Taquahashi, Y, Ogawa, Y, Takagi, A, Tsuji, M, Morita, K, Kanno, J.	An improved dispersion method of multi-wall carbon nanotube for inhalation toxicity studies of experimental animals.	J Toxicol Sci	38	619-628	2013
Ohnishi M, Umeda Y, Katagiri T, Kasai T, Ikawa N, Nishizawa T, Fukushima S.	Inhalation carcinogenicity of 1,1,1-trichloroethane in rats and mice.	Inhal Toxicol	25	298-306	2013
Igarashi K, Kitajima S, Aisaki K, Tanemura K, Taquahashi Y, Moriyama N, Ikeno E, Matsuda N, Saga Y, Blumberg B, Kanno J.	Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence.	J Toxicol Sci	37	373-380	2012
Hang NT, Lien LT, Kobayashi N, Shimbo T, Sakurada S, Thuong PH, Hong LT, Tam DB, Hijikata M, Matsushita I, Hung NV, Higuchi K, Harada N, Keicho N.	Analysis of factors lowering sensitivity of interferon-gamma release assay for tuberculosis.	PLoS One	6	e23806	2011
Nagano, K., Nishizawa, T., Eitaki, Y., Ohnishi, M., Noguchi, T., Arito, H. and Fukushima, S.	Pulmonary Toxicity in Mice by 2- and 13-week Inhalation Exposures to Indium-tin Oxide and Indium Oxide Aerosols.	J Occup Health	53,	234-239	2011

別添５

III. 研究成果の刊行に関する一覧表

CHAPTER 11

Application of Percellome Toxicogenomics to Food Safety

J. KANNO,[a] K. AISAKI,[a] K. IGARASHI,[a] N. NAKATSU,[a,b] Y. KODAMA,[a] K. SEKITA,[a] A. TAKAGI[a] AND S. KITAJIMA[a]

[a]National Institute of Health Sciences, Tokyo 158-8501, Japan; [b]National Institute of Biomedical Innovation, Osaka 567-0085, Japan

11.1 Introduction

The approaches employed to assess chemical toxicity are seldom applied to food safety assessment for a number of reasons. In the first place, routine toxicity assessment involves administration of a pure compound to experimental animals at different doses up to the maximal tolerated dose (MTD) to determine the no observed (adverse) effect level [NO(A)EL], while food is a complex mixture of chemicals. Secondly, the sensitivity of routine toxicity tests is inadequate for foods. The maximal amount of the test substance applied is limited by guidelines to 5% of the diet or, in the case of oral gavage, 10 $mL \cdot kg^{-1}$ (approximately 10 $g \cdot kg^{-1}$ for liquids), in order not to jeopardize reliability (especially with respect to chronic toxicity and carcinogenesis) by inducing malnutrition. For example, low body weight alone can result in tumors at low incidences.[1–3] When an item of food is found to be non-toxic at the dose of 10 g kg^{-1}, this value is taken as NOEL and the tolerable daily intake (TDI) calculated, using a safety factor of 1/100, to be 100 $mg \cdot kg^{-1}$. Thus the grams or even tens of grams per kg body weight that we consume daily are not allowed in routine toxicity testing and, moreover, according to such standard approaches, if a food showed any toxicity in routine testing, such food would be highly toxic.

Issues in Toxicology No. 11
Hormone-Disruptive Chemical Contaminants in Food
Edited by Ingemar Pongratz and Linda Vikström Bergander

Published by the Royal Society of Chemistry, www.rsc.org

Even though flavorings are added to food and other consumed products at relatively low concentrations, these substances are often not subject to toxicity testing at the MTD from other reasons. One practical consideration is that the odor of many flavorings at MTD is too strong to be tolerated by the test animal or the staff that care for them. Moreover, in some cases, the amount of a flavoring required to conduct an MTD test is more than its annual production and/or prohibitively costly. At the practical dose levels, flavoring does not exert any detectable histopathological effect on experimental animals. However, such doses do stimulate, at least, olfactory (and perhaps other) receptors. Thus, more sensitive approaches to assessing the toxicity of food and flavors are highly desirable. One approach is to use particularly sensitive animal models, such as transgenic mice to evaluate certain biological phenomena, including carcinogenic potential. Another strategy is to detect early molecular changes that occur prior to morphological alterations and/or other symptoms. Toxicogenomics is one example of the latter.

Our Percellome Toxicogenomics Project is designed to identify dynamic and extensive networks of genes whose time- and dose-dependent patterns of expression in response to a chemical, or a mixture of chemicals such as food, allows its toxic effects to be predicted. Here, we describe this project briefly, employing a flavoring compound, estragole, as a peroxisome proliferator-activated receptor alpha (PPAR-alpha) agonist whose effects are similar to those of clofibrate. Among the more than 100 chemicals tested, 45 are food related (Table 11.1), including food additives, components of food, functional health foods, agrochemicals, and other food contaminants (*e.g.* that might leach out of packaging materials).

11.2 Materials and Methods

Forty-eight male C57BL/6 Cr Slc or C57BL/6J mice (SLC, Hamamatsu, Charles River Japan, Atsugi, Japan) were maintained for two weeks in an enclosed environment (diet: CRF-1, Oriental Yeast Co.) with a 12-h photoperiod (starting at 8:00 a.m.) and then, at 12 weeks of age, a single oral administration of the test chemical at 10:00 ~ 10:20 a.m. and at the doses indicated in Table 11.1. At 2, 4, 8, and 24 hours later, the livers of three animals at each dose were collected. To minimize circadian oscillations in the transcriptome, 12 animals were processed at each time-point at a rate of 2.5 min from initiation ether anesthesia to immersion of the liver and other organ samples into RNAlater solution (Ambion, TX, USA), thereby keeping the total elapsed to within 30 min. Prior to analysis, the blocks of liver tissue were soaked in RNAlater overnight at 4 °C. This solution was then replaced with RLT buffer (Qiagen, Germany), the tissue homogenized, and a small aliquot of each homogenate treated with DNAse-free RNase A, followed by proteinase K; the DNA concentration was then determined with PicoGreen fluorescent dye (Molecular Probes, USA). Next, on the basis of its DNA content, each sample was spiked with the same relative amount of GSC cocktail (containing

Table 11.1 Food-related chemicals in Percellome Database.

