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Novel potential of tunicamycin as an activator of the aryl hydrocarbon receptor – dioxin responsive element signaling pathway

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Abstract **Tunicamycin is a well-known inhibitor of protein glycosylation and used as an inducer of endoplasmic reticulum (ER) stress. We found that tunicamycin induced expression of cytochrome P450 1A1 in a dose-dependent manner. Like dioxin, the transcriptional induction was associated with dose-dependent activation of the dioxin responsive element (DRE). This effect was independent of inhibition of protein glycosylation or induction of ER stress. Pharmacological and genetic inhibition of the aryl hydrocarbon receptor (AhR) significantly attenuated activation of DRE by tunicamycin. These results elucidated the novel potential of tunicamycin as an activator of the AhR – DRE signaling pathway.**

Keywords: Tunicamycin; Cytochrome P450; Dioxin responsive element; Aryl hydrocarbon receptor

1. Introduction

Tunicamycin has been extensively used as inhibitor of lipid carrier-dependent protein glycosylation and, currently, is widely used as an inducer of endoplasmic reticulum (ER) stress [1]. Tunicamycin was originally purified from *Streptomyces lysosuperificus* as an antibiotic that inhibits cell wall polymer synthesis [2]. However, its therapeutic usefulness is limited because of toxicity in mammals. For example, tunicamycin is hepatotoxic and causes a periportal pattern of damage resulting in denudation of hepatocytes into blood vessels, and formation of pulmonary and cerebral emboli [3]. ER stress may be involved in the toxic effects of tunicamycin, but underlying mechanisms have not been identified yet.

Cytochrome P450 1A1 (CYP1A1) is a member of the multigene family of xenobiotic metabolizing enzymes [4]. Beside its physiological role in the detoxification of xenobiotics, CYP1A1 is also responsible for metabolic activation of polycyclic aromatic hydrocarbons and aromatic amines, leading to generation of genotoxic substances [5]. For example, benzo[*a*]pyrene induces CYP1A1 which in turn metabolizes benzo[*a*]pyrene *per se* to reactive, carcinogenic intermediates. Mice having the higher CYP1 levels were, therefore, more susceptible to various aromatic hydrocarbons-induced pathologies including liver injury [6].

Expression of *cyp1a1* in response to aromatic hydrocarbons is regulated by the aryl hydrocarbon receptor (AhR), the ligand-activating transcription factor. Binding of xenobiotic ligands [*e.g.*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)] to AhR triggers nuclear translocation of AhR and causes binding of the AhR-ligand complex to the AhR nuclear translocator in the nuclei. This molecular event results in binding of the complexes to the dioxin responsive element (DRE, also termed as xenobiotic responsive element; XRE) in the 5′ flanking region of various target genes including *cyp1a1* [7].

Recently, we unexpectedly found that tunicamycin induced expression of *cyp1a1* in a murine hepatoma cell line Hepa-1c1c7. In the present report, we elucidate molecular mechanisms involved in this novel potential of tunicamycin. Our study especially focuses on involvement of distinct cellular events that can be triggered by tunicamycin; *i.e.*, protein hypoglycosylation, ER stress and activation of the AhR – DRE signaling pathway.

2. Materials and methods

2.1. Cells and reagents

Murine hepatoma cell line Hepa-1c1c7 was purchased from American Type Culture Collection (ATCC; Manassas, VA). The reporter cell line HeXS34 was established by transfection of Hepa-1c1c7 cells with pXRE-SEAP, as described previously [8]. pXRE-SEAP encodes secreted alkaline phosphatase (SEAP) under the control of four copies of XRE/DRE [8]. 2,3,7,8-TCDD and 2-deoxy-glucose were obtained from Wako Pure Chemical Industries (Osaka, Japan), and other reagents were purchased from Sigma–Aldrich, Japan (Tokyo, Japan). All experiments were performed using α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 1% fetal bovine serum.

