

HBCDs in fish and food as is done for other harmful residual chemicals.

Acknowledgements

This work was supported by a Scientific Research Grant from the Ministry of Health, Welfare, and labor of Japan.

References

1. Nakagawa R., Murata S., Ashizuka Y., Hori T., Yasutake D., Tobiishi K. and Sasaki K. *Organohalogen Com.*69:2007;69: 1985.
2. Nakagawa R., Ashizuka Y., Hori T., Yasutake D., Tobiishi K. and Sasaki K. *Organohalogen Com.*68:2006;68: 2166.
3. <http://gsar.cerij.or.jp/SHEET/F2001.71.pdf>.
4. Law R.J., Berduder P., Allchin C.R., and Barry J. *Environ. Sci. Technol.*, 40, 2006: 2177.

Table 1 HBCD levels of marine products from four regions of Japan

Region		Fat (%)	α -HBCD	β -HBCD	γ -HBCD	Σ HBCDs	Σ HBCDs
			ng/g ww	ng/g ww	ng/g ww	ng/g ww	ng/g lw
K	Median	1.2	0.04	ND	ND	0.04	1.4
	Max	20.4	0.73	ND	0.27	1.0	40
	Min	0.2	0.00	ND	ND	ND	ND
S	Median	0.9	0.05	ND	ND	0.06	11
	Max	12.7	18.3	2.4	57	77	1700
	Min	0.3	0.00	ND	ND	ND	ND
N	Median	1.3	1.94	ND	1.1	2.9	153
	Max	13.7	14	0.35	18	24	3300
	Min	0.3	0.07	ND	0.08	0.15	12
T	Median	2.4	2.1	ND	1.5	3.6	170
	Max	3.4	3.2	ND	2.3	5.5	230
	Min	1.3	1.2	ND	0.80	2.2	110

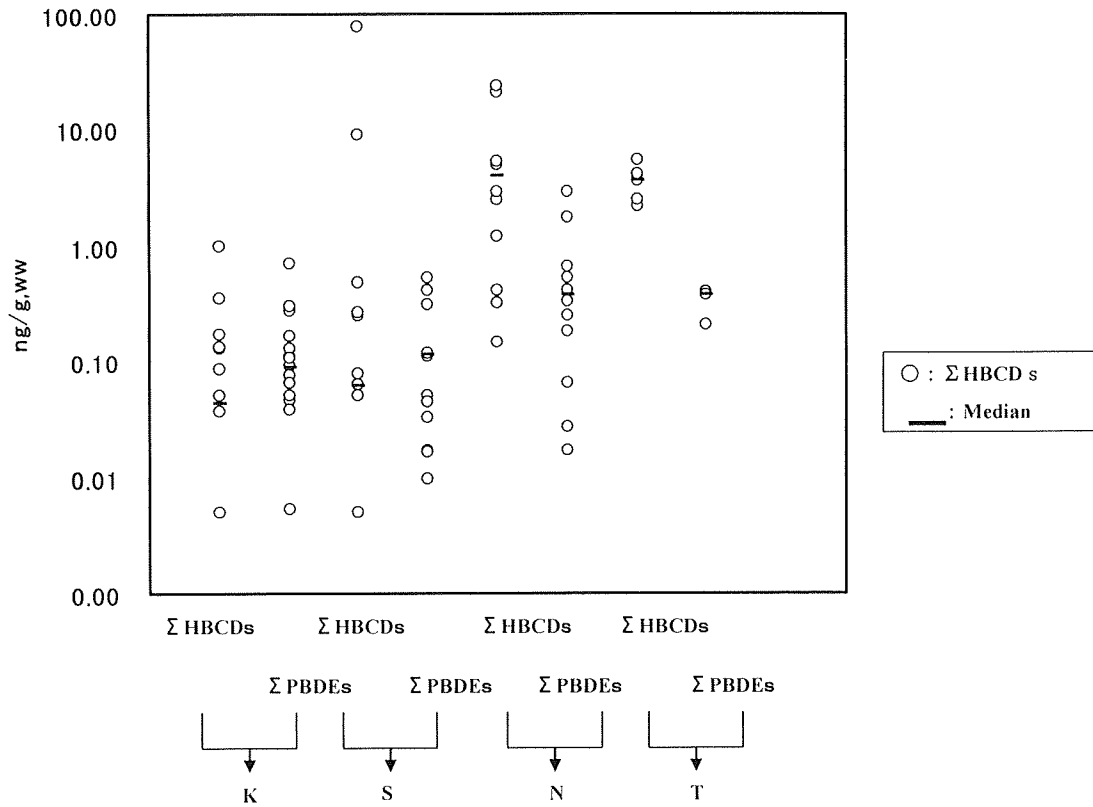


Fig.1 Distribution of Σ HBCDs and Σ PBDEs in marine products from four regions of Japan

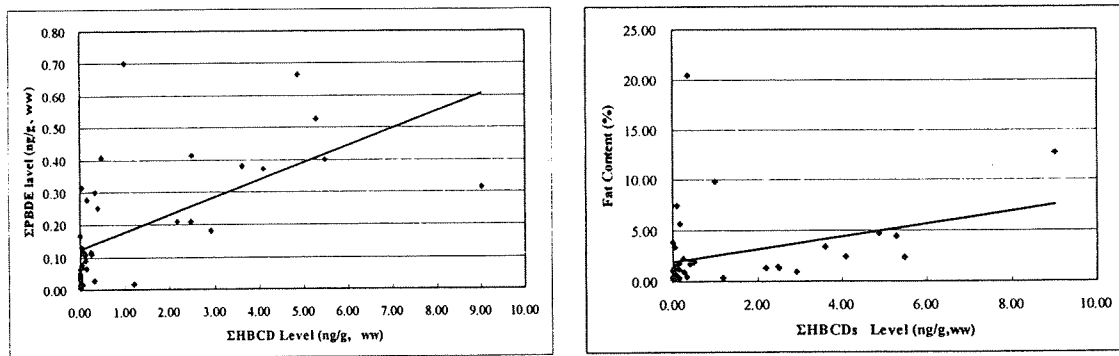


Fig.2 Correlations between Σ HBCDs and Σ PBDEs and between Σ HBCDs and fat content (%) in marine products in Japan



Review

Influence of food polyphenols on aryl hydrocarbon receptor-signaling pathway estimated by *in vitro* bioassay

Yoshiaki Amakura^{a,b,*}, Tomoaki Tsutsumi^b, Kumiko Sasaki^b, Masafumi Nakamura^c,
Takashi Yoshida^a, Tamio Maitani^b

^a College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan

^b Division of Foods, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

^c Hiyoshi Corporation, 908 Kitanosho-cho, Omihachiman, Shiga 523-8555, Japan

Received 26 April 2007; received in revised form 8 June 2007

Available online 14 September 2007

Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the toxic and biological actions of many aromatic environmental pollutants such as dioxins. We investigated AhR activation by some vegetable constituents, including flavonoids, tannins, and related polyphenols, using an AhR-based *in vitro* bioassay for dioxins. Among the compounds tested, marked AhR activation was exhibited by isoflavones such as daidzein, resveratrol (a stilbene) structure, some flavanones such as naringenin, and flavones such as baicalein. On the other hand, some flavones such as apigenin, flavonols such as quercetin, and anthraquinones such as emodin, showed notable inhibitory effects on the *in vitro* activation of AhR induced by the dioxin [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)]. In addition, AhR-mediated interactions between AhR and some plant extracts, including those from vegetables, fruits, herbs, and teas, were tested by using the AhR-based bioassay. Of the samples tested, some leafy green vegetables, citrus fruits, and herbs that contain food polyphenolics showed AhR-based interactions at high concentrations. On the basis of these findings, we discuss the implications of polyphenols on the AhR-signaling pathway.

© 2007 Elsevier Ltd. All rights reserved.

Keywords: Polyphenol; Aryl hydrocarbon receptor; Vegetable food; *In vitro* bioassay; Dioxin

Contents

1. Introduction	3118
2. AhR activation by polyphenol constituents determined using <i>in vitro</i> bioassay	3119
3. Inhibitory effects of polyphenol constituents on the AhR-mediated activity induced by TCDD.	3123
4. Interactions between some plant food extracts and AhR determined by <i>in vitro</i> bioassay	3125
5. Concluding remarks	3127
Acknowledgements	3128
References	3128

* Corresponding author. Address: College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan. Tel.: +81 89 925 7111; fax: +81 89 925 7162.

E-mail address: amakura@cc.matsuyama-u.ac.jp (Y. Amakura).

1. Introduction

The aryl hydrocarbon receptor (AhR), which is also referred to as a dioxin receptor, is a basic helix–loop–helix (bHLH)- and Per-Arnt-Sim (PAS)-containing transcription factor. It is present in numerous animal species, including humans and tissues, and activates gene expression in a ligand-dependent manner (Schmidt and Bradfield, 1996; Ma, 2001; Denison et al., 2002; Mimura and Fujii-Kuriyama, 2003) (Fig. 1a). The prototype ligand is known as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an archetypal dioxin known as one of the most potent congeners. Other known ligands are environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and persistent organochlorine pollutants (POPs), including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) (Safe, 1986, 1990; Whitlock, 1993; Denison et al., 2002). Unliganded AhR is present in the cytosol of most cells, forming a complex with a dimer of 90-kDa heat shock protein (Hsp90), an X-associated protein 2 (XAP2), and a 23-kDa co-chaperone protein (p23). Ligand binding to AhR leads to nuclear translocation, followed by release of its associated protein subunits (Hsp90, XAP2, and p23) and heterodimerization with the AhR nuclear translocator protein (Arnt). This AhR/Arnt heterodimer binds to DNA sequences, called xenobiotic responsive elements

(XRE), which are distributed in the enhancer regions of dioxin-responsive genes, and regulate the expression of target genes including drug-metabolizing enzymes, such as cytochrome P450 (CYP) 1A1. Accordingly, this DNA interaction is highly correlated with the initial step of subsequent toxicity events including carcinogenicity, developmental and reproductive toxicity, and immunological impairment that are known as dioxin toxicity effects (Landers and Bunce, 1991; Poellinger, 2000; Denison and Nagy, 2003; Mimura and Fujii-Kuriyama, 2003; Mandal, 2005; Schwarz and Appel, 2005) (Fig. 1b).