Food-related Chemicals (including agrochemicals)	*CAS No.*	*Dose (mg/kg)*	*Vehicle*	*Percellome study No. TTG*
Acephate	30560-19-1	0, 0.7, 2, 7, 20, 70 mg/kg	MC 0.5%	67, 109
Alpha lipoic acid	1077-28-7	0, 10, 30, 100 mg/kg	MC 0.5%	128
Aluminum ammonium sulfate	7784-26-1	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	155
Aluminum lactate	18917-91-4	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	163
Aluminum sulfate	10043-01-3	0, 100, 300, 1000 mg/kg	MC 0.5%(WAKO)	178
Bisphenol A	1980-5-7	0, 30, 100, 300 mg/kg	DMSO 0.1% + MC 0.5%	47
Caffeine	1958-8-2	0, 3, 10, 30 mg/kg	MC 0.5%	59
Carbaryl	63-25-2	0, 2, 7, 10, 20, 30, 100 mg/kg	MC 0.5%	68, 166
Chlorpyrifos	2921-88-2	0, 3, 10, 30 mg/kg	MC 0.5%(WAKO)	165
Citric acid-calcium salt	813-94-5	0, 100, 300, 1000 mg/kg	MC 0.5%	39
Coenzyme Q10	303-98-0	0, 30, 100, 300 mg/kg	corn oil	86
Curcumin	458-37-7	0, 70, 200, 700 mg/kg	corn oil	132
DEHP (di(2-ethylhexyl)phthalate)	117-81-7	0, 200, 700, 2000 mg/kg (*cf.* MEHP)	corn oil	98
Dexamethasone	1950-2-2	0, 10, 30, 100 ug/kg	DMSO 0.1% + MC 0.5%	62
Estragole	140-67-0	0, 10, 30, 100 mg/kg	corn oil	147
Ethanol	64-17-5	0, 150, 500, 1500 mg/kg (d = 0.79)	MC 0.5%	78
Food Red No.104	18472-87-2	0, 10, 30, 100 mg/kg	MC 0.5%(WAKO)	156
Forskolin	66575-29-9	0, 1, 3, 10 mg/kg	MC 0.5%	60
Genistein	446-72-0	0, 10, 30, 100 mg/kg	DMSO 0.1% + MC 0.5%	48
Glycyrrhizin2K	1405-86-3	0, 3, 10, 30 mg/kg	DMSO 0.1% + MC 0.5%	110
Hydroxycitric Acid	27750-10-3	0, 100, 300, 1000 mg/kg	MC 0.5%	38

Indigo	482-89-3	0, 30, 100, 300 mg/kg	corn oil	57
Maltol	118-71-8	0, 10, 30, 100 mg/kg	corn oil	133
MEHP (mono(2-ethylhexyl)phthalate)	4376-20-9	0, 70, 200, 700 mg/kg (*cf.* MEHP)	corn oil	104
Methanol	67-56-1	0, 30, 100, 300 mg/kg (*cf.* Formalin, EtOH)	MC 0.5%	79
Methoprene	40596-69-8	0, 0.3, 1, 3 mg/kg	DMSO 0.1% + corn oil	66
Methyl dihydro jasmonate	24851-98-7	0, 10, 30, 100 mg/kg	corn oil	135
Nerolidol	cis: [3790-78-1], trans:[40716-66-3]	0, 5.9, 17.7, 59.1 mg/kg	corn oil	134
Paraquat dichloride	1910-42-5	0, 1, 3, 10 mg/kg	MC 0.5%	61
Pentachlorophenol	87-86-5	0, 10, 30, 100 mg/kg	MC 0.5%	16
Permethrin	52645-53-1	0, 10, 30, 100 mg/kg	corn oil	97
Phytol	7541-49-3	0, 10, 30, 100 mg/kg	corn oil	136
Pyriproxyfen	95737-68-1	0, 0.3, 1, 3 mg/kg	DMSO 0.1% + corn oil	87
Sodium Dehydroacetate	4418-26-2	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	154
Tebufenozide	112410-23-8	0, 0.3, 1, 3 mg/kg	DMSO 0.1% + corn oil	88
Tributyltin	56573-85-4	0, 30,100, 300 ug/kg	DMSO 0.5% + MC 0.5%	85
Tributyltin chloride	1461-22-9	0, 3,10, 30 ug/kg	DMSO 0.1% + MC 0.5%	49
Verbenone	18308-32-5	0, 10, 30, 100 mg/kg	corn oil	148
Warfarin (sodium salt)	81-81-2	0, 10, 30, 100 mg/kg	MC 0.5%	81
FD&C Blue No.1 (brilliant blue)	3844-45-9	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	152
FD&C Blue No.2 (Indigo carmine)	860-22-0	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	139
Food Red No.102	2611-82-7	0, 100, 300, 1000 mg/kg	MC 0.5%(WAKO)	177
FD&C Red No.40	25956-17-6	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	140
FD&C Green No.8 (pyranine conc)	6358-69-6	0, 30, 100, 300 mg/kg	MC 0.5%(WAKO)	176
FD&C Yellow No.5 (Food Yellow No.4 Japan)	1934-21-0	0, 100, 300, 1000 mg/kg	MC 0.5%(WAKO)	161

MC: methylcellulose

five *Bacillus subtilis* RNA sequences corresponding to the Affymetrix GeneChip probe sets AFFX-ThrX-3_at, AFFX-LysX-3_at, AFFX-PheX-3_at, AFFX-DapX-3_at, and AFFX-TrpnX-3_at), and subsequently processed in accordance with the Affymetrix Standard protocol for application to Mouse 430 2.0 GeneChips. After conversion of the raw read-outs to Percellome data,[4] the values obtained (with four doses and five time-points, using 2-h vehicle value as 0-h) were plotted on the three-dimensional graphs (*cf.* http://toxicomics.nihs.go.jp/db/) (Figure 11.1).

