Available online at www.sciencedirect.com

ScienceDirect

Toxicology in Vitro 22 (2008) 1050–1061

Toxicology in Vitro

www.elsevier.com/locate/toxinvit

Screening and detection of the *in vitro* agonistic activity of xenobiotics on the retinoic acid receptor

Ryo Kamata [a,*], Fujio Shiraishi [a], Jun-ichi Nishikawa [b], Junzo Yonemoto [a], Hiroaki Shiraishi [a]

[a] *Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan*
[b] *School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kubancho, Nishinomiya, Hyogo 663-8179, Japan*

Received 4 October 2007; accepted 4 January 2008
Available online 12 January 2008

Abstract

The retinoic acid receptors (RARs) play key roles in various biological processes in response to endogenous retinoic acids. However, excessive embryonic exposure to specific ligands for each subtype of the RAR was reported to induce specific developmental abnormalities. We measured the RAR agonistic activity of 543 chemicals using an assay system adopting yeast cells transfected with the human RARγ and a coactivator. Eighty-five of the 543 chemicals, including 16 organochlorine pesticides, 14 styrene dimers, 9 monoalkylphenols and 6 parabens, exhibited RARγ agonistic effects in this assay. In particular, monoalkylphenols having a 6–9 carbon alkyl group *para* to the phenolic hydroxyl group possessed high affinity for the RARγ, and their activities were 1.363–0.446% of that of *all-trans* RA. *para*-Alkylphenols chlorinated at the *ortho* position also were about as active or more active than their unchlorinated analogs. In addition, all tested styrene dimers showed positive effects, and the activity of 1-phenyltetralin, the strongest in this category, was 1.169% that of *all-trans* RA. A number of chemicals having binding affinity for the RARγ were revealed in this study (both newly identified and confirmed), further comprehensive studies of *in vitro* and *in vivo* effects via the RARs are required for the reliable risk assessment of chemicals. *In vitro* receptor binding studies represent an important step in hazard identification and suggest a potential mechanism of action, which can be an important step in risk assessment and in particular for screening studies to identify potential toxicity and inform mechanistic studies.

Keywords: Retinoic acid receptor; Two-hybrid yeast assay; Monoalkylphenol; Styrene dimer; Paraben; Organochlorine pesticide

1. Introduction

The retinoic acid (RA) receptors (RARs) are, like the steroid hormone and thyroid hormone receptors (TR), nuclear receptors that respond to specific natural ligands, *all-trans* RA and 9-*cis* RA. Both RAs, which are oxidative metabolites of vitamin A, are essential for cellular proliferation, development and differentiation in vertebrates and therefore play crucial roles in normal growth and homeostasis. However, both deficiency and excess of vitamin A are harmful. Vitamin A deficiency during gestation results in diverse embryonic malformations in various organs (Zile, 1998, 2001), while excess RA has been reported to trigger teratogenic actions in the developing embryo via the RARs. Ligands specific for each subtype of the RARs (α, β and γ) have been reported to induce specific deformities in rodent embryos (Elmazar et al., 1996, 2001). A ligand for the α-subtype causes defects of the ear, mandible and limb, a β-subtype ligand causes defects of the urinary system and liver, and a γ-subtype ligand causes ossification deficiencies and defects of the sternebrae and vertebral body.

We have developed several yeast two-hybrid systems transduced with the ligand binding domains of nuclear receptors and a coactivator for the receptors for detecting and measuring the activity of chemicals (Nishikawa et al., 1999; Shiraishi et al., 2000). In prior works, it has been found that there are a number of industrial/environmental

* Corresponding author. Tel.: +81 29 8502873; fax: +81 29 8502870.
E-mail address: kamata.ryo@nies.go.jp (R. Kamata).

0887-2333/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2008.01.002

compounds with the capability to activate or inactivate nuclear receptors such as the estrogen receptors (ERs) and the TRs, and some compounds show unexpected activity; i.e., activity that would not be readily predicted from the structure of the compounds (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). These studies indicated that chemicals encountered in daily life or through accidental exposure bind with nuclear receptors and suggested potential toxicity, mechanisms of action and therefore potential risks to human health. In our laboratory, yeast assays with nuclear receptors derived from the Japanese medaka fish (*Oryzias latipes*) as well as the human have also demonstrated the receptor activation of environmental water samples taken from contaminated rivers, lakes and seas (Mispagel et al., 2005; unpublished data, Shiraishi et al., 2006) and thereby show that organisms in the environment may be exposed to potentially harmful chemicals and/or bioactive substances originating from human activity.

The objective of the present study was to screen a wide range of xenobiotic and other compounds for agonistic effects on the RAR and to quantify their activities. We prepared a yeast two-hybrid system to detect transcriptional activation via the human RARγ and assessed 543 compounds including industrial chemicals, agrochemicals, natural compounds, medicines and cosmetic chemicals. An assay for each human RAR subtype was derived from previously reported yeast assays and optimized for high-throughput screening. Because the yeast cells transfected with the γ-subtype showed the lowest luminescence intensity when inactivated and the highest reactivity to an endogenous ligand, *all-trans* RA, we selected the RARγ type of assay for the present investigation. As the RAR agonistic effect was detected in different categories of chemicals, we provide the measured activities of the positively-reacting compounds grouped according to chemical structure.

2. Materials and methods

2.1. Compounds

The 543 compounds examined in this study are listed in Table 1, grouped according to their intended uses and chemical structures. The compounds were purchased from Accu Standard, Inc. (New Haven, CT, USA), Acros Organics N.V. (Geel, Belgium), Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany), Cosmo Bio Co., Ltd. (Tokyo, Japan), Dr. Ehrenstorfer GmbH (Augsburg, Germany), GL Science, Inc. (Tokyo, Japan), Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Katayama Chemical Industries Co., Ltd. (Osaka Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Maruzen Petrochemical Co., Ltd. (Tokyo, Japan), MP Biochemicals (Solon, OH, USA), PerkinElmer, Inc. (Wellesley, MA, USA), Scientific Polymer Products, Inc. (Ontario, NY, USA), Sigma–Aldrich Corp. (St. Louis, MO, USA), Steraloids, Inc. (Newport, RI, USA), Tocris Bioscience (Ellisville, MO, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Toronto Research Chemicals Inc. (North York, ON, Canada) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or were gifts from researchers who had synthesized them for other purposes.

2.2. Yeast two-hybrid assay

The transcriptional agonistic activities of compounds to the RAR were measured with a reporter assay using yeast cells (*Saccharomyces cervisiae* Y190). An expression plasmid for the ligand binding domain of the human RARγ and the coactivator pGAAD424-TIF-2 was introduced into yeast cells that carried the β-galactosidase reporter gene (Nishikawa et al., 1999). The assay was performed using a chemiluminescent reporter gene method (for β-galactosidase) employing a 96-well culture plate, based on a yeast two-hybrid estrogenicity assay (Shiraishi et al., 2000, 2003).

Yeast cells were preincubated for 24 h at 30 °C with shaking in modified SD medium lacking tryptophan and leucine (5.8% yeast nitrogen base, 0.75% dextrose, 0.013% L-valine, 0.00435% L-phenylalanine, 0.00261% L-isoleucine, L-lysine HCl and L-tyrosine, 0.00174% L-adenine hemisulfate salt, L-arginine HCl, L-histidine HCl monohydrate, L-methionine and L-uracil) and the cell density was adjusted to an absorbance of 1.65–1.80 at 595 nm. A dimethylsulfoxide (DMSO) solution of each test compound was stored at −80 °C until just before examination and was serially twofold diluted with the medium. An aliquot of the diluted solution (120 μl) was poured to two wells of a black 96-well culture plate for chemiluminescence measurement, and then the yeast cell suspension (60 μl) was added. At least seven serial two-fold concentrations of each chemical from 10 μM to 156 nM were tested; lower concentrations were tested for chemicals showing high RAR agonistic activity. The solution in every well contained 1% DMSO. After vortex mixing, the plate was incubated at 30 °C under conditions of high humidity for 4 h. A solution (80 μl) for inducing chemiluminescence from released β-galactosidase, consisting of reaction buffer (30 μl) containing GalactLux substrate (AURORA GAL-XE, MP Biochemicals) and a 1:1 mixture (50 μl) of zymolyase 20 T and 100 T solutions for enzymatic digestion (Kirin Brewery Co, Ltd., Tokyo, Japan), was added to each well. The plate was incubated at 37 °C for 1 h and then placed in a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan), and a light emission accelerator solution (AURORA GAL-XE, 50 μl) was added to each well using the luminometer pump. The chemiluminescence produced by released β-galactosidase in each well was measured.

All test compounds were evaluated in a minimum of three separate assays which were performed in duplicate. For comparative estimates of the ability of test compounds to activate the RAR, *all-trans* RA, an endogenous agonist

Table 1
List of 543 compounds tested in a yeast two-hybrid assay for the RARγ

Compounds		
Industrial chemicals (252)	Bisphenols and related chemicals (Continued)	Parabens (13)
Aromatic hydrocarbons (10)	4-Hydroxyphenyl isopropanol	3-Hydroxybenzoic acid
Biphenyl	4-Hydroxyphenyl isobutyl methyl ketone	4-Hydroxybenzoic acid
2-Terphenyl	6-Hydroxy-1-(4-hydroxyphenyl)-1,3,3-trimethylindanc	Methyl 4-hydroxybenzoate
3-Terphenyl	4-Cumylphenol	Ethyl 4-hydroxybenzoate
4-Terphenyl	2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman	Propyl 4-hydroxybenzoate
n-Butylbenzene	6-Hydroxy-3-(4-hydroxyphenyl)-1,1,3-trimethylindane	Isopropyl 4-hydroxybenzoate
n-Octylbenzene	2,4-Bis(4-hydroxycumyl)phenol	Butyl 4-hydroxybenzoate
n-Nonylbenzene	4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman	Isobutyl 4-hydroxybenzoate
1,3-Diphenylpropane	4-Propenylphenol	n-Amyl 4-hydroxybenzoate
Benzylbiphenyl	4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene	n-Hexyl 4-hydroxybenzoate
Triphenylmethane	2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman	Benzyl 4-hydroxybenzoate
		2-Ethylhexyl 4-hydroxybenzoate
Polycyclic aromatic hydrocarbons (14)	Dyes (21)	n-Dodecyl 4-hydroxybenzoate
Naphthalene	Azobenzene	
Acenaphthene	N-Acetyl-5-hydroxy-tryptamine	Phenols and related chemicals (37)
Fluorene	Acid Alizarin Violet N	4-Cyanophenol
Phenanthrene	1-Acetyl-4-(4-hydroxyphenyl)-piperazine	4-Hydroxybenzaldehyde
Anthracene	Anthrarobin	4-Methoxyphenol
Pyrene	Anthraflavic acid	2-Cyclopentylphenol
Fluoranthene	cis-1,3-O-Benzylideneglycerol	4-Cyclopentylphenol
Chrysene	Chlorophenol Red	2,4-Dichlorophenol
Benz[a]anthracene	2,5-Dihydroxyphenylacetic γ-lactone	3,4-Dichlorophenol
Benzo[a]pyrene	4-(2,4-Dinitroanilino)-phenol	4-Hydroxycinnamic acid
Benzo[e]pyrene	6-Fluoro-4-hydroxy-coumarin	2-Hydroxybiphenyl
Benzo(k)fluoranthene	Hoechst 33258	4-Hydroxybiphenyl
Benzo(b)fluoranthene	6-Hydroxy-1,3-benzoxathiol-2-one	4-Cyclohexylphenol
Dibenz(a,h)anthracene	2-Hydroxy-9-fluorenone	4-Benzylphenol
	4-Hydroxyindole	4-Hydroxybezophenone
Arsenic compounds (13)	4-Hydroxy-6-methyl-2-pyrone	Phenyl salicylate
Sodium meta-arsenite	3-Hydroxy-1H-phenalen-1-one	2-Iodophenol
Dimethyl arsinic acid	2-(4-Hydroxyphenyl)-5-pyrimidinol	4-Iodophenol
Methyl arsonic acid	1-Hydroxypyrene	1,1-Bis(4-hydroxyphenyl)-propane
Arsenic(III)oxide	Indigo, carmine	4-(1-Adamantyl)phenol
Phenyldimethylarsine oxide	Indigo, synthetic	2-(1-Adamantyl)-4-methylphenol
Phenylmethylarisine oxide		2,4-Dibromophenol
Phenyl arsonic acid	Metals (5)	2,6-Dibromophenol
Diphenylmethylarsine oxide	Tributyltin(IV)chloride	3-(4-Hydroxyphenyl)propionic acid N-hydroxysuccinimide ester
Diphenyl arsinic acid	Triphenyltin(IV)chloride	Hexestrol
Triphenyl arsine	Dibutyltin(IV)dichloride	4,4-Bis-(4-hydroxyphenyl)valeric acid
Sodium arsenate (dibasic) 7H2O	Lead (II) chloride	Phenolphthalein
Triphenyl arsine oxide	Lead (II) acetate trihydrate	4,4'-(1,3-Adamantane-diyl)diphenol
10,10'-Oxybis(phenoxyarsine)		2,4,6-Tribromophenol
	Monoalkyl phenols and related chemicals (27)	3,5-Diiodosalicylic acid
Bisphenols and related chemicals (37)	2-n-Propylphenol	Butylphenoxy acetic acid
Octafluoro-4,4'-biphenol	3-n-Propylphenol	2,3-Dichlorophenoxy acetic acid
2,2',6,6'-TetramethylbisphenolA	4-n-Propylphenol	2-Chloro-4-butylphenoxy acetic acid
4,4'-Thiodiphenol	2-Isopropylphenol	4-Nonylphenyl 2-hydroxyethyl ether
Bisphenol A	3-Isopropylphenol	2,6-Dichloro-4-butylphenoxy acetic acid
2,2',6,6'-Tetrabromobisphenol A	4-Isopropylphenol	2-Chloro-4-nonylphenyl 2-hydroxyethyl ether
2,2',6,6'-Tetrachlorobisphenol A	4-n-Butylphenol	2-Chloro-4-octylphenoxy acetic acid
Bisphenol B	2-s-Butylphenol	4-Nonylphenyl 2-(2hydroxy-ethyloxy)ethyl ether
2-Chlorobisphenol A	4-s-Butylphenol	2,6-Dichloro-4-nonylphenyl 2-hydroxyethyl ether
2,2'-Dichlorobisphenol A	2-t-Butylphenol	
2,6-Dichlorobisphenol A	3-t-Butylphenol	Phthalates (9)
2,2',6-Trichlorobisphenol A	4-t-Butylphenol	Diethyl phthalate
4,4'-Methylenebisphenol (Bisphenol F)	4-n-Pentylphenol	Di-n-propyl phthalate
4,4'-Sulfonyldiphenol (Bisphenol S)	4-t-Pentylphenol	Di-n-butyl phthalate
3,4-(1-Methyl-1-phenylethyl)diphenol	4-n-Hexylphenol	Di-n-pentyl phthalate
2,4'-Isopropylidenediphenol	4-n-Heptylphenol	Benzyl n-butyl phthalate
4,4'-(1,3-Dimethylbutylidene)bisphenol	4-n-Octylphenol	Dicyclohexyl phthalate
Bisphenol E	4-t-Octylphenol	Di-n-hexyl phthalate
2,4'-Dihydroxydiphenyl sulfone (Bisphenol S iso.)	4-n-Nonylphenol	Dibenzyl terephthalate
2,2'-Isopropylidenediphenol	4-Nonylphenol (mixed isomers)	Di-2-ethylhexyl phthalate
4,4'-Cyclohexyliden bisphenol	4-Dodecylphenol (mixed isomers)	
4,4'-(1-Ethyl-2-methyltrimethylene)bisphenol		
4,4'-Diisobutylethylidenediphenol	2-Chloro-4-butylphenol	
	2,6-Dichloro-4-butylphenol	
4-Hydroxyacetophenone	2-Chloro-4-octylphenol	
4-Hydroxy-4'-isopropoxy diphenylsulfone	2,6-Dichloro-4-octylphenol	
4,4'-Isopropoxy diphenylsulfone	2-Chloro-4-nonylphenol	
4-[1-(4-Hydroxyphenyl)-1-methylethyl]-1,2-benzoquinone	2,6-Dichloro-4-nonylphenol	

