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# Application of a New Genotoxicity Test System with Human Hepatocyte Cell Lines to Improve the Risk Assessment in the Drug Development

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## 1. Introduction

### 1.1 Current situation in the development of pharmaceuticals

A typical testing scheme for a small-molecule therapeutics (outlined in Fig. 1) begins with a large number of compounds and high-throughput assays (Kramer et al., 2007). As the number of viable lead molecules is reduced, incrementally more predictive but lower throughput assays identify those leads with the most drug-like properties and optimal in vitro and in vivo efficacy. Confirmed hit compounds identified in high-throughput screens are evaluated for potency, selectivity, ADME (absorption, distribution, metabolism and excretion), physical and chemical properties, and activity in relevant animal models (Fig. 1). This testing paradigm typically delivers drug-like compounds that have promising pharmacokinetic parameters and efficacy in preclinical models within a 1–2-year cycle time. Compounds that successfully meet preclinical efficacy, ADME, pharmacokinetics and safety criteria are nominated as candidates for formal development. Historically, the move from discovery to development consisted of a discreet hand-off from the ‘discovery’ organization to the ‘development’ organization, and little preclinical safety assessment was performed on lead molecules beyond a few basic in vitro toxicity assays. As toxicity is a primary cause for compound attrition and long development (Kola & Landis, 2004), companies in the past 5–10 years have increasingly integrated safety assessment principles into earlier phases of the drug discovery process.

Also as shown in Fig.1, the costs of R & D for a drug in 2001 were of the order of US \$802 million (DiMasi et al., 2003); current estimates are closer to about US \$900 million; Considerably more of these costs are incurred later in the pipeline, and most of the attrition occurs during full clinical development (Phases II and III). In the other literature, it has been estimated that the average cost associated with the discovery and preclinical evaluation of a single drug candidate were US \$620 million (Rawlins, 2004).

Kola and Landis researched the reason why compounds undergo attrition and how this has changed over time (Kola. & Landis, 2004). In 1991, adverse pharmacokinetic and bioavailability results were the most significant cause of attrition and accounted for ~40% of all attrition. However, in 2000 the major causes of attrition in the clinical trials were lack of efficacy (accounting for approximately 30% of failures) and safety (toxicology in preclinical

development and safety in clinical development accounting for a further approximately 30%). As a result, many companies developing small-molecule therapeutics have adopted a strategy that includes the earlier incorporation of preclinical safety assessment before advancement into regulated preclinical studies.