The highest dose that, 24 hours after a single oral gavage, did not influence body weight or cause macroscopic changes in internal organs, microscopic alterations in liver observed with H&E staining, and/or abnormal behavior was employed as the maximal dose. In general, the maximal doses in the Percellome studies are 10- to 100-fold lower than the reported MTDs.

The Percellome data set was first screened with an RSort program,[5] which identifies the number of peaks on a surface, distinguishes between the surfaces for 45,101 probe sets simply on the basis of their shapes, includes which of the peaks is significantly different ($p<0.05$) from the corresponding control (vehicle) values, as evaluated by Student's *t*-test, and excludes probe sets with less than one copy per liver cell. Thereafter, taking into consideration the *t*-test values and ± 1 standard deviation, the surface shape was checked manually by

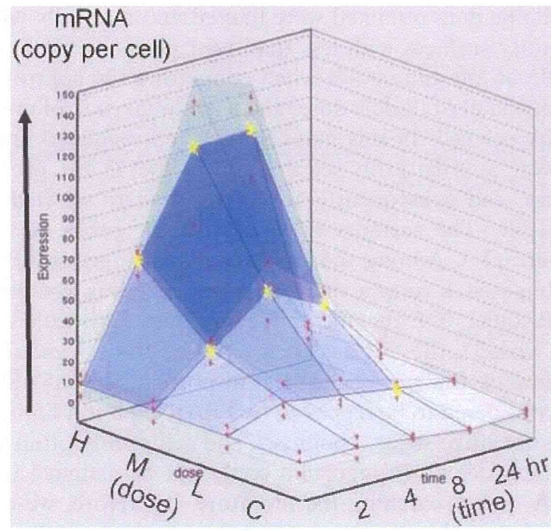

Figure 11.1 A three-dimensional representation of Percellome data. The dose- and time-dependent alterations in the expression of a probe set of genes is expressed as the absolute number of mRNA molecule per cell on the vertical axis. The mean of data from three different animals at each time and dose is represented, along with the SD (colored more lightly). Asterisks indicate $p<0.05$ from concurrent control by student's *t*-test; small crosses represents data for each animal.

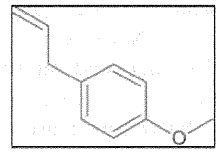

Figure 11.2 The chemical structure of estragole.

S.K. and/or J.K. Another original program referred to as Percellome Explorer [5] was utilized to compare pairwise the RSort results of all evaluations in the Percellome project with lists of shared probe sets.

The flavor compound estragole (1-allyl-4-methoxybenzene; CAS no. 140-67-0) (Figure 11.2) is a major component of tarragon, as well as of oils obtained from basil and other plants. We found that pure estragole (98%) from Sigma-Aldrich (catalog no. A29208-25G, lot no. 05202AH) at a maximal dose of 100 mg kg^{-1} caused no observable effects after 24 hours, so the Percellome investigation was conducted at doses of 0, 10, 30, and 100 mg kg^{-1} dissolved in corn oil (Sigma-Aldrich, C8267) and delivered by gavage.

11.3 Results

First, the Percellome data obtained were filtered automatically with the RSort program to identify surfaces with less than four peaks in the 4 x 4 matrix, a *p*-value of <0.05 at any time-point when comparing the control value to the corresponding maximal or middle dose, and a maximal peak of more than three copies of mRNA per cell. In this manner, 1214 up-regulated and 244 down-regulated sets were automatically selected out of the 45,101 probe sets in the mouse GeneChip, and subsequently 167 of those were selected manually as biologically feasible. The surfaces of several of the probe sets selected are depicted in Figure 11.3. Among those up-regulated, Ingenuity Pathway Analysis (IPA) highlighted a certain number genes encoding proteins associated with the PPAR-alpha. Of the metabolic enzymes up-regulated, Cyp4a10, Cyp4a14, and Cyp4a31, the last has been reported to be regulated by PPAR-alpha. Expression of the up-regulated transporters Slc16a5, Slc25a20, and Slc27a1 is also considered to be regulated by PPAR-alpha. A limited number of genes related to oxidative stress, apoptosis, and cell proliferation were induced (Table 11.2). The genes down-regulated could not be assigned to any known pathway by IPA or by searching the literature. Therefore, we conclude that administration of a single gavage of estragole up-regulates the expression of genes controlled by PPAR-alpha.

11.4 Discussion

Among others, PPAR-alpha regulates a set of genes whose products participate in the β-oxidation of fatty acids, as well as, in rodents at least, peroxisome

