hepatoma Cell Line HepG2-A10 for a Reporter Gene Assay of Arylhydrocarbon Receptor Activators

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To establish a human hepatic cell line for a convenient reporter gene assay of arylhydrocarbon receptor (AhR) activators, the chimera plasmid containing xenobiotic responsible element (XRE), minimal SV40 promoter, and luciferase reporter gene and the expression vector pRC/CMV containing a neomycin-resistant gene were co-transfected into a human hepatoma cell line, HepG2. Then, antibiotic (G418)-resistant HepG2 cells were selected and cloned. A cell clone, HepG2-A10, showed the highest responsibility to 3-methylcholanthrene (MC)-mediated induction of luciferase among the clones obtained. Expression levels of luciferase activity in HepG2-A10 cells were increased in a dose- and time-dependent manner by treatment with either MC, an AhR-ligand type activator, or omeprazole (OME), a non-AhR ligand type activator. In addition, expression levels of cytochrome P4501A subfamily genes (*CYP1A1* and *CYP1A2*) were also increased in a dose- and time-dependent manner by treatment with MC. The present findings demonstrate that a newly established human HepG2-A10 is a useful cell line for a convenient reporter gene assay of AhR activators.

Key words: AhR, reporter gene assay, HepG2, CYP1A, omeprazole

Introduction

Arylhydrocarbon receptor (AhR) exists in a cytosol as an inactive form, which is a complex with heat shock protein 90 (Hsp90). The AhR is activated through release from Hsp90 by AhR activators, moves into a nucleus, and forms a complex with nuclear AhR nuclear translocator (ARNT). The resultant AhR/ARNT complex binds to a xenobiotic responsible element (XRE) in 5'-upstream of the genes, including cytochrome P4501 (CYP1) family genes, and consequently activates the genes (1).

CYP1 family genes code the drug-metabolizing enzyme proteins such as CYP1A1 and CYP1A2, and these enzymes are responsible for not only the detoxifi-

cation of xenobiotics but also metabolic activation of carcinogens including arylhydrocarbons (2,3) and aromatic amines (4–6). Interestingly, there are species-, strain-, and sex-differences in induction of CYP1A subfamily enzyme(s) among experimental animals, and the differences are closely correlated with those in their carcinogenic susceptibilities (4–6). Furthermore, incidences of the carcinogenesis and endocrine disruption by AhR activators, such as arylhydrocarbons and dioxins, are known to be lower in AhR-null mice than the wild-type mice (7–10).

Species-differences between humans and experimental rodents in characteristics of AhR and AhR-activation pathway are also known. For example, indirubin induces more efficiently AhR-dependent transactivation of reporter gene in the yeast expressing human AhR than in the yeast expressing mouse AhR (11). Then, omeprazole (OME), a benzimidazole derivative, which indirectly activates AhR and consequently up-regulates an expression of CYP1 family genes (12,13), induces more efficiently the gene expression in human hepatocytes than in rodent hepatocytes (14,15). Therefore, to precisely detect AhR activators for humans, establishment of a convenient method with a human cell line is necessary. However, there are few cell lines established for such purpose (16–18).

Recently, we have established a rat hepatic cell line KanR2-XL8 for a convenient reporter gene assay of AhR activators (19). In the present study, we further tried to establish a human cell line for the reporter gene assay of AhR activators and succeeded in the establishment.

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Table 1. Sequences of the XRE regions used in the HepG2-based cell lines established for reporter gene assay of AhR activators

Cell line	Plasmid	Sequence of XRE region†	Location‡
HepG2-A10 101L	XRE-Luc (19)* pL1A1N (16)	5'-ctcttctCACGCaaactc-3'	Rat <i>CYP1A1</i> – 1024/– 1007
		5'-agctaggCACGCaaatac-3'	Human <i>CYP1A1</i> – 1392/– 1375
		5'-ccacagGCGTGgaccgaa-3'	– 1266/– 1249
		5'-gaagccaCACGCagacct-3'	– 1210/– 1193
		5'-ccaacccCACGCcgcgcg-3'	– 1120/– 1101
		5'-cccttcGCGTGactgca-3'	– 1064/– 1047
		5'-tccttctCACGCaagcc-3'	– 992/– 975
		5'-gccggcgCACGCaagcta-3'	– 904/– 887
		5'-cgcttctCACGCgagccg-3'	– 506/– 491
		5'-gcattctCACGCccagcg-3'	– 405/– 388
		5'-aaggagGCGTGgccacac-3'	– 61/– 44
		5'-gcagtGCGTGatctcag-3'	Human <i>CYP1A1</i> – 2142/– 2125
		5'-attacagGCGTGagccacc-3'	– 1882/– 1865
		5'-ttGCGTGcga-3'	
HepG2-luc	Not termed (17)	5'-gcagtGCGTGatctcag-3'	Human <i>CYP1A1</i> – 2142/– 2125
DRE12.6	DRE12.6 (18)	5'-ttGCGTGcga-3'	– 1882/– 1865