The lack of TCDD toxicity in AhR knockout mice, along with the ability of AhR to act as a ligand-dependent transcription factor, indicated that AhR mediates the toxic and biological effects of TCDD (Mimura et al., 1997). Recently, research on the structure and physiological functions of AhR cell cycle regulation has been reported (Bock and Köhle, 2006; Harper et al., 2006; Pandini et al., 2007; Goryo et al., 2007), and the characterization of AhR has gradually been clarified. However, AhR is still relatively poorly understood, because its physiological ligand, mechanisms, and functions remain largely unknown. The present functional role of AhR was derived mainly based on studies using environmental contaminants such as dioxins. Environmental contaminants that are prototype AhR ligands are artificial products that have appeared recently. Therefore, AhR might primarily function in human health

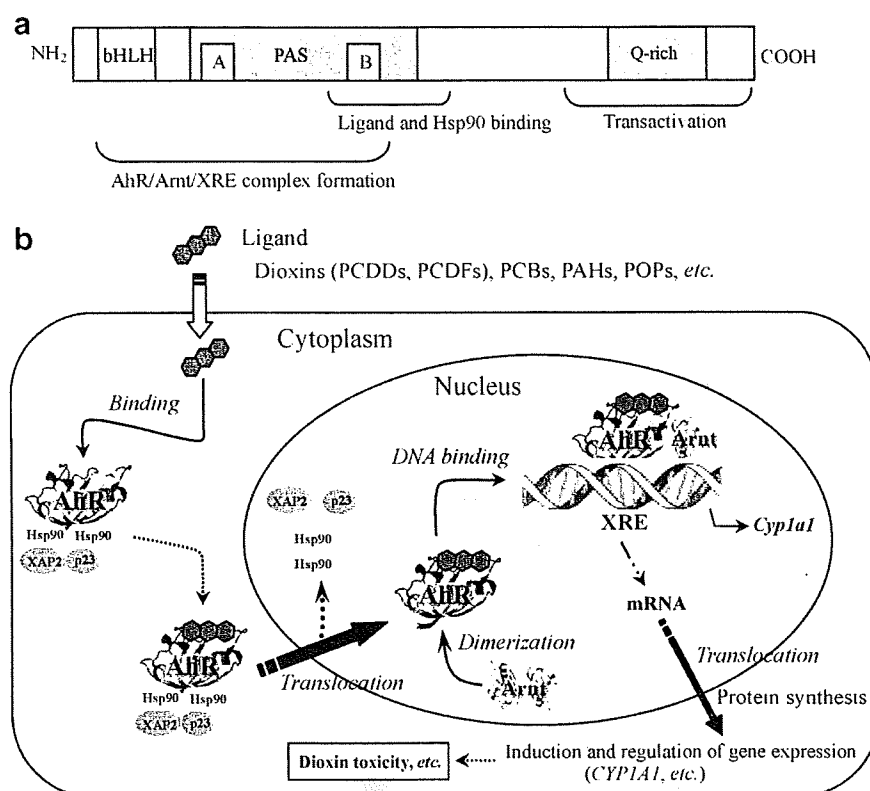


Fig. 1. (a) Domain structure of the aryl hydrocarbon receptor (AhR). bHLH, basic helix–loop–helix; PAS, Per-Arnt-Sim domain; Q-rich, glutamine rich region; Arnt, AhR nuclear translocator protein; XRE, xenobiotic-response element. The PAS domain contains two structural repeats (PAS A and PAS B). (b) Mechanistic model of the AhR signaling pathway. See text for detailed description.

as a regulatory receptor for exogenous natural products such as food constituents.

To better understand AhR's inherent physiological role and significance, information on the interactions between it and compounds such as food constituents related to people's lives is needed, and more fundamental data is required. Recently, numerous investigations have been carried out to search for the agonists and antagonists of AhR contained in natural products rather than in environmental contaminants (Denison et al., 2002; Denison and Nagy, 2003). Regarding AhR agonists, AhR transformation has reportedly been induced by the following: tryptophan and its metabolites (Heath-Pagliuso et al., 1998), carotenoids (β -apo-8'-carotenal, canthaxanthin, and astaxanthin) (Gradelet et al., 1997), berberine (Vrzal et al., 2005), indole-3-carbinol (Bjeldanes et al., 1991; Chen et al., 1996), bilirubin, biliverdin (Phelan et al., 1998), flavonoids (Ashida et al., 2000), and others. Recently, tunicamycin, a well-known antibiotic, has been identified as an activator of the AhR-XRE signal pathway (Horikawa et al., 2006). Indigo and indirubin were also shown to be endogenous AhR agonists present in human urine (Adachi et al., 2001; Sugihara et al., 2004). The AhR agonist activity of the extracts of dietary herbal supplements, vegetables, and fruits were also assessed (Jeuken et al., 2003). AhR agonists are noted as physiological regulatory factors, while the toxicity of a dioxin-like compound is doubtful.

The following have been reported to function as AhR antagonists that involve inhibition of the AhR-signaling pathway: flavonoids (apigenin, luteolin, kaempferol, quercetin, galangin, etc.) (Reiners et al., 1999; Ciolino et al., 1999; Ciolino and Yeh, 1999; Ashida et al., 2000; Fukuda et al., 2004; Ishida et al., 2005; Hamada et al., 2006), catechins (Williams et al., 2000; Ashida et al., 2000; Fukuda et al., 2004), curcumin (Ciolino et al., 1998a), resveratrol (Ciolino et al., 1998b; Casper et al., 1999), and lutein (Fukuda et al., 2004). Recently, the suppressive effects of anthocyanidins and/or anthocyanins and extracts of black tea,

molokhia, and propolis on the dioxin-induced transcription of AhR have also been investigated (Park et al., 2004, 2005; Fukuda et al., 2005; Mukai et al., 2005; Nishimi et al., 2006). These studies suggest that AhR antagonists might protect against dioxin toxicity.

This review summarizes our recent investigations, which include the interaction between polyphenol constituents and AhR as determined by *in vitro* bioassay. The influence of polyphenols on the AhR-signaling pathway with regard to human health are also discussed.

2. AhR activation by polyphenol constituents determined using *in vitro* bioassay

The *in vitro* AhR-inducing potencies of plant constituents, mainly polyphenol compounds, that are present in vegetables, fruits, teas, and herbs (Amakura et al., 2003a) were investigated. For identification of AhR-activating compounds, an *in vitro* reporter gene assay, called the chemical activated luciferase gene expression (CALUX) assay (Denison et al., 1998), was used. The mechanistic outline of the assay is depicted in Fig. 2a. This assay, which uses mouse hepatoma cells (Hepa 1c1c7) containing a stably transfected AhR-responsive luciferase reporter gene, detects dioxin-like compounds based on their ability to activate AhR. Since the response for a sample containing dioxin-like compounds can be correlated with dioxin levels in the CALUX assay, this assay has recently been applied as an alternative screening method to determine dioxin levels (Tsutsumi et al., 2003). With this assay, TCDD showed an appreciable, dose-dependent increase in luciferase activity (Fig. 3). The concentrations of a test compound producing luciferase activity equal to 25% and 50% of the maximal response to TCDD were calculated and expressed as EC_{TCDD25} and EC_{TCDD50} , respectively. EC_{25} and EC_{50} of TCDD were determined to be 1.3×10^{-5} and $3.0 \times 10^{-5} \mu\text{M}$, respectively. Dose-response curves plotted

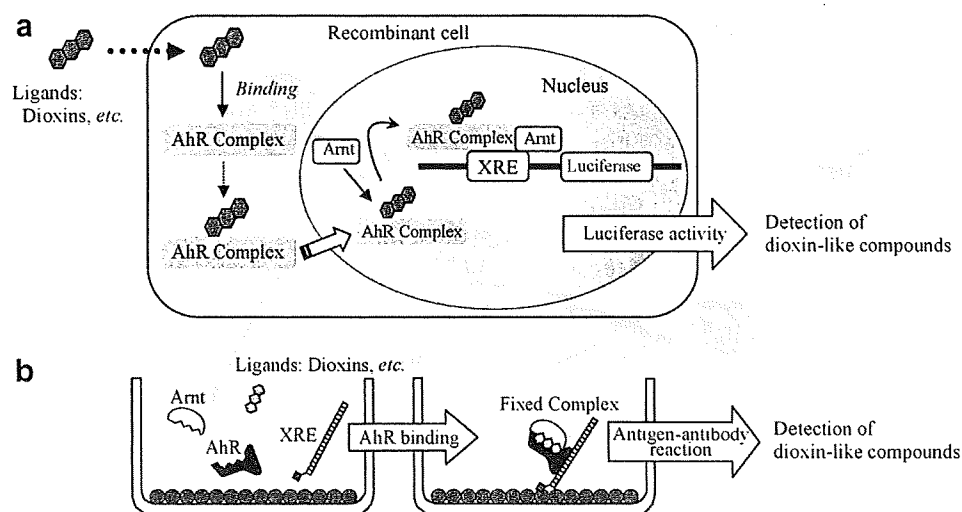


Fig. 2. Outline of mechanistic models of AhR-mediated *in vitro* bioassay used in this study. (a) CALUX assay, (b) Ah-immunoassay.

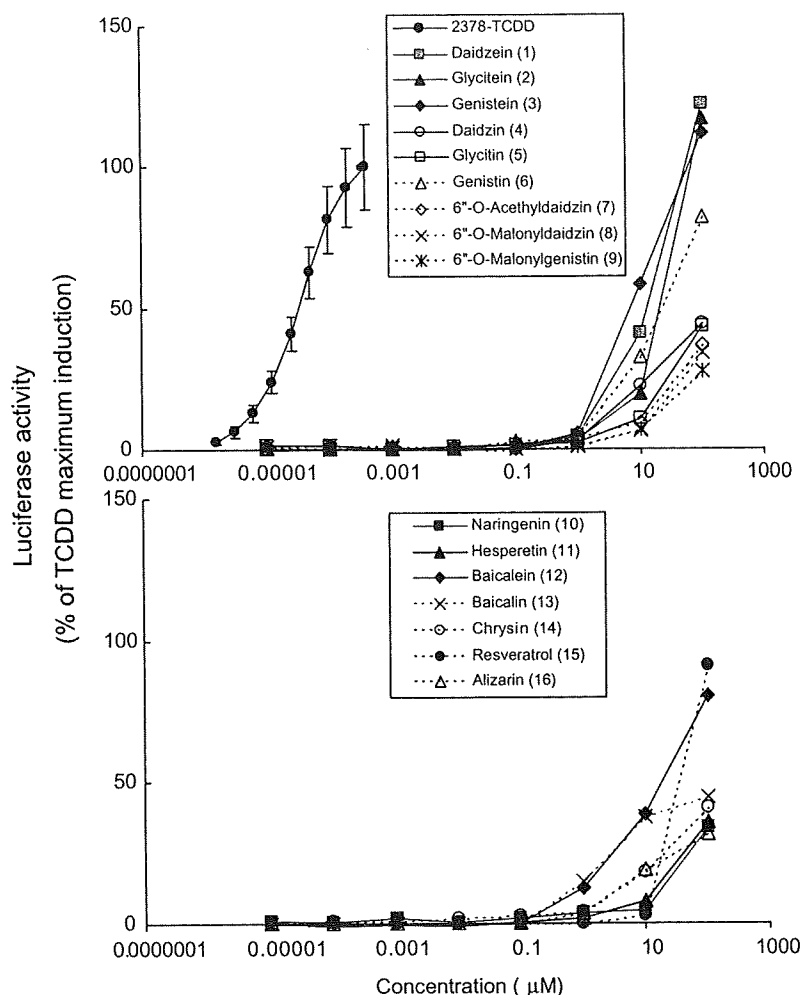


Fig. 3. Concentration–response curves of positive vegetable polyphenols and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for the induction of luciferase activity in the CALUX assay. Each point represents the mean of at least three replicates (40 experiments in the case of TCDD).

on a log scale for some samples are also shown in Fig. 3. Most tested compounds showed no luciferase induction at the concentration level of 100 µM, but some showed luciferase activity at higher concentrations. Of the samples tested, isoflavones produced responses that reached maximal TCDD levels (Fig. 3 and Table 1). EC_{TCDD25} (EC_{TCDD50}) values for daidzein (1), glycitein (2), and genistein (3) were 3.0 (7.9), 4.2 (20.6), and 2.4 (7.0) µM, respectively. Their glycosides showed lower AhR responses than the corresponding aglycones [EC_{TCDD25} : 12.0, 20.0, and 4.2 µM for daidzin (4), glycitin (5), and genistin (6), respectively], and their acetylates or malonylates showed much lower induction (EC_{TCDD25} : 32.0, 48.0, and 98.0 µM for 6''-acetyl daidzin (7), 6''-malonyl daidzin (8), and 6''-malonyl genistin (9), respectively).