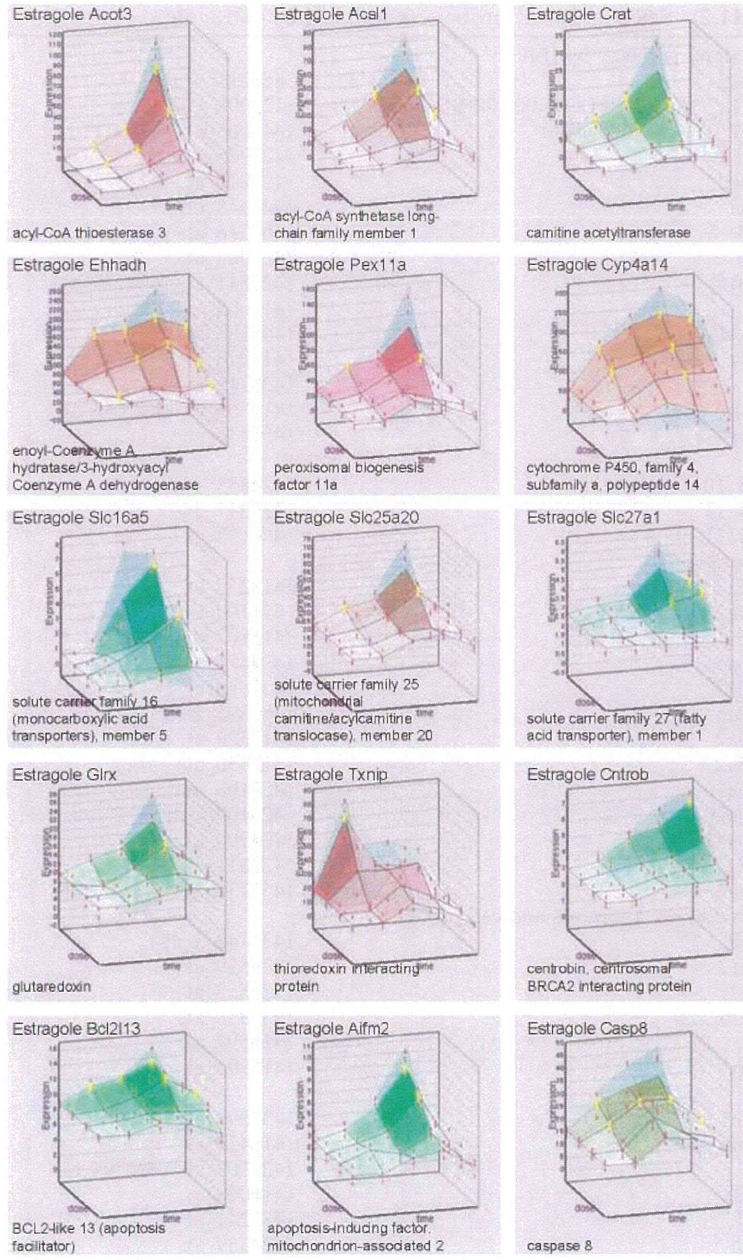

Figure 11.3 Representative three-dimensional surfaces of probe sets induced by estragole; Acot3, Acsl1, Crat, Ehhadh, Pex11a, Cyp4a14, Slc16a5, Slc25a20, and Slc27a1 are considered to be regulated by PPAR-alpha; the findings with respect to genes related to oxidative stress, apoptosis, and cell proliferation are also shown (*cf.* Table 11.2).

Table 11.2 Selected list of probe sets induced by estragole.

PPAR alpha pathway by IPA	
Acaa1a	1456011_x_at
Acot1 /// Acot2 /// LOC100044830	1422997_s_at
Acot1 /// LOC100044830	1449065_at
Acot3	1422925_s_at
Acot4	1422076_at, 1422077_at
Acox1	1444518_at
Acsl1	1460316_at
Acsl5	1428082_at
Agpat6	1422841_at, 1450776_at
Crat	1417008_at
Ehhadh	1448382_at
Pdk4	1417273_at
Pex1	1428716_at
Pex11a	1419365_at, 1449442_at
Pxmp4	1455438_at
P450s	
Cyp4a10 /// Cyp4a31	1424853_s_at
Cyp4a14	1423257_at
Cyp4a31	1440134_at
Transporters	
Slc12a7	1418257_at
Slc14a2	1426109_a_at
Slc16a1	1415802_at
Slc16a5	1434473_at
Slc22a5	1421848_at
Slc22a5	1450395_at
Slc23a2	1417329_at
Slc23a2	1417330_at
Slc25a20	1423108_at
Slc25a20	1423109_s_at
Slc25a42	1424790_at
Slc27a1	1422811_at
Slc29a3	1455731_at
Oxidative stress, apoptosis and cell proliferation	
Aifm2	1431143_x_at
Bcl2l13	1429539_at
Casp8	1424552_at
Cntrob	1433958_at
Glrx	1416592_at
Txnip	1415996_at
Others	
Dnaic1	1437093_at, 1437094_x_at
Fabp2	1418438_at
Gyk	1422703_at, 1422704_at
Klf10	1416029_at
Klf11	1437241_at
Oplah	1424359_at
Paqr7	1435312_at, 1460674_at

proliferation. Agonists of this receptor are carcinogenic towards rodent liver *via* mechanisms related to peroxisome proliferation.[6] At the same time, since peroxisome proliferation leads to lowering of serum triglyceride levels, PPAR-alpha is a molecular target for hypolipidemic drugs, such as clofibrate.

Here, we found that estragole induces a considerable number of genes known to be regulated by PPAR-alpha. The Percellome Explorer (PE) program, which automatically compares probe set lists, has identified clofibrate (81 probe sets), followed by di(2-ethylhexyl) phthalate (DEHP) (126 probe sets) as the two chemicals causing changes most similar to those evoked by estragole upon testing with the same experimental protocol (Figure 11.4) (the absolute numbers of common probe sets were normalized to the product of the numbers of altered probe sets in each study). The PE program also compares probe sets on the basis of time, in the present case 2 hours after administration. Such early-responding genes are considered to be direct or almost direct targets of a receptor(s) that is activated by the test chemicals. Again, the similarity of its pattern to those of clofibrate and DEHP[7,8] confirms that one of the primary receptors that binds estragole is the PPAR-alpha. Isozyme 4 of pyruvate dehydrogenase kinase (Pdk4), shown in Figure 11.4, has already been reported to be regulated by this latter compound. Ingenuity pathway analysis was then applied for comparison with reported information (Figure 11.5). Two hours after administration, TXNIP, Klf10, and KLF11 did not appear to be directly regulated by PPAR-alpha, even though these three genes have recently been reported to be under the regulation of this receptor,[9,10] albeit with no clear relationship to liver function. Dynein, axonemal, and intermediate chain 1 (Dnaic1) is induced 8 and 24 hours after administration of estragole and clofibrate, respectively, as well as by DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) at later time-points (Figure 11.6). Dnaic1 appears to play an important role in peroxisome biogenesis.[11,12] This delayed induction, compared to that of early-response genes such as Pdk4, might indicate that several mediators are located between PPAR-alpha and this gene. The nature of the pathway, together with a literature search, indicate that HNF4A and PEX13[13] may be involved, since the latter is induced slightly by estragole (not significantly) and clearly by clofibrate, DEHP, and MEHP (Figure 11.6).