2.2. Transient transfection

Using lipofectamine 2000 (Invitrogen), Hepa-1c1c7 cells were transiently co-transfected with pGL3-XRE-Luc [9] together with pEFBOS or pEFBOS-AhR(Arg39Ile) encoding a dominant-negative mutant of AhR [10]. pGL3-XRE-Luc encodes firefly luciferase under the control

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Abbreviations: ER, endoplasmic reticulum; CYP1A1, cytochrome P450 1A1; AhR, aryl hydrocarbon receptor; 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DRE, dioxin responsive element; SEAP, secreted alkaline phosphatase; XRE, xenobiotic responsive element; GRP78, 78-kD glucose-regulated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AhRDN, dominant-negative mutant of AhR

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of consensus sequences of XRE/DRE. Forty eight hours after the transfection, the cells were treated with tunicamycin (10 μg/ml) or 2,3,7,8-TCDD (100 pM) for 6 h and subjected to luciferase assay, as described later. Assays were performed in quadruplicate.

2.3. Northern blot analysis

Total RNA was extracted by the single-step method, and Northern blot analysis was performed as described before [11]. cDNAs for CYP1A1 [12], SEAP (BD Biosciences, Palo Alto, CA) and 78-kDa glucose-regulated protein (GRP78) [12] were used to prepare radio-labeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MO).

2.4. Luciferase assay

Activity of luciferase was evaluated by Luciferase Assay System (Promega, Madison, WI). In brief, according to the manufacturer's protocol, cells were lysed by lysis buffer, and activity of luciferase was evaluated in the presence of luciferin using a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan).

2.5. Statistical analysis

Data were expressed as means ± S.E. Statistical analysis was performed using the non-parametric Mann–Whitney *U* test to compare data in different groups. *P* value <0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Induction of cyp1a1 mRNA by tunicamycin

We first examined a dose-dependent effect of tunicamycin on the expression of *cyp1a1*. Hepa-1c1c7 cells were exposed to 0–5 μg/ml of tunicamycin for 6 h and subjected to Northern blot analysis. As shown in Fig. 1A, expression of *cyp1a1* mRNA was induced by tunicamycin in a dose-dependent manner. Substantial induction was observed at concentrations higher than 0.5 μg/ml and peaked to maximum at 2.5–5 μg/ml. Time-lapse experiments revealed that the induction of *cyp1a1* was transient; *i.e.*, it was peaked at 3 h and declined thereafter (Fig. 1B).

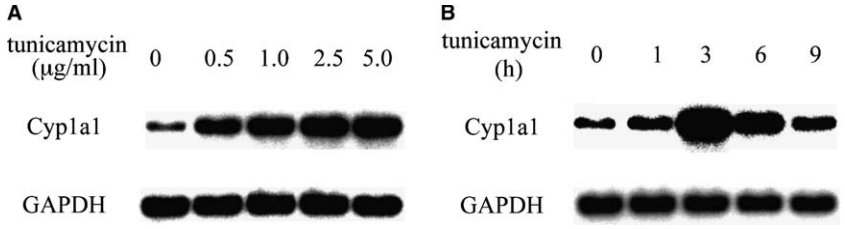

Fig. 1. Dose- and time-dependent induction of cytochrome P450 1A1 (CYP1A1) mRNA by tunicamycin. Hepa-1c1c7 cells were exposed to either indicated concentrations of tunicamycin for 6 h (A) or 5.0 μg/ml tunicamycin for indicated time periods (B), and expression of *cyp1a1* was examined by Northern blot analysis. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown at the bottom as a loading control.

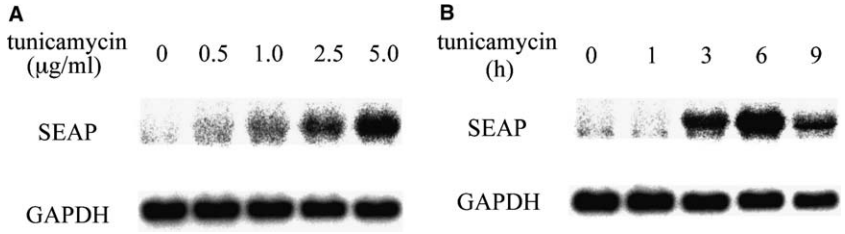

Fig. 2. Dose- and time-dependent activation of the dioxin responsive element (DRE) by tunicamycin. Hepa-1c1c7-derived HeXS34 cells that express secreted alkaline phosphatase (SEAP) under the control of DRE consensus sequences were exposed to either indicated concentrations of tunicamycin for 6 h (A) or 5.0 μg/ml tunicamycin for indicated time periods (B), and expression of *SEAP* was examined by Northern blot analysis.

