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Original Article

Effects of benzotriazole ultraviolet stabilizers on rat PXR, CAR and PPARα transcriptional activities

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ABSTRACT — Benzotriazole ultraviolet stabilizers (BUVSs) are widely used as ultraviolet filters in various consumer and industrial products. For this purpose, we examined the effects of 10 BUVSs and benzotriazole itself on transcriptional activation mediated by nuclear receptors: pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor alpha (PPARα). UV-090 and UV-9 showed rat PXR-agonistic activity in the concentration range of 1-30 μM in reporter gene assay using simian kidney COS-1 cells. UV-090 showed the highest activity (REC_{20} value: 3.85×10^{-6} M). UV-090 was also positive in rat CAR activation assay, while UV-P showed inverse agonistic activity towards CAR. In the presence of the CAR agonist artemisinin (10 μM), UV-P also showed dose-dependent CAR-antagonistic activity in the concentration range of 10-30 μM. UV-090 and UV-9 activated rat PPARα. Overall, these results suggest that UV-090, UV-9 and UV-P modulate PXR, CAR and/or PPARα activation.

Key words: Benzotriazole UV stabilizer, CAR, Nuclear receptor activation, PPARα, PXR

INTRODUCTION

Many kinds of benzotriazole ultraviolet stabilizers (BUVSs; (2-hydroxyphenyl)benzotriazole derivatives) are widely used as ultraviolet filters in consumer and industrial products. They are often added to plastic products, including food packaging products, to prevent light-induced degradation and yellowing. This has led to increasing environmental contamination with BUVSs; for example, a series of BUVSs has been found in municipal waste-water, sewage sludge and coastal waters, and BUVSs have also been detected in foods, such as mussels, fish and marine mammals (Kim *et al.,* 2011a, 2011b; Nakata *et al.,* 2009, 2010, 2012; Ruan *et al.,* 2012; Lu *et al.,* 2016). 2-(Benzotriazole-2-yl)-4-methylphenol (UV-P) was detected at the level of 8 ng/L in Chinese river waters (Liu *et al*., 2014). BUVSs were also detected in house dust and in soil (Kim *et al.,* 2012; Lai *et al.,* 2014), as well as in human urine and breast milk (Lee *et al.,* 2015). One of the BUVSs, 2-(2'-hydroxy-3', 5'-di-*tert*-butylphenyl)benzotriazole (UV-320), was declared a "Class I specified chemical substance" under the Japanese Law concerning Examination and Regulation of Manufacture of Chemical Substances in 2007, and its pro-

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duction, use and import are prohibited. BUVSs were shown to have low acute toxicity in aquatic organisms and rats (Hirata-Koizumi *et al.,* 2007; Kim *et al.,* 2011c; Seeland *et al.,* 2012), but their chronic effects in marine organisms or mammalian species remain largely unknown.

Endocrine-disrupting action is a problematic activity of various environmental pollutants. Among BUVSs, UV-P, 2-(3-allyl-2-hydroxy-5-methylphenol)-2H-benzotriazole (UV-9), 2-(3-allyl-2-hydroxy-5-methylphenol)-2-H-benzotriazole (UV-090) and 2-(2-hydroxy-3-*tert*-butyl-5-methylphenol)-5-chlorobenzotriazole (UV-326) exhibit significant agonistic activities towards human aryl hydrocarbon receptor (AhR), whereas 2-[(3,5-bis(1-methyl-1-phenylethyl)-2-hydroxyphenyl)benzotriazole (UV-234), 5-chloro-2-(3,5-di-*tert*-butyl-2-hydroxyphenyl)benzotriazole (UV-327), 2-(3,5-di-*tert*-amyl-2-hydroxyphenyl)benzotriazole (UV-328) and 2-(2-hydroxy-5-*tert*-octyl-phenyl)benzotriazole (UV-329) are inactive (Nagayoshi *et al.,* 2015). Fent *et al.* (2014) showed that UV-P and UV-326 induce AhR signalling in zebrafish embryos. BUVSs are not estrogenic (Kawamura *et al.,* 2003), but UV-P showed antiandrogenic activity in a yeast assay system (Fent *et al.,* 2014).

Pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor α (PPARα) are members of the nuclear receptor superfamily, and are each composed of four modules: the modulator domain, which has a ligand-independent transcription activation function (AF-1); the DNA-binding domain (DBD); the hinge region; and the ligand-binding domain (LBD). The differences and similarities of target genes regulated by the three receptors can be illustrated by the induction of cytochrome P450 (P450) genes in response to PXR, CAR or PPARα activation. There is a significant overlap of ligand specificity in the P450 genes regulated by PXR and CAR: activation of both receptors induces CYP2B, CYP2C and CYP3A gene expression (Maglich *et al.,* 2002). In contrast, PPARα primarily induces expression of CYP4A (Kroetz *et al.,* 1998). PXR agonists show great structural diversity, including steroids, xenobiotics, clinical drugs and components of medicinal herbal extracts. Most of the known PXR agonists are also ligands of the closely related xeno-sensing nuclear receptor, CAR. Some compounds have both PXR and CAR activities, whereas others are PXR agonists, but not CAR agonists; i.e., CAR is less promiscuous than PXR (Kretschmer and Baldwin*,* 2005; Timsit and Negishi, 2007). CAR differs from the other two nuclear receptors, firstly because it shows activity in the absence of ligand activation, and secondly because further activation can be achieved either through direct ligand binding or via indirect intracellular metabolic pathways (Kawamoto *et al.,* 1999; Rencurel *et al.,* 2005). Many CAR activators are not ligands, but act indirectly, e.g., via dephosphorylation (Mutoh *et al.,* 2013).

These receptors regulate not only P450, but also phase II enzymes such as uridine diphospho-glucuronosyltransferases (UGTs), glutathione-*S*-transferases (GSTs) and sulfotransferases (SULTs) (Kast *et al*., 2002; Kodama and Negishi, 2013; Bigo *et al.,* 2013). Activation or inactivation of these receptors is also able to cause endocrine disruption indirectly. Various endogenous hormones are metabolized by P450, UGTs and SULTs, and induction of these enzymes mediated by PXR, CAR and PPARα could lead to altered levels of active hormones. For example, CAR agonists reduce thyroid hormone levels by inducing UGTs (Schraplau *et al.,* 2015). In addition, phthalates are PPARγ agonists, and have been reported to reduce serum estradiol levels by inhibiting the gene expression of aromatase, which catalyzes the conversion of testosterone to estrogen (Lovekamp-Swan and Davis, 2003). Thus, it is important to investigate the activities of environmental chemicals towards nuclear receptors that regulate hormonal activities.

In the present study, we focused on the effects of 10 BUVSs, *i.e.* UV-P, UV-9. UV-090, UV-234, UV-326, UV-327, UV-328, UV-329, UV-360 and UV-571, as well as benzotriazole (BTZ), on transcriptional activation mediated by rat PXR, rat CAR and rat PPARα (Fig. 1).

MATERIALS AND METHODS

Materials

The sources, CAS numbers, purity and abbreviations of the compounds tested in the present study are listed in Table 1. 5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN; > 97% pure) and artemisinin (> 97% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), respectively. Bezafibrate (BZF; > 99.3% pure) and dimethyl sulfoxide (DMSO; > 99.5% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells and plasmids

COS-1 cells derived from African green monkey kidney were obtained from RIKEN BioResource Center (Ibaraki, Japan). The cells were routinely cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic mixture (Anti-Anti) and 1% MEM non-essential amino acids (MEM NEAA) at 37°C, in an atmosphere of 5% CO_2/95% air under saturating humidity, as described