Among several flavanones, naringenin (10) and hesperetin (11) elicited agonist-like AhR-mediated activity (EC_{TCDD25} : 53.0 and 38.0 µM). Their glycosides (naringin and hesperidin) and flavanonols including (+)-taxifolin and (+)-fustin did not induce activity at the EC_{TCDD25} level. The tendency for glycosides to weaken these activities was similar to that of isoflavones. Of the flavones, baicalein

(12) and baicalin (13) induced the production of luciferase activity (EC_{TCDD25} : 2.8 and 3.2 µM). Chrysin (14) slightly induced the activity at 14.0 µM in EC_{TCDD25} . On the other hand, apigenin (17), luteolin (18), and others slightly induced the activity at high concentrations in the order of 10–100 µM, but no luciferase induction reached the EC_{TCDD25} level. Also, flavonols, myricetin (21), morin (22), and others slightly activated luciferase at high concentration on the order of 100 µM.

Based on these findings, the structure–activity correlations in the activation of AhR by flavonoids suggested that the level of activity depends on the molecular size, polarity, and structure of isoflavones and flavanones. Isoflavones such as daidzein (1), glycitein (2), and genistein (3) had similar AhR-inducing potencies, while their glycosides and 6''-*O*-acylates showed lower induction levels. Similarly, flavanone glycosides had weaker activity than their corresponding aglycones such as naringenin (10) and hesperetin (11), indicating that the increase in the molecule's polarity clearly weakened the activity. Flavanone 3-ols and flavan 3-ols such as (+)-catechin and (–)-epicatechin showed poor induction of luciferase activity. Therefore,

Table 1
Relative responses of the reporter gene system to some polyphenolic constituents (data from Amakura et al., 2003a)

	EC _{TCDD25} (μM) ^a	EC _{TCDD50} (μM) ^a
2,3,7,8-TCDD	1.3×10^{-5} (EC ₂₅)	3.0×10^{-5} (EC ₅₀)
<i>Isoflavones</i>		
Daidzein (1)	3.0	7.9
Glycitein (2)	4.2	20.6
Genistein (3)	2.4	7.0
Daidzin (4)	12.0	– ^b
Glycitin (5)	20.0	–
Genistin (6)	4.2	22.6
6''-Acetyldaidzin (7)	32.0	–
6''-Malonyldaidzin (8)	48.0	–
6''-Malonylgenistin (9)	98.0	–
<i>Flavanones</i>		
Naringenin (10)	53.0	–
Hesperetin (11)	38.0	–
<i>Flavones</i>		
Baicalein (12)	2.8	18.8
Baicalin (13)	3.2	–
Chrysin (14)	14.0	–
<i>Others</i>		
Resveratrol (15)	7.3	34.3
Alizarin (16)	30.0	–

Each value is the mean of at least three replicates.

^a Concentration producing luciferase activity equal to 25% (or 50%) of the maximal response to TCDD. Calculated from the slope of the linear portion of each dose–response curve near the origin.

^b No luciferase induction to the EC_{TCDD50} level observed.

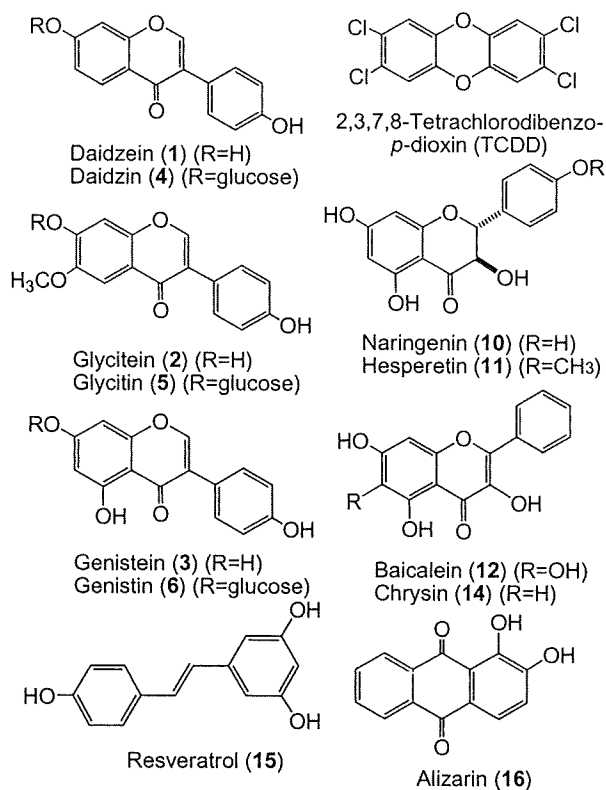


Fig. 4. Structures of TCDD and some tested polyphenols.

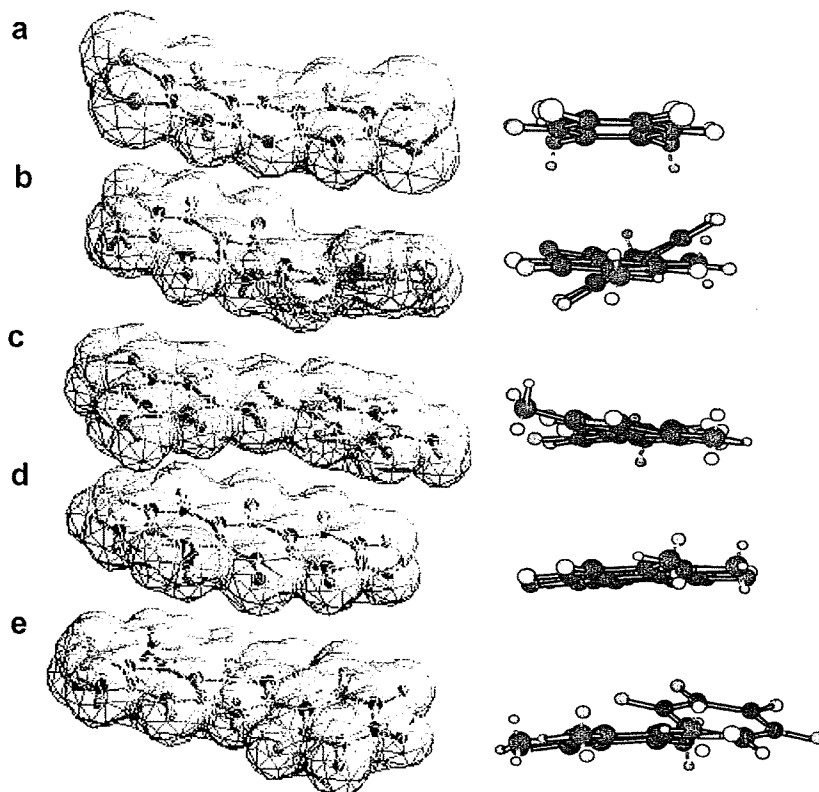


Fig. 5. Molecular models of AhR-activating polyphenols obtained as MM2-minimized structures, (a) TCDD; (b) daidzein (1); (c) resveratrol (15); (d) alizarin (10); (e) baicalein (12). Energy-minimized structures were calculated by molecular mechanics using MM2 in CS Chem 3D.

the hydroxyl group at C-3 of the C-ring may also contribute to weakening the activity. Baicalein (**12**), baicalin (**13**), and chrysin (**14**), which induced AhR activation, each possess two or three hydroxyl groups at C-5, -6, and -7 of the A-ring, but none on the B-ring. Other flavones, apigenin (**17**), luteolin (**18**), and vitexin, which weakly induced the activity, each have a hydroxyl group on the B-ring. As for flavones, hydroxyl groups on the B-ring reduced the activation, while the hydroxyl groups on the A-ring had a negligible influence. Similarly, flavonols, which possess hydroxyl group(s) on the B-ring, poorly induced activation, with the exception of myricetin (**21**) and morin (**22**), each of which caused a slight induction at the 100 μM level. Thus, in naturally occurring flavones and flavonols, hydroxyl group(s) on the B-ring and in C-3 of the C-ring may reduce activation.

Among the other compounds tested, resveratrol (**15**), having a *trans*-stilbene structure, showed strong AhR-inducing potency comparable to the maximum induction

of TCDD at a high concentration level [7.3 (34.3) μM in $\text{EC}_{\text{TCDD}25}$ ($\text{EC}_{\text{TCDD}50}$)], while analogues possessing longer carbon chains [rosmarinic acid, curcumin (**35**)] showed significantly lower AhR inducing capabilities. Previously, the AhR ligand activity of *trans*-stilbene was reported, so the present result further demonstrated that the molecular size in the *trans*-stilbene is important for this activity (Kato et al., 2002). Among the anthraquinones, alizarin (**16**) showed AhR activation (30.0 μM in $\text{EC}_{\text{TCDD}25}$), whereas emodin (**32**) and aloe-emodin (**33**) exhibited only weak AhR activity. Although they each possess a structure similar to that of TCDD, substituents ($-\text{OH}$, $-\text{CH}_3$, and $-\text{CH}_2\text{OH}$) in emodin (**32**) and aloe-emodin (**33**) may contribute less than in alizarin (**16**) to the activity. Condensed and hydrolyzable tannins, phenolcarboxylic acids, rosmarinic acid, and curcumin (**35**) induced little production of luciferase at $\text{EC}_{\text{TCDD}25}$ levels.

Fig. 5 depicts molecular models of TCDD and several compounds that induced luciferase activity [daidzein (**1**),

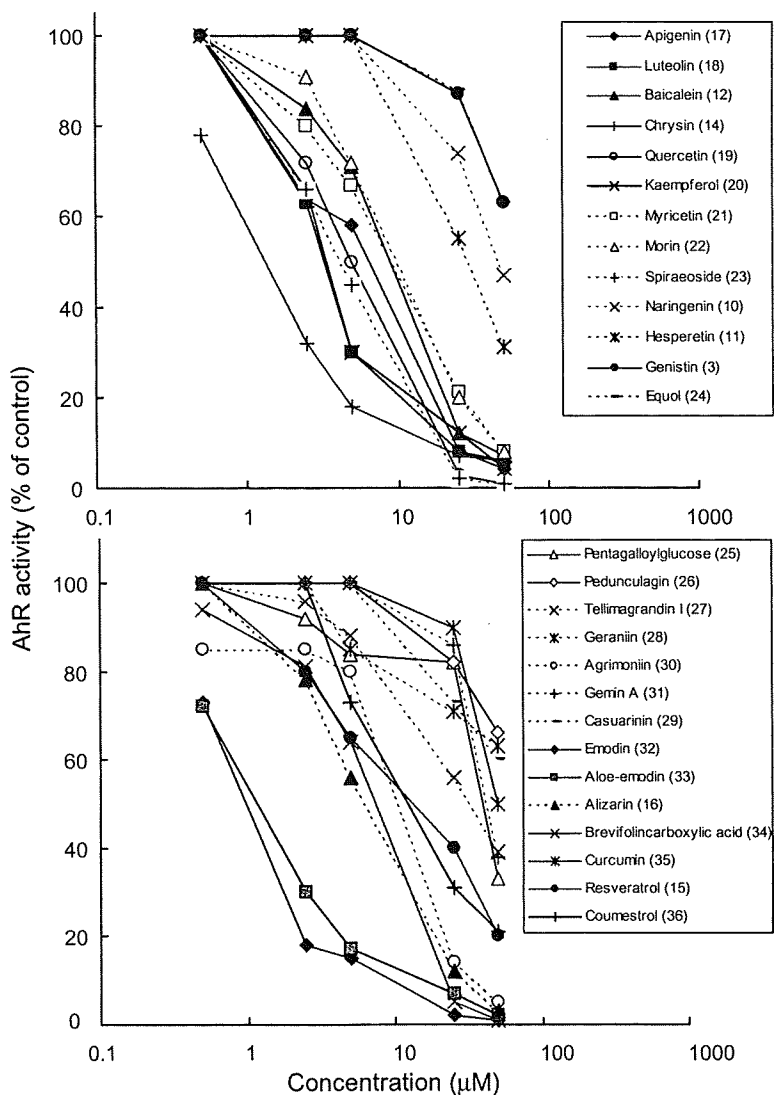


Fig. 6. Dose-dependent inhibitory effect of some vegetable polyphenols on AhR-activation induced by TCDD in Ah-I. Each point represents the mean of two or three replicates.

resveratrol (15), alizarin (16), and baicalein (12)]; these models were each analyzed for their minimum energy conformation. The AhR has been found to favor hydrophobic molecules with quasi-planar structures and to accommodate a ligand-binding pocket (Safe, 1986; Landers and Bunce, 1991; Denison and Nagy, 2003). As shown in Fig. 5, these active compounds have molecular sizes and planar structures similar to those of TCDD.

