INHIBITORY MECHANISMS OF TYROSINE KINASE INHIBITORS, HERBIMYCIN A, AND TYRPHOSTIN COMPOUNDS ON THE FUNCTION OF ARYL HYDROCARBON RECEPTOR IN CACO-2 CELLS

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Introduction

Aryl hydrocarbon receptor (AhR) is an endogenous target molecule for dioxin and related compounds. Nowadays, AhR is thought of as a key intermediary in the induction of a number of toxicities[1,2]. AhR is a ligand-activated transcription factor that recognizes dioxins as ligands. In the absence of a ligand, AhR forms a cytoplasmic complex with the molecular chaperone heat shock protein 90 (HSP90), the co-chaperone p23, and the hepatitis B virus X-associated protein 2 (XAP-2). This complex contributes mainly to the stability and cytoplasmic retention of AhR. Ligand binding induces conformational changes in AhR that are followed by the release of AhR from the cytoplasmic complex and its translocation into the nucleus. Within the nucleus, active AhR forms a heterodimer with AhR nuclear translocator (ARNT) and binds to the xenobiotic-responsive element (XRE). The AhR/ARNT heterodimer induces the transcriptional activation of a number of genes, which includes that for cytochrome P-450 1A1 (CYP1A1). CYP1A1 is a drug-metabolizing enzyme that catalyzes the conversion of AhR ligands into inactive metabolites or reactive intermediates. Therefore, AhR/CYP1A1 is thought of as a system that can detoxify a number of xenobiotic compounds[3,4].

Previously, we[5] and other groups[6,7] have shown that the activation of AhR is regulated through multiple pathways. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), which is the prototypical agonist of AhR, induces the transcriptional activation of *CYP1A1* in human and mouse cell lines. In contrast, omeprazole activates AhR without direct binding, and induces the transcription of *CYP1A1* in human but not in mouse cell lines. The transcriptional activation of *CYP1A1* that is induced by omeprazole is prevented by tyrosine kinase inhibitors, herbimycin A, or some of the tyrphostin compounds, whereas TCDD-induced *CYP1A1* transcription is not affected. These findings imply that the activation of AhR that is induced by omeprazole is mediated by tyrosine kinase activity. In contrast, the induction of CYP1A1 by the AhR agonist 2,3,7,8-tetrachlorodibenzofuran (TCDF) is inhibited by the tyrosine kinase inhibitors herbimycin A and genistein in human keratinocytes[8]. These findings suggest that AhR may be regulated by a putative tyrosine kinase in a manner that is specific to a particular cell type and inducer. However, the precise molecular mechanism by which this occurs has not yet been elucidated. In addition, it has been reported that the mobilization of intracellular calcium is involved in the activation of AhR[9–12]. For example, oltipraz induces an increase in the concentration of intracellular calcium and transcription of *CYP1A1* in Caco-2 cells. The induction of *CYP1A1* is suppressed when extracellular or intracellular calcium is chelated[10]. Although it is unclear how these intrinsic systems are integrated, elucidation of the molecular mechanism that regulates the function of AhR might provide information about the physiological role of AhR and the toxicity of compounds related to dioxin.

Materials and Methods

Cell culture: Human colon-carcinoma-derived cell line Caco-2 was maintained and treated as described previously[13].

RT-PCR: Total RNA was isolated using the acid guanidium phenol chloroform method[14]. Five micrograms of total RNA was reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). cDNAs encoding GAPDH and CYP1A1 were amplified with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) as described previously[13].

Immunoblot analysis: Whole-cell homogenates were prepared, and subcellular fractionation into cytoplasmic fractions and nuclear extracts was performed, as described previously[13]. Protein concentrations were determined

using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein samples were separated by SDS-PAGE and transferred to Hybond-P PVDF membrane (Amersham Biosciences, Bucks, UK). The membrane was blocked in blocking buffer (5% non-fat dried milk, 0.2% Tween 20 in Tris-buffered saline, pH 8.0). For the detection of AhR protein, the membrane was incubated with anti-AhR rabbit antibody (Biomol Research Laboratories, Plymouth Meeting, PA, USA), and then with HRP-conjugated anti-rabbit goat antibody (Dako, Glostrap, Denmark). For the detection of GAPDH protein, the membrane was probed with anti-GAPDH rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Specific signals were detected using the ECL system (GE Healthcare Biosciences, Tokyo, Japan).