*Reference number.

†Sequences of the XRE regions in plasmids are shown, and the underlined parts represent the core sequence of XRE.

‡Locations of the XRE regions in the rat and human *CYP1A1* promoters are referred from NCBI database (rats: M14633, human: D10855).

Materials and Methods

Chemicals: 3-Methylcholanthrene (MC), omeprazole (OME), and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). G418 was obtained from Invitrogen (Garlsbad, CA, USA). Reporter Lysis Buffer was purchased from Promega (Madison, WI, USA). PicaGene Luminescence Reagent was obtained from NipponGene (Toyama, Japan).

Stable transfection of reporter plasmid: A human hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS and used as a host cell. The reporter plasmid XRE-Luc (19) and the neomycin-resistant plasmid pRC/CMV (Invitrogen) were co-transfected into HepG2 cells by use of Lipofectamine reagent (Invitrogen). After 48 h, transfection efficiency of a reporter plasmid XRE-Luc was ascertained by the luciferase assay as described in the "Luciferase Assay" section. HepG2 cells with a reporter plasmid were selected and then, cloned after the 3-week-incubation in the DMEM supplemented with 5% FCS and G418 (400 µg/mL). Ten cell clones with a reporter plasmid XRE-Luc were obtained. In addition, sequence of an XRE region in the plasmid XRE-Luc is compared with those in the plasmids constructed by other investigators (Table 1).

Luciferase assay: Luciferase assay was performed according to the method as previously described (19). In brief, XRE-Luc-integrated HepG2 clones were seeded into a 24-well plate (5 × 10⁴ cells/well), cultured for 48 h, and treated with MC or OME for the indicated times. Thereafter, 1 × Reporter Lysis Buffer (100 µL) was added to each well. After 15 min, the resultant cell lysates were frozen at –80°C for 30 min, thawed at room temperature, and centrifuged at 20,000 g for 5 min. The protein concentration in the resultant super-

natant was measured with a BCA-protein Assay Kit (Pierce, Rockford, IL, USA) and adjusted to 1 mg protein per mL with phosphate-buffered saline (pH 7.4). A portion (10 µL) of the supernatant was mixed with 50 µL of PicaGene Luminescence Reagent, and amount of light product was immediately measured with a Luminescencer-PSN (ATTO, Tokyo, Japan). Luciferase activity was represented as a luminescence unit per mg protein. A cell clone, HepG2-A10, which showed the highest responsibility to MC-mediated induction of luciferase among the ten clones, was selected and used for further experiments.

RT-PCR analysis: RT-PCR was performed according to the method as previously described (19). Briefly, HepG2-A10 cells were incubated in the medium containing MC for the indicated times. After the chemical treatment, total RNA was isolated from the cells with Isogen (NipponGene). A portion (4 µg) of total RNA was converted to cDNA using poly d(N)₆ primer (Pharmacia Biotech, Piscataway, NJ, USA) and MMLV Reverse Transcriptase (Invitrogen) in an RT reaction mixture (20 µL). PCR was performed in a total reaction mixture (25 µL) containing RT reaction mixture (0.8 µL), the corresponding primer sets (12.5 pmol), and 0.625 unit of AmpliTaq Gold™ (Applied Biosystems, Norwalk, CT, USA).