Isoflavones such as daidzein (1), the so-called phytoestrogens, exhibited AhR activation in this *in vitro* experimental system. Southeast Asian and Japanese people often consume soybeans and soybean-derived products, which contain isoflavones in abundance. It is also known that these soy isoflavones may have some health-enhancing properties (Murkies et al., 1998; Setchell and Cassidy, 1999). Therefore, it is considered that a modest AhR-inducer including foods may perform some beneficial regulatory role in the homeostasis, proliferation, and differentiation of cells in animals (Harper et al., 2006).

Isoflavones are known to be mostly metabolized in the body, and their metabolites may not induce AhR because equol, which is regarded as the final metabolite of isoflavones, only slightly induced AhR activity at high concentrations. On the other hand, it has also been reported that two soy isoflavones, daidzein (1) and genistein (2), appear to be incorporated into tissues after ingestion of baked soybean powder by humans (Watanabe et al., 1998). Since the daily intake and serum excretions of daidzein (1) and genistein (2) by Japanese has been assessed [daily intake of daidzein (1) and genistein (2) were *ca.* 20 and 30 mg/day, respectively; serum excretions were *ca.* 120 and 475 nM, respectively] (Yamamoto et al., 2001), it is clear that they are incorporated intact into the body. Considering the bio-

availability of isoflavones in these reports, it can be inferred that, in the quantities typically consumed, these isoflavones might not function as agonists to AhR, while a large excess intake of AhR-inducers such as isoflavones may merit attention as a risk factor for health.

3. Inhibitory effects of polyphenol constituents on the AhR-mediated activity induced by TCDD

To evaluate the effect of polyphenol constituents on the AhR pathway induced by TCDD, we used an AhR-based bioassay for dioxins, the Ah-Immunoassay (Ah-I; Paracelsian, USA), which previously proved sensitive as a preliminary experimental model (Amakura et al., 2003b). The Ah-I kit method is a receptor-binding assay using cytosol containing AhR extracted from mammalian liver cells. It immunologically measures the dioxin level by utilizing an antigen-antibody reaction (Fig. 2b). This technique detects the reactivity of AhR with dioxins and dioxin-like compounds on an ELISA plate without using living cells. It is useful for screening the biological toxicity of dioxins (Kobayashi et al., 2002).

Fig. 6 shows the dose–response curves plotted on a log scale for some individual samples (see Figs. 4 and 7 for structures). Most of the compounds inhibited AhR activation by TCDD at high concentrations around the 50 μM level. Some showed marked inhibitory effects at low concentrations of 0.5–2.5 μM . The concentrations showing AhR activity equal to 70% of the maximal response to TCDD in controls were calculated and expressed as EC₇₀ values. Table 2 shows the EC₇₀ values of the compounds on AhR-based bioassay activation. The inhibitory effects

Table 2

Inhibitory effects of some polyphenolic constituents on TCDD-induced activation of AhR estimated using the AhR-based bioassay (data from Amakura et al. (2003b))

	EC ₇₀ (μM) ^a		EC ₇₀ (μM)
<i>Flavones</i>		<i>Hydrolyzable Tannins</i>	
Apigenin (17)	1.9	Pentagalloylglucose (25)	29.6
Luteolin (18)	1.8	Pedunculagin (26)	42.0
Baicalein (12)	5.1	Tellimagrandin I (27)	12.4
Chrysin (14)	0.7	Geraniin (28)	27.3
<i>Flavonols</i>		Casuarinin (29)	29.3
Quercetin (19)	2.7	Agrimoniin (30)	6.4
Kaempferol (20)	2.1	Gemin A (31)	31.5
Myricetin (21)	4.3	<i>Others</i>	
Morin (22)	5.3	Emodin (32)	0.6
Spiraeside (23)	2.1	Aloe-emodin (33)	0.5
<i>Flavanones</i>		Alizarin (16)	3.2
Naringenin (10)	27.7	Brevifolincarboxylic acid (34)	3.9
Hesperetin (11)	14.6	Curcumin (35)	35.4
<i>Isoflavones</i>		Resveratrol (15)	3.9
Genistein (3)	40.8	Coumestrol (36)	5.6
Equol (24)	41.2		

Each value is the mean of at least three replicates.

^a Concentration producing AhR activity equal to 70% of the maximal response to TCDD. Calculated from the slope of the linear portion of each dose–response curve near the origin.

of most samples were weak, less than the EC₇₀ level even at high concentrations (inhibitory effect *ca.* 20%).

The flavones and flavonols in Table 2 had strong inhibitory potencies (EC₇₀: 0.7–5.3 μM) against AhR activation induced by TCDD. However, three flavonol 3-*O*-glycosides, quercitrin, rutin, and isoquercitrin, did not inhibit activity to the EC₇₀ level. In contrast, spiraeoside (**23**) (a quercetin 4'-*O*-glucoside) showed a strong inhibitory effect, comparable to that of the aglycone [quercetin (**19**)]. This suggested that the inhibitory effects of flavonols might be

little influenced by glycosidation of the B-ring. Among the flavanones, hesperetin (**11**) and naringenin (**10**) showed inhibitory effects with EC₇₀ values of 14.6 and 27.7 μM, while their 7-*O*-glycosides (naringin, hesperidin, taxifolin, and fustin) did not inhibit activity to the same level. The tendency of glycosides to weaken these activities was similar to those of flavones and flavonols. The isoflavones did not show an inhibitory effect on AhR activation at the EC₇₀ level, and they slightly inhibited activity at high concentrations of 25–50 μM (*ca.* 10–20% inhibition), although

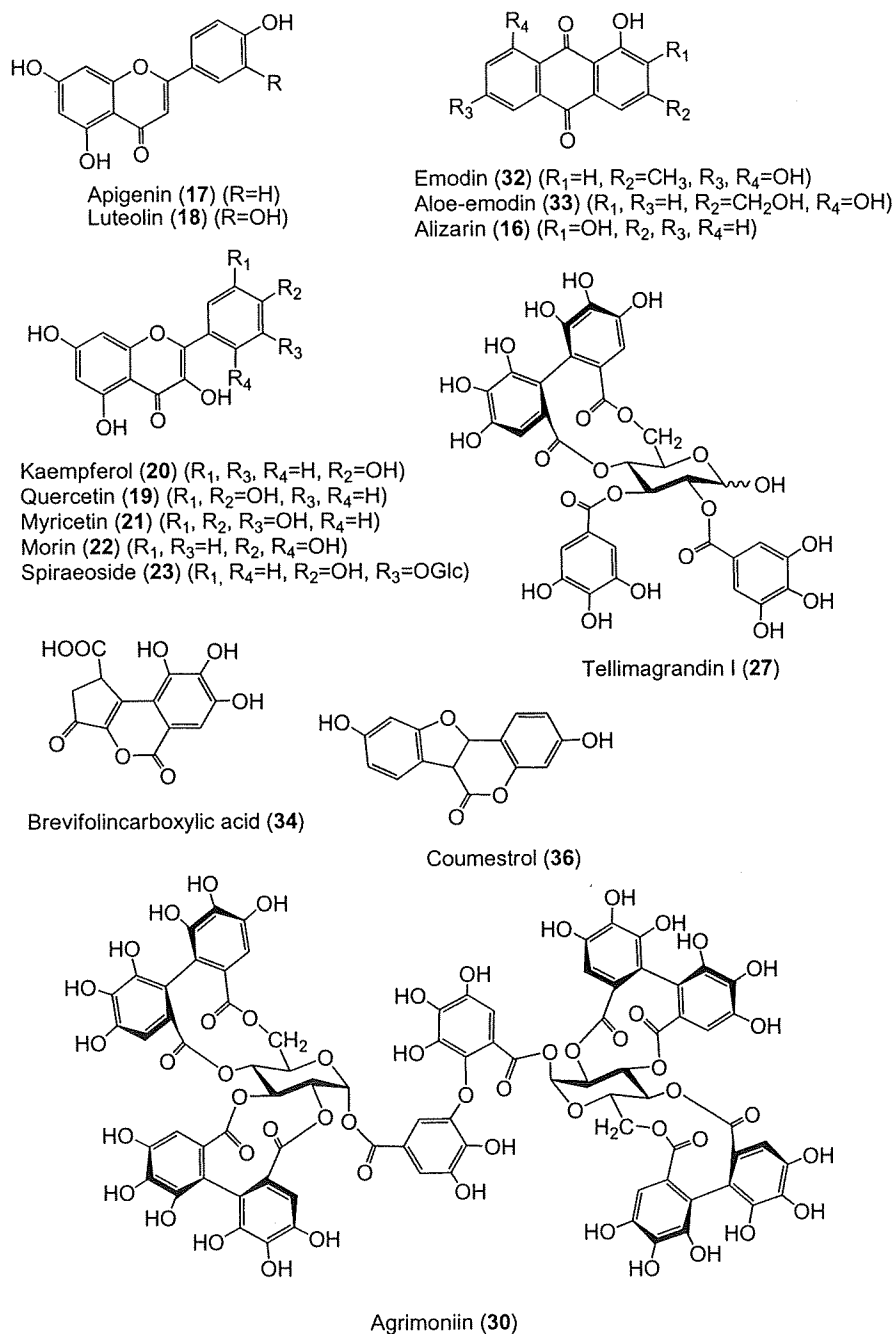


Fig. 7. Structures of some tested polyphenols.

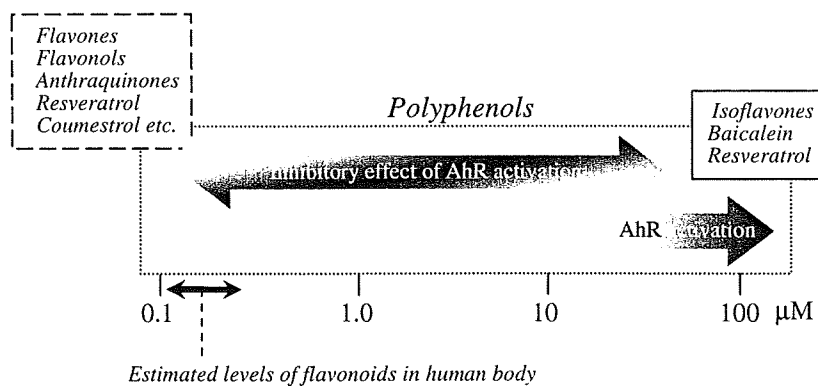


Fig. 8. Estimated ranges of AhR activation and inhibitory effects on AhR activation by TCDD of some polyphenols.

genistein (**3**) had a slight inhibitory effect at 40.8 μM in EC_{70} and equol inhibited activity at 41.2 μM .

Anthraquinones showed remarkable inhibition of AhR activation, comparable to flavones and flavonols [EC_{70} values of aloe-emodin (**33**), emodin (**32**), and alizarin (**16**) were 0.5, 0.6, and 3.2 μM , respectively]. Resveratrol (**15**), brevifolincarboxylic acid (**34**), coumestrol (**36**), and curcumin (**35**) also inhibited activation in this assay (EC_{70} values were 3.9, 3.9, 5.6, and 35.4 μM , respectively). Among these, resveratrol (**15**) and curcumin (**35**) were reported to show antagonist effects on AhR (Ciolino et al., 1998a, b; Casper et al., 1999). As they also showed inhibitory effects in our assay system, they would be promising candidates as prophylactic agents for the prevention of dioxin toxicity.