Results and Discussion

To measure the transcriptional activity of AhR in Caco-2 cells, the amount of CYP1A1 mRNA was determined by quantitative RT-PCR. Oltipraz induces a transient increase in *CYP1A1* transcription, as reported previously by Morel *et* al.[10] and our data (not shown), therefore, RT-PCR experiments were carried out at 8 hours after induction, the time of maximum induction by oltipraz. The transcription of *CYP1A1* was significantly greater in cells treated with TCDD, omeprazole, or oltipraz than in control cells. To determine whether tyrosine kinase activity is required for CYP1A1 induction by each inducer, we treated cells with herbimycin A before induction. Herbimycin A completely inhibited induction of CYP1A1 by omeprazole or oltipraz, and that by TCDD (Fig. 1A). In response to the finding that herbimycin A completely suppressed CYP1A1 induction by each of the inducers tested, further experiments were limited to induction by TCDD, which is the best-studied model of AhR activation. To identify the crucial step for AhR activation that is sensitive to herbimycin A, the nuclear localization of AhR that is induced by TCDD was analyzed by subcellular fractionation and immunoblotting. Fig. 1B indicates that TCDD induced a significantly greater accumulation of AhR in the nucleus than occurred in the controls. In contrast, in cells treated with herbimycin A, the amount of AhR protein was diminished significantly in the cytoplasmic fraction and nuclear extract in the presence and absence of TCDD. This result indicates that herbimycin A induces down-regulation of total AhR protein and abrogates TCDD-induced nuclear translocation of AhR.

Although herbimycin A has been used as a general inhibitor of tyrosine kinase in the current and previous studies[5,6,8], it is also known to be an inhibitor of HSP90 chaperone activity[15]. The effect of herbimycin A on the stability of AhR protein was compared with geldanamycin, which is used as a specific inhibitor of HSP90 chaperone activity[16]. As shown in Fig. 1E, treatment with herbimycin A and geldanamycin reduced the amount of AhR protein in whole-cell homogenates (Fig. 1E, left panels). In light of the fact that molybdate stabilizes the interaction between AhR and HSP90 and increases the amount of AhR protein[17], we determined whether the stabilization of the AhR/HSP90 complex by molybdate reversed the effect of herbimycin A. The amount of AhR protein increased in cells that were treated with 5–20 mM molybdate, and fell to lower than the original levels at higher concentrations (Fig. 1C). Despite the effect of molybdate on the stability of AhR protein, molybdate did not affect the destabilization of AhR protein that was induced by herbimycin A and geldanamycin (Fig. 1D). It has been reported previously that activation of AhR or inhibition of HSP90 activity induces degradation of AhR protein, through ubiquitination and the 26S proteasome-mediated pathway[18,19]. Fig 1E shows that the amount of AhR protein fell decreased significantly in cells treated with TCDD, and that the protein was completely absent following treatment with herbimycin A or geldanamycin. In contrast, the reduction of AhR protein was prevented completely by pretreatment with the 26S proteasome inhibitor MG-132 (Fig. 1E). These findings indicate that the inhibitory effect of herbimycin A on HSP90 activity resulted in the degradation of AhR protein that is mediated by 26S proteasome. This conclusion is also supported by the results of one of our previous studies, which indicates that herbimycin A induces the transcription of HPS70, which is known to be a marker of HSP90 inhibition[13].

Although the current findings indicate that the inhibitory effect of herbimycin A on the function of AhR is mediated by inhibition of HSP90 activity and degradation of AhR protein, previous studies indicate that herbimycin A failed to suppress *CYP1A1* transcription induced by TCDD[5,6,8]. To render the conditions of current and previous experiments comparable, the transcription of *CYP1A1* was determined after the treatment of cells with TCDD for 8 and 24 hours. As shown in Fig. 2A, the transcription of *CYP1A1* that was induced by TCDD at 8 hours was significantly inhibited by pretreatment with either herbimycin A or geldanamycin. In contrast, the suppressive effects

were not observed in samples that were treated for 24 hours (Fig. 2A). The amount of AhR protein after the same treatment correlated with the transcriptional activity of AhR; AhR protein was completely absent at 8 hours but levels had recovered at 24 hours, although not to the original levels (Fig. 2B). These findings indicate that the effect of herbimycin A is transient and that the lability of herbimycin A can account for the conflicting results that were obtained under different experimental conditions[5,6,8].