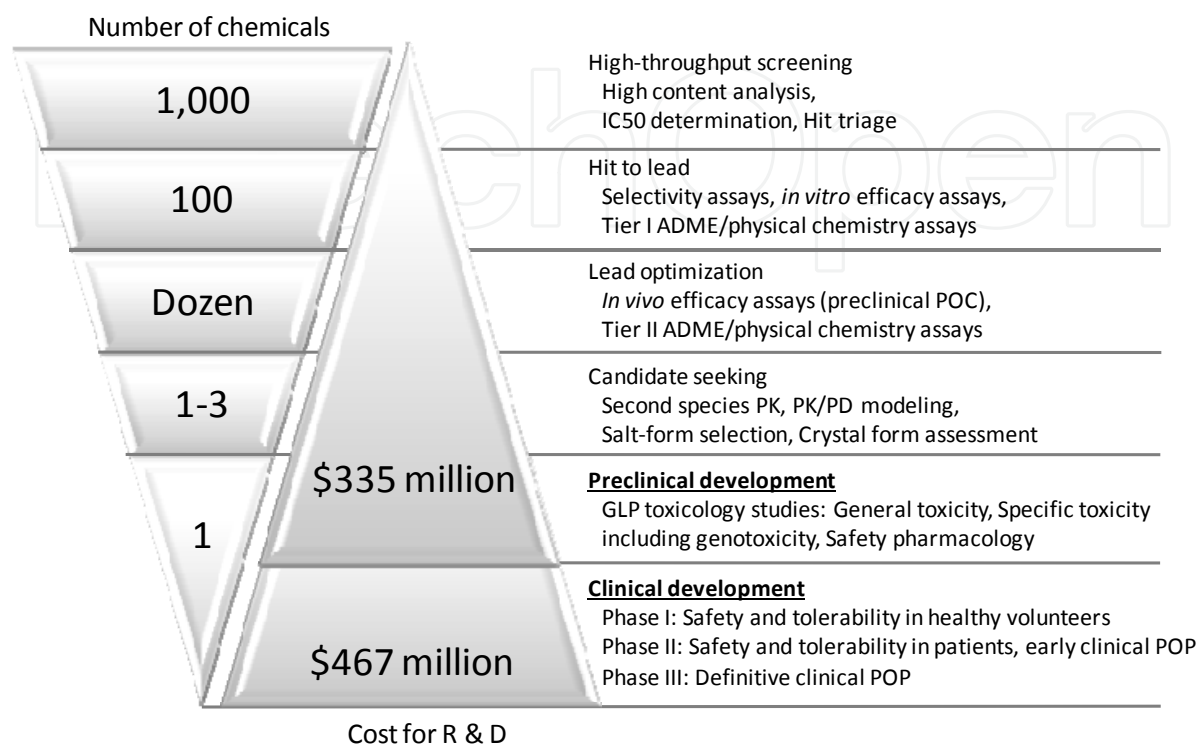


Fig. 1. A typical testing scheme in the development for a small-molecule therapeutics. PK/PD, pharmacokinetic/pharmacodynamic; POC, proof-of-concept; POP, proof-of-principle. ADME; absorption, distribution, metabolism and excretion, GLP; good laboratory practice.

1.2 Safety assessment of pharmaceutical candidates before administration to humans according to the regulatory guidance

Currently, drug companies tend to perform a fairly standard package of nonclinical studies before commencing First-In-Man (FIM) clinical trial investigations with pharmaceuticals. The non-clinical safety study recommendations for the marketing approval of a pharmaceutical usually include single and repeated dose toxicity studies, reproduction toxicity studies, genotoxicity studies, and local tolerance studies. For drugs that have special cause for concern or are intended for a long duration of use, an assessment of carcinogenic potential must be included. Other non-clinical studies include pharmacology studies for safety assessment (safety pharmacology) and pharmacokinetic (ADME) studies. For the conventional, chemically-synthesized small molecules, such a package of studies is in agreement with international regulatory guidance as given by the International Conference on Harmonization (ICH M3-R2) (Table 1). The genotoxic potential has to be assessed comprehensively before administration to humans regardless for both chemically-synthesized small molecules and biotechnology-derived pharmaceuticals. According to the current international guidelines on genotoxicity testing of pharmaceutical candidates (ICH S2A, S2B and M3), a standard battery of tests has to be performed. This

battery generally includes (i) an in vitro test for gene mutation in bacteria; (ii) an in vitro test in mammalian cells with cytogenetic evaluation of chromosomal damage and/or a test that detects gene mutations; (iii) an in vivo test for chromosomal damage using rodent hematopoietic cells. For compounds giving negative results in all 3 of the assays, the completion of this test battery is generally considered to provide a sufficient level of safety in demonstrating the absence of genotoxic activity.

### **1.3 Recent achievement in the in vitro genotoxicity testing**

The most widely used in vitro genotoxicity test is the Ames test (Ames et al., 1975). The relatively simplicity and low cost of the test make it a valuable screening tool for mutagens. However, DNA is naked in the prokaryote and the form of DNA is different from that in eukaryote. Thus, the test using mammalian cell lines has been developed. Chromosomal alterations are quite common in malignant neoplasm, as such the detection of chromosomal abnormalities by test chemicals is considered an excellent test for the assessment of carcinogenic potential. In mammalian cell lines, most of the test systems used the same lines as used in the genotoxicity test.