Among the many isozymes of cytochrome P-450, Cyp4a10, Cyp4a14, and Cyp4a31 are induced significantly by estragole, as well as by clofibrate and DEHP. On the other hand, Cyp1a2, which is induced slightly by phenobarbital, was not up-regulated by estragole. All three of these Cyp4a's are considered to be regulated by PPAR-alpha. Whereas DEHP also induces Cyp2b10 and Cyp51, estragole and clofibrate do not. Phenobarbital also induces Cyp2b10 and Cyp51, but not the Cyp4a's. Thus, the present Percellome analysis indicates (Figure 11.7) that DEHP activates at least two receptors, both PPAR-alpha and the constitutive androstane receptor (NR1I3 or CAR), which regulates Cyp2b[14] and probably Cyp51.[15,16] In contrast, estragole and clofibrate activate PPAR-alpha, but not CAR.

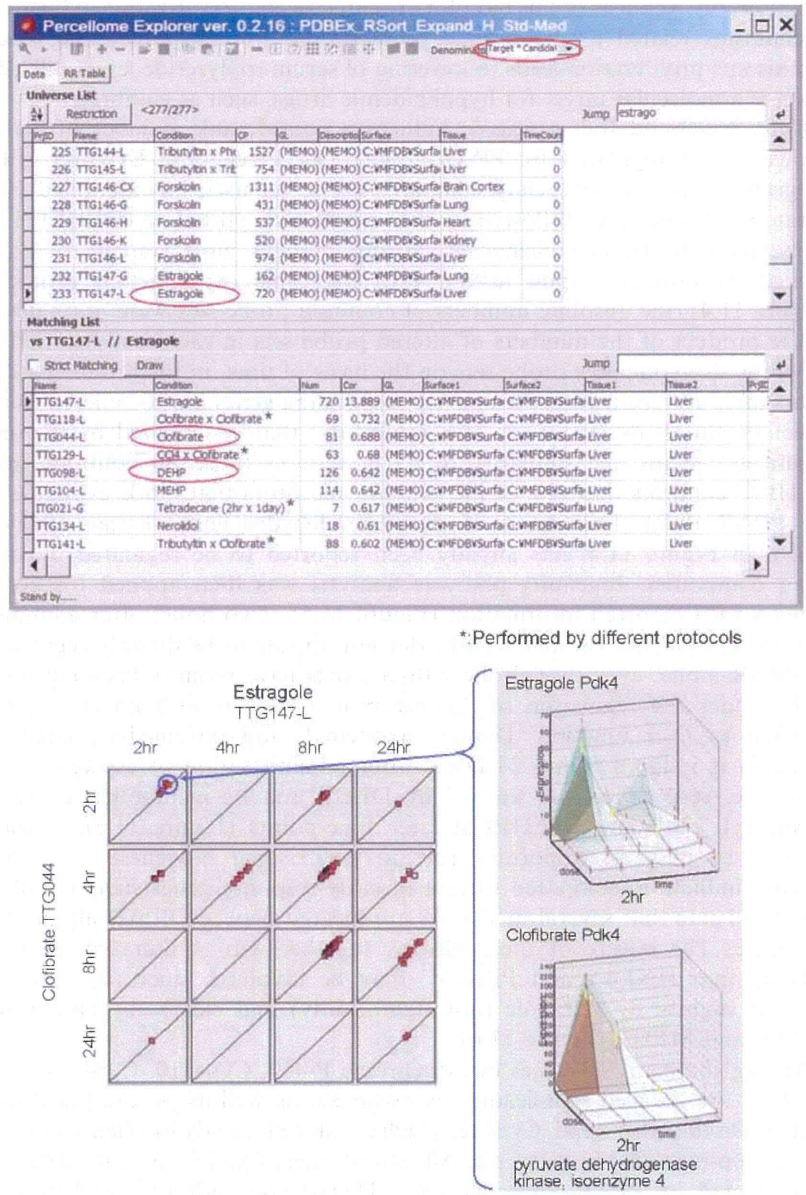

Figure 11.4 Comparison of the similarity between various Percellome studies utilizing the Percellome Explorer program. The upper figure shows the calculations that identify clofibrate and DEHP as the top chemicals most similar to estragole. The lower figure illustrates the similar peak times of the probe sets common to estragole and clofibrate, using Pdk4 (1417273_at) as an example.