In light of the fact that the inhibitory effect of herbimycin A on HSP90 was sufficient to suppress the transcriptional activity of AhR, tyrphostins, which are more selective inhibitors of tyrosine kinases, were used to determine the contribution of putative tyrosine kinase activity to the activation of AhR. Tyrphostins are a series of compounds that are structurally similar to the side chain of tyrosine and designed to compete with the substrate recognition site in a subset of tyrosine kinases. The transcription of *CYP1A1* that was induced by TCDD was attenuated by pretreating cells with tyrphostins; tyrphostin B44 and B48 inhibited *CYP1A1* transcription strongly, whereas tyrphostin A1, which is used as a negative compound, did not inhibit it significantly (Fig. 3A). Tyrphostin B48 and A1 inhibit *CYP1A1* transcription in a dose-dependent manner and the inhibitory effect of A1 compound was significant at higher concentrations (Fig. 3B). After the cells had been treated with tyrphostins and TCDD, they were subjected to subcellular fractionation to detect the nuclear localization of AhR protein. The amount of AhR protein in the cytoplasmic fraction was not affected by any treatment that was used in the study. Treating cells with tyrphostin B46 or B48 resulted in the complete inhibition of the nuclear localization of AhR protein that was induced by TCDD (Fig. 3C). These findings indicate that some tyrphostins prevent the nuclear localization of AhR protein, which results in the suppression of the transcriptional activity. Due to the fact that the amount of AhR protein in the nuclear extract is much less than that in the cytoplasmic fraction, the total amount of AhR protein was not reduced significantly by treatment with tyrphostins.

In conclusion, the transcriptional activity of AhR and subcellular localization was analyzed. It was determined that herbimycin A inhibited the HSP90 activity that resulted in the destabilization of AhR protein. In contrast, tyrphostin B48 inhibited the nuclear localization of AhR induced by TCDD that resulted in the suppression of the transcriptional activity without altering the cytoplasmic AhR protein. Although further work needs to be done to elucidate putative tyrosine kinase and its substrate, which take part in the activation of AhR, the approaches used here enabled the identification of the step of AhR regulation that is inhibited by tyrosine kinase inhibitors.