On the other hand, some hydrolyzable tannins in Table 2, which are large molecules with molecular weights of 1000–2000 higher than the phenolics mentioned above, inhibited AhR activation with EC_{70} of 6.4–42.0 μM , while condensed tannins showed no inhibitory effects. As tannins are known to form complexes with various proteins, to function as enzyme inhibitors, and so on (Haslam, 1989), their inhibitory effects in this assay system may partly be ascribed to a non-specific binding to AhR. Further study will be required to elucidate these effects.

In Section 2, we described the AhR-mediated activity of vegetable polyphenolics using the CALUX assay. The active compounds were classified into the so-called phytoestrogens, and their structural characteristics were similar to those of TCDD. Flavones such as chrysin (**14**) showed remarkable activation in the AhR-based assay as well as strong inhibitory effects on AhR-mediated activity induced by TCDD at the EC_{70} level, suggesting that they may be both agonists and antagonists of AhR, depending on the amount. On the other hand, flavones such as apigenin (**17**) and flavonols were regarded as strong antagonists of AhR because they showed little AhR activation. Most isoflavones had slight inhibitory effects, less than the EC_{70} level, whereas genistein (**3**) and equol (**24**) showed inhibition at the EC_{70} level. Anthraquinones, brevifolincarboxylic acid (**34**), and coumestrol (**36**), each of which

slightly activated AhR at high concentrations, also produced inhibitory effects on AhR activity at the EC_{70} level.

Some reports have measured the amounts of flavonoid ingestion and absorption in the human body. For example, the daily consumption of flavonols was recently estimated at only 20–35 mg/day (Manach et al., 2005). Another paper reported that the ingestion of 8, 20, and 50 mg quercetin (**19**) resulted in concentrations of 0.14, 0.22, and 0.29 μM quercetin (**19**) in plasma, respectively (Yamamoto et al., 2001). Another study, performed on ten healthy volunteers, showed that ingestion of 68 ± 13 mg quercetin equivalents from onion resulted in a maximum quercetin (**19**) concentration in plasma of 0.74 ± 0.15 μM (Hollman et al., 1997). Taking into account the average intake (ca. 20–35 mg/day) and the absorption amount (ca. 0.1–0.3 μM) of flavonoids in general, it can be suggested that flavonoids might act as antagonists of AhR at usual intake levels.

Fig. 8 summarizes the estimated levels and inhibitory effects of AhR activation by TCDD of some low-molecular-weight polyphenols. Thus, some vegetable polyphenolics with low molecular weights and planar structures exhibited the properties of agonistic and/or antagonistic effects of AhR in the *in vitro* bioassays, and it can be inferred that they may have an antagonistic function in our usual dietary intake.

4. Interactions between some plant food extracts and AhR determined by *in vitro* bioassay

The influences of aqueous alcohol extracts of some plant foods on the AhR-signaling pathway were also investigated (Amakura et al., 2002, 2004, 2005). The *in vitro* AhR-inducing potencies of 39 plant food extracts including vegetables, fruits, herbs, and teas were determined by the CALUX assay. The induction of luciferase by each extract is shown in Fig. 9. Among the vegetables, shungiku (*Glebionis coronaria*) and spinach extracts induced production of luciferase activity of about 50% relative to TCDD maximum induction at concentrations of 1 mg/ml (plant extract dissolved in DMSO), while the other extracts did not

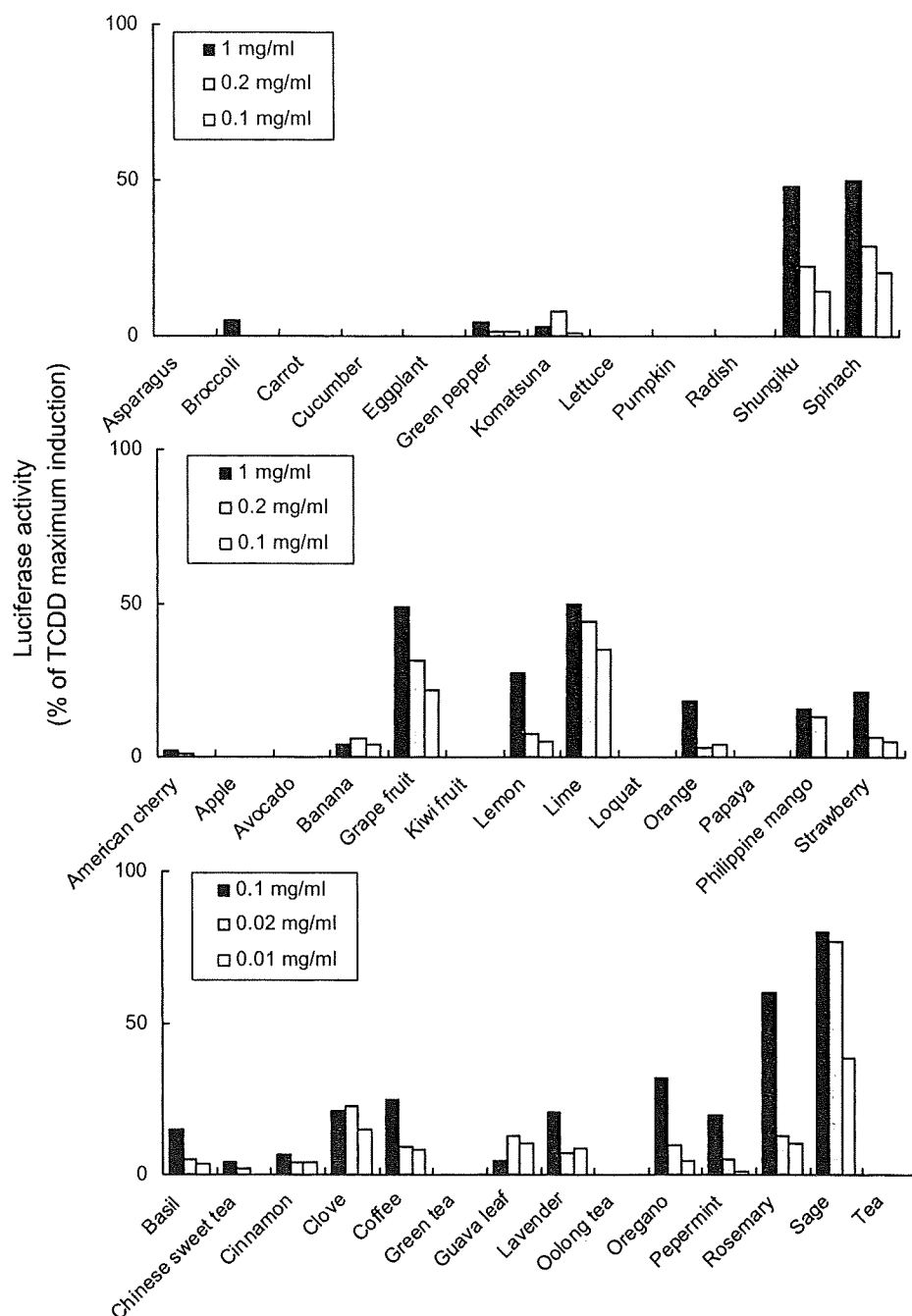


Fig. 9. Vegetable, fruit, and herb extracts tested for the induction of luciferase activity in the CALUX assay. The final sample concentrations added were 0.1, 0.2, and 1 mg/ml (or 0.01, 0.02, and 0.1 mg/ml for dried herbs) as extracts in three steps. Values represent the mean of triplicate determinations and are expressed relative to the activity obtained with TCDD.

induce luciferase activity (below 10%). Of the fruits, citrus fruits such as grapefruit and lime induced luciferase activity. Among the dried herbs and teas, sage and rosemary induced luciferase activity about 80% and 70%, respectively, at 0.1 mg/ml. On the other hand, in the assay using Ah-I to measure the inhibitory effects of these extracts on AhR activation by TCDD, green leafy vegetables such as spinach and komatsuna, citrus such as orange and grape-

fruit, and herbs such as sage and peppermint showed marked inhibitory effects (Fig. 10).

The agonistic and antagonistic effects of green leafy vegetables, citrus, and sage, as indicated by the induction of luciferase activity and the inhibitory effect on AhR-induced activation by TCDD, respectively, could be due to the flavonoids and/or related ingredients in these extracts.

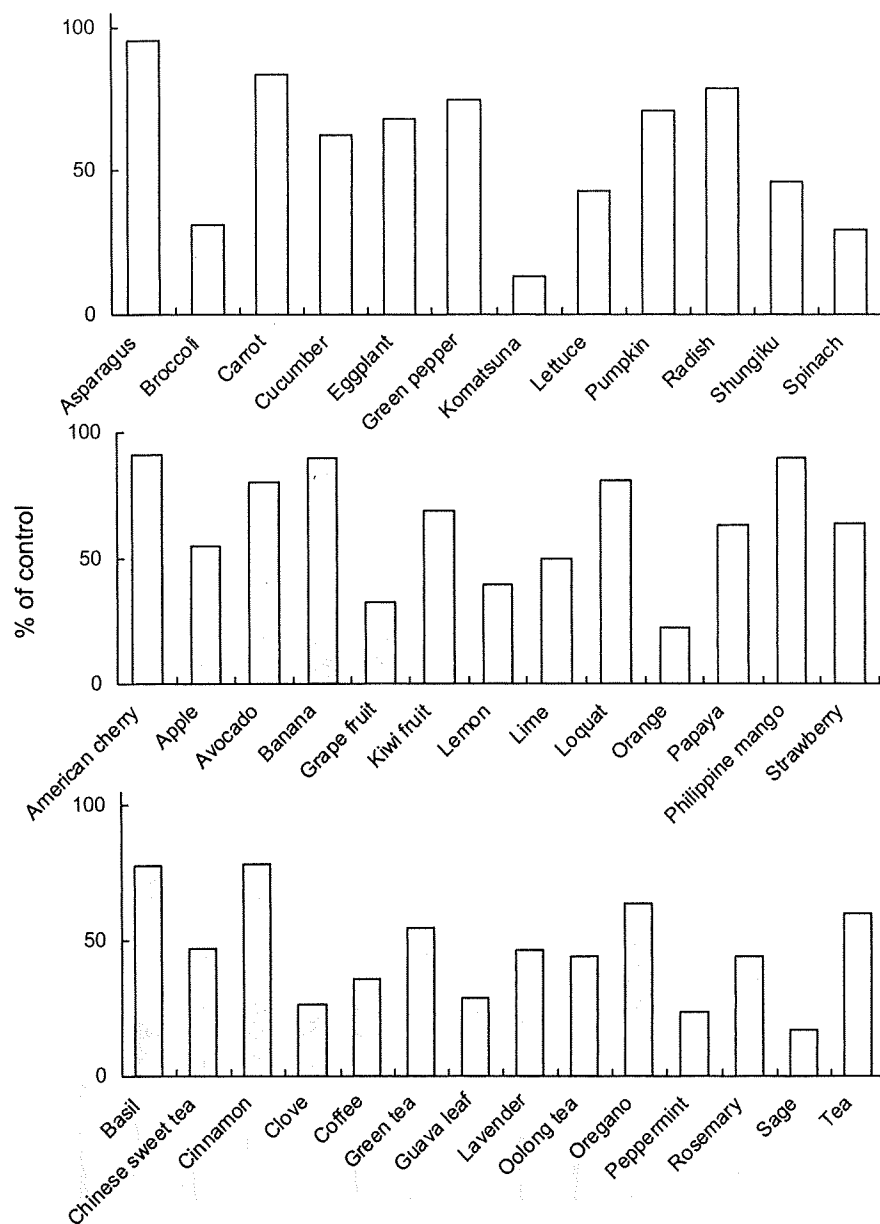


Fig. 10. Inhibitory effect of vegetable, fruit, and herb extracts on AhR induction by TCDD. Sample extracts at final concentrations of 500 $\mu\text{g/ml}$ were used. The values indicate AhR binding activity. The value obtained without the addition of the sample solution was considered 100% of the control.