Table 1 (*continued*)

Compounds		
Industrial chemicals (Continued)	Agrochemicals (124)	Organophosphates (Continued)
Styrene dimers (14+1)	Amides (2)	EPN
1,4-Diphenyl-2-butene	Mepronil	Ethion
1-Methyl-1-phenylindan	Flutolanil	Glyphosate
1-Methyl-3-phenylindan		Iprobenfos
1-Phenyltetralin	Benzimidazoles (2)	Isofenphos
2,3-Diphenyl-1-butene	Carbendazim	Isofenphos oxon
2,4-Diphenyl-1-butene	Thiabendazole	Isoxathion
cis-1,4-Diphenyl-1-butene		Isoxathion oxon
trans-1,4-Diphenyl-1-butene	Carbamates (12)	Leptophos
cis-2,4-Diphenyl-2-butene	Aldicarb	Malathion
trans-2,4-Diphenyl-2-butene	Benfuracarb	MEP oxon
cis-1,2-Diphenylcyclobutane	Benomyl	Parathion
trans-1,2-Diphenylcyclobutane	Carbaryl (NAC)	Phenthoate
trans-1,3-Diphenyl-1-butene	Mancozeb	Piperophos
trans-1,3-Diphenylcyclobutane	Maneb	Prothiophos
Polystyrene standard (mixed styrene dimer isomers)	Methomyl	Thenylchlor
	Metiram	Tolclofos-methyl
Styrene trimer (16)	Molinate	
1,2,4-Triphenylcyclohexane	Thiobencarb	Pyrethroids (16)
1,3,5-Triphenyl-1-hexene	Zineb	Allethrin
1,3,5-Triphenylcyclohexane	Ziram	cis-Permethrin
1,4,5-Triphenyl-1-hexene		Cycloprothrin
1a-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Diphenyl ethers (7)	Cyfluthrin
1a-Phenyl-4a-(2-phenylethyl)tetralin	Acifluorfen	Cyhalothrin
1a-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Aclonifen	Cypermethrin
1e,2e,4a-Triphenylcyclohexane	Bifenox	Esfenvalerate
1e-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Chlomethoxynil	Ethofenprox
1e-Phenyl-4a-(2-phenylethyl)tetralin	Chlomitrofen (CNP)	Fenpropathrin
1e-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	CNP-amino	Fenvalerate
1-Methyl-1,2,4-triphenylcyclopentane	Nitrofen	Flucythrinate
1-Methyl-1,3,4-triphenylcyclopentane		Fluvalinate
1-Methyl-3-phenyl-2-(1-phenylethyl)indan	Organochlorines (30)	Permethrin
2,4,6-Triphenyl-1-hexene	Aldrin	Resmethrin
2,4,6-Triphenyl-2-hexene	β-Benzene hexachloride (β-BHC)	Tralomethrin
	Chlordane	trans-Permethrin
Others (35)	cis-Chlordane	
4-Nitrotoluene	trans-Chlordane	Triazines (4)
1-Naphthol	Chlordecone	Atrazine
2-Naphthol	o,p'-DDD	Dimethametryn
4-Hydroxy-1-indanone	o,p'-DDE	Metribuzin
5,6,7,8-Tetrahydro-1-naphthol	o,p'-DDT	Simazine (CAT)
5,6,7,8-Tetrahydro-2-naphthol	p,p'-DDD	
Sodium pyrithione	p,p'-DDE	Ureas (2)
1,2-Benzothiazol-3-one	p,p'-DDT	Diuron
Biphenyl ether	Dicofol	Pencycuron
2-n-Octyl-4-isothiazolin-3-one	Dieldrin	
N,N',N''-Trishydroxyethylhexahydro-s-triazine	α-Endosulfan	Others (22)
Benzyl-2-naphthylether	β-Endosulfan	Alachlor
4-Nonylcatechol	Endrin	Amitrole
1-(4-Hydroxyphenyl)-1-nonanol	Fthalide	Bromofenoxim
Methyl o-benzoylbenzoate	Heptachlor	Buprofezin
1,2-Bis(3-methylphenoxy)ethane	cis-Heptachlor epoxide	Captan
Triphenylborane	trans-Heptachlor epoxide	Chlorothalonil
1-Nitropyrene	Hexachlorobenzene	Coumachlor
2-Iodobenzoic Acid	1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	Dazomet
Dipyrithione	Methoxychlor	1,2-Dibromo-3-chloropropane (DBCP)
6-Bromoharman	Mirex	Dichlofluanid
Phenyl-1-hydroxy-2-naphthoate	trans-Nonachlor	(2,4-Dichlorophenoxy)acetic acid
β-Naphthoflavone	Oxychlordane	2-(3-Chlorophenoxy)-propionic Acid
Zinc pyrithione	Pentachlorophenol (PCP)	1,3-Dichloropropene
Triphenylborane-pyridine complex	Toxaphene	Fluazifop-butyl
6,8-Dibromoharman	2,4,5-Trichlorophenoxyacetic Acid	Oxadiazon
meso-Stilbene dibromide		Pendimethalin
Copper quinolate	Organophosphates (27)	Probenazole
Di(2-ethylhexyl)adipate	Bromophos-ethyl	Pyroquilon
Octachlorostyrene	Bromopropylate	Tebuconazole
3,6,8-Tribromoharman	Butamifos	Tebufenozide
Triphenylborane-octadecylamin complex	Chlorpyrifos	Trifluralin
Perfluorobutansulfonate (potassium salt)	Chlorpyrifosmethyl	Vinclozolin
Chlorhexidine Hydrochloride	Cyanofenphos	
1,2,5,6,9,10-Hexabromocyclododecane	Diazinon	
	Diazinon oxon	
	Dichlofenthion	
	Edifenphos	

(*continued on next page*)

Table 1 (*continued*)

Compounds		
Natural compounds and related chemicals (109)	Natural compounds (continued)	Medicines and cosmetic-related chemicals (58)
Natural compounds (72)	Melatonin	Medicines (30)
cis-Stilbene	Naringenin	Allyl-thiourea
trans-Stilbene	Naringin	Amiodarone
Azulene	Progesterone	5α-Androstane
Dibenzyl	Resveratrol	Benzophenone
Acridine	Retene	Benzoyl peroxide (BPO)
Flavone	9-cis-Retinoic Acid	Camphorquinone
Biochanin A	13-cis-Retinoic acid	Cinnarizine
Quercetin Dihydrate	all-trans-Retinal	Clofibrate
Genistein	all-trans-Retinoic acid	Clomiphene
17β-Estradiol	all-trans-Retinol	Cyproterone acetate
Zearalenone	3,5-Diiodo-L(+)-tyrosine dihydrate	Dexamethasone
Daidzein	T3 (3,5,3'-Triiodothyronine)	Dienestrol
β-Estradiol 3-Sulfate	T4 (3,3',5,5'-Tetraiodothyronine)	Diethylstilbestrol (DES)
β-Estradiol 3,17-Disulfate	3,3',5'-Triiodo-L-Thyronine	Dimethylaminoethyl methacrylic acid
β-Estradiol 17-(β-D-Glucuronide)	Vitamin A acetate	Dimetyl-p-toluidine
β-Estradiol 3-(β-D-Glucuronide)		Ethynylestradiol
Testosterone	Natural product-related chemicals (37)	Flutamide
Kaempferol	6,8-Dichlorochrysin	Hydroxypropionic Acid
Abietic Acid	6,8-Dichloroapigenin	4-Hydroxytamoxifen
Phloretin	3',8-Dichlorodaidzein	ICI 182780
Apigenin	3',5',8-Trichlorodaidzein	3-Iodo-L-tyrosine
4',5,7-Trihydroxyflavanone	6,8-Dichlorogenistein	Methyltrienolone
Coumestrol	6,8-Dichloronaringenin	Mibolerone
Genistin	6,8-Dichlorocatechin	5-Propyl-2-thiouracil
Daidzin	2-Chloroestrone (E1)	6-n-Propyl-2-thiouracil
17α-Estradiol	4-Chloroestrone (E1)	Spironolactone
Chrysin	2,4-Dichloroestrone (E1)	Tamoxifen
Luteolin	2,4,16,16-Tetrachloroestrone (E1)	3,3',5,5'-Tetraiodothyroacetic acid
Indole-3-Carbinol	10-Chloro-1,4-estradiene-3,17-dione	Thiamazole
Hesperetin	2-Chloro-17β-estradiol (E2)	3,3',5-Triiodothyroacetic acid
β-Sitosterol	4-Chloro-17β-estradiol (E2)	
Equol	2,4-Dichloro-17β-estradiol (E2)	Cosmetic-related chemicals (28)
Enterolactone	2-Chloroestriol (E3)	4-Aminobenzoic Acid
Formononetin	4-Chloroestriol (E3)	2-phenoxyethanol
β-Ecdysterone	2,4-Dichloroestriol (E3)	Ethyl 4-aminobenzoate
α-Ecdysterone	2-Chloro-17α-ethinylestradiol (EE2)	2-Ethylhexyl-4-p-dimethylamino-benzoate
Juvabione	4-Chloro-17α-ethinylestradiol (EE2)	2-Hydroxyethyl salicylate
Cyasterone	2,4-Dichloro-17α-ethinylestradiol (EE2)	4-t-Butylphenyl salicylate
Murisuterone A	4-Androstene-3,17-dione	4-Octylphenyl salicylate
Allylisothiocyanate	16,16-Dichloro-4-androstene-3,17-dione	Salicylic Acid 2-Ethylhexyl Ester
Catechin	1,4-Androstadiene-3,17-dione	2,4-Dihydroxy-benzophenone
Dehydroabietic acid	4,6-Cholestadien-3-one	2-Hydroxy-4-methoxy-benzophenone
5α-Dihydrotestosterone	2-Bromoestrone	2,2'-Dihydroxy-4-methoxy-benzophenone
Estriol	4-Bromoestrone	2,2',4,4'-Tetrahydroxybenzophenone
Estrone	2,4-Dibromoestrone	2,2'-Dihydroxy-4,4'-dimethoxy-benzophenone
Fisetin hydrate	2-Bromo-17β-estradiol	2-Hydroxy-4-n-octyloxy-benzophenone
Flavanone	4-Bromo-17β-estradiol	2-Hydroxy-4-methoxy-benzophenone-5-sulfonic acid
Galangin	2,4-Dibromo-17β-estradiol	4-t-Butyl-4'-methoxy-dibenzoylmethane
Harmol hydrochloride dihydrate	2-Bromoestriol	2-(2'-Hydroxy-5'-methylphenyl)-benzotriazole
Hesperidin	4-Bromoestriol	2-(2'-Hydroxy-5'-tert-butylphenyl)-benzotriazole
Hinokitiol	2,4-Dibromoestriol	2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-benzotriazole
Hydrocortisone	2-Bromo-17α-ethinylestradiol	2-(2'-Hydroxy-3',5'-di-tert-amylphenyl)-benzotriazole
6-Hydroxyflavanone	4-Bromo-17α-ethinylestradiol	2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole
7-Hydroxyflavanone	2,4-Dibromo-17α-ethinylestradiol	2-(5-Chloro-2-benzotriazolyl)-6-tert-butyl-p-cresol
16α-Hydroxyestrone		3-(4-Methylbenzylidane)-camphor
11-Ketotestosterone		2-Ethylhexyl-4-Methoxycinnamate
		(±)-α-Tocopheryl Acetate
		Octamethylcyclotetrasiloxane
		Decamethylcyclopentasiloxane
		Dodecamethylcyclohexasiloxane