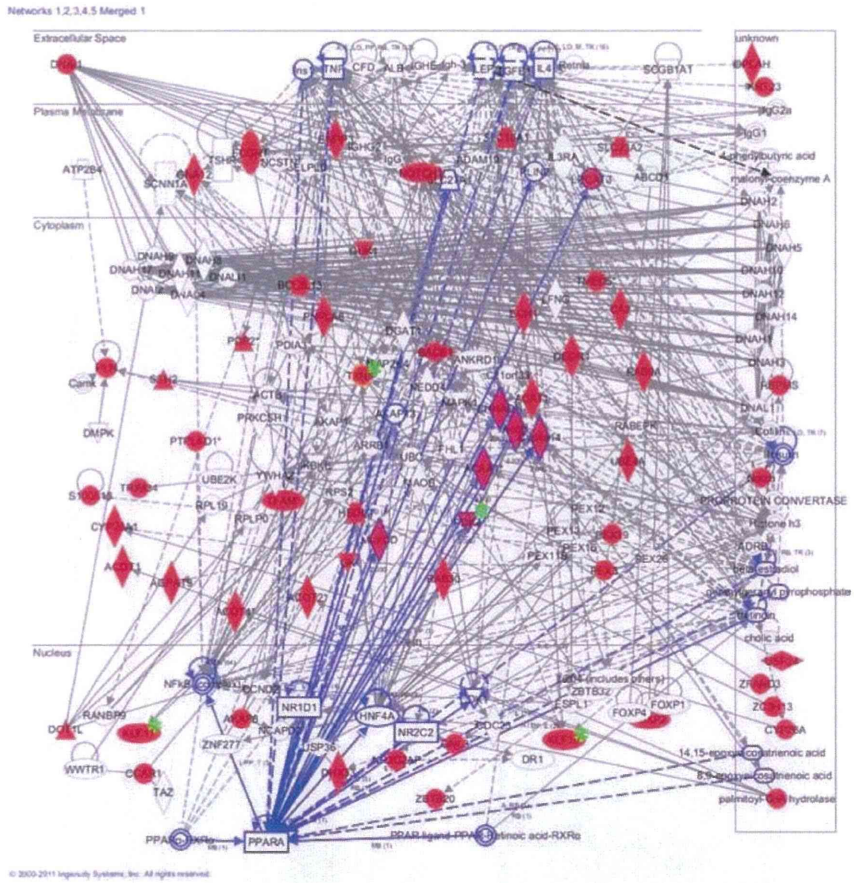

Figure 11.5 Ingenuity pathway analysis of the probe sets common to estragole and clofibrate. Those elevated by both chemicals are highlighted in red. Among the four 2-h probe sets (green asterisk), only Pdk4 appears to be a direct target, whereas TXNIP, Klf10, and Klf11 appear to be influenced indirectly.

Expression of the drug and/or metal transporters Slc12a7, Slc14a2, Slc16a1, Slc16a5, Slc22a5, Slc23a2, Slc25a20, Slc25a42, Slc27a1, and Slc29a3 appeared here to be altered by estragole. Again, PE analysis reveals that most of these same Slc transporters are also induced by DEHP and clofibrate (not shown). Among these, only Slc27a1 has so far been reported to be regulated by PPAR-alpha.[17,18] However, expression of most of these Slc transporters, including Slc27a1, peaked after 8 hours, indicating that these genes may be activated indirectly.

Our novel finding that estragole activates PPAR-alpha signaling may help elucidate the mechanism(s) underlying estragole-induced carcinogenesis, *i.e.*

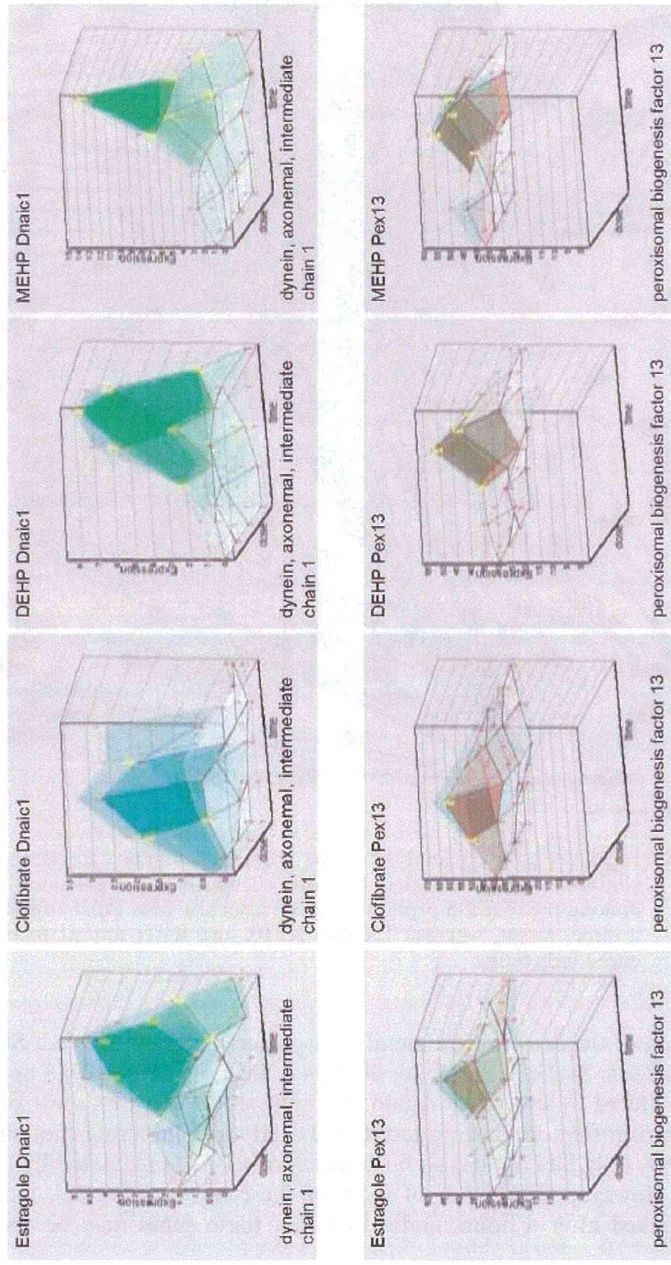

Figure 11.6 The surface of Dnaic1 and Pex13 for estragole, clofibrate, DEHP, and MEHP. The late response may be mediated by Pex13.

peroxisome proliferation may be involved. Indeed, estragole increases liver weight at a dose lower than the carcinogenic dose.[19]

An important advantage in determining the actual average number of mRNA molecules per cell is that the responses obtained in different studies can be compared directly. As shown in Figures 11.6 and 11.7, the magnitude of the up-regulation of PPAR-alpha-inducible genes by estragole was comparable to that of clofibrate, since, at the same doses (*i.e.* 0, 10, 30, and 100 $mg \cdot kg^{-1}$) employed, estragole appears to be as potent as clofibrate in activating PPAR-alpha signaling.