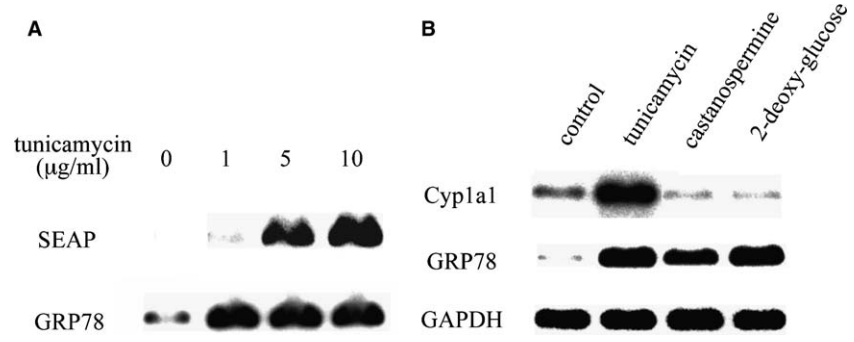

Fig. 3. Lack of involvement of endoplasmic reticulum stress and protein hypoglycosylation in the effects of tunicamycin. (A) HeXS34 cells were treated with 1–10 μg/ml tunicamycin for 6 h, and expression of *SEAP* and *grp78* was examined by Northern blot analysis. (B) HeXS34 cells were treated with 5 μg/ml tunicamycin, 2 mM castanospermine or 6 mg/ml 2-deoxy-glucose for 6 h, and expression of *cyp1a1* and *grp78* was examined.