of RAR, was used as a standard. A DMSO solution of *all-trans* RA was stored in a shielding container at −80 °C until just before examination to prevent degradation, and seven serial twofold concentrations of *all-trans* RA were examined for every culture of yeast cells. A dose-response curve of the luminescence intensity of each compound was described, and two activity values were calculated from the power approximate expression. The EC × 10 was defined as the concentration of a test solution producing luminescence intensity 10 times that of the blank control, and the REC20 (20% relative effective concentration) was the concentration showing 20% of the activity of 10^{-8} M *all-trans* RA. Activity relative to RA was then calculated by dividing the REC20 of *all-trans* RA by that of a test compound. As there were no high volatile compounds in this study, loss of compounds by volatilization over an incubation period was not taken into consideration.

3. Results

3.1. Assay characteristics

The sensitivity and reproducibility of the RAR yeast assay were assessed using the endogenous ligand, *all-trans*

RA. As a chemiluminescence method is used in which an artificial substrate for β-galactosidase is added to sensitively detect transcriptional activation via the human RAR, the large amount of substrate did not allow luminescence intensity to reach a plateau, even at high concentrations of *all-trans* RA (Fig. 1). Therefore, the half-maximal effective concentration (EC_{50}) commonly used in this kind of *in vitro* assay was not reasonable to evaluate the agonistic ability of test chemicals in the present method. Instead of the EC_{50}, the EC × 10 of *all-trans* RA as defined above was 5.41 ± 1.73 nM (mean ± SD, 22 experiments, Table 2). Chemiluminescence intensity at 10^{-8} M *all-trans* RA was 21.2 ± 7.2 times that of the blank control, and then the REC20 was 2.19 ± 0.20 nM (Table 2).

3.2. Positive substances

Eighty-five of the 543 tested compounds, at their highest concentrations (10 μM), exhibited transcriptional agonistic activity via the human RARγ of at least 20% that of 10^{-8} M *all-trans* RA. Table 2 lists the positive substances in order of RARγ activation potency grouped into categories as for Table 1. The range of molecular weights was 164 (4-*n*-pentylphenol) to 444 (*trans*-nonachlor), and the range of total carbon number was 7 (chlorpyrifosmethyl) to 20 (*all-trans* retinol). Many of the tested organochlorine pesticides, styrene dimers, monoalkylphenols and parabens were found to be RARγ active.

3.3. Organochlorine pesticides

Sixteen of the tested 30 organochlorines had a positive effect on RARγ transfected yeast cells. γ-BHC was the most potent in this category and the activities of this compound and seven other organochlorines were over 0.1% (1/1000) of that of *all-trans* RA. The numbers of chlorine atoms in the active compounds were 6–9, while organochlorines out of this range had no effect on the RARγ.

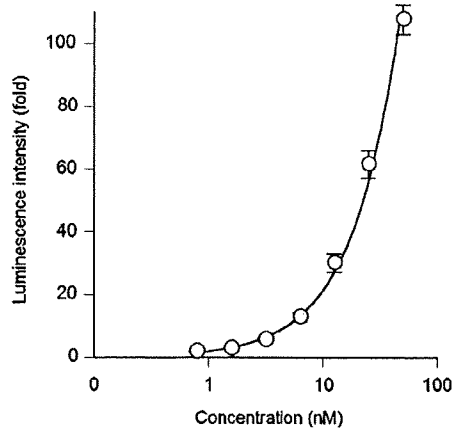

Fig. 1. Response of a yeast two-hybrid assay transfected with the human RARγ and the coactivator to the endogenous ligand, *all-trans* retinoic acid. Values are presented as n-fold induction over the vehicle control and as the mean ± SE of eight independent duplicate experiments.

3.4. Styrene dimers and trimers

All tested styrene dimers and a styrene dimer mixture of unspecified composition exhibited agonistic activity on the RARγ (Fig. 2), but styrene trimers had no effect. 1-Phenyltetralin and 1-methyl-3-phenylindan were particularly active styrene dimers with activities of over 0.6% of that of *all-trans* RA, while five other compounds had agonistic activities over 0.1%.

3.5. Monoalkylphenols

There were several highly active monoalkylphenols with 4-*n*-heptylphenol being the most potent compound tested in this study. All active monoalkylphenols had their alkyl chains *para* to the phenolic hydroxyl group, and the five most active compounds in this category had an alkyl group containing 6–9 carbons and their activities were over 0.4% of that of *all-trans* RA. The ranking of phenols having a linear alkyl group was heptyl (7 carbons) > hexyl (6) > octyl (8) > pentyl (5) > nonyl (9) > dodecyl (12) (Fig. 3). Branching of the alkyl group altered the potency of the phenols (Fig. 4). Comparison of REC20 values showed that an unspecified mixture of isomers of 4-nonylphenol was 4.7 times as active as 4-*n*-nonylphenol and that 4-tert-octylphenol was 2.2 times as active as 4-*n*-octylphenol. However, 4-tert-pentylphenol was 2.8-fold weaker than 4-*n*-pentylphenol. Moreover, six ring-chlorinated monoalkylphenols exhibited positive effects and had about the same or more potency than their unchlorinated analogs.

3.6. Parabens and other chemicals

Some alkyl *p*-hydroxybenzoates (parabens) with an alkyl group of 4–8 carbons were also positive to the RARγ. Of six positives, *n*-hexyl 4-hydroxybenzoate, *n*-pentyl 4-hydroxybenzoate and benzyl 4-hydroxybenzoate showed the highest agonistic activities with values over 0.1% that of *all-trans* RA. Phenols having a cyclic hydrocarbon side-chain *para* to the phenolic hydroxyl group (except 2-(1-adamantyl)-4-methylphenol), a hydroxyethyl ether of 4-nonylphenol and its chlorinated derivative were also positive to the RARγ, but with the exception of 4-(1-adamantyl)phenol, their activities were under 0.1% of that of *all-trans* RA. There were six active substances in the group that we have categorized as 'bisphenol-related compounds', namely, chlorinated bisphenol A and compounds found as impurities in industrial grade bisphenol A, but their activities, with the exception of 4-cumylphenol, were comparatively low. As shown in Table 2, there were also several active substances in the other categories, but most of these had a low potency for the RARγ. However, three diphenyl ethers, aclonifen, nitrofen and chlornitrofen, and the

Table 2
Responsiveness of a RARγ yeast assay to active compounds

Compounds	CAS No.	EC×10 (± SD, ×10^{-6} M)	REC20 (± SD, ×10^{-6} M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (55)				
Aromatic hydrocarbons (4)				
2-Terphenyl	84-15-1	5.03 ± 2.43	1.77 ± 1.14	0.165
n-Octylbenzene	2189-60-8	20.09 ± 6.88	4.51 ± 2.52	0.065
1,3-Diphenylpropane	1081-75-0	8.68 ± 0.81	4.74 ± 1.10	0.062
Triphenylmethane	519-73-3	19.73 ± 4.76	5.34 ± 1.10	0.055
Bisphenols and related chemicals (6)				
2,2'-Dichlorobisphenol A	-	18.95 ± 7.05	6.15 ± 1.61	0.048
2,2',6-Trichlorobisphenol A	-	16.66 ± 5.30	7.85 ± 1.56	0.037
4-Cumylphenol	599-64-4	2.73 ± 0.58	1.68 ± 0.67	0.174
2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman	-	6.53 ± 0.44	3.97 ± 1.33	0.074
2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman	-	7.26 ± 0.96	4.57 ± 2.15	0.064
4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman	472-41-3	6.98 ± 1.33	5.11 ± 2.00	0.057
Monoalkyl phenols and related chemicals (15)				
4-n-Heptylphenol	1987-50-4	0.49 ± 0.26	0.21 ± 0.11	1.363
4-t-Octylphenol	140-66-9	0.78 ± 0.41	0.29 ± 0.13	0.997
4-n-Hexylphenol	2446-69-7	0.69 ± 0.23	0.42 ± 0.13	0.695
4-Nonylphenol (mixed isomers)	84852-15-3	1.36 ± 0.70	0.62 ± 0.25	0.476
4-n-Octylphenol	1806-26-4	1.70 ± 0.56	0.66 ± 0.41	0.446
4-n-Pentylphenol	14938-35-3	3.43 ± 0.70	1.85 ± 0.44	0.159
4-n-Nonylphenol	104-40-5	4.61 ± 1.01	2.92 ± 1.10	0.100
4-Dodecylphenol (mixed isomers)	27193-86-8	5.45 ± 0.84	3.62 ± 0.72	0.081
4-t-Pentylphenol	80-46-6	9.92 ± 2.29	5.24 ± 1.33	0.056
2-Chloro-4-octylphenol	-	0.61 ± 0.37	0.23 ± 0.05	1.286
2,6-Dichloro-4-octylphenol	-	0.70 ± 0.16	0.28 ± 0.14	1.041
2-Chloro-4-nonylphenol	-	1.70 ± 0.96	0.69 ± 0.06	0.422
2,6-Dichloro-4-nonylphenol	-	2.77 ± 1.25	1.35 ± 0.41	0.217
2,6-Dichloro-4-butylphenol	-	4.64 ± 0.32	3.05 ± 0.96	0.096
2-Chloro-4-butylphenol	-	17.33 ± 4.12	7.94 ± 1.04	0.037
Parabens (6)				
n-Hexyl 4-hydroxybenzoate	1083-27-8	1.24 ± 0.26	0.75 ± 0.26	0.391
n-Amyl 4-hydroxybenzoate	6521-29-5	2.95 ± 0.26	1.92 ± 0.58	0.153
Benzyl 4-hydroxybenzoate	94-18-8	3.18 ± 0.55	2.26 ± 0.64	0.130
Isobutyl-4-hydroxybenzoate	4247-02-3	5.54 ± 0.93	4.08 ± 1.36	0.072
2-Ethylhexyl 4-Hydroxybenzoate	5153-25-3	5.58 ± 0.76	4.27 ± 0.90	0.069
Butyl-4-hydroxybenzoate	94-26-8	10.35 ± 2.66	6.37 ± 1.80	0.046
Phenols and related chemicals (7)				
4-(1-Adamantyl)phenol	29799-07-3	1.30 ± 0.80	0.54 ± 0.33	0.547
2-Chloro-4-nonylphenyl 2-hydroxyethyl ether	-	4.93 ± 1.33	3.21 ± 0.31	0.091
4-Cyclohexylphenol	1131-60-8	5.48 ± 1.00	3.23 ± 1.06	0.091
2-(1-Adamantyl)-4-methylphenol	41031-50-9	4.90 ± 0.68	3.82 ± 0.55	0.077
4-Nonylphenyl 2-hydroxyethyl ether	-	7.61 ± 2.34	4.35 ± 0.76	0.067
4-Benzylphenol	101-53-1	9.39 ± 3.55	4.74 ± 1.07	0.062
Hexestrol	5635-50-7	22.01 ± 3.51	7.00 ± 2.18	0.042

aromatic hydrocarbon 2-terphenyl showed agonistic activities over 0.1% of that of *all-trans* RA.