The primer sets used for determinations of expression levels of the target genes were as follows: *CYP1A1*, 5'-TGGATGAGAACGCCAATGTC-3' (forward) and 5'-TGGGTTGACCCATAGCTTCT-3' (reverse) (20); *CYP1A2*, 5'-ACAGCACTTCCCTGAGAGTA-3' (forward) and 5'-TCTGGATCTTCTCTGTATC-3' (reverse) (20); *GAPDH*, 5'-TGTTGCCATCAATGACCCCTTC-3' (forward) and 5'-AGCATCGCCCCACTTGATTTTG-3' (reverse) (21). PCR amplification

protocol consisted of the preactivation of AmpliTaq Gold™ for 10 min at 95°C, 16–35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C for *CYP1A1* and *CYP1A2* or 60°C for *GAPDH*, and the elongation for 1 min at 72°C with a GeneAmp PCR system model 9600 (Perkin-Elmer Norwalk, CT, USA). The resultant PCR products were separated by an agarose-gel electrophoresis and visualized by ethidium bromide staining under UV light. Amount of each PCR product was densitometrically quantified with a computer using Kodak 1D Image Analysis Software (Macintosh 1D version 2.02). Expression level of each gene was normalized to that of *GAPDH*.

Results

Establishment of human hepatic cell lines for screening of AhR activators: The reporter plasmid XRE-Luc (19) and the pRC/CMV vector containing the neomycin-resistant gene were co-transfected into a human hepatoma cell line HepG2, and thereafter, G418-resistant 10 clones stably expressing a luciferase reporter gene were obtained. Among the 10 clones, a clone HepG2-A10 was selected as the most responsive clone to MC-mediated induction of luciferase reporter gene (data not shown) and used for further experiments.

MC-mediated induction of luciferase: A time-dependency for MC-mediated induction of luciferase was examined in HepG2-A10 cells (Fig. 1A). The cells were treated with MC at 10^{-6} M, and the luciferase activities were measured at 3, 6, 12 and 24 h later. Significant increase in luciferase activity was observed even at 3 h later. The increase occurred in a time-dependent manner up to 12 h, and thereafter, the increased level was retained up to 24 h.

A concentration-dependency for the induction of luciferase by MC was further examined. When luciferase activity was measured at 6 h after the chemical treatment, it increased in a concentration-dependent manner over a range from 10^{-8} M to 10^{-6} M (Fig. 1B).

MC-mediated activation of AhR-regulated genes: As AhR-regulated genes, CYP1A subfamily genes, *CYP1A1* and *CYP1A2*, were selected, and culture period-dependent changes in the expression of the genes after treatment with MC were examined by RT-PCR in HepG2-A10 cells. Representative expression patterns of CYP1A subfamily genes after the MC treatment were shown in Fig. 2A.

In the cells treated with MC at a concentration of 10^{-6} M, clear increases in expression levels of the CYP1A subfamily genes occurred within 1 h, and their levels reached at a maximum 6 h later. Thereafter, gene expression levels of *CYP1A1* and *CYP1A2* were gradually reduced (Fig. 2B). In addition, although culture period-dependent increases in the expression of the