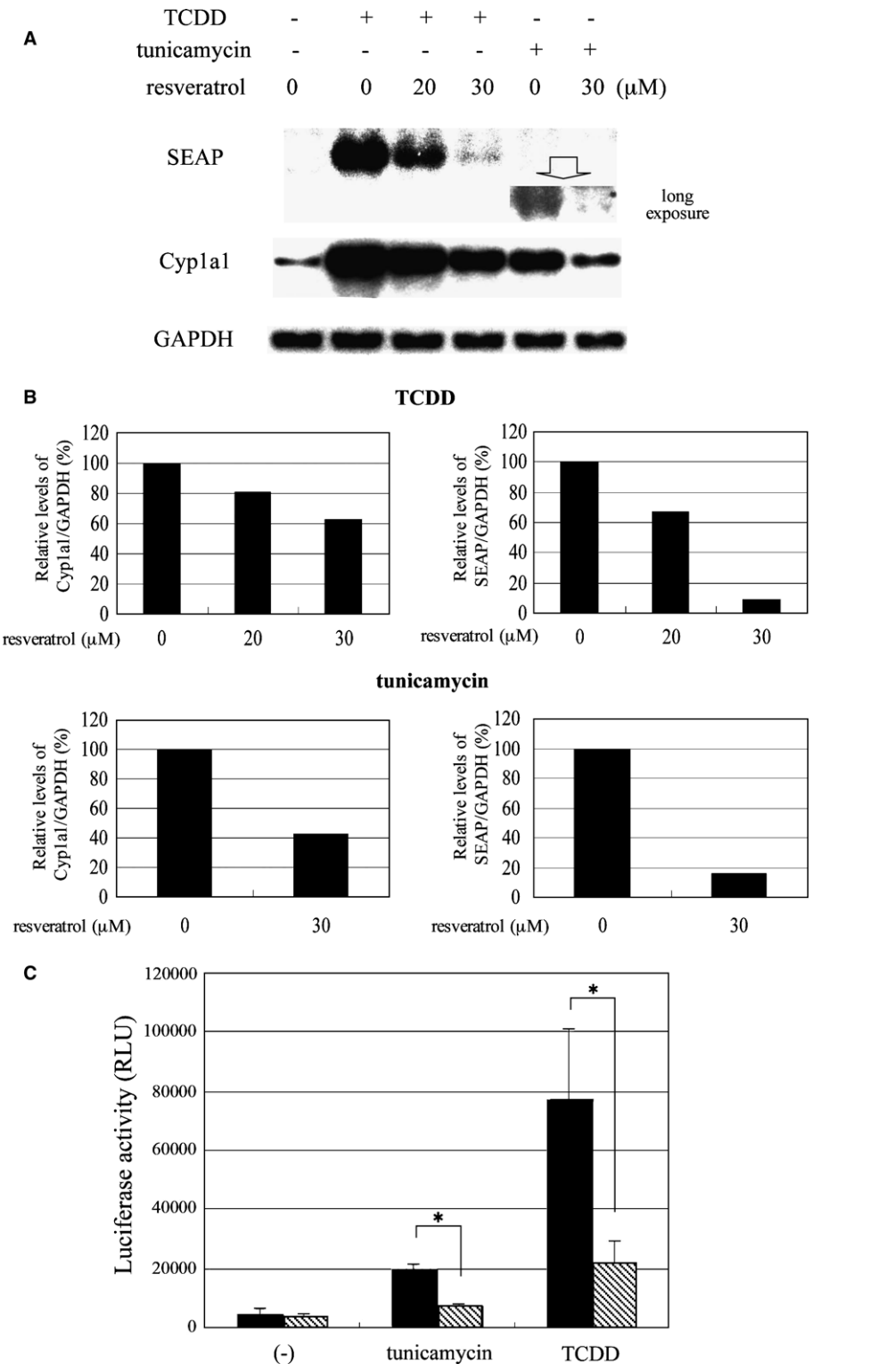

Fig. 4. Involvement of aryl hydrocarbon receptor (AhR) in the activation of DRE and induction of *cyp1a1* by tunicamycin. (A and B) Effects of resveratrol on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)- and tunicamycin-induced activation of DRE and expression of *cyp1a1*. HeXS34 cells were pretreated with 0–30 μM resveratrol for 30 min, exposed to 25 pM 2,3,7,8-TCDD or 5 μg/ml tunicamycin for 3 h and subjected to Northern blot analysis of *SEAP* and *cyp1a1*. Intensity of individual signals normalized by the levels of GAPDH is shown in (B). (C) Hepa-1c1c7 cells were transiently co-transfected with pGL3-XRE-Luc together with pEFBOS (closed bars) or pEFBOS-AhR (Arg39Ile) encoding a dominant-negative mutant of AhR (shaded bars). Forty-eight hours after the transfection, the cells were treated with tunicamycin (10 μg/ml) or 2,3,7,8-TCDD (100 pM) for 6 h and subjected to luciferase assay. Assays were performed in quadruplicate. Data are expressed as means ± S.E. Asterisks indicate statistically significant differences ($P < 0.05$). RLU, relative light unit.

3.2. Activation of DRE by tunicamycin

The 5′-flanking region of the *cyp1a1* gene contains DREs. These DREs are essential for transcriptional induction of *cyp1a1* by aromatic hydrocarbons including dioxins [4,7]. We next examined whether tunicamycin can trigger activation of DRE. For this purpose, Hepa-1c1c7-derived HeXS34 cells were used. This reporter line expresses *SEAP* under the control of the DRE/XRE consensus sequences [8]. As shown in Fig. 2A, tunicamycin induced activation of DRE in a dose-dependent manner, which was closely correlated with the expression level of *cyp1a1* (Fig. 1A). The activation was peaked at 6 h and declined thereafter (Fig. 2B). The similar result was also obtained using a different Hepa-1c1c7-derived reporter cells, HeDS49 [13], that express *SEAP* under the control of the truncated promoter of the mouse *cyp1a1* gene that contains 4 DREs (data not shown).

