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*Letter*

# Induction of CYP3A4 mRNA by cooked food-derived carcinogenic heterocyclic aromatic amines in human HPL-A3 cells

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**ABSTRACT** — The effects of six cooked food-derived heterocyclic carcinogenic amines (HCAs), including 2-amino-9*H*-pyrido[2,3-*b*]indole acetate (AαC) and its 3-methyl derivative (MeAαC), on cytochrome P450 3A (CYP3A) induction were examined using a human hepatoblastoma cell-derived HPL-A3 cell line, previously established for a luciferase-reporter gene assay for the detection of a CYP3A4 inducer. AαC and MeAαC, but not the four other HCAs, enhanced luciferase activity. Quantitative RT-PCR further confirmed the significant induction of *CYP3A4* mRNA by AαC and MeAαC. Since *CYP3A4* gene expression is primarily regulated by the pregnane X receptor (PXR) and occasionally by other receptors, such as the aryl hydrocarbon receptor and vitamin D receptor, the effects of siRNAs for these regulator genes on AαC- and MeAαC-mediated increases in luciferase activity were investigated further. PXR siRNA, but not other siRNAs, significantly reduced AαC- and MeAαC-induced luciferase activities. These results demonstrate for the first time that AαC and MeAαC, among the six HCAs tested, increase CYP3A4 mRNA via PXR activation in human hepatoblastoma-derived HPL-A3 cells.

**Key words:** Heterocyclic aromatic amines, AαC, MeAαC, CYP3A4 induction, Human PXR

## INTRODUCTION

Humans are exposed to many cooked food-derived carcinogenic heterocyclic amines (HCAs) via diet (Sugimura *et al*., 2004). Therefore, the International Agency for Research on Cancer (IARC) has registered ten types of HCAs in the "IARC monographs on the identification of carcinogenic hazards to humans" (IARC, 1993). Exposure to HCAs should be avoided to prevent cancer (Sugimura *et al*., 2004).

Cytochrome P450s (CYPs) are the most abundant in the liver and are generally involved in the detoxification of various chemicals, including drugs and pesti-

cides. CYPs are rarely involved in the metabolic activation of procarcinogens (Rendic and Guengerich, 2021). Carcinogenic aromatic amines, including HCAs, reportedly induce their metabolic activation enzyme(s), especially CYP1A2, in the rodent liver (Degawa *et al.*, 1986, 1989). Moreover, the species difference in the animal susceptibility to aromatic amine-induced carcinogenesis is, at least in part, dependent on the difference in the aromatic amine-mediated increase of its activation enzyme(s), including CYPs, in the target organ of each animal (Degawa *et al.*, 1985, 1987, 1988, 1990, 1992). Although 2-amino-9*H*-pyrido[2,3-*b*]indole acetate (AαC) reportedly forms AαC-DNA adducts in human liver tis-

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sue through a CYP3A4-mediated pathway (Baranczewski and Möller, 2004), limited research has been conducted on the CYP inducibility of HCAs. Interestingly, hepatocarcinogenic 2-acetylaminofluorene (2-AAF) induces CYP1A and CYP3A enzymes in the rodent liver (Tateishi *et al.*, 1999). CYP3A subfamily enzymes are induced via agonist (chemical)-mediated pregnane X receptor (PXR) activation (LeCluyse, 2001). PXR-dependent induction of CYP3A mRNAs by 2-AAF occurred in rat primary hepatocytes (Sparfel *et al.*, 2003) and PXR-transfected human HepG2 cells (Anapolsky *et al.*, 2006).

Therefore, we examined the CYP3A inducibility of HCAs, including AαC, using a human hepatoblastoma cell-derived HPL-A3 cell line, previously established for a PXR-based luciferase reporter gene assay for the detection of CYP3A4 inducers. In this study, six HCAs together with 2-AAF were selected as test chemicals. CYP3A inducibility of AαC and its 3-methyl derivative MeAαC was observed among the six HCAs examined.

## MATERIALS AND METHODS

### Chemicals

3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P-2), 2-amino-6-methylpyrido[12-*a*:3',2'-*d*]imidazole hydrochloride (Glu-P-1), 2-amino-pyrido[12-*a*:3',2'-*d*]imidazole hydrochloride (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole acetate (AαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole acetate (MeAαC) were kindly donated by the Division of Biochemistry of the National Cancer Center Institute (Tokyo, Japan) and used (Fig. 1). 2-Acetylaminofluorene (2-AAF) was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Rifampicin (RIF) and dimethyl-sulfoxide (DMSO) were purchased from Wako Pure Chemical Co., Ltd. (Tokyo, Japan). All chemicals were dissolved in DMSO and stored at −80°C until use.