4. Discussion

The reactivity, reproducibility and dose-dependency of the RARγ yeast assay, using *all-trans* RA, as a standard, were satisfactory for assessing a wide range of chemicals as described here. The EC_{50} of *all-trans* RA in a reporter gene assay using RARγ-cotransfected HeLa cells and the IC_{50} in a competitive binding assay using RARγ-transfected COS-1 cells were reported to be 2.5 nM and 8 ± 1 nM, respectively (Bernard et al., 1992; Allenby et al., 1994). Although there is no simple comparison between these reports and our results, the responsivity of our assay system, which can be represented as EC × 10 or REC20 values, is within a similar range. Taking into account its simplicity and rapidity, application of this assay using easily managed yeast cells to the toxicological evaluation of chemicals is appropriate and reasonable as a step

Table 2 (*continued*)

Compounds	CAS No.	EC×10 (± SD, ×10^{-6} M)	REC20 (± SD, ×10^{-6} M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (continued)				
Styrene dimers (14+1)				
1-Phenyltetralin	-	0.65 ± 0.02	0.25 ± 0.16	1.169
1-Methyl-3-phenylindan	-	1.22 ± 0.13	0.46 ± 0.28	0.632
trans-1,2-Diphenylcyclobutane	20071-09-4	3.37 ± 0.80	1.33 ± 0.75	0.220
1-Methyl-1-phenylindan	79034-12-1	4.77 ± 1.98	2.16 ± 0.81	0.135
cis-1,2-Diphenylcyclobutane	7694-30-6	5.43 ± 2.03	2.20 ± 1.12	0.133
2,4-Diphenyl-1-butene	16606-47-6	4.62 ± 0.69	2.32 ± 0.52	0.126
2,3-Diphenyl-1-butene	-	5.51 ± 2.81	2.40 ± 1.35	0.122
cis-1,4-Diphenyl-1-butene	-	6.22 ± 2.78	2.95 ± 1.38	0.099
trans-1,3-Diphenyl-1-butene	-	6.04 ± 1.91	2.99 ± 1.39	0.098
trans-1,3-Diphenylcyclobutane	-	7.09 ± 2.15	3.23 ± 1.39	0.091
1,4-Diphenyl-2-butene	-	8.29 ± 4.00	3.54 ± 1.62	0.083
cis-2,4-Diphenyl-2-butene	-	8.95 ± 2.77	3.89 ± 1.76	0.075
trans-1,4-Diphenyl-1-butene	-	8.62 ± 3.77	4.09 ± 1.84	0.072
trans-2,4-Diphenyl-2-butene	-	14.33 ± 4.49	6.83 ± 2.40	0.043
Polystyrene standard (mixed styrene dimer isomers)	-	7.54 ± 2.96	3.97 ± 0.77	0.074
Others (2)				
Octachlorostyrene	29082-74-4	21.93 ± 9.01	4.77 ± 3.47	0.061
Benzyl-2-naphthylether	613-62-7	21.98 ± 6.32	6.79 ± 0.37	0.043
Agrochemicals (22)				
Carbamate (1)				
Thiobencarb	28249-77-6	16.74 ± 1.64	5.48 ± 0.39	0.054
Diphenyl ethers (3)				
Aclonifen	74070-46-5	2.74 ± 0.62	1.33 ± 0.82	0.220
Nitrofen	1836-75-5	4.45 ± 1.31	2.18 ± 0.61	0.134
Chlornitrofen (CNP)	1836-77-7	7.77 ± 3.31	2.82 ± 1.22	0.104
Organochlorines (16)				
1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	58-89-9	0.89 ± 0.27	0.44 ± 0.14	0.668
Endrin	72-20-8	1.22 ± 0.79	0.85 ± 0.21	0.346
Heptachlor	76-44-8	2.09 ± 0.77	0.87 ± 0.37	0.336
Oxychlordane	27304-13-8	3.87 ± 0.99	1.90 ± 0.03	0.154
Chlordane	57-74-9	3.23 ± 1.89	1.93 ± 1.15	0.152
Toxaphene	8001-35-2	3.35 ± 1.64	2.59 ± 0.96	0.113
cis-Heptachlor epoxide	1024-57-3	3.46 ± 1.82	2.85 ± 0.60	0.103
cis-Chlordane	5103-71-9	5.63 ± 4.03	2.94 ± 1.33	0.100
trans-Chlordane	5103-74-2	7.15 ± 3.70	3.56 ± 1.80	0.082
β-Endosulfan	33213-65-9	9.06 ± 0.87	4.36 ± 2.71	0.067
Dieldrin	60-57-1	7.47 ± 4.60	4.70 ± 1.80	0.062
trans-Nonachlor	39765-80-5	7.74 ± 5.15	4.92 ± 0.30	0.060
Aldrin	309-00-2	8.88 ± 6.14	5.43 ± 0.91	0.054
β-Benzene hexachloride (β-BHC)	319-85-7	12.85 ± 3.15	5.69 ± 0.77	0.052
α-Endosulfan	959-98-8	8.02 ± 2.67	6.07 ± 2.40	0.048
trans-Heptachlor epoxide	1024-57-3 (trans)	8.49 ± 5.06	6.30 ± 1.58	0.047
Organophosphates (2)				
Cyanofenphos	13067-93-1	8.83 ± 1.51	3.42 ± 1.89	0.086
Chlorpyrifosmethyl	5598-13-0	13.81 ± 3.47	7.08 ± 2.11	0.041
Natural compounds and related chemicals (7)				
all-trans-Retinol	68-26-8	2.81 ± 0.17	1.67 ± 0.77	0.175
Flavanone	487-26-3	10.65 ± 1.14	4.23 ± 0.26	0.069
cis-Stilbene	645-49-8	10.16 ± 5.57	4.99 ± 2.58	0.059
Dibenzyl	103-29-7	17.95 ± 3.31	6.66 ± 2.10	0.044
4-Chloro-17β-estradiol	-	16.23 ± 3.77	4.35 ± 1.75	0.067
2,4-Dichloro-17β-estradiol	-	13.26 ± 1.52	4.79 ± 1.38	0.061
4-Bromo-17β-estradiol	-	12.49 ± 4.72	5.52 ± 3.91	0.053
Medicines and cosmetic-related chemicals (1)				
Cosmetic-related chemical (1)				
3-(4-Methylbenzylidane)-camphor	36861-47-9	15.98 ± 4.04	5.92 ± 1.85	0.050

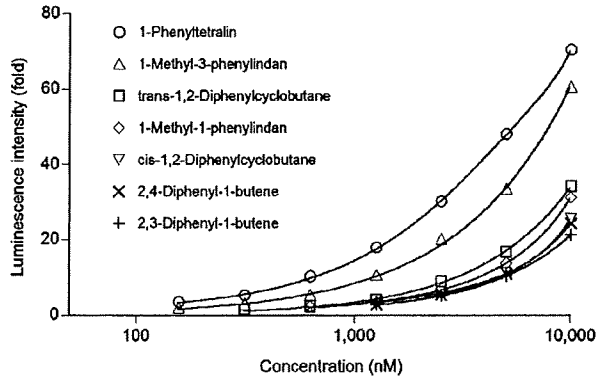

Fig. 2. Dose-response curves of styrene dimers in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

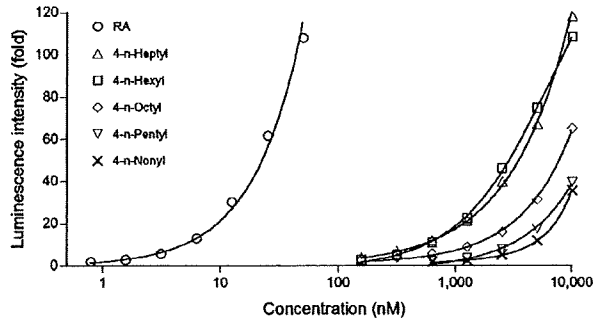

Fig. 3. Dose-response curves of monoalkylphenols having a linear alkyl group in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

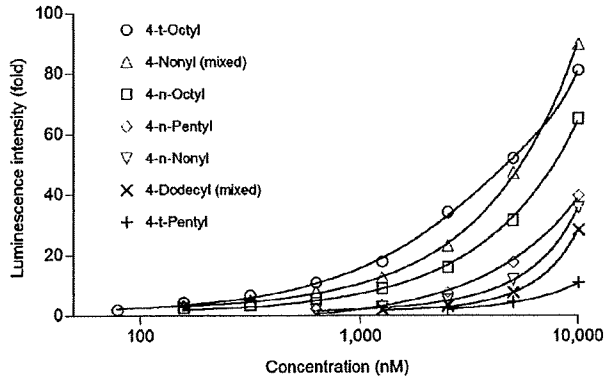

Fig. 4. Activity comparison of monoalkylphenols having a linear alkyl group with a branched group in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

in hazard identification with implications for mechanism of action. As RAs are known to undergo isomerization and oxidation when exposed to light and air (Bempong et al., 1995), some of the variability in the activity of *all-trans* RA can probably be attributed to the lability of the reagent to, in particular, photodegradation. However, the ready availability of an endogenous ligand for use in the assay more than compensates for this instability.

Many of the compounds active to the RARγ (organochlorines and styrene dimers were notable exceptions) were *para*-alkyl-substituted phenols. Phenols of this type are manufactured on a very large scale for many industrial purposes and have been reported to possess estrogenic activity, with the degree of activity depending on the length and branching of the alkyl substituent. Of the 4-*n*-alkylphenols, 4-*n*-nonylphenol had the highest binding affinity for the human ER (Tabira et al., 1999). 4-Alkylphenols having an alkyl group composed of 3–12 carbons, including branched groups (both secondary and tertiary), exhibited ER transactivation activity in a recombinant yeast assay (Routledge and Sumpter, 1997). There were some differences in the responsivities of the present RAR assay to alkyl and other phenols from those of the ER. The most potent activator of the RARγ was 4-*n*-heptylphenol and phenols with four or less carbons in their alkyl groups had no effect regardless of its position or branching. Alkylphenols having a branched alkyl group with many carbons, such as the mixed isomers of nonylphenol and tert-octylphenol, were more potent than analogs with a linear alkyl group, whereas of the pentylphenols with less carbon atoms in their alkyl groups, tert-pentylphenol is weaker than its analog with a linear alkyl group. This suggests that the overall length of a side-chain but not the actual number of carbons it contains may influence the potency of alkylphenols to the RARγ and that there may be an optimal length.

Ring-chlorinated alkylphenols had somewhat higher activity than their unchlorinated analogs, and bisphenol A, which had no positive effect on the RARγ, acquired agonistic activity by chlorination. This parallels observations in previous studies of estrogenicity where the estrogenic activities of chlorinated bisphenol A were stronger both *in vitro* and *in vivo* than those of the unchlorinated compound (Takemura et al., 2005; unpublished data, Shiraishi et al., 2000). Halogenation of chemicals might thus activate them or boost their actions on nuclear receptors. This is also indicated by observations in the present study that chlorination and bromination activated 17β-estradiol (E2) to the RARγ to a small extent. These findings suggest that not only in industrial manufacturing but in effluent processing, halogenation treatment does not reduce the unexpected activity of chemicals on nuclear receptors, but on the contrary might enhance the harmful effects.