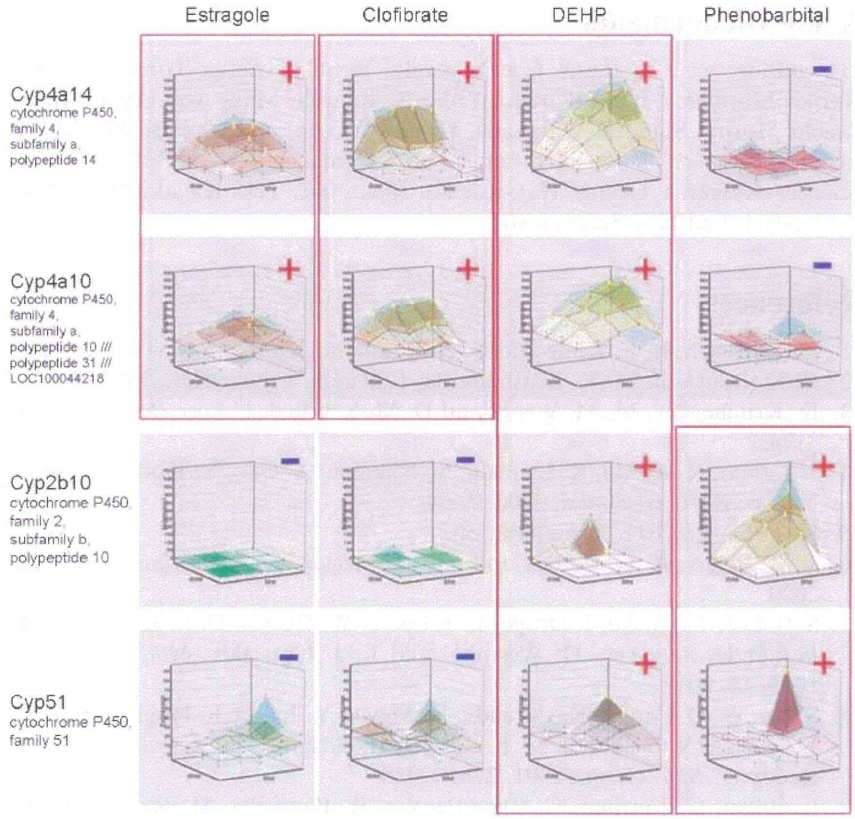

Figure 11.7 Percellome analysis of representative P450s induced by estragole, clofibrate, DEHP, and phenobarbital. DEHP appears to induce P450s *via* at least two different pathways, *i.e.* PPAR-alpha and CAR, whereas estragole, clofibrate, and phenobarbital induce only PPAR-alpha or CAR, respectively.

11.5 Conclusions

Our present observations that estragole appears to be as potent an agonist of PPAR-alpha as clofibrate (on a $mg \cdot kg^{-1}$ basis) should now be confirmed by actual binding and signaling studies. If confirmed, the hepatocarcinogenic potential of this compound should be reevaluated accordingly. Although recent reports on estragole carcinogenicity suggest involvement of its metabolites[20] or glucocorticoid pathways,[21] our Percellome data support neither the involvement of such pathways or pronounced genotoxicity (which can be monitored indirectly as an enhancement in DNA repair and responses to oxidative stress). Interestingly, DEHP and Wyeth 14,643, well-characterized non-genotoxic rodent hepatocarcinogens that evoke tumors through peroxisome proliferation, gave mutation in Lac Z transgenic mice.[22]

Acknowledgements

The authors wish to thank Nae Matsuda, Kenta Yoshiki, Tomoko Ando, Noriko Moriyama, Yuko Kondo, Yuko Nakamura, Maki Abe, Ayako Imai, Koichi Morita, Shinobu Watanabe, Hisako Aihara, and Chiyuri Aoyagi for technical support. This study was supported financially by MHLW Health Sciences Research Grants H18-Kagaku-Ippan-001, H15-Kagaku-002, H14-Toxico-001, and H13-Seikatsu-012.

References

1. H. P. Rusch, B. E. Kline and C. A. Baumann, *Cancer Res.*, 1945, **5**, 431.
2. R. K. Boutwell, M. K. Brush and H. P. Rusch, *Cancer Res.*, 1949, **9**, 741.
3. D. Kritchevsky, M. M. Weber and D. M. Klurfeld, *Cancer Res.*, 1984, **44**, 3174.
4. J. Kanno, K. Aisaki, K. Igarashi, N. Nakatsu, A. Ono, Y. Kodama and T. Nagao, *BMC Genomics*, 2006, **7**, 64.
5. K.-I. Aisaki, 2011, in preparation.
6. J. M. Peters, R. C. Cattley and F. J. Gonzalez, *Carcinogenesis*, 1997, **18**, 2029.
7. S. S. Lee, T. Pineau, J. Drago, E. J. Lee, J. W. Owens, D. L. Kroetz, P. M. Fernandez-Salguero, H. Westphal and F. J. Gonzalez, *Mol. Cell. Biol.*, 1995, **15**, 3012.
8. S. Yu, W. Q. Cao, P. Kashireddy, K. Meyer, Y. Jia, D. E. Hughes, Y. Tan, J. Feng, A. V. Yeldandi, M. S. Rao, R. H. Costa, F. J. Gonzalez and J. K. Reddy, *J. Biol. Chem.*, 2001, **276**, 42485.
9. L. Billiet, C. Furman, C. Cuaz-Perolin, R. Paumelle, M. Raymondjean, T. Simmet and M. Rouis, *J. Mol. Biol.*, 2008, **384**, 564.
10. M. Rakhshandehroo, G. Hooiveld, M. Muller and S. Kersten, *PLoS One*, 2009, **4**, e6796.
11. C. B. Brocard, K. K. Boucher, C. Jedeszko, P. K. Kim and P. A. Walton, *Tra c*, 2005, **6**, 386.