### Cell line and culture

The HPL-A3 cell line, previously established for a human PXR-based luciferase reporter gene assay (Sekimoto *et al.*, 2012), was used for this study. HPL-A3 cells were cultured at 37°C in a 5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum, 2 mM L-glutamine solution (Nacalai Tesque Inc., Kyoto, Japan), and 1 × antibiotic-antimycotic mixed stock solution (Nacalai Tesque Inc.).
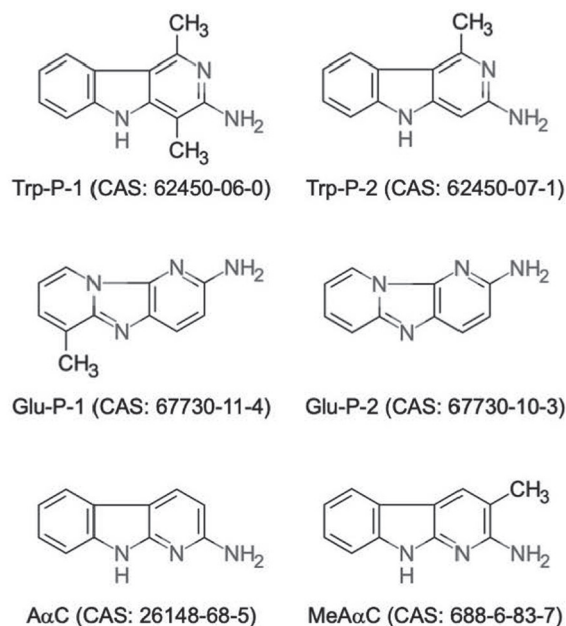


**Fig. 1.** Chemical structures of the HCAs used in this study.

## Measurement of luciferase activity

An aliquot ($1 \times 10^4$ cells/well) of HPL-A3 cells was precultured in a 96-well cell culture plate for 48 hr, treated with a test compound for 24 hr, and lysed with 1 × reporter lysis buffer (Promega, Madison, WI, USA). The cell lysate was prepared by incubating for 15 min at room temperature, followed by freezing at −80°C for 30 min. The resultant cell lysate was thawed and used to measure luciferase activity and protein concentration. Luciferase activity was measured using a previously reported method (Tomita *et al.*, 2022). Briefly, 10 µL of the cell lysate was mixed with 190 µL of luciferase working reagent containing D-luciferin and coenzyme A, and the resultant luminescence intensity was measured using AB-2350 Pherios (ATTO, Tokyo, Japan). The amount of protein in each cell lysate was determined using a bicinchoninic acid (BCA)-protein assay kit (Takara Bio Inc., Shiga, Japan). The luminescence intensity was normalized to the amount of protein.

## siRNA treatment

Silencer® Select siRNA for human PXR (ID number s16910, Ambion, Austin, TX, USA), human AhR (ID number s1199, Ambion), human VDR (ID number s114777, Ambion), and negative control siRNA #1 (Ambion) were separately transfected into HPL-A3 cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA,

USA) and the reverse transfection method. Briefly, 0.2 µL of Lipofectamine RNAiMAX was added to 20 µL of serum-free DMEM containing 1 pmol of each siRNA and mixed for 15 min at room temperature. The resultant siRNA-Lipofectamine RNAiMAX complex was added to 100 µL of HPL-A3 cell suspension ($10^5$ cells/mL in DMEM) in a 96-well cell culture plate, and the cells were precultured for 48 hr at 37°C in a 5% $CO_2$ atmosphere. Next, individual siRNA-transfected HPL-A3 cells were treated with the test compound for 24 hr, and luciferase activity was measured as described above.

### Quantitative RT-PCR analysis

Total RNA was isolated from HPL-A3 cells treated with a test compound or vehicle alone using Sepazol (Nacalai Tesque Inc.) and used to determine the mRNA levels of *CYP3A4*, *CYP3A7,* and *GAPDH*. Total RNA (1 µg) was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Norwalk, CT, USA) along with a random hexamer. Quantitative RT-PCR was performed with Fast SYBR green master mix (Applied Biosystems) in a total reaction mixture (10 µL) containing 0.3 µL of RT-reaction mixture and 2 pmol of each primer. The primer sequences used were as follows: *CYP3A4*, 5′-TGAGAAATCTGAGGCGGGAAGC-3′ (forward) and 5′-CGATGTTCACTCCAAATCATGTGC-3′ (reverse) (Zhuo *et al.*, 2004); *CYP3A7*, 5′-GATCTCATCCCAAACTTGGCCG-3′ (forward) and 5′-CATAGGCTGTTGACAGTCATAAATA-3′ (reverse) (Zhuo *et al.*, 2004); and *GAPDH*, 5′-TGTTGCCATCAATGACCCCTTC-3′ (forward) and 5′-AGCATCGCCCCACTTGATTTTG-3′ (reverse) (Tian *et al.*, 2004). The amplification protocol for *GAPDH* consisted of pre-activation with the enzyme for 20 sec at 95°C, followed by 40 cycles of denaturation for 3 sec at 95°C and annealing/extension for 30 sec at 55°C. Moreover, the amplification protocol for *CYP3A4* and *CYP3A7* consisted of pre-activation with the enzyme for 20 sec at 95°C, followed by 40 cycles of denaturation for 3 sec at 95°C, annealing for 30 sec at 55°C, and extension for 60 sec at 72°C. PCR amplification was performed using the Step-One Real-Time PCR System (Applied Biosystems). The amount of each mRNA was calculated using the comparative threshold cycle (ddCt) method. The amount of each gene was normalized to that of *GAPDH*, used as the internal standard.