5. Concluding remarks

As part of a study on food function and safety, various food polyphenols were assessed for their effects on AhR in relation to toxic dioxins, using *in vitro* assays of AhR antagonistic and agonistic effects. In the CALUX assay, isoflavones, resveratrol (15), and some flavonoids activated AhR (agonistic effect). Then, a screening of the inhibitory effects of food polyphenols on TCDD-induced AhR activation was conducted using Ah-I, demonstrating that flavones, flavonols, anthraquinones, coumestrol (36), brevifolincarboxylic acid (34), and resveratrol (15) remarkably inhibited activation (antagonistic effect). In addition, aqueous alcohol extracts of consumables such as some green

leafy vegetables, citrus fruits, and herbs that are rich in flavonoids were shown to interact with the AhR-signaling pathway in *in vitro* bioassays (CALUX assay and Ah-I) (agonistic effect at high concentrations and antagonistic effect at low concentrations).

To discuss the utility of food polyphenols for humans, it is necessary to understand not only their functional effects but also the influences of polyphenols on health and safety. Some vegetable polyphenolics with low molecular weights and planar structures exhibited properties of agonistic and/or antagonistic effects of AhR in the *in vitro* bioassays. However, in light of the bioavailability of such polyphenols, it can be inferred that they may have an antagonistic function in our usual dietary intake. The

AhR for polyphenols in usual intake might function biodefensively to protect the incorporation of foreign chemical compounds such as dioxin. On the other hand, the large excessive intake of foods that contain AhR-activators may be conducive to dioxin-like toxicity, therefore it may be necessary to pay attention to how much of these foods people eat. The results suggest that a well-balanced meal is also important in preventing dioxin-like toxicity.

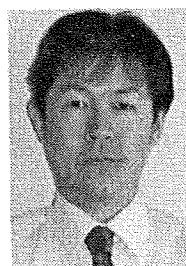
Acknowledgements

This work was supported by a Health Sciences Research Grant from Ministry of Health, Labour and Welfare of Japan.

References

- Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller, C.A., Kato, T., Saeki, K., Matsuda, T., 2001. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J. Biol. Chem.* 276, 31475–31478.
- Amakura, Y., Tsutsumi, T., Nakamura, M., Kitagawa, H., Fujino, J., Sasaki, K., Yoshida, T., Toyoda, M., 2002. Preliminary screening of the inhibitory effect of food extracts on activation of the aryl hydrocarbon receptor induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biol. Pharm. Bull.* 25, 272–274.
- Amakura, Y., Tsutsumi, T., Nakamura, M., Kitagawa, H., Fujino, J., Sasaki, K., Toyoda, M., Yoshida, T., Maitani, T., 2003a. Activation of the aryl hydrocarbon receptor by some vegetable constituents determined using in vitro reporter gene assay. *Biol. Pharm. Bull.* 26, 532–539.
- Amakura, Y., Tsutsumi, T., Sasaki, K., Yoshida, T., Maitani, T., 2003b. Screening of the inhibitory effect of vegetable constituents on the aryl hydrocarbon receptor-mediated activity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biol. Pharm. Bull.* 26, 1754–1760.
- Amakura, Y., Tsutsumi, T., Nakamura, M., Sasaki, K., Yoshida, T., Maitani, T., 2004. Interaction of some plant food extracts with aryl hydrocarbon receptor determined by in vitro reporter gene assay. *Nat. Med.* 58, 31–33.
- Amakura, Y., Tsutsumi, T., Sasaki, K., Nakamura, M., Ito, H., Hatano, T., Yoshida, T., Maitani, T., 2005. Interaction of the aryl hydrocarbon receptor with several constituents from spinach and komatsuna extracts determined using in vitro bioassay. *J. Health Sci.* 51, 715–719.
- Ashida, H., Fukuda, I., Yamashita, T., Kanazawa, K., 2000. Flavones and flavonols at dietary levels inhibit a transcription of aryl hydrocarbon receptor induced by dioxin. *FEBS Lett.* 476, 213–217.
- Bjeldanes, L.F., Kim, J.Y., Grose, K.R., Bartholomew, J.C., Bradfield, C.A., 1991. Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Proc. Natl. Acad. Sci. USA* 88, 9543–9547.
- Bock, K.W., Köhle, C., 2006. Ah receptor: Dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem. Pharmacol.* 72, 393–404.
- Casper, R.F., Quesne, M., Rogers, I.M., Shirota, T., Jolivet, A., Milgrom, E., Savouret, J., 1999. Resveratrol has antagonist activity on the aryl hydrocarbon receptor: Implications for prevention of dioxin toxicity. *Mol. Pharmacol.* 56, 784–790.
- Chen, I., Safe, S., Bjeldanes, L., 1996. Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem. Pharmacol.* 51, 1069–1076.
- Ciolino, H.P., Daschner, P.J., Wang, T.T., Yeh, G.C., 1998a. Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem. Pharmacol.* 56, 197–206.
- Ciolino, H.P., Daschner, P.J., Yeh, G.C., 1998b. Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res.* 58, 5707–5712.
- Ciolino, H.P., Daschner, P.J., Yeh, G.C., 1999. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem. J.* 340, 715–722.
- Ciolino, H.P., Yeh, G.C., 1999. The flavonoid galangin is an inhibitor of CYP1A1 activity and an agonist/antagonist of the aryl hydrocarbon receptor. *Br. J. Cancer* 79, 1340–1346.
- Denison, M.S., Brouwer, A., Clark, G.C., 1998. US Patent #5,854,010.
- Denison, M.S., Pandini, A., Nagy, S.R., Baldwin, E.P., Bonati, L., 2002. Ligand binding and activation of the Ah receptor. *Chem. Biol. Interact.* 141, 3–24.
- Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann. Rev. Pharmacol. Toxicol.* 43, 309–334.
- Fukuda, I., Sakane, I., Yabushita, Y., Kodoi, R., Nishiumi, S., Kakuda, T., Sawamura, S., Kanazawa, K., Ashida, H., 2004. Pigments in green tea leaves (*Cameria sinensis*) suppress transcription of the aryl hydrocarbon receptor induced by dioxin. *J. Agric. Food Chem.* 52, 2499–2506.
- Fukuda, I., Sakane, I., Yabushita, Y., Sawamura, S., Kanazawa, K., Ashida, H., 2005. Black tea theaflavins suppress dioxin-induced transformation of the aryl hydrocarbon receptor. *Biosci. Biotechnol. Biochem.* 69, 883–890.
- Goryo, K., Suzuki, A., Del Carpio, C.A., Siizaki, K., Kuriyama, E., Mikami, Y., Kinoshita, K., Yasumoto, K., Rannug, A., Miyamoto, A., Fujii-Kuriyama, Y., Sogawa, K., 2007. Identification of amino acid residues in the Ah receptor involved in ligand binding. *Biochem. Biophys. Res. Commun.* 354, 396–402.
- Gradelet, S., Astorg, P., Pineau, T., Canivenc, M.C., Siess, M.H., Leclerc, J., Lesca, P., 1997. Ah receptor-dependent CYP1A induction by two carotenoids, canthaxanthin and β -apo-8'-carotenol, with no affinity for the TCDD binding site. *Biochem. Pharmacol.* 54, 307–315.
- Hamada, M., Satsu, H., Natsume, Y., Nishiumi, S., Fukuda, I., Ashida, H., Shimizu, M., 2006. TCDD-induced CYP1A1 expression, an index of dioxin toxicity, is suppressed by flavonoids permeating the human intestinal Caco-2 cell monolayers. *J. Agric. Food Chem.* 54, 8891–8898.
- Harper, P.A., Riddick, D.S., Okey, A.B., 2006. Regulating the regulator: Factors that control levels and activity of the aryl hydrocarbon receptor. *Biochem. Pharmacol.* 72, 267–279.
- Haslam, E., 1989. *Plant Polyphenols*. Cambridge University Press, Cambridge, UK.
- Heath-Pagliuso, S., Rogers, W.J., Tullis, K., Seidel, S.D., Cenijn, P.H., Brouwer, A., Denison, M.S., 1998. Activation of the Ah receptor by tryptophan and tryptophan metabolites. *Biochemistry* 37, 11508–11515.
- Hollman, P.C.H., van Trijp, J.M.P., Buysman, M.N.C.P., van der Gaag, M.S., Mengelers, M.J.B., de Vries, J.H.M., Katan, M.B., 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* 418, 152–156.
- Horikawa, K., Oishim, N., Nakagawa, J., Kasai, A., Hayakawa, K., Hiramatsu, N., Takano, Y., Yokoichi, M., Yao, J., Kitamura, M., 2006. Novel potential of tunicamycin as an activator of the aryl hydrocarbon receptor-dioxin responsive element signaling pathway. *FEBS Lett.* 580, 3721–3725.
- Ishida, T., Naito, R., Mutoh, J., Takeda, S., Ishii, Y., Yamada, H., 2005. The plant flavonoid, quercetin, reduces some forms of dioxin toxicity by a mechanism distinct from aryl hydrocarbon receptor activation, heat-shock protein induction and quenching oxidative stress. *J. Health Sci.* 51, 410–417.
- Jeuken, A., Keser, B.J.G., Khan, E., Brouwer, A., Koeman, J., Denison, M.S., 2003. Activation of the Ah receptor by extracts of dietary herbal