3.3. Lack of involvement of protein hypoglycosylation and ER stress in the effects of tunicamycin

Tunicamycin inhibits transfer of *N*-acetylglucosamine-1-phosphate from uridine 5-diphosphate-*N*-acetylglucosamine to dolichol monophosphate in the first step of glycoprotein synthesis and thereby blocks formation of protein-carbohydrate linkages of the N-glycosidic type [14]. Accumulation of hypoglycosylated proteins in the ER subsequently causes ER stress and induces the so-called unfolded protein responses including expression of GRP78 [1]. We examined whether protein hypoglycosylation or ER stress is causative of induction of *cyp1a1* and activation of DRE by tunicamycin. First, HeXS34 cells were treated with different concentrations of tunicamycin, and the levels of *SEAP*and *grp78*, an endogenous marker of ER stress, were compared by Northern blot analysis. As shown in Fig. 3A, the level of ER stress indicated by *grp78* was not in parallel with the activation level of DRE indicated by *SEAP*. For example, 1 μg/ml of tunicamycin fully induced ER stress whereas activation of DRE was not obvious. This result indicated that activation of DRE by tunicamycin may be independent of ER stress. To confirm this conclusion, HeXS34 cells were treated with different glycosylation inhibitors, castanospermine and 2-deoxy-glucose, and expression of *cyp1a1* and *grp78* was examined. Like tunicamycin, both castanospermine and 2-deoxy-glucose substantially induced ER stress indicated by expression of *grp78*. However, both agents did not cause induction of *cyp1a1* (Fig. 3B). These results suggested that neither protein hypoglycosylation nor ER stress was causative of activation of the DRE pathway by tunicamycin.

3.4. Involvement of AhR in the activation of DRE and expression of cyp1a1 by tunicamycin

In general, DRE is activated via binding of AhR-xenobiotics complexes [4,7]. We tested whether functional AhR is required for the effects of tunicamycin on the activation of DRE and expression of *cyp1a1*. For this purpose, we used resveratrol, a pure antagonist of AhR [15]. As shown in Fig. 4A and B, 2,3,7,8-TCDD induced activation of DRE and expression of *cyp1a1* in HeXS34 cells. This induction was inhibited by the treatment with resveratrol modestly at 20 μM and markedly at 30 μM. Like its suppressive effect on 2,3,7,8-TCDD, expression of *cyp1a1* triggered by tunicamycin was substantially inhibited by the treatment with resveratrol, and it was associated with suppression of DRE activation evidenced by attenuated *SEAP* mRNA.

To further confirm the involvement of AhR in the effects of tunicamycin, Hepa-1c1c7 cells were transiently transfected with a dominant-negative mutant of AhR (AhRDN) together with a luciferase-based DRE reporter plasmid. Northern blot analysis confirmed high levels of expression of AhRDN in the transfected cells (data not shown). Using these cells, the effect of tunicamycin on DRE was retested. Transfection with AhRDN did not affect the basal luciferase activity. As expected, functional suppression of AhR by AhRDN significantly inhibited activation of DRE triggered by 2,3,7,8-TCDD (35.0 ± 17.5% *vs.* 100% in control, $P < 0.05$). AhRDN also substantially inhibited activation of DRE triggered by tunicamycin (39.7 ± 19.8% *vs.* 100% in control, $P < 0.05$) (Fig. 4C). These results provided additional evidence that tunicamycin has the potential for activation of the AhR – DRE signaling pathway.

4. Discussion

Tunicamycin has been used for many years as an inducer of ER stress as well as an inhibitor of protein glycosylation in the fields of cell biology and biochemistry. In the present report, we elucidated the novel potential of tunicamycin as an activator of the AhR – DRE signaling pathway. A number of previous reports showed crucial roles of the AhR – DRE pathway in various pathologies caused by halogenated and polycyclic aromatic hydrocarbons [4,7]. These environmental substances cause toxic effects via induction of DRE-regulated genes that inhibit mitosis, induce apoptosis and generate toxic metabolites. DREs are also located in the 5′-regulatory regions of a variety of inflammation-associated genes including: (1) interleukins and chemokines; (2) cytokine receptors and adhesion receptors; (3) enzymes involved in synthesis of nitric oxide, prostaglandins and lipoxygenases; and (4) molecules involved in the NF-κB pathway [16]. Therefore, the current finding that tunicamycin has the potential for activating the DRE – AhR pathway raises a possibility that this agent may affect not only protein glycosylation and ER function but also influence a diverse range of other cellular events. For example, *in vivo* toxicity of tunicamycin in the liver and the lung [3] could be caused by the activation of the AhR – DRE pathway. It should be emphasized that the triggering effect of tunicamycin on the AhR – DRE pathway must be considered carefully if this agent is used as a "selective" inhibitor of protein glycosylation and/or inducer of ER stress under *in vitro* and *in vivo* situations.