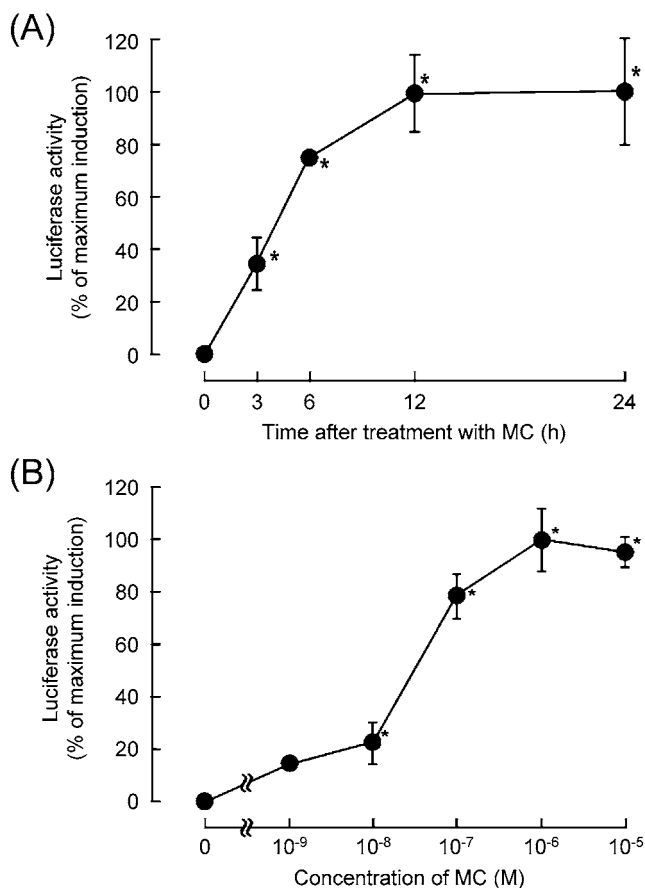


Fig. 1. Time- and concentration-dependent changes in luciferase activity after treatment of HepG2-A10 cells with MC. HepG2-A10 cells were treated with MC (10^{-6} M) for the indicated times (A) or at the indicated concentrations of MC for 6 h (B), and the luciferase activity was measured as described in “Materials and Methods”. The values of luciferase activities shown represent ratios to a maximum luciferase activity in each experiment (A or B). The data shown represent mean \pm SD in each experimental group ($n=4$). *Significant differences ($p<0.01$) from the corresponding controls assayed by ANOVA and Dunnett’s test.

CYP1A subfamily genes were also observed in HepG2-A10 cells treated with a vehicle alone (control), their increased levels were much lower than those in MC-treated cells at the corresponding culture periods (data not shown).

Subsequently, concentration effects of MC on the expression of CYP1A subfamily genes were examined at 6 h after the chemical treatment. Representative expression patterns and levels of CYP1A subfamily genes after MC treatment were shown in Figs. 3A and 3B, respectively. Expression levels of the *CYP1A1* and *CYP1A2* genes were linearly increased in a concentration-dependent manner over a range from 10^{-9} M to 10^{-7} M. In addition, no significant change in expression level of the *GAPDH* gene was observed at any concentrations of MC examined.

Responsibility of HepG2-A10 cells toward a non-

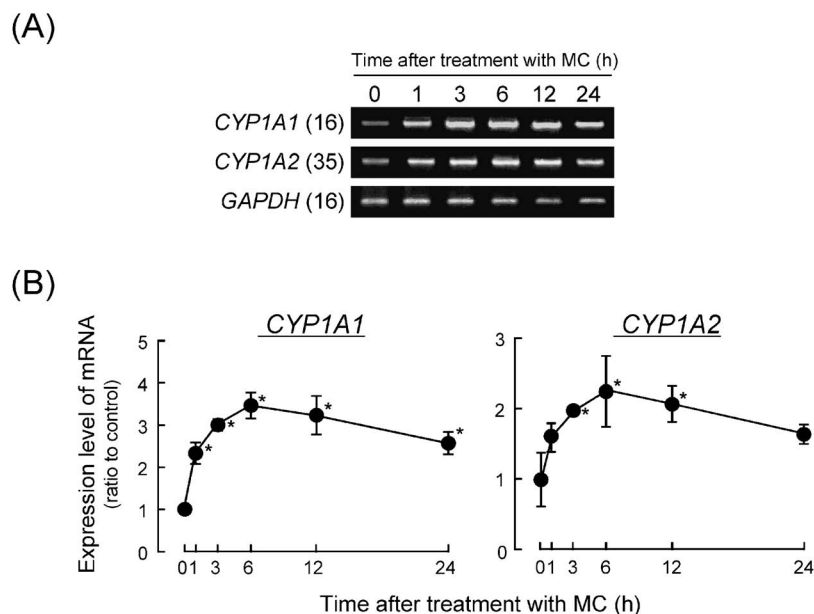


Fig. 2. Time-dependent changes in gene expression levels of CYP1A subfamily enzymes after treatment of HepG2-A10 cells with MC. HepG2-A10 cells were treated with MC (10^{-6} M) for the indicated times. Total RNA was prepared from MC-treated cells and used for RT-PCR analysis. RT-PCR products from four individual samples in each experimental group were mixed, and the mixture was subjected to agarose gel-electrophoresis (A). The values in parentheses represent the number of PCR cycles performed. Expression levels of the *CYP1A1* and *CYP1A2* genes were measured using four individual samples in each experimental group, calculated on the basis of that of the *GAPDH*, and represented as ratios to the corresponding controls (B). The data shown represent mean \pm SD in each experimental group ($n=4$). *Significant differences ($p<0.01$) from the corresponding controls assayed by ANOVA and Dunnett's test.