We present here the first evidence that styrene dimers but not trimers have the ability to transactivate the RAR. The dimer 1-phenyltetralin was one of the most active chemicals revealed in this study. Furthermore, it was apparent that the fewer carbons linking the benzene rings of the dimers, the higher the agonistic activity (Figs. 2 and 5). Therefore, limited overall molecular length seems

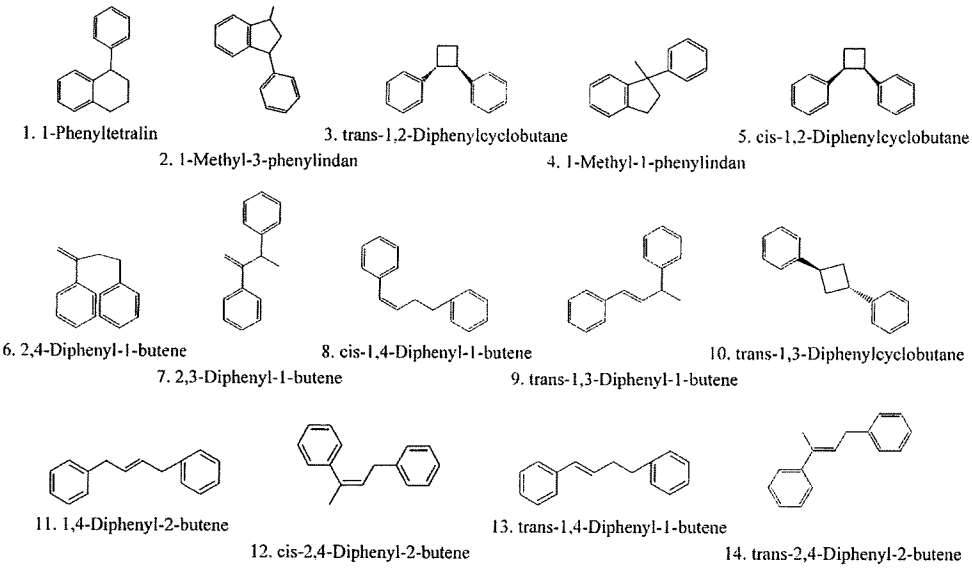

Fig. 5. Structural formulas of styrene dimmers.

to correlate with potency in styrene dimers. The lack of activity of styrene trimers may also arise from their length or size as the upper limit for the molecular weights of positive compounds (except halogenated compounds and organophosphates) was 286.5 (*all-trans* retinol), whereas the styrene trimers have molecular weights of 312. It is interesting that 2-terphenyl and 1,3-diphenylpropane have about the same activity as styrene dimers with similar structures (trans-1,2-diphenylcyclobutane and trans-2,4-diphenyl-2-butene, respectively). The binding affinity of these chemicals for the RAR may depend on molecular length, like alkylphenols as discussed above. Styrene oligomers also have binding affinity for the human ER (Ohyama et al., 2001; Kitamura et al., 2003). Importantly, it has been reported that styrene trimers exhibit *in vivo* disruption of endocrine systems and that embryonic exposure obstructs genital organ development and disrupts the endocrine function of male rat offspring (Ohyama et al., 2007). Polystyrene resins contain substantial amounts of styrene dimers and trimers, and extraction tests with polystyrene food containers have shown that these compounds leak into the food, water and oil with which they are in direct contact (Kawamura et al., 1998a,b,c). Because all styrene dimers tested in this study had a positive effect on the RARγ and selective ligands for the RAR subtypes are teratogenic in the developing embryo, the developmental toxicity of styrene oligomers via the RAR as well as the ER should be investigated.

Organochlorine pesticides were previously reported to transactivate RARβ and γ, but not RARα in human RAR reporter cell lines (Lemaire et al., 2005). The tested five organochlorines had dose-dependent effects on the RARγ and the order of potency was endrin > dieldrin > aldrin > chlordane > endosulfan, although even the activity of endrin was only approximately 1/12000 of that of the standard RAR agonist, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid. Our results indicated that many organochlorines, including those five compounds examined, also have the potential to activate the RARγ. The present yeast assay detected the activities of these compounds at lower concentrations than the reported cell line. In addition, our assay results indicated that binding affinity for the RAR partially depends on the number of chlorine atoms in the molecule and that there seems to be an optimal range of their chlorine number. Many studies have shown that a number of pesticides, including organochlorines, possess estrogenic activity (Hodges et al., 2000; Kojima et al., 2004), but their potencies for the ER do not correspond with those for the RAR. For example, the ranking of the activities of organochlorines for the ERs in Chinese hamster ovary cells are *o,p'*-DDT (4.5×10^{-8} M, test compound concentration showing 20% of the agonistic activity of 10^{-10} M 17β-estradiol) > β-BHC (3.5×10^{-7} M) > methoxychlor (5.6×10^{-7} M) for the ERα, and β-BHC (1.1×10^{-7} M) > *o,p'*-DDT (1.2×10^{-7} M) > δ-BHC (1.1×10^{-6} M) for the ERβ (Kojima et al., 2004), whereas none of these compounds showed a significant effect on the RARγ in our assay system. Because persistent and/or harmful pesticides such as organochlorines are now prohibited in developed countries, but still used in developing regions, detailed investigations of their toxicity via nuclear receptors, as well as a global restriction on their use, are required.

The present study illustrated that a number of compounds possessed unexpected transcriptional activation effects on the human RARγ in a yeast two-hybrid system, as we have already shown for the ERs and TRs (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). It is common for xenobiotics to have low affinity for nuclear receptors relative to the natural ligands, as we

reported here. However, there are a number of examples (principally from the ER, which is better studied than the RAR) where man-made compounds produce adverse effects in living organisms via nuclear receptors. Agonistic ligands for nuclear receptors such as the RARs and ERs have been confirmed the ability to experimentally induce developmental abnormalities (Elmazar et al., 1996, 2001; Kamata et al., 2006), and there are medically or environmentally documented cases of their teratogenicities (Lammer et al., 1985; Fry, 1995; Sumpter, 1998). For example, in previous studies, the REC20 of 2,2′,4′6′-tetrachlorobiphenyl-4-ol, one of monohydroxylated polychlorinated biphenyls, was 24 nM in our human ERα yeast assay, and its activity relative to E2 was 1.7% (Arulmozhiraja et al., 2005). *In ovo* exposure to this compound at a dose of 100 ng/g egg or more caused shortening of the oviduct in female Japanese quails and a dose of 500 ng/g or more caused a reduction testis weight in males after sexual maturation (Kamata et al., 2006). This dose range in ovo was approximately 100 times higher than a positive control, diethylstilbestrol, producing *in vitro* effect equivalent to E2. Recently, the existence of environmental pollutants having binding affinity for less researched but important nuclear receptors, such as the retinoid X receptors (RXR) and peroxisome proliferator-activated receptors (PPAR), and consequential disorders in organisms has been reported (Nishikawa et al., 2004; Abbott et al., 2007; Takacs and Abbott, 2007). Both tributyltin and triphenyltin used in antifouling paints exhibited agonistic activity in yeast assays transfected with the human RXR (α, β or γ) at a concentration of 10 nM or more, and this concentration was somewhat lower than that of 9-*cis* RA, the natural ligand of RXR (Nishikawa et al., 2004). One injection of 1 μg triphenyltin/g wet weight induced the differentiation and growth of male genital tracts in female gastropods, *Thais clavigera*, which was also stronger than 9-*cis* RA (Nishikawa et al., 2004). Thus, these reports are typical cases that *in vitro* affinity of chemicals for nuclear receptors is well correlated with their *in vivo* potential, and therefore, measurement of the relative activity of chemicals using *in vitro* assays seems to be valuable to estimate the *in vivo* effects of them.

There is great concern that substances released into the environment and/or used in everyday life may influence human and wildlife health. Detection of chemicals with an affinity for the various receptors is an important step in hazard identification. This data can be used to direct and prioritize additional research and *in vivo* studies on chemicals that bind nuclear receptors for possible receptor-mediated toxicity and endocrine disrupting activity. As described here, yeast assay systems including a RAR assay are useful for high-throughput screening of substances active to nuclear receptors.

5. Conflict of interest

There are no conflicts of interests involved in this study.

Acknowledgement

The authors thank Ms. Miho Yamasaki for skilful technical assistance.

References

Abbott, B.D., Wolf, C.J., Schmid, J.E., Das, K.P., Zehr, R.D., Helfant, L., Nakayama, S., Lindstrom, A.B., Strynar, M.J., Lau, C.S., 2007. Perfluorooctanoic acid (PFOA)-induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha. Toxicological Sciences 98, 571–581.

Allenby, G., Janocha, R., Kazmer, S., Speck, J., Grippo, J.F., Levin, A.A., 1994. Binding of 9-*cis*-retinoic acid and *all-trans*-retinoic acid to retinoic acid receptors α, β, and γ. Retinoic acid receptor gamma binds *all-trans*-retinoic acid preferentially over 9-*cis*-retinoic acid. Journal of Biological Chemistry 269, 16689–16695.

Arulmozhiraja, S., Shiraishi, F., Okumura, T., Iida, M., Takigami, H., Edmonds, J.S., Morita, M., 2005. Structural requirements for the interaction of 91 hydroxylated polychlorinated biphenyls with estrogen and thyroid hormone receptors. Toxicological Sciences 84, 49–62.

Bempong, D.K., Honigberg, I.L., Meltzer, N.M., 1995. Normal phase LC–MS determination of retinoic acid degradation products. Journal of Pharmaceutical and Biomedical Analysis 13, 285–291.

Bernard, B.A., Bernardon, J.M., Delescluse, C., Martin, B., Lenoir, M.C., Maignan, J., Charpentier, B., Pilgrim, W.R., Reichert, U., Shroot, B., 1992. Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor gamma. Biochemistry and Biophysical Research Communications 186, 977–983.

Elmazar, M.M., Reichert, U., Shroot, B., Nau, H., 1996. Pattern of retinoid-induced teratogenic effects: possible relationship with relative selectivity for nuclear retinoid receptors RAR alpha, RAR beta, and RAR gamma. Teratology 53, 158–167.

Elmazar, M.M., Ruhl, R., Nau, H., 2001. Synergistic teratogenic effects induced by retinoids in mice by coadministration of a RARα- or RARγ-selective agonist with a RXR-selective agonist. Toxicology and Applied Pharmacology 170, 2–9.

Fry, D.M., 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. Environmental Health Perspectives 103 (Suppl. 7), 165–171.

Hodges, L.C., Bergerson, J.S., Hunter, D.S., Walker, C.L., 2000. Estrogenic effects of organochlorine pesticides on uterine leiomyoma cells *in vitro*. Toxicological Sciences 54, 355–364.

Kamata, R., Takahashi, S., Shimizu, A., Morita, M., Shiraishi, F., 2006. *In ovo* exposure quail assay for risk assessment of endocrine disrupting chemicals. Archives of Toxicology 80, 857–867.

Kawamura, M., Nishi, K., Maehara, T., Yamada, T., 1998a. Migration of styrene dimers and trimers from polystyrene containers into instant foods. Journal of the Food Hygiene Society of Japan 39, 390–398.

Kawamura, M., Sugimoto, N., Takeda, Y., Yamada, T., 1998b. Identification of unknown substances in food contact polystyrene. Journal of the Food Hygiene Society of Japan 39, 110–119.

Kawamura, Y., Kawamura, M., Takeda, Y., Yamada, T., 1998c. Determination of styrene dimers and trimers in food contact polystyrene. Journal of the Food Hygiene Society of Japan 39, 199–205.

Kitamura, S., Ohmegi, M., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N., Ohta, S., 2003. Estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. Environmental Health Perspectives 111, 329–334.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese hamster ovary cells. Environmental Health Perspectives 112, 524–531.

Lammer, E.J., Chen, D.T., Hoar, R.M., Agnish, N.D., Benke, P.J., Braun, J.T., Curry, C.J., Fernhoff, P.M., Grix Jr., A.W., Lott, I.T., et al.,

1985. Retinoic acid embryopathy. New England Journal of Medicine 313, 837 841.

Lemaire, G., Balaguer, P., Michel, S., Rahmani, R., 2005. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. Toxicology Applied Pharmacology 202, 38 49.

Mispagel, C., Shiraishi, F., Allinson, M., Allinson, G., 2005. Estrogenic activity of treated municipal effluent from seven sewage treatment plants in Victoria, Australia. Bulletin of Environmental Contamination and Toxicology 74, 853 856.

Morohoshi, K., Yamamoto, H., Kamata, R., Shiraishi, F., Koda, T., Morita, M., 2005. Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by *in vitro* assays. Toxicology in Vitro 19, 457 469.

Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environmental Science and Technology 38, 6271 6276.

Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., Nishihara, T., 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicology and Applied Pharmacology 154, 76 83.

Ohyama, K., Satoh, K., Sakamoto, Y., Ogata, A., Nagai, F., 2007. Effects of prenatal exposure to styrene trimers on genital organs and hormones in male rats. Experimental Biology and Medicine (Maywood) 232, 301 308.