12. C. Kural, H. Kim, S. Syed, G. Goshima, V. I. Gelfand and P. R. Selvin, *Science*, 2005, **308**, 1469.
13. D. T. Odom, N. Zizlsperger, D. B. Gordon, G. W. Bell, N. J. Rinaldi, H. L. Murray, T. L. Volkert, J. Schreiber, P. A. Rolfe, D. K. Gifford, E. Fraenkel, G. I. Bell and R. A. Young, *Science*, 2004, **303**, 1378.
14. H. Ren, L. M. Aleksunes, C. Wood, B. Vallanat, M. H. George, C. D. Klaassen and J. C. Corton, *Toxicol. Sci.*, **113**, 45.
15. J. G. Dekeyser, E. M. Laurenzana, E. C. Peterson, T. Chen and C. J. Omiecinski, *Toxicol. Sci.*, **120**, 381.
16. C. Xu, C. Y. Li and A. N. Kong, *Arch. Pharm. Res.*, 2005, **28**, 249.
17. G. A. Francis, E. Fayard, F. Picard and J. Auwerx, *Annu. Rev. Physiol.*, 2003, **65**, 261.
18. M. Lemoine, J. Capeau and L. Serfaty, *PPAR Res.*, 2009, **2009**, 906167.
19. FAO/WHO, 69th joint meeting, *Safety Evaluation of Certain Food Additives*, WHO Food Additives Series, International Programme on Chemical Safety, World Health Organization, Geneva, 2009.
20. Y. Ishii, Y. Suzuki, D. Hibi, M. Jin, K. Fukuhara, T. Umemura and A. Nishikawa, *Chem. Res. Toxicol.*, **24**, 532.
21. V. I. Kaledin, M. Y. Pakharukova, E. N. Pivovarova, K. Y. Kropachev, N. V. Baginskaya, E. D. Vasilieva, S. I. Ilnitskaya, E. V. Nikitenko, V. F. Kobzev and T. I. Merkulova, *Biochemistry (Moscow)*, 2009, **74**, 377.
22. M. E. Boerrigter, *J. Carcinog.*, 2004, **3**, 7.

15

STANDARDIZATION OF GENE-EXPRESSION INFORMATION FOR THE SAFETY EVALUATION: ACTIVITIES IN JAPAN

Ken-ichi Aisaki and Jun Kanno

Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan

Applications of Toxicogenomics in Safety Evaluation and Risk Assessment, First Edition.
Edited by Darrell R. Boverhof and B. Bhaskar Gollapudi.

15.1 INTRODUCTION

Recent developments in cDNA microarray and quantitative PCR methodologies have demonstrated their applicabilities to medicine, food (including food additives and supplements), and industrial chemicals for the evaluation of their efficacies and safety. In the United States, the Food and Drug Administration (FDA) has started to accept voluntarily submitted gene-expression data for the drug approval processes. The European Medicines Agency (EMEA) is also considering a guideline for such processes. In Japan, the regulatory agencies involved in the approval of medicines have not been officially taking action or developing guidelines for the use of genomics data. An exception is a Ministry of Health, Labor, and Welfare (MHLW) funded research group that has been preparing to educate corporations, government, and consumers on the urgency of this matter. The general thought among Japanese chemical corporations is that the safety evaluation of toxicogenomics is second only to the safety evaluation of medicine. Here we report the study results by the above-mentioned research group, which was organized by scientists who specialize in mRNA measurements.

15.2 TECHNICAL INFORMATION ESSENTIAL FOR RNA STANDARDIZATION

For the mRNA to be utilized for the safety evaluation of new chemical substances, the standardization techniques should meet the following conditions:

1. Be commonly applicable to quantitative PCR, any kind of microarray, and new techniques such as comprehensive high-throughput sequencing.
2. Be based on a universal scale independent of the time and place of measurement
3. Be highly reproducible
4. Be easy to conduct

Among various methodologies proposed, currently available, and most fitting to these conditions would be the Percellome method, which measures mRNA in a copy number per one cell basis (Kanno et al., 2006). Conditions 1 through 3 have been satisfied, and for condition 4, a manual on the use of robotics is available. In addition a database with over 100 chemicals for rodent liver has been prepared and will be publically accessible in the near future.

15.3 INFORMATION ON DOMESTIC AND INTERNATIONAL ACTIVITIES

There exists more than one activity domestically and internationally for mRNA standardization.

15.3.1 Domestic Activities

15.3.1.1 The Toxicogenomics Project of NIHS

The Toxicogenomics Project (TGP 1: 2002–2006, TGP 2: 2007–2012) is a joint research project of the government partnering fifty-fifty with the private sector. It is run by the National Institute of Health Sciences (NIHS) and an alliance of 17 Japanese pharmaceutical companies. The mission is to streamline the safety evaluation process for pharmaceuticals. Rat liver was chosen as a primary research target because the member companies already had their own reference data. The project adopted the Percellome method, which was championed by a group of scientists of the NIHS (led by Jun Kanno). Up to 150 chemicals were monitored for rat liver transcriptome responses after acute dosing (3, 6, 9, and 24 h after single oral administration) and repeated dosing (3, 7, 14, and 28 days of repeated oral dosage), with four dose levels. The project included a parallel examination of responses using in vitro assays of rat and human hepatocyte culture systems (Affymetrix GeneChip, Rat Expression 230A, Rat Genome 230 2.0 Array and Human Genome U133 Plus 2.0 Array). In 2005 the entire project was moved from the NIHS location to the newly established National Institute of Biomedical Innovation (NIBIO) in the Osaka region. Because of that point the member pharmaceutical companies had their own cumulative data (which were non–Percellome data), to save resources, it was decided to move away from the Percellome quality control and analysis, and instead adopt a common normalization such as the 75 percentile global normalization with superficial quality control. However, the sample processing protocol was not changed, including essential processes of the Percellome method, and retaining Percellome spike data. A repeating of arrays due to bad data quality was discovered by Percellome quality control and is no longer performed (later validation of the data revealed that most of the data were of adequate Percellome quality). For the in vitro studies, on the other hand, the TGP confirmed that the Percellome method is appropriate for normalizing in vitro data, and thus this methodology has remained. In 2007, the second five-year-project was started, again as a government–private sector joint research project by NIBIO, an alliance of 13 Japanese pharmaceutical companies and 2 groups of scientists, including Kanno's group, charged with the development of informatics using the database established in the first five-year project (http://www.tgp.nibio.go.jp/). Because the project is of a proprietary nature due to the membership contracts with the investing companies, the data will gradually become available to the public.

15.3.1.2 The Percellome Project

The Percellome Project to develop a mouse transcriptome began in 2003, one year after to the TGP project was initiated. This Percellome Project, which is supported by grants from the MHLW and other public institutions, has as its mission facilitating the chemical safety processes. The project is run by the Division of Cellular and Molecular Toxicology, Biological Safety Research Center of the NIHS (headed by Jun Kanno). The first study (designated as TTG1) ran from 2003 through