### Statistical analysis

GraphPad Prism 7 software was used for statistical analyses (GraphPad Software, San Diego, CA, USA). Non-repeated measures of analysis of variance (ANOVA), followed by Dunnett's test, were used to evaluate the data.

## RESULTS AND DISCUSSION

RIF (Lehmann *et al.*, 1998) and 2-AAF (Anapolsky *et al.*, 2006) induce the CYP3A enzyme via human PXRs. Therefore, we first investigated whether RIF and 2-AAF increased luciferase activity in HPL-A3 cells and confirmed that RIF (100 µM) and 2-AAF (30 µM) showed significant capacities for luciferase induction via PXR activation.

Therefore, six HCAs, such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, and MeAαC, together with 2-AAF were selected as test chemicals. Among these HCAs, Trp-P-1, Trp-P-2, AαC, and MeAαC, as well as 2-AAF, have an indole skeleton in their chemical structures. The capacities of these six HCAs for PXR-based luciferase induction were examined using HPL-3 cells at a chemical concentration range (0.1–100 µM). AαC (100 µM) and MeAαC (100 µM) showed significant capacities for luciferase induction, whereas four other HCAs, including Trp-P-1 and Trp-P-2, showed no such capacity at any concentration examined (Table 1). This indicates that the indole skeleton in HCA is not a necessary and sufficient element to show a capacity for human PXR activation.

Subsequently, we examined the effects of AαC and MeAαC on the induction of *CYP3A4* mRNA in HPL-A3 cells using quantitative RT-PCR (Fig. 2). RIF was used as a positive control for *CYP3A4* mRNA expression (Lehmann *et al.*, 1998). RIF significantly induced *CYP3A4* mRNA at 0.1 and 10 µM. Likewise, AαC and MeAαC showed similar capacities at 100 µM. In addition, the patterns of induction of *CYP3A7* mRNA by these compounds were similar to those of *CYP3A4* mRNA (data not shown). These findings strongly suggest that AαC and MeAαC induce *CYP3A* mRNAs via PXR activation. However, *CYP3A4* mRNA is reportedly induced not only through PXR activation but also through activation of the aryl hydrocarbon receptor (AhR) (Gerbal-Chaloin *et al.*, 2006) or vitamin D receptor (VDR) (Thompson *et al.*, 2002). Furthermore, we have previously reported that all the HCAs examined herein showed definite capacities for human AhR activation (Sekimoto *et al.*, 2016). HPL-A3 cells expressed VDR and PXR, and VDR-mediated luciferase induction occurred in HPL-A3 cells (Sekimoto *et al.*, 2012).

Therefore, we examined the contribution of PXR, AhR, and VDR to HCA-mediated luciferase induction in HPL-A3 cells using specific siRNAs. RIF-, AαC-, and MeAαC-induced luciferase activities were significantly suppressed by PXR siRNA but not by other siRNAs (Fig. 3). Surprisingly, the luciferase-inducing activities of RIF, AαC, and MeAαC were significantly enhanced by VDR siRNA, as observed in our previous experiment on PXR-

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**Table 1.** Effects of HCAs on induction of luciferase via PXR-mediated activation in HPL-A3 cells.

| Reagent | Concentrations | | | | |
|---|---|---|---|---|---|
| | 0.1 μM | 1 μM | 10 μM | 30 μM | 100 μM |
| RIF | - | 1.12 ± 0.39 | 3.18 ± 1.86 | - | 19.47 ± 11.6** |
| 2-AAF | - | 1.22 ± 0.20 | 1.77 ± 0.70 | 3.29 ± 2.14* | - |
| Trp-P-1 | 1.04 ± 0.34 | 0.99 ± 0.47 | 1.24 ± 0.85 | (Cell death) | (Cell death) |
| Trp-P-2 | 1.00 ± 0.38 | 0.86 ± 0.21 | 1.00 ± 0.22 | (Cell death) | (Cell death) |
| Glu-P-1 | - | 1.19 ± 0.90 | 1.01 ± 0.35 | - | 1.03 ± 0.24 |
| Glu-P-2 | - | 0.92 ± 0.15 | 0.89 ± 0.43 | - | 1.24 ± 0.68 |
| MeAαC | - | 1.18 ± 0.35 | 1.51 ± 0.52 | - | 3.40 ± 1.72* |