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Figure 1

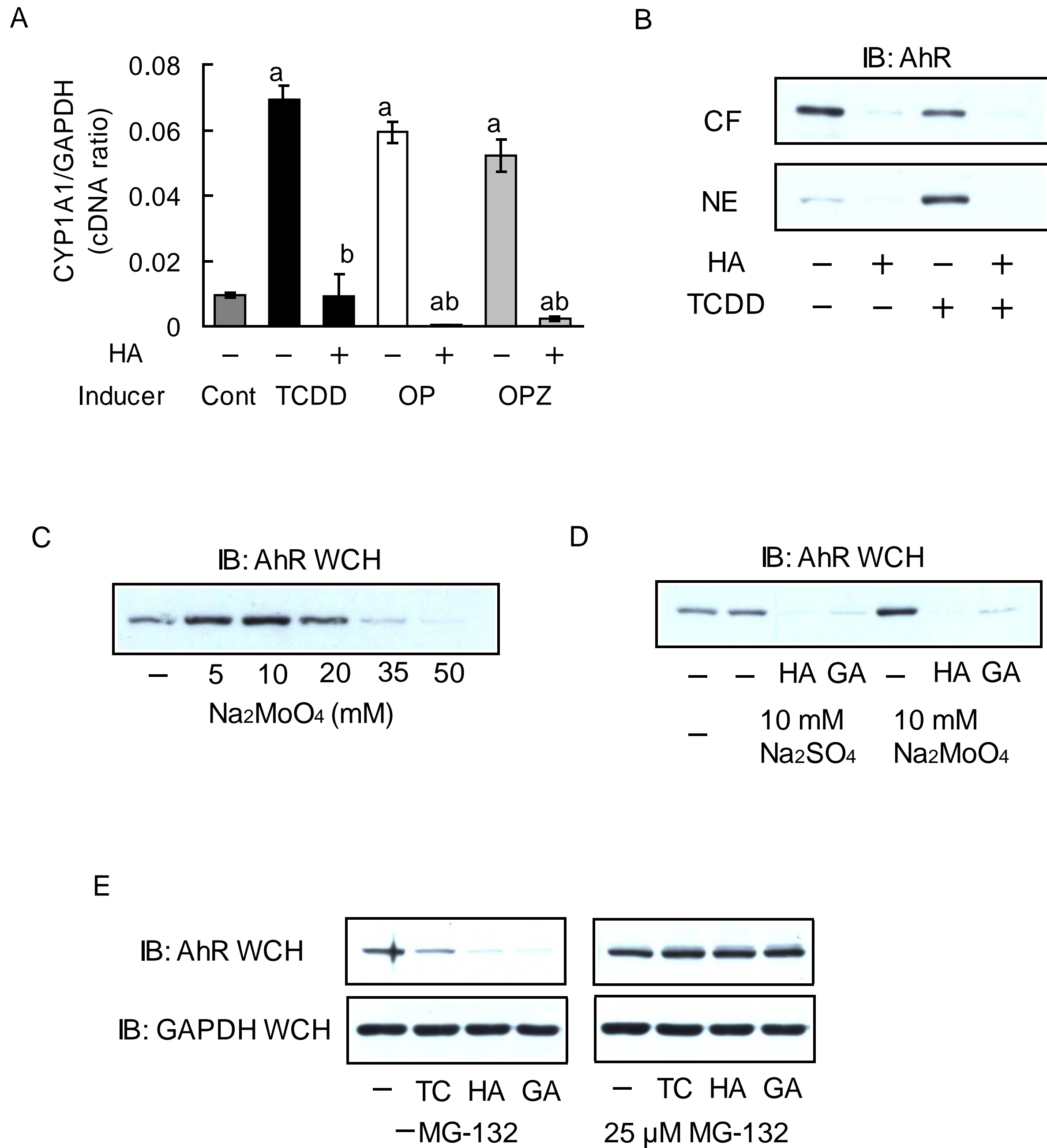

Figure 2

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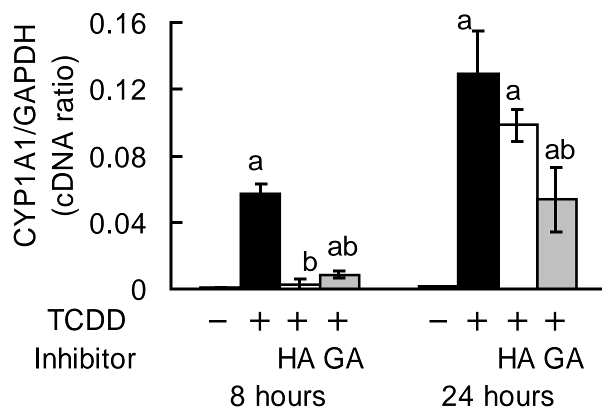

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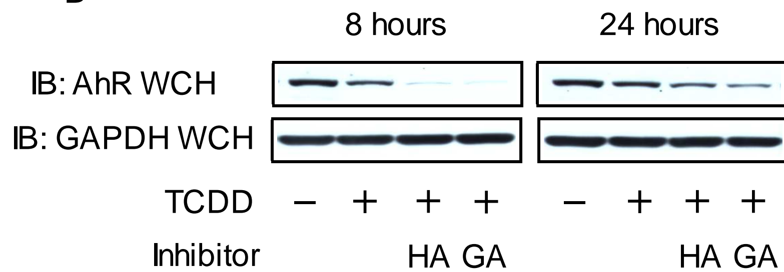
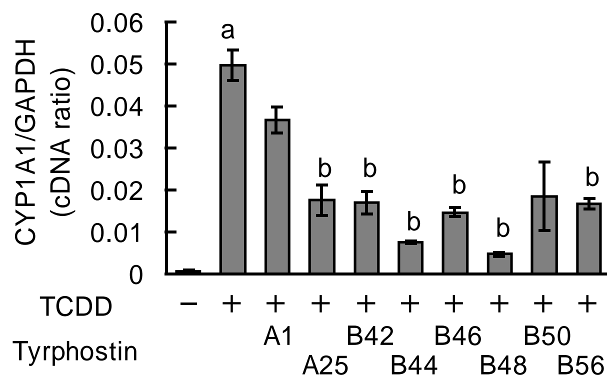


Figure 3

A



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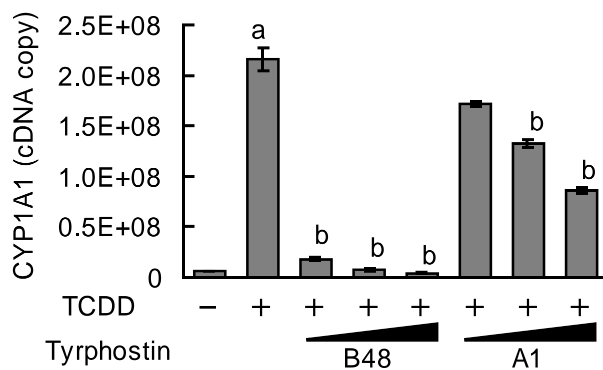