- supplements, vegetables, and fruits. *J. Agric. Food Chem.* 51, 5478–5487.
- Kato, T., Matsuda, T., Matsui, S., Mizutani, T., Saeki, K., 2002. Activation of the aryl hydrocarbon receptor by methyl yellow and related congeners: structure–activity relationships in halogenated derivatives. *Biol. Pharm. Bull.* 25, 466–471.
- Kobayashi, Y., Lundquist, A., Uechi, T., Ashieda, K., Sasaki, K., Hughes, B., Kaise, T., 2002. Dioxin screening in environmental samples using the Ah-Immunoassay. *Organohalogen Compd.* 58, 337–340.
- Landers, J.P., Bunce, N.J., 1991. The Ah receptor and the mechanism of dioxin toxicity. *Biochem. J.* 276, 273–287.
- Ma, Q., 2001. Induction of CYP1A1 The AhR/DRE paradigm: Transcription, receptor regulation, and expanding biological roles. *Curr. Drug Metab.* 2, 149–164.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 81, 230–242.
- Mandal, P.K., 2005. Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. *J. Comp. Physiol. B* 175, 221–230.
- Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., Fujii-Kuriyama, Y., 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* 2, 645–654.
- Mimura, J., Fujii-Kuriyama, Y., 2003. Functional role of AhR in the expression of toxic effects by TCDD. *Biochim. Biophys. Acta* 1619, 263–268.
- Mukai, R., Fukuda, I., Hosokawa, K., Nishiumi, S., Kaneko, A., Ahida, H., 2005. Anthocyanins fail to suppress transformation of aryl hydrocarbon receptor induced by dioxin. *Biosci. Biotechnol. Biochem.* 69, 896–903.
- Murkies, A.L., Wilcox, G., Davis, S.R., 1998. Clinical review 92: Phytoestrogens. *J. Clin. Endocrinol. Metab.* 83, 297–303.
- Nishiumi, S., Yabushita, Y., Fukuda, I., Mukai, R., Yoshida, K., Ashida, H., 2006. Molokhia (*Corchorus olitorius* L.) extract suppresses transcription of the aryl hydrocarbon receptor induced by dioxins. *Food Chem. Toxicol.* 44, 250–260.
- Pandini, A., Denison, M.S., Song, Y., Soshilov, A.A., Bonati, L., 2007. Structural and functional characterization of the aryl hydrocarbon receptor ligand binding domain by homology modeling and mutational analysis. *Biochemistry* 46, 696–708.
- Park, Y.K., Fukuda, I., Ahida, H., Nishiumi, S., Guzman, J.P., Sato, H.H., Pastore, G.M., 2004. Suppression of dioxin mediated aryl hydrocarbon receptor transformation by ethanolic extracts of propolis. *Biosci. Biotechnol. Biochem.* 68, 935–938.
- Park, Y.K., Fukuda, I., Ashida, H., Nishiumi, S., Yoshida, K., Daugsch, A., Sato, H.H., Pastore, G.M., 2005. Suppression effects of ethanolic extracts from propolis and its main botanical origin on dioxin toxicity. *J. Agric. Food Chem.* 53, 10306–10309.
- Phelan, D., Winter, G.M., Rogers, W.J., Lam, J.C., Denison, M.S., 1998. Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. *Arch. Biochem. Biophys.* 357, 155–163.
- Poellinger, L., 2000. Mechanistic aspects—the dioxin (aryl hydrocarbon) receptor. *Food Addit. Contam.* 17, 261–266.
- Reiners Jr., J.J., Clift, R., Mathieu, P., 1999. Suppression of cell progression by flavonoids: dependence on the aryl hydrocarbon receptor. *Carcinogenesis* 20, 1561–1566.
- Safe, S.H., 1986. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* 26, 371–399.
- Safe, S.H., 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* 21, 51–88.
- Schmidt, J.V., Bradfield, C.A., 1996. Ah receptor signaling pathways. *Ann. Rev. Cell Develop. Biol.* 12, 55–89.
- Schwarz, M., Appel, K.E., 2005. Carcinogenic risks of dioxin: Mechanistic considerations. *Regul. Toxicol. Pharmacol.* 43, 19–34.
- Setchell, K.D.R., Cassidy, A., 1999. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* 129, 758S–767S.
- Sugihara, K., Kitamura, S., Yamada, T., Okayama, T., Ohta, S., Yamashita, K., Yasuda, M., Fujii-Kuriyama, Y., Saeki, K., Matsui, S., Matsuda, T., 2004. Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo. *Biochem. Biophys. Res. Commun.* 318, 571–578.
- Tsutsumi, T., Amakura, Y., Nakamura, M., Brown, D.J., Clark, G.C., Sasaki, K., Toyoda, M., Maitani, T., 2003. Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail fish. *Analyst* 128, 486–492.
- Vrzal, R., Zdražilová, A., Ulrichová, Bláha, L., Giesy, J.P., Dvořák, Z., 2005. Activation of the aryl hydrocarbon receptor by berberine in HepG2 and H4IIE cells: Biphasic effect on CYP1A1. *Biochem. Pharmacol.* 70, 925–936.
- Watanabe, S., Yamaguchi, M., Sobue, T., Takahashi, T., Miura, T., Arai, Y., Mazur, W., Wähälä, K., Adlercreutz, H., 1998. Pharmacokinetics of soybean isoflavones in plasma, urine, feces of men after ingestion of 60 g baked soybean powder (kinako). *J. Nutr.* 128, 1710–1715.
- Whitlock Jr., J.P., 1993. Mechanistic aspects of dioxin action. *Chem. Res. Toxicol.* 6, 754–763.
- Williams, S.N., Shih, H., Guenette, D.K., Brackney, W., Denison, M.S., Pickwell, G.V., Quattrochi, L.C., 2000. Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A1 gene expression. *Chem. Biol. Interact.* 128, 211–229.
- Yamamoto, S., Sobue, T., Sasaki, S., Kobayashi, M., Arai, Y., Uehara, M., Adlercreutz, H., Watanabe, S., Takahashi, T., Itoi, Y., Iwase, Y., Akabane, M., Tsugane, S., 2001. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. *J. Nutr.* 131, 2741–2747.



Yoshiaki Amakura, Ph.D. Associate Professor, Department of Pharmacognosy, College of Pharmaceutical Sciences, Matsuyama University. Ph.D. in Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Okayama University in 1997; 1999–2004, Researcher, National Institute of Health Sciences (NIHS), Japan; 2004–2006, Senior Researcher, NIHS; 2006–Present, Associate Professor, Faculty of Pharmaceutical Sciences, Matsuyama University.



Tomoaki Tsutsumi, Ph.D. Senior Researcher, Division of Foods, National Institute of Health Sciences (NIHS), Japan. Ph.D. in Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Science University of Tokyo in 1998; 1998–1999, Assistant Professor, The Jikei University School of Medicine; 1999–2005, Researcher, NIHS; 2005–Present, Senior Researcher, NIHS.



Masafumi Nakamura, Bioassay Team Leader, Measurement Section, Analytical Research Division, Engineering Department, HIYOSHI Corporation. B.S. in Agriculture, Shimane University in 1995; 1996–2000, Researcher, HIYOSHI Corporation; 2000–Present, Bioassay Team Leader, HIYOSHI Corporation (<http://www.calux.jp/english/index.html>).



Kumiko Sasaki, Ph.D. Chief Researcher, Division of Foods, National Institute of Health Sciences (NIHS), Japan. M.S. in Agriculture, Graduate School of Agriculture, Kyusyu University in 1969; Ph.D. in Agriculture, Kyusyu University in 1985; 1969–1985, Researcher, NIHS; 1985–1993, Senior Researcher, NIHS; 1993–2007, Section Chief, NIHS.



Takashi Yoshida, Ph. D. Professor, Department of Pharmacognosy, College of Pharmaceutical Sciences, Matsuyama University. M.S. in Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University in 1964; Ph.D. in Pharmaceutical Sciences, Kyoto University in 1969; 1965–1970, Research Associate, Faculty of Pharmaceutical Sciences, Kyoto University; 1970–1993, Associate Professor, Faculty of Pharmaceutical Sciences, Okayama University; 1993–2005, Professor, Faculty of Pharmaceutical Sciences, Okayama University; 2005–Present, Professor, College of Pharmaceutical Sciences, Matsuyama University. He is now also an Emeritus Professor of Okayama University.



Tamio Maitani, Ph.D. Director, Division of Foods, National Institute of Health Sciences (NIHS), Japan. Ph.D. in Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University in 1979; 1976–1984, Research Scientist and Senior Research Scientist, Division of Basic Medical Sciences, National Institute for Environmental Studies; 1982–1983, Research Fellow, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center (Professor C.D. Klaassen); 1984–1989, Senior Researcher, Division of Foods, NIHS; 1989–2000, Section Chief, Division of Food Additives, NIHS; 2000–2002, Director, Division of Food Additives, NIHS; 2002–Present, Director, Division of Foods, NIHS.

Dioxin Concentrations in Commercial Health Tea Materials in Japan

Yoshiaki Amakura,^{*,a} Tomoaki Tsutsumi,^b
Kenji Tanno,^c Koichi Nomura,^c
Toshihiko Yanagi,^c Yoichi Kono,^c
Morio Yoshimura,^a Tamio Maitani,^b
Rieko Matsuda,^b and Takashi Yoshida^a

^aCollege of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan,

^bDivision of Foods, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan and

^cJapan Food Research Laboratories, 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

(Received October 27, 2008; Accepted February 4, 2009)

This study determined the concentrations of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dioxin-like PCBs) in five selected plant materials [dokudami (from *Houttuynia* herb), rose hip (from *Rosa* fruit), ebisugusa (from *Cassia* seed), rooibos, and tochu (from *Eucommia* leaf)] used as health teas in Japan. The toxic equivalent (TEQ) levels for dioxins in the samples ranged from < 0.001 to 0.27 pg-TEQ/g weight, when undetectable and trace amounts were taken as zero. The mean of total TEQ level in commercial tea materials was estimated as 0.08 pg-TEQ/g ($n = 5$). The total TEQ in these samples was mainly dominated by the levels of PCDD/Fs (representing *ca.* 80% of the total TEQ).

Key words—dioxin, polychlorinated dibenzo-*p*-dioxin, polychlorinated dibenzofuran, dioxin-like polychlorinated biphenyl, health tea, food

INTRODUCTION

In Japan, foods have been generally recognized as the main route of human intake of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (PCBs), which are referred to col-

lectively as dioxins, contributing to more than 95% of the daily intake of these compounds.¹⁾ Thus, it is very important to survey the levels of dioxins in various kinds of foodstuffs in order to evaluate the risk to humans. For food safety and security, we have been regularly performing daily intake surveys of dioxins by total diet studies and dioxin-pollution surveys of individual foods in Japan. From previous results, fishery products have been regarded as the main source of dioxins in the Japanese diet.^{2,3)} In addition, the levels of dioxins in retail fish and shellfish including dioxin contamination in the edible parts of Japanese common squid and saury were elucidated.^{4,5)}

In recent years, the demand for functional foods, including health teas, has expanded significantly.⁶⁾ Functional foods tend to be concentrates of selected foods; therefore, they may have a risk of excessive intake of harmful chemical contaminants in the foods even if those chemicals in individual food are at such a small level that they have no influence on health routinely. Previously, the levels of dioxin contaminants in fish oil supplements on the Japanese market were reported.⁷⁾ As a part of these ongoing studies on the actual situation of dioxin contamination in functional foods in Japan, it is reported herein on the dioxin levels in health teas, which have not been described previously with the exception of studies on green tea leaf.^{8,9)}

MATERIALS AND METHODS

Samples and Reagents—Five tea materials [sample 1: dokudami (from *Houttuynia* herb; aerial part of *Houttuynia cordata*), sample 2: ebisugusa (from *Cassia* seed; seed of *Cassia obtusifolia* or *Cassia tora*), sample 3: rooibos (from the leaves of *Aspalathus linearis*), sample 4: rose hip (from the fruit of *Rosa* spp.) and sample 5: tochu (from the leaves of *Eucommia ulmoides*)] were obtained from stores in Japan (in 2006). Reagents used in this study were the same as described in the previous paper.¹⁰⁾

Extraction and Cleanup Procedure—The method of extraction and cleanup followed that of the tentative guideline for the analysis of dioxins in foods in Japan.¹¹⁾ Briefly, 50 g of homogenized tea material was spiked with a mixture of ¹³C-labeled internal quantitative standards [seventeen PCDD/Fs and twelve dioxin-like PCBs that have toxic equivalency factor (TEF) values proposed by the World Health Organization (WHO)], then extracted twice

*To whom correspondence should be addressed: College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan. Tel.: +81-89-925-7111; Fax: +81-89-926-7162; E-mail: amakura@cc.matsuyama-u.ac.jp

by shaking with acetone-*n*-hexane (1 : 1, total 200–360 ml) for 1 hr at room temperature, and filtered (0.8 μ m filter, GPF filter paper of Kiriyama, Tokyo, Japan). After extraction, sulfuric acid treatment and column chromatography (CC) procedures (silver nitrate-silica gel CC, alumina CC, and activated carbon mixed silica gel CC) were carried out for cleanup, and each fraction obtained was spiked with ^{13}C -labeled recovery standards before analysis by high-resolution gas chromatography (HRGC)/high-resolution mass spectrometry (HRMS). The details were described in the previous paper.¹⁰⁾

Dioxin Analysis—Dioxin analysis was performed under HRGC/HRMS conditions using an Agilent 6890 plus gas chromatograph (Agilent Technologies, Santa Clara, CA, U.S.A.) coupled to a Micromass Autospec mass spectrometer (Micromass, Manchester, U.K.). The approximate limits of quantification in this study were; 0.01 pg/g for Tetra (Te)CDDs, Penta (Pe)CDDs, Tetra (Te)CDFs, and Penta (Pe)CDFs; 0.02 pg/g for Hexa (Hx)CDDs, Hepta (Hp)CDDs, Hexa (Hx)CDFs, and Hepta (Hp)CDFs; 0.05 pg/g for Octa (O)CDD and Octa (O)CDF; 0.1 pg/g for non-*ortho* PCBs; and 1 pg/g for mono-*ortho* PCBs. The toxic equivalent (TEQ) values for congeners were calculated using the WHO-TEFs.¹²⁾ The total TEQ in a sample was calculated based on the assumption that all isomer concentrations lower than the limits of quantification were equal to zero.