Ohyama, K.I., Nagai, F., Tsuchiya, Y., 2001. Certain styrene oligomers have proliferative activity on MCF-7 human breast tumor cells and binding affinity for human estrogen receptor. Environmental Health Perspectives 109, 699 703.

Routledge, E.J., Sumpter, J.P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. Journal of Biological Chemistry 272, 3280 3288.

Shiraishi, F., Okumura, T., Nomachi, M., Serizawa, S., Nishikawa, J., Edmonds, J.S., Shiraishi, H., Morita, M., 2003. Estrogenic and thyroid hormone activity of a series of hydroxy-polychlorinated biphenyls. Chemosphere 52, 33 42.

Shiraishi, F., Shiraishi, H., Nishikawa, J., Nishihara, T., Morita, M., 2000. Development of a simple operational estrogenicity assay system using the yeast two-hybrid system. Journal of Environmental Chemistry 10, 57 64.

Sumpter, J.P., 1998. Xenoendorine disrupters-environmental impacts. Toxicology Letters 102 103, 337 342.

Tabira, Y., Nakai, M., Asai, D., Yakabe, Y., Tahara, Y., Shinmyozu, T., Noguchi, M., Takatsuki, M., Shimohigashi, Y., 1999. Structural requirements of *para*-alkylphenols to bind to estrogen receptor. European Journal of Biochemistry 262, 240 245.

Takacs, M.L., Abbott, B.D., 2007. Activation of mouse and human peroxisome proliferator-activated receptor (α, β/δ, γ) by perfluorooctanoic acid and perfluorooctane sulfonate. Toxicological Sciences 95, 108 117.

Takemura, H., Ma, J., Sayama, K., Terao, Y., Zhu, B.T., Shimoi, K., 2005. *In vitro* and *in vivo* estrogenic activity of chlorinated derivatives of bisphenol A. Toxicology 207, 215 221.

Zile, M.H., 1998. Vitamin A and embryonic development: an overview. Journal of Nutrition 128, 455S 458S.

Zile, M.H., 2001. Function of vitamin A in vertebrate embryonic development. Journal of Nutrition 131, 705 708.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.33, No.4, 431-445, 2008

Original Article

Screening of toxicological properties of 4-methylbenzoic acid by oral administration to rats

Mariko Shirota, Takayuki Seki, Kazumi Tago, Hiroyasu Katoh, Hideki Marumo, Mami Furuya, Tomoko Shindo and Hiroshi Ono

Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan

(Received April 3, 2008; Accepted May 26, 2008)

ABSTRACT — Oral toxicity of 4-methylbenzoic acid in male and female Sprague-Dawley rats was profiled through a twenty-eight-day repeated dose toxicity study (the 28-day study) and a screening test for reproductive/developmental toxicities (the reproduction/developmental study) conducted under Organisation for Economic Co-operation and Development (OECD) test guidelines. Daily administration of 4-methylbenzoic acid, at a dose level of 0, 100, 300 or 1,000 mg/kg, did not show any adverse effect on reproductive organs of animals in the 28-day study. In the reproductive/developmental study, however, 1,000 mg/kg/day of the compound reduced epididymal weights and increased incidence of cauda epididymal oligo/azoospermia. While the compound did not affect estrous cycle or mating performances, 1,000 mg/kg of the compound reduced fertility. Furthermore, 300 mg/kg or more of the compound increased pre-implantation loss, which resulted in a decrease in the number of offspring, and reduced body weight gain of the dams during the latter period of gestation. From these results, the no-observed-effect-level (NOEL) for reproductive/developmental toxicities is considered to be 100 mg/kg, whereas 1,000 mg/kg did not show any effect on neonates. In the 28-day study, NOEL is considered to be 300 mg/kg for male and female rats, since 1,000 mg/kg of the compound caused, in both sexes, a few minor changes, such as temporal salivation, a slight increase in food consumption and a moderate increase in blood aspartate aminotransferase (AST) activity. Thus, 4-methylbenzoic acid has the potential for reproductive toxicity, with diverse adverse effects on the epididymis, after repeated administration, observed in the two studies.

Key words: 4-methylbenzoic acid, Epididymis, OECD test guideline, Reproductive toxicant, Repeated dose toxicity, Rats

INTRODUCTION

Faced with an enormous number of existing chemicals that lacked hazard information, the Organisation for Economic Co-operation and Development (OECD) decided, in 1990, to undertake an investigation of such chemicals in cooperation with its member countries. They gave priority to high production volume (HPV) chemicals in collecting the data for an initial assessment of the hazard. A project was launched in 1990 to complete the dossiers of screening information data sets (SIDS) of toxicity for HPV chemicals through testing, and the work has been done cooperatively in Japan, as well as in other countries. Since 1991, we have participated in the testing under the auspices of the Ministry of Health (, Labour) and Welfare.

4-Methylbenzoic acid (*p*-toluic acid) was selected from the OECD List of HPVs (OECD, 2004). 4-Methylbenzoic acid is produced at levels greater than 1,000 tones per year and is used in the manufacture of dye stuffs, colorants and paints; agrochemicals; and anticorrosive additives, as well as being an intermediate in the manufacture of polyethylene terephthalate. Although oral LD_{50} values in rats and mice have been reported as 3,113 mg/kg and 2,115 mg/kg, respectively (Mineshita *et al.*, 1978), its toxicological properties are little known. The present study was performed to profile the oral toxicity of 4-methylbenzoic acid according to the standard protocols, "Repeated Dose 28-day Oral Toxicity Study" (the 28-day study) and "Reproduction/Developmental Toxicity Screening Test" (the reproduction/developmental study), in rodents (OECD, 1997a, 1997b).

Correspondence: Mariko Shirota (E-mail: shirota.m@fdsc.or.jp)

MATERIALS AND METHODS

Test substance

4-Methylbenzoic acid (CAS No. 99-94-5, purity 98.95%) was supplied by Toray (Tokyo, Japan), and was kept at room temperature until use. To prepare dosing samples, the compound was finely ground in a mortar, at first. Then, the ground compound for each dose was suspended in a 0.5% sodium carboxymethylcellulose solution, and each dose was adjusted to a constant volume of 5 ml/kg. The stability of the suspended compound in the vehicle and the content and uniformity of the compound of each dose were confirmed in Hatano Research Institute.

The doses used in the studies were determined based on a preliminary, 7-day, repeated dose, oral toxicity study of the compound, in which a dose level of 1,000 mg/kg did not show any toxic effects. This was considered sufficient for the highest dose, and the doses used in the present studies were set at 0, 100, 300 and 1,000 mg/kg.

Animals

Male and female rats of the Sprague-Dawley (Crj: CD(SD)) strain were purchased from Charles River Japan (Atsugi Breeding Center, Atsugi, Kanagawa, Japan). For the 28-day study, the animals were purchased at 4 weeks of age, and after an 8-day quarantine period, the animals were divided into 4 groups of each sex according to a stratified allocation based on body weight measured on the day before initial dosing. For the reproduction/developmental study, the animals were purchased at 7 weeks of age and quarantined for 7 days. After the quarantine period, the female rats were monitored for estrous cycle by observing daily vaginal smears for 2 weeks. At 10 weeks of age, the females revolving on a regular 4-day estrous cycle and the males were divided into each 4 groups by the same method as used in the 28-day study.

These animals were kept individually in metallic cages with metal-meshed floors, except copulated females in the reproduction and developmental study, which were kept in flat-bottomed plastic cages with bedding materials (Paper Clean, Nippon SLC, Hamamatsu, Japan) from 18 days after copulation. The animal rooms were maintained with a light-dark cycle of 12-hr (lights on 7 hr), and temperature and relative humidity were controlled to 21.0-25.0°C and to 40.0-75.0%, respectively, with air ventilation at 15 complete changes per hour. The animals were supplied with solid rodent chow (CE-2, CLEA Japan, Tokyo, Japan) and tap water, *ad libitum*.

Experimental design

All procedures described here were approved by the Committee on Animal Experiments of Hatano Research Institute, Food and Drug Safety Center.

The 28-day study

Groups given 0 and 1,000 mg/kg of 4-methylbenzoic acid comprised 10 animals of each sex, including 5 animals of each sex for the 2-week recovery study after the 28-day administration of the compound. The groups given 100 and 300 mg/kg comprised 5 animals of each sex.

Administration was begun at 5 weeks of age, and the initial day of the administration was designated as Day 1 of treatment. All the animals received daily administration of the compound by gavage for 28 days at a fixed time every day, and the initial day of recovery was designated as Day 1 of recovery.

Signs of toxicity were daily observed. Detailed clinical observations were made as specified in the OECD test guideline 407 (OECD, 1997a) in all animals prior to the initial administration, and once a week thereafter until the end of the recovery period. The observers were unaware of the treatment of each animal. Findings were recorded using a scoring system. In addition to the detailed observation of clinical signs, a 4-item neurobehavioral test battery assessing auditory and visual functions was administered at the last week of treatment.

Body weight was measured 3 times during the first week of the treatment, and twice a week thereafter. Food consumption was determined weekly.

Urinalysis was performed at the final week of treatment and at the final week of the recovery study. Urine of all animals was collected for 4 and 24 hr in a metabolic cage, and was examined for pH, occult blood, protein, ketone bodies, urobilinogen and bilirubin, semi-quantitatively using a urine test strip analyzer (Clinitek 200+, Bayer Medical, Tokyo, Japan), and for its color, turbidity and sediments. The volume and weight of the 24-hr urine was measured, and the specific gravity of the urine was calculated.

Necropsy of the animals was performed after 18-24 hr of fasting on the day following final treatment and on the day following the recovery period. Under anesthesia with pentobarbital sodium, blood samples were collected from the abdominal caval vein by syringe, with sodium citrate as an anticoagulant, for determination of coagulation times; with EDTA-2K potassium, for hematological examination; and with heparin, for blood chemical examination. Then, the animals were killed by exsanguination from the axillary artery. After gross observation, dissected organs, such as brain, thymus, heart, liver, kidneys, spleen, testes, adrenals and epididymides, were weighed.

In addition to these organs, spinal cord, lungs, bronchi, stomach, ileum, colon, seminal vesicles, ovaries, uterus, vagina, urinary bladder, thyroid gland, femoral marrow, mesenteric lymph nodes, mandibular lymph nodes and ischiadic nerves were dissected out and fixed in buffered formalin for histopathological examination. The testes were fixed in Bouin's solution, with post fixation in buffered formalin. These organs/tissues were then processed for paraffin embedded block, and sections cut from the blocks were stained with hematoxylin-eosin (HE).

Hematological examination was carried out using automatic blood analysis apparatus (CELL-DYN3500SL, Abbot Diagnostics, IL, USA) for cell counts (erythrocytes (RBCs), leucocytes (WBCs) and platelets), hemoglobin concentration and differential WBC counts. Hematocrit, mean concentration of hemoglobin in the RBC (MCHC) and mean content of hemoglobin in the RBC were calculated. The prothrombin time (PT) and activated partial thromboplastin time were measured using a fully automatic analyzer for blood coagulation (CA-1000, Toa Medical Electronics, Saitama, Japan).

In the blood chemical examination, plasma concentrations of total protein, albumin, total cholesterol, glucose, blood urea nitrogen, creatinine, triglyceride, total bilirubin, inorganic phosphorus and calcium and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GTP) were determined using a centrifugal automatic blood chemical analyzer (COBAS-FARA, Roche Diagnostics, Basel, Switzerland). Ratios of albumin to globulin were calculated, and plasma concentrations of sodium and potassium were measured using an automated electrolyte analyzer (EA05, A & T, Yokohama, Japan).

The reproduction/developmental study

The study consisted of 4 dosing groups, and each group comprised 13 males and 13 females. Administration was begun at 10 weeks of age, 2 weeks prior to mating in both, males and females, and was performed by gavage at a fixed time every day. The initial day of the administration was designated as Day 1 of treatment. Mating was performed by co-housing one male and one female from the same treatment group for a maximum of 2 weeks and was confirmed by observation of a copulatory plug or by the presence of sperm in a vaginal specimen. After confirmation of mating, each female was housed individually, and the day on which mating was confirmed was designated as Day 0 of gestation. Administration was continued during the mating period and gestation period, until 3 days after delivery for females. For males, the administration was continued through and after the mating period, for a total of 42 days. For females that had copulated but did not deliver or for females that had not copulated, the administration was continued until 25 days after copulation or to Day 52 of treatment, respectively.

Clinical conditions were observed daily, and body weights and food consumptions were measured weekly. After confirming copulation, the females were weighed on Days 0, 7, 14 and 20 of gestation, and their food consumptions were determined on Days 0-1, 7-8, 14-15 and 20-21 of gestation. When the females delivered live fetuses, they were weighed on Days 0 (the day of delivery) and 4 of lactation, and their food consumptions were determined on Day 3-4 of lactation.