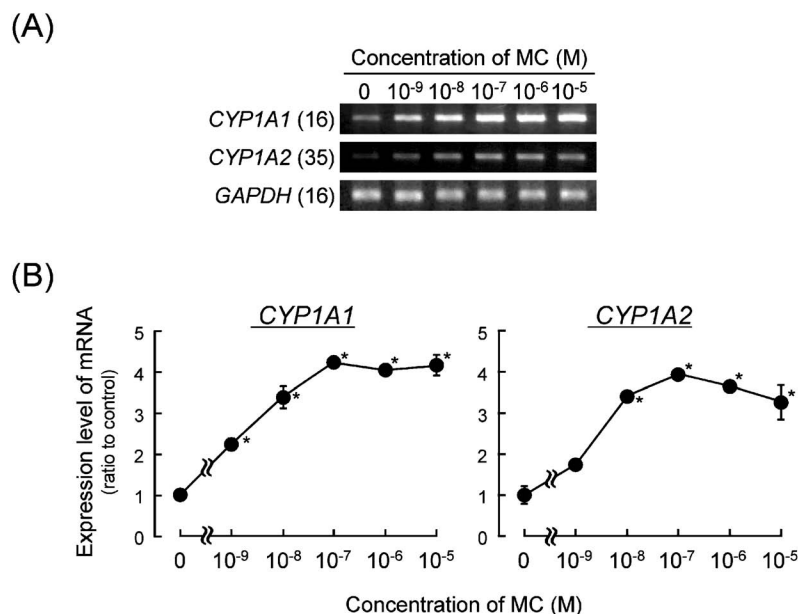


Fig. 3. Concentration-dependent changes in the expression of *CYP1A1* and *CYP1A2* after treatment of HepG2-A10 cells with MC. HepG2-A10 cells were treated with the indicated concentrations of MC for 6 h. Total RNA was prepared from MC-treated cells and used for RT-PCR analysis. RT-PCR products from four individual samples in each experimental group were mixed, and the mixture was subjected to agarose gel-electrophoresis (A). The values in the parentheses (Fig. 3A) represent the number of PCR cycles performed. The expression levels of *CYP1A1* and *CYP1A2* were measured using four individual samples in each experimental group, calculated on the basis of that of the *GAPDH*, and compared to the corresponding controls (B). The data represent mean \pm SD in each experimental group ($n=4$). *Significant differences ($p<0.01$) from the corresponding controls assayed by ANOVA and Dunnett's test.