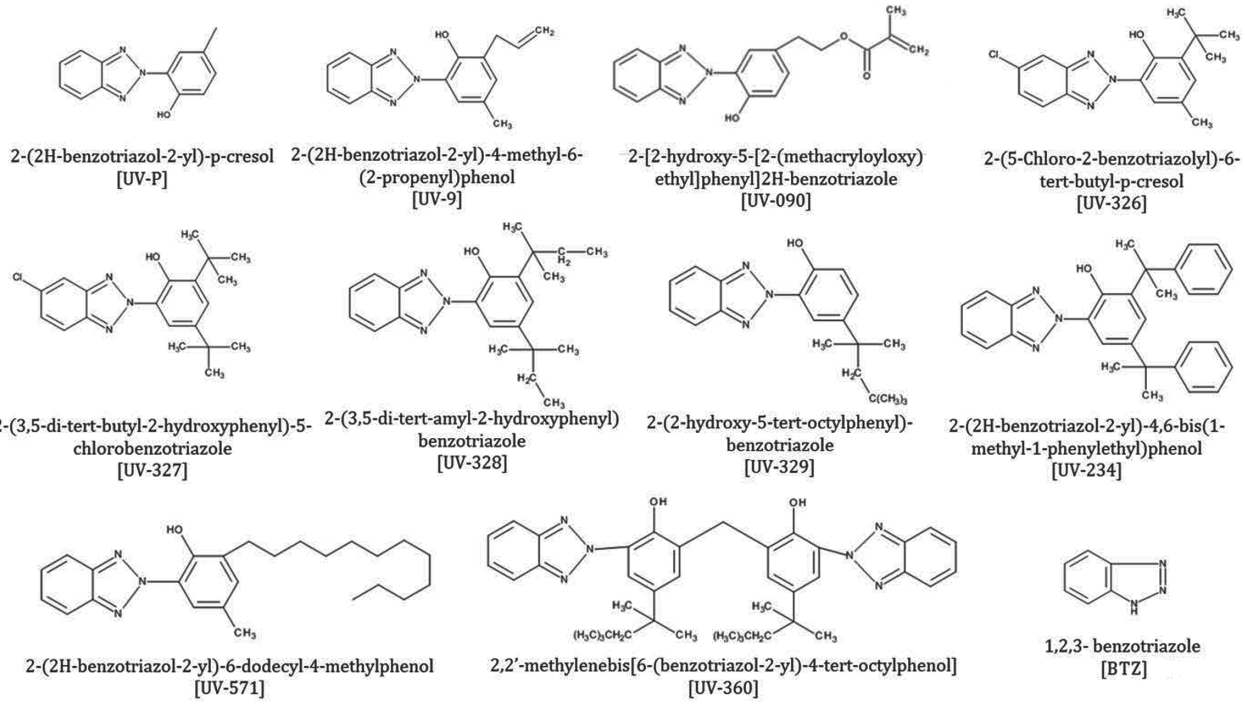

Fig. 1. Structures of BUVSs used in this study.

Table 1. Abbreviation, CAS number, source and purity of BUVSs and benzotriazole tested in this study.

Compound	Abbreviation	CAS number	Source	Purity (%)
2-(2-Hydroxy-5-methylphenyl)benzotriazole	UV-P	2440-22-4	Wako	97.0
2-(3-Allyl-2-hydroxy-5-methylphenyl)-2*H*-benzotriazole	UV-9	2170-39-0	Sigma	99.0
2-[2-Hydroxy-5-[2-(methacryloyloxy)ethyl]phenyl]-2*H*- benzotriazole	UV-090	96478-09-0	TCI	> 98.0
2-(2-Hydroxy-3-tert-butyl-5-methylphenyl)-5-chlorobenzo triazole	UV-326	3896-11-5	TCI	> 98.0
2-(3,5-Di-*tert*-butyl-2-hydroxyphenyl)-5-chlorobenzo triazole	UV-327	3864-99-1	TCI	> 98.0
2-(3,5-Di-*tert*-amyl-2-hydroxyphenyl) benzotriazole	UV-328	25973-55-1	TCI	> 98.0
2-(2-Hydroxy-5-*tert*-octylphenyl)-benzotriazole	UV-329	3147-75-9	TCI	> 98.0
2-[3,5-Bis(1-methyl-1-phenylethyl)-2-hydroxyphenyl]-2*H*- benzotriazole	UV-234	70321-86-7	TCI	> 98.0
2,2'-Methylenebis[2-hydroxy-5-(1,1,3,3-tetramethylbutyl)-1,3-phenylene] bis(2*H*-benzotriazole)	UV-360	103597-45-1	TCI	> 98.0
2-(2*H*-Benzotriazol-2-yl)-6-dodecyl-4-methylphenol	UV-571	125304-04-3	Sigma	> 98.0
1,2,3-Benzotriazole	BTZ	95-14-7	TCI	> 98.0

Wako: Wako Pure Chemical Industries, Ltd. (Osaka, Japan), TCI: Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Sigma: Sigma-Aldrich (St. Louis, MO, USA).

in our previous report (Fujino *et al*., 2016). Plasmids used in this study were as described previously (Fujino *et al.,* 2016).