HPL-A3 cells were precultured for 48 hr and treated with HCAs at the indicated concentrations for 24 hr. RIF and 2-AAF were used as positive controls and 0.1% DMSO was used as the vehicle control. After chemical treatment for 24 hr, cell lysates were prepared from HPL-A3 cells treated with each chemical or vehicle alone and used for the determination of luciferase activity. The data represent the ratios to the control, and the values represent the mean ± standard deviation (n = 3). Significant differences from vehicle controls were assessed using ANOVA and Dunnett's test: *$P < 0.05$, ** $P < 0.01$.
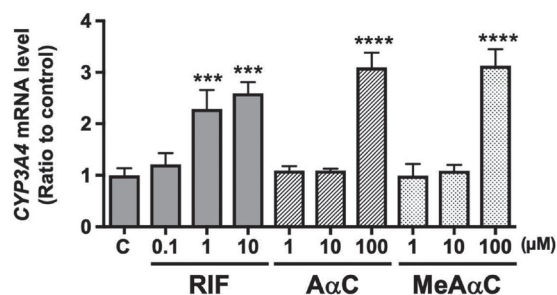


**Fig. 2.** Effects of AαC and MeAαC on *CYP3A4* mRNA expression in HPL-A3 cells. HPL-A3 cells were precultured for 48 hr and treated with each chemical at the indicated concentrations for 24 hr. RIF and 0.1% DMSO were used as positive and vehicle controls, respectively. Total RNA was extracted from HPL-A3 cells treated with a chemical or vehicle alone (control, C) and used for quantitative RT-PCR analysis. Each column represents the mean of each experimental group, and each bar represents the standard deviation (n = 3). Significant differences from vehicle controls were assessed using ANOVA and Dunnett's test: ***$P < 0.001$, **** $P < 0.0001$.

**Fig. 3.** Effects of PXR, AhR, and VDR siRNAs on the increase of luciferase activity by AαC or MeAαC in HPL-A3 cells. Each gene-specific siRNA was transfected to HPL-A3 cells using a reverse transfection method, and the siRNA-transfected cells were precultured for 48 hr. After preculture, individual siRNA-transfected cells were treated with a chemical or vehicle alone for 24 hr at the indicated concentrations. RIF and 0.1% DMSO were used as the positive and vehicle controls, respectively. After chemical treatment, cell lysates were prepared and used to measure the luciferase activity. The luciferase activities in AαC- or MeAαC-treated cells were assessed as ratios to those in vehicle control group transfected with negative control siRNA (control). Each column represents the mean of each experimental group, and each bar represents the standard deviation (n = 3). Significant differences between the control siRNA- and gene-specific siRNA-transfected groups were assessed using ANOVA and Dunnett's test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

dependent activation of the CYP3A4 enhancer (Sekimoto *et al.*, 2012). These findings indicate that the chemical-mediated induction of CYP3A mRNAs via PXR activation might be suppressed by the activation of VDR by endogenous vitamin D or vitamin D-like substances in the culture medium and used cells.

In conclusion, we demonstrated for the first time that among the six HCAs examined, AαC and MeAαC induced *CYP3A* mRNAs expression via PXR activation in human HPL-A3 cells, although we have previously
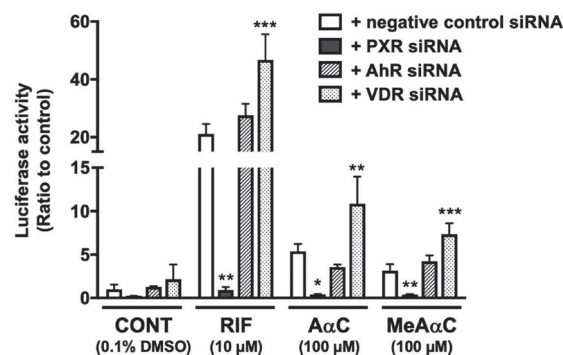
reported that all HCAs used herein showed definite capacities for the induction of *CYP1A* mRNAs via AhR activation (Sekimoto *et al.*, 2016). PXR activation is reportedly involved in the suppression of carcinogenesis through

pathways involving cell proliferation, oxidative stress, and immune functions (Xing *et al.*, 2020; Yoshinari and Shizu, 2022). Further investigation concerning the HCA-mediated activation of PXR and AhR would be useful for understanding its toxicity expression mechanism and prevention of chemical carcinogenesis.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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