The estrous cycle was monitored daily until copulation. The females that had copulated were allowed to deliver spontaneously and to nurse their own pups until Day 4 of lactation. During the lactation period, the number and sex of dead and live pups were recorded for each dam, and the external morphology and general condition of the live pups were examined daily. The dead pups were examined for external and visceral abnormalities, when possible.

All the males were killed for necropsy on the day after Day 42 of treatment by exsanguination under sodium pentobarbital anesthesia. Maternal animals were similarly killed on Day 4 of lactation, while their offspring were killed for necropsy on the same day by ether inhalation. The females that had not copulated during the mating period and the females that had copulated but did not deliver fetuses were also similarly killed.

At necropsy, males were examined grossly for abnormalities, and the testes and epididymides were dissected, weighed and fixed in Bouin's solution, with post fixation with a buffered formalin solution for processing for histopathological examination. The other reproductive organs, such as ventral prostate and seminal vesicles with coagulating glands, were also dissected and fixed in a buffered formalin solution for histopathological examination. Females were examined grossly for abnormalities. Organs with lesions were dissected for histopathological examination. Ovaries, uterus and vagina from dams were dissected to determine the number of corpora lutea and implantation sites. Then, these organs were processed for histopathological examination. The absence of implantation sites in the uterus of females that had not copulated and females that had copulated but did not deliver fetuses was confirmed under a stereomicroscope. Live pups were euthanized by ether inhalation and were examined for of external and visceral abnormalities.

Statistical analysis

Fisher's direct probability test was applied to analysis

of the incidence of animals in which the estrous cycle was altered, the copulation rate, the fertility index, the incidence of offspring with morphological abnormalities, and the incidence of histopathological findings. Graded findings in the histopathological examination were analyzed using the Mann-Whitney U-test. Data from the urinalysis urine quality test were analyzed using a chi-square test.

The other data were analyzed with multiple comparisons when comparing the data from more than 2 groups with those from the control. When comparing the data from a single group with those from the control, Student's t-test or Aspin-Welch's t-test was applied, following an F-test. In the multiple comparisons, an analysis of variance (ANOVA) test or Kruskal-Wallis's rank test was applied, following examination of the uniformity of variations by Bartlett's method. Significant differences from the control were determined by Dunnett's test. A p value of less than 0.5% was judged a significant difference.

RESULTS

Effects of repeated dosing in the 28-day study

Neither, death nor moribund condition, was observed in any animal, but a few male and female animals in the 1,000 mg/kg group showed temporary salivation after dosing. Except that, there were no clinical signs related to the treatment, and the scores of the detailed observation of clinical signs in the compound treated groups were comparable to those in the control (data not shown). In addition, no abnormalities were observed in the neurobehavioral test at the last week of treatment (data not shown).

Changes in body weight and food consumption are illustrated in Figs. 1 and 2, respectively. While body weights of males and females in the compound treated groups changed similarly to those in the control group at any period, food consumption of females in the 1,000 mg/kg treated group was slightly greater than in the control group, from Day 7 to 8 of treatment. Whereas the food consumption of females in this group tended to be greater thereafter, until the end of the treatment, no differences in food consumption were observed in males among any groups throughout the study.

As summarized in Table 1, urine volume measured on the Day 23 of treatment was larger in the males given 300 mg/kg or more of the compound and in females given 1,000 mg/kg when compared with those in the control. In addition, urine specific gravities were decreased in the males of these groups. These changes accompanied by an increase in water consumption, which was confirmed by an observation of water feeding bottles placed in the metabolic cages. Urinalysis showed some minor changes and was not suggestive of any toxic effects (data not shown).

The hematology data are presented in Table 2. At the end of treatment period, platelet counts were slightly lower in the 1,000 mg/kg treated females, but were statistically insignificant when compared with those in the control. No changes were observed in any of the hematology parameters of males at the end of treatment period. At the end of recovery period, there were statistically significant differences between the males of compound treated and

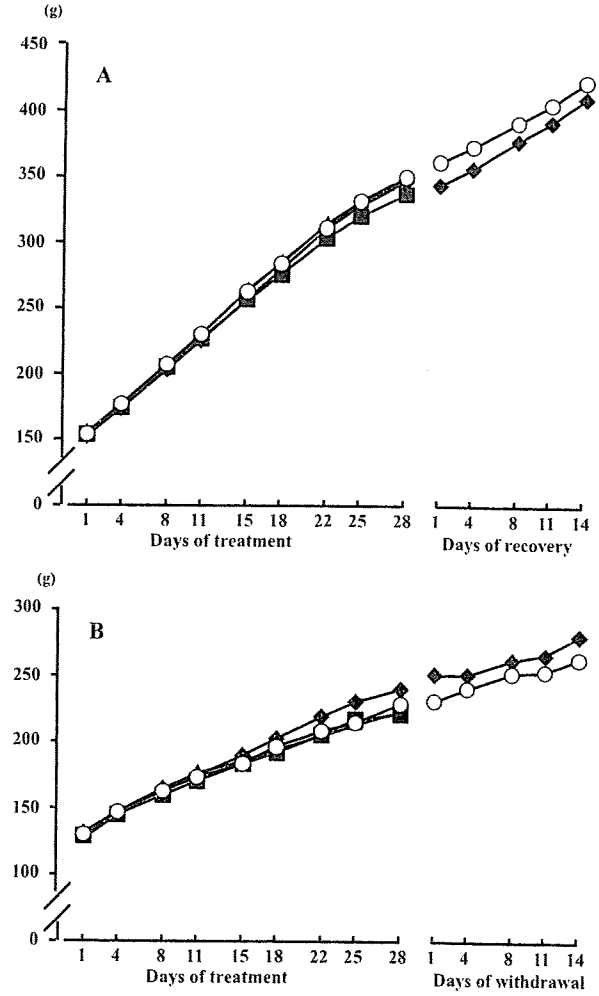

Fig. 1. Body weight changes of male (A) and female (B) rats treated orally with 4-methylbenzoic acid for 28-days at dose levels of 0 (○), 100 (■), 300 (▲) and 1,000 mg/kg/day (♦) in the repeated dose 28-day oral toxicity study. Values during the treatment period represent the average for 5 animals in the 100 or 300 mg/kg-treated group and for 10 animals in the 0 or 1,000 mg/kg-treated group. Those during the recovery period represent the average for 5 animals.

control groups, and were not suggestive any toxic effects.

The blood chemistry data are presented in Table 3. At the end of treatment period, a moderate increase in AST activity and a slight decrease in total protein concentration were observed in the 1,000 mg/kg-treated females, compared with those of control. In those data from males and those at the end of the recovery period from animals of both sexes, no significant differences were observed between the compound treated and control groups.

No significant differences from the control were observed in absolute organ weights or in organ weights relative to body weight in any of the compound treated groups of males (Table 4) or females (data not shown).

No abnormalities suggestive of any toxic effects were observed in any organs or tissues on gross examination at necropsy or histopathological examination (data not shown).

Effects of repeated dosing in the reproduction/ developmental study

As found in the 28-day study, temporary salivation was observed in a few animals given 1,000 mg/kg (data not shown). Except that, there were no clinical signs relating to the treatment.

Changes in body weight and food consumption of males are shown in Figs. 3 and 4, respectively. The compound did not affect body weight increase or food consumption, at any dose level.

Changes in body weight and food consumption of females are shown in Figs. 5 and 6, respectively. The compound did not affect body weights until Day 14 of gestation, at any dose level, while food consumption of the 1,000 mg/kg treated group was slightly higher than that of the control at the beginning of the dosing. During Days 14-20 of gestation, however, maternal body weight gain was reduced in the 300 mg/kg or more treated groups, while the food consumption that was determined during this period (Day 14-15 of gestation) and body weight on

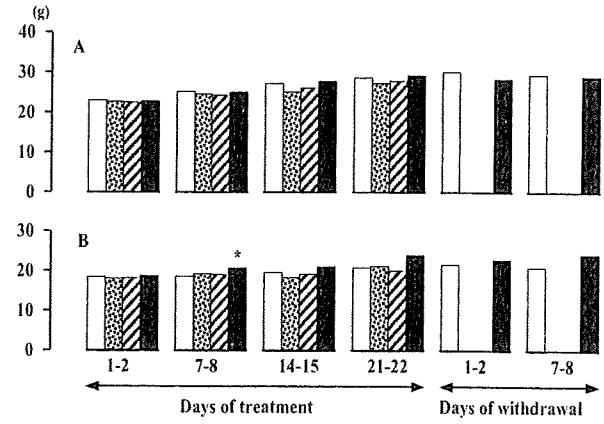

Fig. 2. Changes in food consumption of male (A) and female (B) rats treated orally with 4-methylbenzoic acid for 28-days at dose levels of 0 (open column), 100 (dashed column), 300 (hatched column) or 1,000 mg/kg/day (closed column) in the repeated dose 28-day oral toxicity study. Each column represents average for 5 animals in the 100 or 300 mg/kg-treated group and for 10 animals in the 0 or 1,000 mg/kg-treated group. During the recovery period, it represents the average for 5 animals. * indicates significant difference from control at $p < 0.05$.

Table 1. Urinalysis of rats treated orally with 4-methylbenzoic acid in repeated dose 28-day oral toxicity study

	On Day 23 of treatment				On Day 9 of recovery	
Dose (mg/kg)	0	100	300	1,000	0	1,000
Number of animals (males/females)						
	10/10	5/5	5/5	10/10	5/5	5/5
Urinary volume (ml/24 hr)						
Males	15.6 ± 2.2	15.6 ± 2.1	20.9 ± 4.7*	23.8 ± 4.7**	18.3 ± 4.7	24.3 ± 4.6
Females	11.7 ± 3.5	11.9 ± 3.4	13.0 ± 4.3	22.1 ± 7.5**	13.3 ± 2.8	18.3 ± 5.6
Specific gravity						
Males	1.058 ± 0.008	1.051 ± 0.007	1.043 ± 0.011**	1.045 ± 0.006**	1.056 ± 0.009	1.038 ± 0.006**
Females	1.045 ± 0.012	1.046 ± 0.008	1.043 ± 0.007	1.038 ± 0.009	1.041 ± 0.010	1.044 ± 0.011

Values represent average ± S.D.

* and **, significant difference from control at $p < 0.05$ and 0.01, respectively.

Table 2. Hematology data of rats treated orally with 4-methylbenzoic acid in the repeated dose 28-day oral toxicity study