An important discovery in the understanding of chemical carcinogenesis came from the investigation of the Millers who established that many carcinogens are not intrinsically carcinogenic, but require metabolic activation to be carcinogenic (Miller and Miller, 1947). They demonstrated that azo dyes covalently bind to proteins in liver, leading to the conclusion that carcinogens may bind to proteins that are critical for cell growth control (Miller and Miller, 1947). An additional investigation with other genotoxic carcinogens which requires metabolic activation confirmed that metabolism of the parent compound was necessary to produce a metabolite (activation) that was able to interact with DNA.

In standard in vitro genotoxicity testing, an activation system is included with the purpose of generating electrophilic metabolites that can react with macromolecules including nucleic acids. To address the potential role of metabolism, the induced rat liver S9 has been adopted for in vitro genotoxicity tests as an exogenous activation system for detecting promutagens (Ames et al., 1973, Paolini, 1997). Its initial choice was logical; levels of several cytochrome P450 (CYP) enzymes are elevated after induction, in particular the CYP1A subfamily of enzymes (CYP1A1 and 1A2), which are efficient catalysts of the bioactivation of polycyclic aromatic hydrocarbons and azaarenes, aromatic amines and aflatoxins. These types of compounds were some of the first known and best understood mutagens and the Aroclor 1254-induced rat S9 fraction effectively allowed for their identification as mutagens. Its choice was also logical in that it provided a reliable, robust and readily available bioactivation system at a time when human-derived systems were rare or unavailable. Also, a rodent system can be more easily standardized than an exogenous human derived system that normally would rely on human tissue samples, which are subject to significant biological variation.

### **1.4 Problems in the use of rat liver S9 fraction as a metabolic activation system in vitro genotoxicity testing**

As mentioned in the above sections, the initial choice of rat liver S9 fraction as a metabolic activation system in the in vitro genotoxicity testing was logical. However, it can be questioned if the standard Aroclor-induced rat liver S-9 fraction represents an appropriate surrogate for the metabolic capabilities of humans for the following reasons (Ku et al., 2007; Obach and Dobo, 2008). First, it is now known that the rat and human CYP enzymes can

Menu	Purpose
General Toxicity	
Acute toxicity	To identify doses causing no adverse effect and doses causing major (life-threatening) toxicity.
(Sub) Chronic toxicity	To characterize the toxicological profile of a chemical following repeated administration.
Specific Toxicity	
Genotoxicity	To detect chemicals that induce genetic damage by various mechanisms
Reproductive and developmental toxicity	To reveal any effect of an active chemical on mammalian reproduction and development.
Carcinogenicity	To examine carcinogen that is an agent directly involved in causing cancer.
Immunotoxicology	To detect immune dysfunction resulting from exposure of an organism to a chemical
Local tolerance	To ascertain whether chemicals are tolerated at site in the body.
Safety pharmacology	To investigate the potential undesirable pharmacodynamic effects of a chemical on physiological functions in relation to exposure in the therapeutic range and above.

Table 1. Non-clinical toxicology testing. Toxicological testing is conducted on large numbers of animals of different species in an attempt to predict adverse effects that might be triggered by the drug in humans. Genotoxicity assays are mandatory regulatory studies designed to detect potential mutagens and/or carcinogens.