Reporter gene assay

COS-1 cells were plated in 96-well plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) in D-MEM supplemented with 10% FBS, 1% Anti-Anti and 1% MEM NEAA. They were transfected with expression plasmid, reporter plasmid and internal control plasmid using the jetPEI transfection reagent (PolyPlus Transfection, Illkirch, France). After 24 hr (assay for PXR and PPARα) or 12 hr (assay for CAR), the cells were exposed to various concentrations of test compounds or 0.1% DMSO (vehicle control). After 24 hr incubation with chemicals, cells were harvested with 25 µL of passive lysis buffer (Promega Co., Madison, WI, USA). Luciferase assays were performed using the Dual Luciferase

Assay Kit (Promega Co.) by measuring luminescence with a luminometer, Luminoskan Ascent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Firefly luciferase activity was normalized to renilla luciferase activity of phRL-tk. Results are expressed as means ± standard deviation (SD) from at least three independent experiments performed in duplicate. Details are described in our previous report (Fujino *et al.,* 2016).

All test compounds used were dissolved in DMSO at a concentration of 30 mM. The final DMSO concentration in the culture medium did not exceed 0.1%, and this concentration did not affect cell yields. All compounds were diluted to the predetermined concentrations in appropriate medium immediately before use.

Data analyses and statistics

Data are presented as means ± standard deviation (SD). An analysis of variance (ANOVA) followed by Bonferroni correction was used to evaluate the differences in transcriptional levels between each control group (0.1% DMSO alone) and each of the chemical groups in the PXR, CAR and PPARα assays. A value of $P < 0.05$ was considered significant.

RESULTS

Agonistic activities of BUVSs and BTZ towards PXR and PPARα

The ability of the 10 BUVSs and BTZ to activate rat nuclear receptors PXR and PPARα was examined by reporter gene assay in COS-1 cells. PCN (positive control for PXR) enhanced luciferase activity 2.5-fold compared to DMSO-treated cells (control) at the concentration of 1 μM. Among the test compounds, UV-9 and UV-090 activated PXR. UV-090 showed activity at the concentration of 3 μM compared with the control, and its activity was concentration-dependent, reaching about 2.1-fold at 30 μM. UV-9 showed about 1.7-fold enhancement at 30 μM, compared with the control (Fig. 2A). However, UV-P, UV-234, UV-326, UV-327, UV-328, UV-329, UV-360, UV-571 and BTZ were inactive at 1-30 μM. BZF, a positive control for PPARα, enhanced luciferase activity 2.7-fold at the concentration of 30 μM. UV-090 and UV-9 showed significant PPARα-agonistic activity in the concentration range of 10-30 μM compared with the control (Fig. 2B).

Effect of BUVSs and BTZ on CAR activity

Effect of BUVSs and BTZ on rat nuclear receptor CAR activity was examined by reporter gene assay in COS-1 cells. Artemisinin, a positive control for CAR, enhanced luciferase activity 1.5-fold at the concentration of 30 μM. Among the BUVSs tested, only UV-090 enhanced CAR activity at 10-30 μM. In contrast, UV-P dose-dependently decreased the constitutive activity of CAR in the concentration range of 3-30 μM, indicating that they act as inverse agonists towards CAR (Fig. 3A). The effect was also examined in the presence of a positive agonist for