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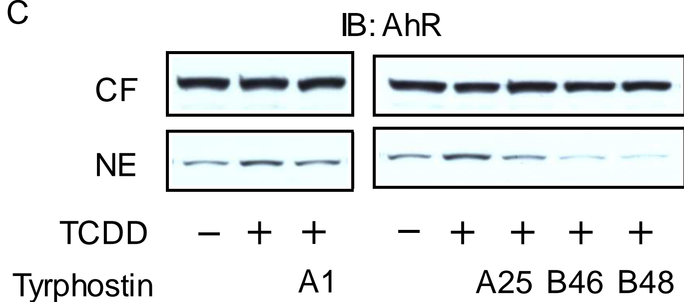

Figure legends

Fig. 1 Herbimycin A suppresses transcriptional activation of *CYP1A1* through the degradation of AhR.
Caco-2 cells were treated with 5 μM herbimycin A (HA+) or vehicle (HA−) for 2 hours and then with 5 nM TCDD, 100 μM omeprazole (OP), 50 μM oltipraz (OPZ) or vehicle (Cont) for a further 8 hours. Cells were harvested and subjected to RT-PCR to quantify the copy number of CYP1A1 and GAPDH cDNAs. The data were expressed as means ± SD of the ratio of CYP1A1 to GAPDH from three independent experiments. The data were statistically analyzed by *t* test: “a” indicates $p < 0.01$ compared with controls and “b” indicates $p < 0.01$ compared with each HA− sample (A). Cells treated with herbimycin A (HA+) or vehicle (HA−) for 2 hours and then with TCDD (TCDD+) or vehicle (TCDD−) for a further 1.5 hours were subjected to biochemical fractionation into cytoplasmic fraction (CF) and nuclear extract (NE). Equal amounts of protein were subjected to immunoblotting to detect AhR protein (B). Cells were treated with increasing concentration (5 to 50 mM) of sodium molybdate (Na_2MoO_4) for 12 hours. Whole-cell homogenates were prepared and subjected to immunoblotting to detect AhR (C). Cells were treated with 10 mM Na_2MoO_4 or control salt (Na_2SO_4) for 12 hours and then with 5 μM herbimycin A (HA), 0.5 μM geldanamycin (GA) or vehicle (−) for a further 3.5 hours. AhR protein was detected as described above (D). Cells were treated with 25 μM MG-132 or control vehicle (−MG-132) for 2 hours and then with 5 nM TCDD, 5 μM herbimycin A (HA), 0.5 μM geldanamycin (GA) or vehicle (−) for a further 8 hours. Whole-cell homogenates were prepared and equal amounts of protein were subjected to immunoblotting to detect AhR and GAPDH protein (E).

Fig. 2 Time-dependent effect of herbimycin A on AhR protein stability and transcriptional activity.
Cells were treated with 5 μM herbimycin A (HA), 0.5 μM geldanamycin (GA) or vehicle for 2 hours and then with 5 nM TCDD (+) or vehicle (−) for a further 8 or 24 hours. Cells were subjected to RT-PCR to detect transcriptional activation of *CYP1A1*. The data were statistically analyzed by *t* test: “a” indicates $p < 0.01$ compared with controls and “b” indicates $p < 0.01$ compared with samples treated with TCDD (A). After the same treatment, cells were harvested and whole-cell homogenates were prepared. Equal amounts of protein were subjected to immunoblotting to detect AhR and GAPDH proteins (B).

Fig. 3 Inhibitory effects of tyrphostin compounds on AhR transcriptional activity and nuclear localization.
Cells were treated with 25 μM tyrphostin compounds (A1, A25, B42, B44, B46, B48, B50 and B56) or vehicle for 2 hours and then with 5 nM TCDD (+) or vehicle (−) for a further 8 hours. Cells were subjected to RT-PCR to quantify CYP1A1 and GAPDH cDNA (A). Cells were treated with 25, 50 and 100 μM tyrphostin A1, B48 or vehicle for 2 hours, and then with 5 nM TCDD (+) or vehicle (−) for a further 8 hours. Cells were subjected to RT-PCR to quantify the copy number of CYP1A1 (B). Cells were treated with 50 μM tyrphostin A1, A25, B46 or B48 for 2 hours and then with 5 nM TCDD for a further 1 hour. Cells were fractionated into cytoplasmic fraction (CF) and nuclear extract (NE). Equal amounts of protein were subjected to immunoblotting to detect AhR protein (C). The data from two independent experiments were expressed as means ± SD and statistically analyzed by *t* test: “a” indicates $p < 0.01$ compared with controls and “b” indicates $p < 0.01$ compared with samples treated with TCDD.