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References

[1] Lee, A.S. (2001) The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem. Sci. 26, 504–510.
[2] Elbein, A.D. (1984) Inhibitors of the biosynthesis and processing of N-linked oligosaccarides. CRC Crit. Rev. Biochem. 16, 21–49.

[3] Finnie, J.W. and O'Shea, J.D. (1989) Acute hepatotoxicity with resultant pulmonary and cerebral embolism in guinea pigs given tunicamycin. Pathology 21, 194–199.
[4] Nebert, D.W. and Gonzalez, F.J. (1987) P450 genes: structure, evolution, and regulation. Annu. Rev. Biochem. 56, 945–993.
[5] Nebert, D.W., Dalton, T.P., Okey, A.B. and Gonzalez, F.J. (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J. Biol. Chem. 279, 23847–23850.
[6] Nebert, D.W. (1989) The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. Crit. Rev. Toxicol. 20, 153–174.
[7] Mimura, J. and Fujii-Kuriyama, Y. (2003) Functional role of AhR in the expression of toxic effects by TCDD. Biochim. Biophys. Acta 1619, 263–268.
[8] Kasai, A., Yao, J., Yamauchi, K., Hiramatsu, N., Hayakawa, K., Meng, Y., Maeda, S. and Kitamura, M. (2006) Influence of cAMP on reporter bioassays for dioxin and dioxin-like compounds. Toxicol. Appl. Pharmacol. 211, 11–19.
[9] Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y. and Kato, S. (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423, 545–550.
[10] Sogawa, K., Numayama-Tsuruta, K., Takahashi, T., Matsushita, N., Miura, C., Nikawa, J., Gotoh, O., Kikuchi, Y. and Fujii-Kuriyama, Y. (2004) A novel induction mechanism of the rat CYP1A2 gene mediated by Ah receptor – Arnt heterodimer. Biochem. Biophys. Res. Commun. 318, 746–755.
[11] Kitamura, M., Suto, T., Yokoo, T., Shimizu, F. and Fine, L.G. (1997) Transforming growth factor-β1 is the predominant paracrine inhibitor of macrophage cytokine synthesis produced by glomerular mesangial cells. J. Immunol. 156, 2964–2971.
[12] Katayama, T., Imaizumi, K., Honda, A., Yoneda, T., Kudo, T., Takeda, M., Mori, K., Rozmahel, R., Fraser, P., George-Hyslop, P.S. and Tohyama, M. (2001) Disturbed activation of endoplasmic reticulum stress transducers by familial Alzheimer's disease-linked presenilin-1 mutations. J. Biol. Chem. 276, 43446–43454.
[13] Kasai, A., Hiramatsu, N., Meng, Y., Yao, J., Takeda, M., Maeda, S. and Kitamura, M. (2004) DRESSA: biosensing of dioxin and dioxin-like chemicals using secreted alkaline phosphatase. Anal. Biochem. 335, 73–80.
[14] Duksin, D. and Mahoney, W.C. (1982) Relationship of the structure and biological activity of the natural homologues of tunicamycin. J. Biol. Chem. 257, 3105–3109.
[15] Ciolino, H.P., Daschner, P.J. and Yeh, G.C. (1998) Resveratrol inhibits transcription of CYP1A1 *in vitro* by preventing activation of the aryl hydrocarbon receptor. Cancer Res. 58, 5707–5712.
[16] Savouret, J.F., Berdeaux, A. and Casper, R.F. (2003) The aryl hydrocarbon receptor and its xenobiotic ligands: a fundamental trigger for cardiovascular diseases. Nutr. Metab. Cardiovasc. Dis. 13, 104–113.