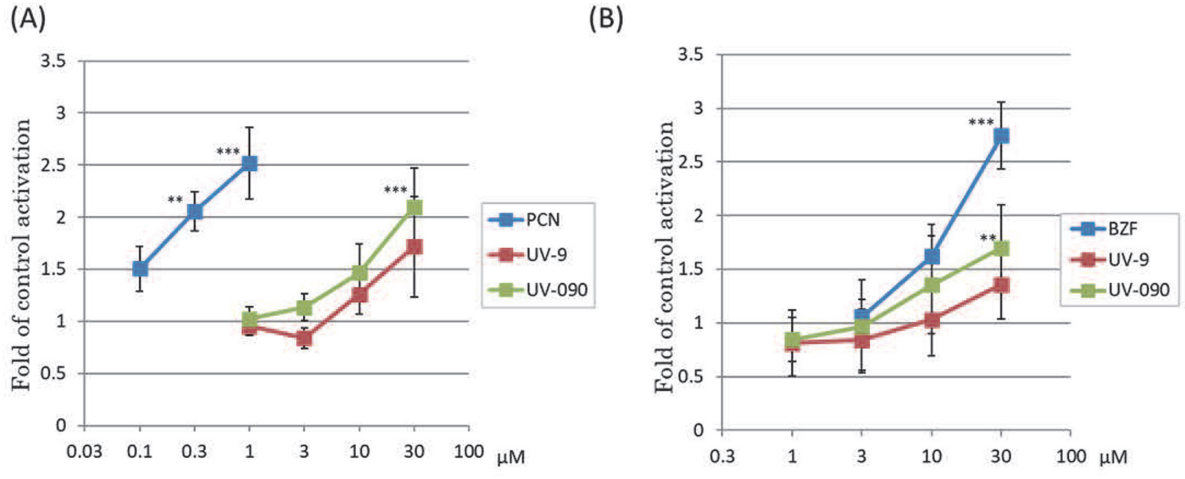

Fig. 2. Agonistic activities of BUVSs towards PXR (A) and PPARα (B) as determined by luciferase reporter assay in COS-1 cells. Nuclear receptor activation of BUVSs was expressed as n-fold induction versus the vehicle control. Each value represents the mean ± SD of 3-6 individual experiments. Significant differences from the vehicle control were expressed. $^{**}p < 0.01$, $^{***}p < 0.005$. Other details are described in Materials and methods. PCN: 5-pregnen-3β-ol-20-one-16α-carbonitrile, BZF: bezafibrate.

CAR, artemisinin (10 μM). UV-P again dose-dependently decreased the activity of artemisinin in the concentration range of 10-30 μM (Fig. 3B).

Relative activity of BUVSs for PXR, CAR and PPARα

To assess the relative activities of BUVSs against PXR, CAR and PPARα, we determined EC_{50} for agonistic activity and IC_{50} for inverse agonistic activity. UV-090 showed the lowest EC_{50} value for PXR (16.64 μM) among the tested compounds, and also showed an EC_{50} value of 10.15 μM for CAR. On the other hand, UV-P showed inverse agonistic activity towards CAR, with an IC_{50} value of 10.18 μM.

DISCUSSION

Various pharmaceuticals and pesticides exhibit agonistic activities towards PXR, CAR and PPARα (Takeuchi *et al.,* 2006; Kojima *et al.,* 2010, 2011; Baldwin and Roling, 2009; Abass *et al.,* 2012), but the activity of sunscreen components, etc., in consumer products has received less attention. In this study, we found that UV-090 and UV-9 showed PXR-agonistic activity, UV-090 showed CAR-agonistic activity, and UV-090 and UV-9 showed PPARα-agonistic activity. In addition, UV-P showed inverse agonistic activity against CAR. However, we could not identify any clear structure-activity relationship. On the other hand, some BUVSs have been reported to show AhR-agonistic activity (Nagayoshi *et al.,* 2015), and UV-P exhibited antiandrogenic activity in a yeast reporter assay (Fent *et al.,* 2014). Thus, 2-hydroxyl and straight-chain 5-alkyl structure may favor affinity for these nuclear receptors, while the bulky *tert*-butyl moiety of BUVSs such as UV-326 and UV-327, and the large substituents in UV-328, UV-329 and UV-234 may hinder binding to these receptors.

Activation of nuclear receptors can induce expression and activity of P450; for example, rat CYP3A1 is primarily regulated by PXR, and also partially regulated by CAR (Gamou *et al.,* 2015), while CYP4A expression is induced by activation of PPARα (Kroetz *et al.,* 1998). Hormone level-regulating phase II drug-metabolizing enzymes such as UGT and SULT are also induced by activation of these nuclear receptors (Bigo *et al.,* 2013; Kodama and Negishi, 2013). Since UV-090 and UV-9 activated PXR, CAR and/or PPARα, they may also induce these drug-metabolizing enzymes. We know no previous report on P450 induction by BUVSs in mammals, although effects on zebrafish elenthero-embryos *in vivo* were reported (Fent *et al.,* 2014). UV-P and UV-326 induced the AhR pathway, and UV-P induced GST (*gstp1*), UGT (*ugt1a*) and P450 (*cyp1a1*) at the mRNA level. Further examination of the effect of BUVSs on drug-metabolizing enzymes related to endocrine-disrupting action in mammals would be desirable.