differ in their substrate specificities and the reactions catalyzed (Guengerich, 1997). Second, with phenobarbital/ 5,6-benzoflavone induction, although the expression levels of CYP1A and 2B enzymes are markedly elevated, others such as CYP3A are affected only in a minor way, whereas others (e.g., CYP2C11) may decrease (Guengerich et al., 1982). Third, the system is set up to favor CYP-mediated metabolism. Some phase II enzymes, such as UDP-glucuronosyltransferases (UGT), glutathione S-transferases (GST), sulfotransferase (SULT), or N-acetyl transferases, are not active in the reduced form of the nicotinamide adenine dinucleotide phosphate (NADPH)-supplemented S9 system (S9 mix) because other cofactors and additives (e.g., uridine diphosphate glucuronic acid, glutathione, acetyl-coenzyme A, etc.) would be needed (Ku et al., 2007; Obach and Dobo, 2008). This can be essential not only for reducing potential false positives (e.g., reactive electrophiles that would be rapidly quenched by conjugation in vivo before being able to cause mutation) but also for false negatives because some conjugation reactions can yield metabolites that are more reactive than their substrate (e.g., sulfation of N-hydroxy-2-acetylaminofluorene or acetylation of N-hydroxylated heterocyclic amines) (Dashwood, 2002; Ku et al., 2007). The rat liver S9 mix may represent an incomplete picture of the metabolism that can occur in vivo (Fig. 2).

To detect those genotoxic potential, some genotoxic metabolites have to be formed in the target cell by endogenous enzymes that are not represented in standard in vitro test systems. One of the major reasons is that certain types of active metabolites (including many



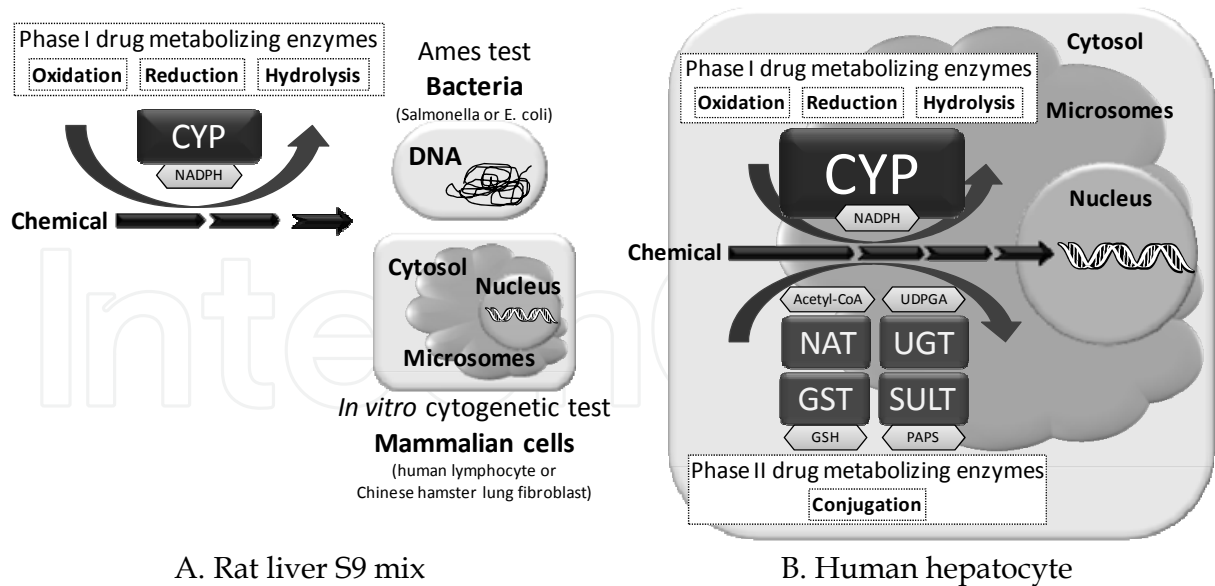


Fig. 2. Detection of genotoxicity using rat liver S9 mix compared to the expression of genotoxicity occurring hepatocyte. A. The detection method of genotoxicity is currently used a rat liver S9 fraction in the *in vitro* genotoxicity testing. B. The expression of genotoxicity is occurred in the hepatocyte. It can be questioned if the standard Aroclor-induced rat liver S-9 fraction represents an appropriate surrogate for the metabolic capabilities of humans. However, in human hepatocyte, the genotoxicity was expressed through the comprehensive metabolic pathway including phase I and phase II drug-metabolizing enzymes. Thus human hepatocyte can be a good genotoxicity test system reflecting human metabolism. In addition, human hepatocyte has complete metabolism consisting oxidation, reduction, hydrolysis and conjugation, whereas rat liver S9 mix is set up to favor CYP-mediated metabolism and the other enzymes present in the system that could be responsible for detoxification of reactive intermediates are not supplemented with the appropriate cofactors (e.g., UGT, GST, methyl transfereases, etc), thus potentially providing an unrealistic metabolic profile.