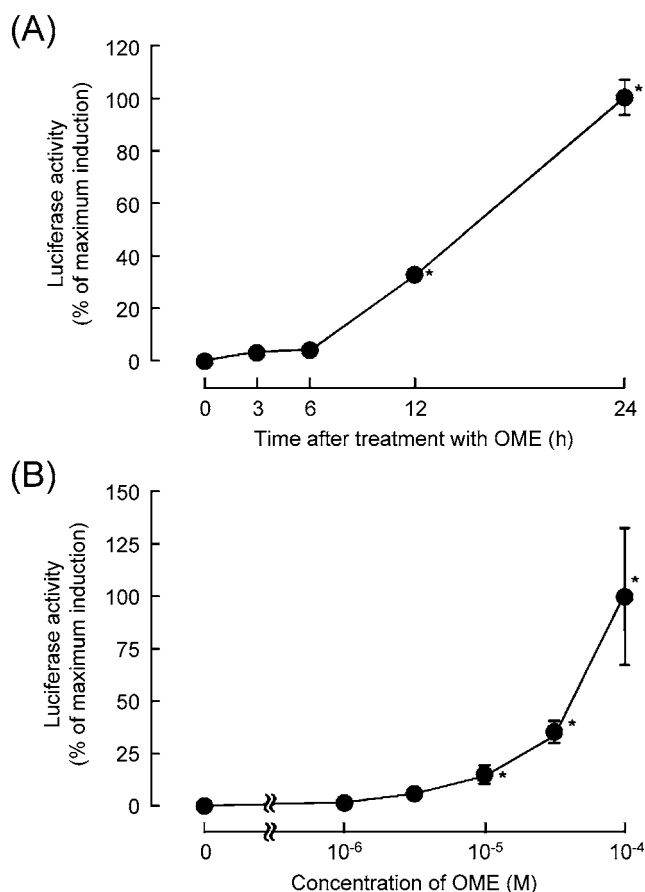


Fig. 4. Time- and concentration-dependent inductions of luciferase activity after treatment of HepG2-A10 cells with OME. HepG2-A10 cells were treated with OME (10^{-4} M) for the indicated times (A) or at the indicated concentrations of OME for 24 h (B), and the luciferase activity was measured as described in "Materials and Methods". The values of luciferase activities shown are the ratios to a maximum luciferase activity in each experiment group (A or B). The data represent mean \pm SD in each experimental group ($n=4$). *Significant differences ($p<0.01$) from the corresponding controls assayed with ANOVA and Dunnett's test.

ligand type AhR activator: OME, a non-ligand type AhR activator (12,13), shows species-difference in AhR activation between humans and rodents (14,15). Therefore, we examined whether or not OME-mediated induction of luciferase occurs in HepG2-A10 cells.

Treatment of HepG2-A10 cells with OME (10^{-4} M) led to induction of luciferase activity in a culture period-dependent manner over a range from 12 h to 24 h (Fig. 4A). Furthermore, a concentration-dependency for the induction of luciferase was examined at 24 h after treatment with OME. The OME-mediated induction occurred in a concentration-dependent manner over a range from 10^{-5} M to 10^{-4} M (Fig. 4B).

Discussion

In the present study, we succeeded in establishment of a cell line HepG2-A10, which showed the highest

responsibility to AhR activators among the established 10 clones stably expressing luciferase gene, by transfection into a human hepatoma cell line HepG2 of a chimera plasmid (XRE-Luc) (19). As AhR-regulated genes, CYP1A subfamily genes such as *CYP1A1* and *CYP1A2* were selected, and changes in their gene expression levels after treatment of MC, an AhR-ligand type activator (1), were examined. MC-mediated increases in expression levels of luciferase and AhR-regulated CYP1A subfamily genes were observed in a time- and concentration-dependent manner. However, there were slight differences in MC-induced expression patterns of the *CYP1A1/1A2* genes and luciferase. These differences would be dependent on those in stability (a half-life) between luciferase and *CYP1A1/1A2* mRNAs. Namely, luciferase would be more stable than the CYP1A mRNAs. In addition, similar differences in MC-induced expression patterns of the *CYP1A1* gene and luciferase are also observed in a rat KanR2-XL8 cell line (19).

Induction of luciferase by OME, a non-ligand type AhR activator (12,13), was also observed in HepG2-A10. Thus, a newly established HepG2-A10 resembles the reported human cell lines, 101L (16) and DRE12.6 (18), in responsibility for AhR activators such as MC and OME. This suggests that only existence of the AhR-binding core sequence (5'-CACGC-3' or 5'-GCGTG-3') in their plasmid XRE regions is important for the AhR-mediated induction of luciferase, because with an exception of the core sequence, sequences of the XRE regions differ among HepG2-A10, 101L, and DRE12.6 cell lines (Table 1).

To date, pGudLuc1.1 (22) and pL1A1N (16) reporter plasmids, which contain the mouse *CYP1A1* promoter (-1301/-849) and human *CYP1A1* promoter (-1612/+292), respectively, are widely used for construction of the cell lines for the detection of AhR activators. However, these promoter regions contain the binding sequences for transcription factors such as peroxisome proliferator-activated receptor α (23), retinoic acid receptor α (24), and/or nuclear factor-1 (25), which might contribute to gene expression of *CYP1A1*. Therefore, the cell line with a plasmid containing the *CYP1A1* promoter might not be adequate for screening of AhR-selective activators. On the other hand, the plasmid "XRE-Luc" used in the present experiment contains only XRE region, and HepG2-A10 with the plasmid would be more adequate for screening of AhR-selective activators than the reported cell lines.

In conclusion, we newly established a human hepatoma cell line HepG2-A10 useful for a convenient reporter gene assay of AhR activators. Further studies with both human HepG2-A10 and rat KanR2-XL8 cell lines, which are established by transfection of a plasmid "XRE-Luc", would contribute to not only screening of

AhR activators but also understanding of species-differences in an AhR activation pathway.

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