	End of treatment								The end of recovery			
	Males				Females				Males		Females	
Dose (mg/kg)	0	100	300	1,000	0	100	300	1,000	0	1,000	0	1,000
RBC ($\times 10^4/\mu l$)	755 ± 53	780 ± 15	770 ± 27	755 ± 18	742 ± 19	771 ± 26	750 ± 25	751 ± 30	797 ± 13	766 ± 22**	749 ± 25	766 ± 24
Hemoglobin (g/dl)	14.9 ± 0.6	15.2 ± 0.2	15.2 ± 0.3	15.0 ± 0.2	14.8 ± 0.3	15.1 ± 0.5	14.9 ± 0.3	14.7 ± 0.5	15.2 ± 0.1	14.6 ± 0.3 **	14.5 ± 0.4	14.4 ± 0.7
Hematocrit (%)	44.8 ± 2.9	46.0 ± 0.7	45.5 ± 1.1	45.2 ± 0.4	44.2 ± 1.0	44.9 ± 1.3	44.4 ± 0.7	43.6 ± 1.9	45.3 ± 0.8	43.5 ± 1.0**	42.6 ± 1.3	42.7 ± 1.9
MCV (fl)	59.4 ± 2.2	58.9 ± 0.8	59.0 ± 1.3	60.0 ± 1.5	59.5 ± 1.1	58.3 ± 1.2	59.2 ± 1.4	58.0 ± 1.1	56.8 ± 1.4	56.8 ± 0.8	56.9 ± 1.2	55.8 ± 2.0
MCH (pg)	19.8 ± 0.8	19.5 ± 0.6	19.8 ± 0.6	19.9 ± 0.5	20.0 ± 0.6	19.6 ± 0.3	19.8 ± 0.6	19.6 ± 0.3	19.1 ± 0.4	19.0 ± 0.3	19.4 ± 0.4	18.8 ± 0.7
MCHC (g/dl)	33.3 ± 0.1	33.0 ± 0.7	33.6 ± 0.3	33.2 ± 0.2	33.6 ± 0.4	33.7 ± 0.3	33.5 ± 0.2	33.8 ± 0.6	33.5 ± 0.6	33.5 ± 0.4	34.0 ± 0.3	33.7 ± 0.3
Platelet ($\times 10^4/\mu l$)	102.9 ± 13.9	104.5 ± 16.3	106.6 ± 8.1	101.6 ± 10.4	96.3 ± 7.0	100.5 ± 9.4	93.3 ± 9.3	83.9 ± 3.1	96.1 ± 19.6	99.3 ± 8.4	101.8 ± 12.7	102.7 ± 10.2
PT (sec)	18.4 ± 3.0	18.9 ± 3.1	17.2. ± 4.0	14.9 ± 1.7	12.6 ± 0.7	12.7 ± 0.5	12.9 ± 0.6	13.2 ± 0.6	16.4 ± 2.7	15.2 ± 1.4	11.9 ± 0.7	11.5 ± 0.7
APTT (sec)	22.1 ± 0.8	22.9 ± 1.7	20.5 ± 2.4	20.1 ± 1.0	18.5 ± 1.0	18.2 ± 1.4	17.8 ± 1.2	16.7 ± 1.4	21.6 ± 1.5	21.0 ± 0.4	17.1 ± 0.8	16.9 ± 0.8
WBC ($\times 100/\mu l$)	90.8 ± 16.7	61.5 ± 20.5	67.7 ± 17.6	74.4 ± 21.7	61.0 ± 18.1	54.9 ± 14.8	46.7 ± 12.4	59.8 ± 7.8	69.4 ± 13.4	65.4 ± 14.2	43.9 ± 2.9	58.8 ± 14.5
Differential leukocyte counts (%)												
Neutrophil	10 ± 5	11 ± 2	10 ± 2	11 ± 3	7 ± 2	6 ± 3	8 ± 4	8 ± 4	13 ± 5	15 ± 3	10 ± 4	10 ± 3
Eosinophil	1 ± 0	1 ± 0	1 ± 0	0 ± 0*	1 ± 0	1 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 1	2 ± 1	1 ± 1
Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Monocyte	3 ± 1	4 ± 1	5 ± 1	4 ± 1	3 ± 1	4 ± 1	3 ± 1	3 ± 1	4 ± 1	4 ± 2	4 ± 1	4 ± 2
Lymphocyte	85 ± 5	85 ± 3	85 ± 3	85 ± 3	89 ± 3	88 ± 3	88 ± 4	88 ± 4	81 ± 4	79 ± 4	85 ± 5	85 ± 5

Values represent average for five animals ± S.D.
* and **, significant difference from control at $p < 0.05$ and 0.01, respectively.

Table 3. Blood chemistry data of rats treated orally with 4-methylbenzoic acid in repeated dose 28-day oral toxicity study

Dose (mg/kg)	The end of treatment								The end of recovery			
	Males				Females				Males		Females	
	0	100	300	1,000	0	100	300	1,000	0	1,000	0	1,000
TP (g/dl)	5.0 ± 0.2	5.2 ± 0.2	5.1 ± 0.1	5.0 ± 0.0	5.4 ± 0.3	5.3 ± 0.3	5.2 ± 0.3	4.8 ± 0.3**	5.6 ± 0.4	5.4 ± 0.1	5.5 ± 0.1	5.7 ± 0.3
Albumin (g/dl)	3.0 ± 0.2	3.0 ± 0.3	3.0 ± 0.1	3.0 ± 0.1	3.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	3.0 ± 0.1	3.2 ± 0.2	3.3 ± 0.4
A/G	1.48 ± 0.15	1.46 ± 0.27	1.38 ± 0.16	1.44 ± 0.08	1.51 ± 0.17	1.48 ± 0.17	1.64 ± 0.11	1.56 ± 0.09	1.14 ± 0.08	1.28 ± 0.14	1.41 ± 0.20	1.37 ± 0.24
BUN (mg/dl)	16 ± 2	17 ± 2	16 ± 3	16 ± 1	23 ± 3	21 ± 2	23 ± 1	20 ± 2	19 ± 3	15 ± 3	21 ± 1	24 ± 3
Creatinine (mg/dl)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.8 ± 0.1
Glucose (mg/dl)	130 ± 13	137 ± 32	1 43 ± 13	155 ± 24	109 ± 13	113 ± 21	118 ± 7	108 ± 10	137 ± 10	129 ± 16	125 ± 12	128 ± 10
T. Cholest.(mg/dl)	34 ± 8	33 ± 4	37 ± 5	40 ± 5	41 ± 8	48 ± 9	45 ± 10	31 ± 8	41 ± 15	38 ± 10	48 ± 9	54 ± 4
Triglyceride (mg/dl)	19 ± 5	22 ± 7	28 ± 14	35 ± 15	11 ± 5	9 ± 3	10 ± 3	9 ± 2	24 ± 13	29 ± 12	13 ± 2	22 ± 12
T. Bil.(mg/dl)	0.07 ± 0.04	0.08 ± 0.03	0.09 ± 0.05	0.07 ± 0.03	0.09 ± 0.03	0.07 ± 0.03	0.09 ± 0.04	0.09 ± 0.03	0.08 ± 0.02	0.09 ± 0.03	1.10 ± 0.04	0.08 ± 0.02
Inorg. P. (mg/dl)	9.0 ± 0.7	8.7 ± 0.8	8.4 ± 0.3	8.6 ± 0.4	7.9 ± 0.8	8.1 ± 0.7	8.2 ± 1.1	8.4 ± 0.5	7.2 ± 0.9	6.5 ± 0.6	7.4 ± 0.9	7.5 ± 0.5
Ca (mg/dl)	8.9 ± 0.1	8.9 ± 0.3	9.0 ± 0.1	9.2 ± 0.2	9.1 ± 0.1	9.1 ± 0.2	9.1 ± 0.2	8.8 ± 0.4	8.9 ± 0.2	8.8 ± 0.1	9.1 ± 0.2	9.2 ± 0.2
Na (mEq/l)	145.8 ± 0.6	145.1 ± 0.9	145.8 ± 1.3	145.5 ± 0.9	145.5 ± 0.8	144.7 ± 0.7	145.6 ± 0.8	146.0 ± 1.4	146.2 ± 0.4	145.9 ± 0.6	143.1 ± 0.8	143.4 ± 0.2
K (mEq/l)	4.48 ± 0.53	4.33 ± 0.36	4.08 ± 0.26	4.29 ± 0.24	4.18 ± 0.39	4.31 ± 0.20	4.20 ± 0.28	4.05 ± 0.38	4.03 ± 0.26	3.96 ± 0.12	4.11 ± 0.28	3.98 ± 0.32
Cl (mEq/l)	109.0 ± 1.7	108.3 ± 1.7	108.1 ± 1.2	107.8 ± 1.4	109.1 ± 1.2	107.5 ± 1.0	109.9 ± 1.1	109.3 ± 2.3	108.2 ± 1.1	108.7 ± 0.6	107.2 ± 0.6	107.8 ± 0.6
ALP (U/l)	441 ± 143	451 ± 58	511 ± 190	459 ± 56	319 ± 65	261 ± 62	299 ± 75	266 ± 46	366 ± 80	374 ± 29	219 ± 36	231 ± 39
ALT (U/l)	31 ± 6	27 ± 4	29 ± 3	37 ± 15	25 ± 2	22 ± 2	22 ± 5	29 ± 5	35 ± 5	32 ± 4	24 ± 3	28 ± 5
AST (U/l)	72 ± 10	66 ± 11	67 ± 7	88 ± 24	69 ± 3	66 ± 5	69 ± 8	94 ± 16*	83 ± 14	66 ± 2	62 ± 3	66 ± 5
γ-GTP (U/l)	0 ± 1	0 ± 1	1 ± 0	1 ± 1	1 ± 0	1 ± 0	1 ± 1	1 ± 0	0 ± 1	1 ± 1	1 ± 1	1 ± 1

Values represent average for five animals ± S.D.

* and **, significant difference from control at $p < 0.05$ and 0.01, respectively.

TP, total protein; A/G, albumin ratio to globulin (TP albumin); BUN, blood urea nitrogen; T. Cholest., total cholesterol; T. Bil., total bilirubin; Inorg. P., Inorganic phosphorus; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ -GTP, γ-glutamyl transpeptidase

Table 4. Organ weights of male rats treated orally with 4-methylbenzoic acid in repeated dose 28-day oral toxicity study

	The end of treatment				End of recovery	
Dose (mg/kg)	0	100	300	1,000	0	1,000
Number of animals	5	5	5	5	5	5
Body weight (g)	307.9 ± 24.7	301.4 ± 32.0	311.2 ± 4.2	311.8 ± 30.7	380.8 ± 43.4	370.2 ± 15.1
Absolute weight						
Brain (g)	1.92 ± 0.07	1.87 ± 0.06	1.89 ± 0.07	1.84 ± 0.07	1.92 ± 0.11	1.91 ± 0.07
Thymus (mg)	587 ± 86	493 ± 61	537 ± 95	535 ± 98	423 ± 42	481 ± 132
Heart (g)	1.10 ± 0.09	1.01 ± 0.06	1.00 ± 0.05	1.06 ± 0.11	1.21 ± 0.15	1.18 ± 0.12
Liver (g)	9.62 ± 0.99	9.97 ± 1.87	9.73 ± 0.46	10.26 ± 1.15	10.95 ± 2.00	10.88 ± 0.46
Kidneys (g)	2.39 ± 0.20	2.42 ± 0.24	2.43 ± 0.17	2.42 ± 0.28	2.72 ± 0.30	2.69 ± 0.27
Spleen (mg)	707 ± 192	611 ± 74	643 ± 85	591 ± 106	743 ± 114	694 ± 93
Testes (g)	2.69 ± 0.01	2.84 ± 0.14	3.00 ± 0.28	2.87 ± 0.35	2.94 ± 0.16	2.95 ± 0.15
Epididymides (g)	0.67 ± 0.04	0.71 ± 0.07	0.70 ± 0.02	0.69 ± 0.10	0.91 ± 0.06	0.90 ± 0.04
Adrenal glands (mg)	50.9 ± 3.6	45.6 ± 5.6	54.6 ± 4.6	47.1 ± 6.3	52.7 ± 6.0	55.8 ± 6.8
Relative weight (g/100 g)						
Brain	0.63 ± 0.04	0.62 ± 0.05	0.61 ± 0.03	0.60 ± 0.06	0.51 ± 0.03	0.52 ± 0.01
Thymus	0.19 ± 0.02	0.16 ± 0.02	0.17 ± 0.03	0.17 ± 0.02	0.11 ± 0.02	0.13 ± 0.04
Heart	0.36 ± 0.04	0.34 ± 0.03	0.32 ± 0.01	0.34 ± 0.02	0.32 ± 0.05	0.32 ± 0.03
Liver	3.12 ± 0.16	3.29 ± 0.36	3.13 ± 0.17	3.29 ± 0.14	2.87 ± 0.32	2.94 ± 0.08
Kidneys	0.78 ± 0.05	0.80 ± 0.05	0.78 ± 0.05	0.78 ± 0.06	0.72 ± 0.09	0.73 ± 0.07
Spleen	0.23 ± 0.04	0.20 ± 0.02	0.21 ± 0.03	0.19 ± 0.03	0.20 ± 0.02	0.19 ± 0.02
Testes	0.88 ± 0.13	0.95 ± 0.11	0.97 ± 0.09	0.92 ± 0.05	0.78 ± 0.07	0.80 ± 0.06
Epididymides	0.22 ± 0.03	0.24 ± 0.02	0.23 ± 0.00	0.22 ± 0.02	0.24 ± 0.02	0.24 ± 0.02
Adrenal glands	0.017 ± 0.002	0.015 ± 0.001	0.018 ± 0.002	0.015 ± 0.001	0.014 ± 0.001	0.015 ± 0.002

Values represent average ± S.D.

the day of delivery (Day 0 of lactation) were higher in the 1,000 mg/kg treated group and in the 300 mg/kg or more treated groups, respectively, compared with those in the control. During the lactation period, maternal body weight gain and food consumption in the 1,000 mg/kg treated group were smaller than those in the control.

Terminal body weight and the weights of testes and epididymides of the male rats treated for 42 days are shown in Table 5. The compound did not affect the terminal body weight, at any dose level. While no differences were observed in the absolute or relative testicular weights between the control and the compound treated groups, those of the epididymides were significantly lower in the 1,000 mg/kg treated group than in the control group. Histopathological examination revealed that there were lumens containing no or few spermatozoa, i.e., oligo/azoospermia, in the cauda epididymis in all the males of this group (Fig. 7), while none of their caput epididymis showed the same abnormality. In addition to this, the number of animals with cell debris in the cauda epididymal lumen was increased in this group, although it was statistically insignificant when compared with that in the