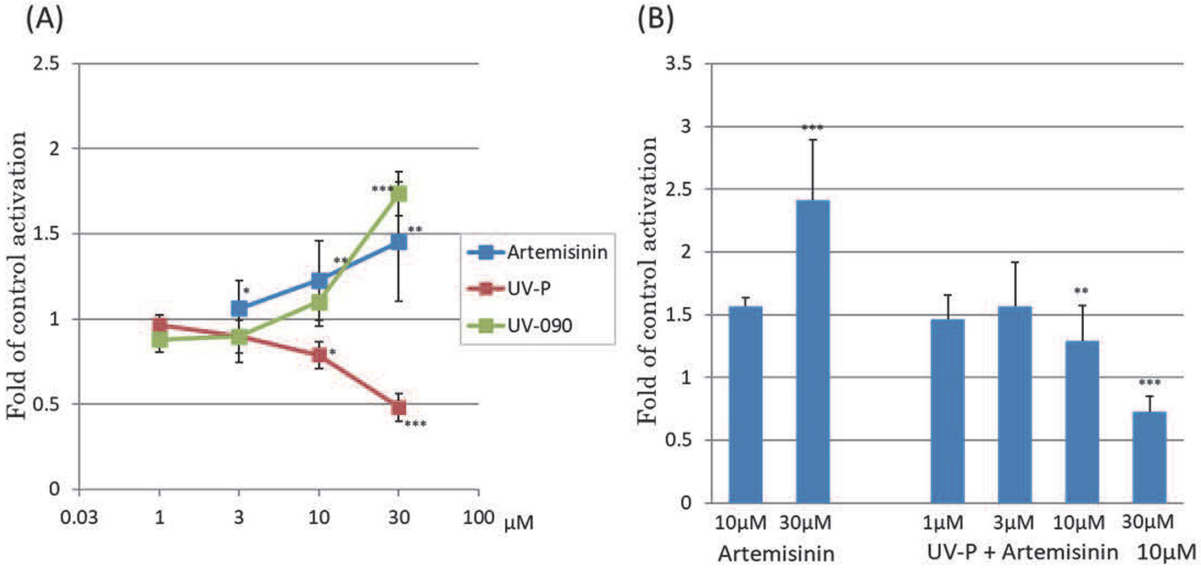

Fig. 3. Influence of BUVSs on CAR activity (A), and effect of UV-P in the presence of artemisinin (10 μM) (B), as determined by luciferase reporter assay in COS-1 cells. The activity was expressed as n-fold induction versus the vehicle control. Each value represents the mean ± SD of 3-6 individual experiments. Significant differences from the vehicle control were expressed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

CAR has constitutive activity, which can be repressed or further activated by ligands (Shan *et al.,* 2004; Jyrkkarinne *et al.,* 2012). We found that UV-P dose-dependently repressed the constitutive activity of rat CAR, while UV-090 activated the constitutive activity of CAR. Furthermore, in the presence of artemisinin (10 μM), an agonist of CAR, UV-P showed antagonistic activity. These results suggest that UV-090 and UV-P could have contrasting effects on CAR-related biological actions.

Hirata-Koizumi *et al.* (2007, 2008, 2016) reported that UV-320 showed gender-dependent hepatotoxicity in rats, inducing CYP4A-specific lauric acid 12-hydroxylase activity. As a possible mechanism, they suggested hepatotoxicity through the PPARα signaling pathway, based on gender-specific enhancement of the expression of PPARα mRNA in rat liver after the administration of UV-320. Since UV-090 and UV-9 activated PPARα in our study, they may induce similar hepatotoxicity.

In this study, we found that UV-090, UV-9 and UV-P showed positive results in reporter gene assay against PXR, CAR and PPARα. The active concentrations of these compounds were higher than those of the positive controls *i.e.* PCN for PXR, artemisinin for CAR, and BZF for PPARα. Some BUVSs have been detected in human urine and breast milk, possibly originating from marine foods and indoor dust (Carpinteiro *et al.,* 2010; Kim *et al.,* 2011a, 2011b, 2012; Lu *et al.,* 2016), but the observed concentrations seem too low to have any direct effect on human health. Nevertheless, our results suggest that at least UV-090, UV-9 and UV-P among BUVSs could alter metabolizing activity towards xenobiotics and endogenous chemicals in the human body, if they were present at sufficiently high concentrations.

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

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