RESULTS AND DISCUSSION

Table 1 shows the concentrations of the sums for PCDDs, PCDFs, and dioxin-like PCBs, as well as the total TEQ, for five plant materials used as health teas. The recoveries calculated for the spiked compounds were always in the range defined as sufficient recovery in the tentative guideline.¹¹⁾ On the

whole, the samples had low dioxin levels around the limit of quantification or below. The highest total concentration in pg/g product weight was 37.15 pg/g in sample 5 (tochu). Subsequently samples 1 (dokudami) and 4 (rose hip) were found to have contamination levels of 17.46 and 11.42 pg/g, respectively. The dioxin concentrations of samples 2 (ebisugusa) and 3 (rooibos) were low, 2.46 and 0.66 pg/g, respectively. The mean of dioxin concentrations of commercial tea materials was 13.83 pg/g ($n = 5$, 3.18 pg/g for PCDDs, 0.79 pg/g for PCDFs, and 9.86 pg/g for dioxin-like PCBs, respectively). The maximum TEQ values corresponded to sample 5 (0.27 pg-TEQ/g) followed by sample 1 (0.13 pg-TEQ/g), and the mean of total TEQ level in commercial tea materials was 0.08 pg-TEQ/g ($n = 5$).

Figure 1 graphically illustrates the TEQ con-

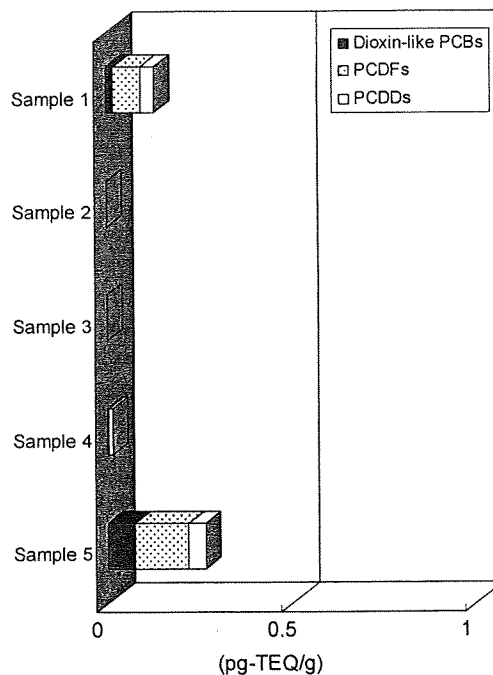


Fig. 1. TEQ Concentrations in Five Commercial Health Tea Materials
Name of samples: see Materials and Methods.

Table 1. Concentrations of PCDD/Fs and Dioxin-like PCBs in Commercial Health Tea Materials in Japan

Tea Materials	Concentration (pg/g prepared weight)				Total TEQ concentration (pg-TEQ/g prepared weight)
	PCDDs	PCDFs	Dioxin-like PCBs	Total	
Sample 1	3.35	1.01	13.1	17.46	0.13
Sample 2	0.16	nd	2.3	2.46	< 0.001
Sample 3	0.06	nd	0.6	0.66	< 0.001
Sample 4	8.50	0.42	2.5	11.42	0.014
Sample 5	3.83	2.52	30.8	37.15	0.2
Average ($n = 5$)	3.18	0.79	9.86	13.83	0.08

nd: not detected. TEQ was calculated as WHO-TEFs. Values below the limit of detection were taken as zero.

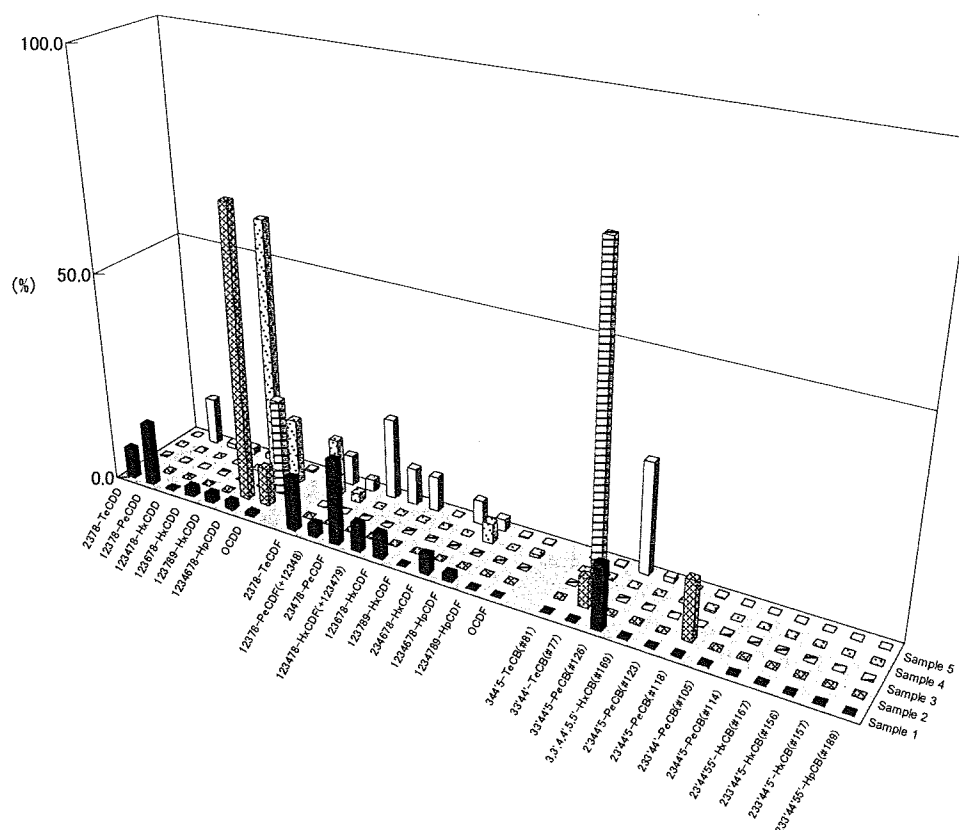


Fig. 2. Percentage Contributions of Congeners Using WHO-TEFs to Total Contamination in Five Commercial Health Tea Materials
Name of samples: see Materials and Methods.

centrations and their components in each health-tea sample. As can be seen, PCDD/Fs were the predominant congeners detected in the samples.

Figure 2 shows the percentage contribution of isomers to the total TEQ levels of tea materials. Total TEQ levels were dominated by 1,2,3,7,8-PeCDD, 2,3,7,8-TeCDF, 2,3,4,7,8-PeCDF, and 3,3',4,4',5-PeCB (#126) in samples 1 and 5; 1,2,3,4,6,7,8-HpCDD, OCDD and 2,3,7,8-TeCDF in sample 4; 1,2,3,4,6,7,8-HpCDD and 2,3',4,4',5-PeCB (#118) in sample 2; and OCDD and 3,3',4,4'-TeCB (#77) in sample 3. It was reported previously that the dioxin concentrations in vegetables reflect in the contamination source.¹³⁾ For example, dioxin analysis of leafy vegetables indicated that 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, and 3,3',4,4',5-PeCB (#126), which are indicator isomers of total TEQ in environmental samples (exhaust gas, ash, and ambient air), are the dominant isomers. In the present study, the dominant isomers contaminating samples 1 and 5 were similar to those found in leafy vegetables. Thus, it is suggested that the dioxin contamination in the teas was caused by absorption or adhesion from the atmosphere to the surface of the plants.

The previous report indicated that the dioxin levels of green tea leaf were also in the range of 0.053–0.856 pg-TEQ/g.^{8,9)} The present TEQ levels for dioxins in health tea materials were < 0.001–0.27 pg-TEQ/g with the roughly same values as those of green tea. Generally, the plant materials for health teas are decocted in boiling water for drinking. As dioxins are insoluble in water, they are considered not to be ingested from health teas.

In conclusion, the levels of dioxins in health tea materials were surveyed for the first time in Japan. The samples analyzed had low dioxin levels similar to those of leafy vegetables.

Acknowledgements This work was supported by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

- 1) Council of Ministries and Agencies on Dioxin Policy, Japan (2005) *Informational Brochure of Dioxins*.
- 2) Tsutsumi, T., Yanagi, T., Nakamura, M., Kono, Y.,

- Uchibe, H., Iida, T., Hori, T., Nakagawa, R., Tobiishi, K., Matsuda, R., Sasaki, K. and Toyoda, M. (2001) Update of daily intake of PCDDs, PCDFs, and dioxin-like PCBs from food in Japan, *Chemosphere*, **45**, 1129–1137.
- 3) Toyoda, M. (2001) The data resulting from dietary intake studies of dioxins and related PCBs in 1999, *Food Sanit. Res.*, **51**, 15–24.
- 4) Tsutsumi, T., Amakura, Y., Yanagi, T., Nakamura, M., Kono, Y., Uchibe, H., Iida, T., Toyoda, M., Sasaki, K. and Maitani, T. (2003) Levels of PCDDs, PCDFs and dioxin-like PCBs in retail fish and shellfish in Japan. *Organohalogen Compd.*, **62**, 93–96.
- 5) Tsutsumi, T., Amakura, Y., Sasaki, K. and Maitani, T. (2007) Dioxin concentrations in the edible parts of Japanese common squid and saury. *Organohalogen Compounds, the Journal of Food Hygienics Society Japnn*, **48**, 8–12.
- 6) Arai, S., Morinaga, Y., Yoshikawa, T., Ichiishi, E., Kiso, T., Yamazaki, M., Morotomi, M., Shimizu, M., Kuwata, T. and Kaminogawa, S. (2002) Recent trends in functional food science and the industry in Japan. *Biosci. Biotechnol. Biochem.*, **66**, 2017–2029.
- 7) Tsutsumi, T., Amakura, Y., Tanno, K., Kono, Y., Sasaki, K. and Maitani, T. (2007) Dioxins and other organohalogen compounds in fish oil supplements on the Japanese market. *Organohalogen Compd.*, **69**, 2371–2374.
- 8) <http://www.mhlw.go.jp/houdou/2004/12/h1227-2.html>
- 9) http://www1.mhlw.go.jp/topics/dioxin_13/tds2.html
- 10) Amakura, Y., Tsutsumi, T., Iida, T., Nakagawa, R., Hori, T., Tobiishi, K., Uchibe, H., Nakamura, M., Yanagi, T., Kono, Y., Toyoda, M., Sasaki, K. and Maitani, T. (2005) Contamination levels of PCDDs, PCDFs and Co-PCBs in commercial baby foods in Japan. *J. Food Hyg. Soc. Jpn.*, **46**, 148–152.
- 11) Environmental Health Bureau (1999) *Provisional guidelines for analysis of polychlorinated dibenzo-p-dioxins, dibenzofurans and coplanar PCBs in foods*, Environmental Health Bureau, Ministry of Health and Welfare, Japan.
- 12) Van den Berg, M., Birnbaum, L., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and Peterson, R. E. (2006) The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol. Sci.*, **93**, 223–241.
- 13) Amakura, Y., Tsutsumi, T., Sasaki, K. and Maitani, T. (2003) Levels of congener distributions of PCDDs, PCDFs and Co-PCBs in Japanese retail fresh and frozen vegetables. *J. Food Hyg. Soc. Jpn.*, **44**, 294–302.