short-life phase-2 metabolites) will not penetrate cell membranes sufficiently. If these types of metabolites are generated extracellularly, most *in vitro* genotoxicity testing showed negative results since the access to nuclear DNA was difficult. Another reason is that the diffusion pathways are longer for externally generated active metabolites resulting in more opportunities for alternative chemical reactions (e.g. with components of S9 or cell membranes) than for metabolites formed in the target cell. Electrophilic metabolites of a chemical bind to serum or S9 proteins (forming protein adducts) and this reduces the rate of binding to DNA to form DNA adducts.

Therefore it is considered that the use of genetically engineered cells is the most reliable remedy to avoid the shortcomings of the extracellular metabolic activation systems such as human S9 and recombinant human CYPs (Fig. 3). To be useful tools for the prediction of drug metabolism and toxicity in the human liver, Yoshitomi et al. established a series of HepG2 transformants expressing the cytochromes 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 with the apparent  $V_{max}$  values for characteristic substrates (Table 2) in a previous work (Yoshitomi et al., 2001). Since most human drug metabolism is catalyzed by CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, this HepG2 transformant system would be

more suitable for the genotoxic assessment of chemicals than the induced rat liver S9 fraction in the routine screening when considering human hepatic metabolism in the future. Therefore in the present thesis, we explored the usefulness of a series of 10 transformants expressing major human CYP isoforms such as CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 in HepG2 cells established previously to assess the genotoxicity of metabolites (Fig.3) (Hashizume et al., 2009; Hashizume et al., 2010).

Name of transformant	Expressed CYP isoform	Catalytic reaction measured	Kinetic analysis		
			Transformant Km (μM)	Human liver microsomes Km (μM) <sup>a</sup>	Transformant Vmax (pmol/min/mg)
Hepc/1A1.4	CYP1A1	7-Ethoxyresorufin O-deethylation	0.25	0.19	56
Hepc/1A2.9	CYP1A2	7-Ethoxyresorufin O-deethylation	0.72	0.39	2
Hepc/2A6L.14	CYP2A6	Coumarin 7-hydroxylation	5.1	2.3	812 000
Hepc/2B6.68	CYP2B6	7-Ethoxycoumarin O-deethylation	81	–	80 000
Hepc/2C8.46	CYP2C8	Taxol 6-hydroxylation	7.4	24	9400
Hepc/2C9.1	CYP2C9	Tolbutamide 4-hydroxylation	45	120	25 000
Hepc/2C19.12	CYP2C19	(S)-Mephenytoin 4'-hydroxylation	8.3	16	140 000
Hepc/2D6.39	CYP2D6	Bufuralol 1'-hydroxylation	17	40	14
Hepc/2E1.3-8	CYP2E1	p-Nitrophenol hydroxylation	88	30	120
Hepc/3A4.2-30	CYP3A4	Testosterone 6β-hydroxylation	96	89	71

Table 2. Characteristics of a series of 10 transformants expressing major human CYP isoforms such as CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 in HepG2 cells (Yoshitomi et al., 2001). a). Iwata et al., 1998.

2. Advantages of HepG2 transformants expressing a series of human CYP isoforms in the in vitro genotoxicity testing

The need for metabolism, especially CYP-mediated one, for in vitro genotoxicity testing has been recognized for many years. Most target cells for genotoxicity assays lack sufficient CYP to activate many promutagens. Therefore, extracellular systems are commonly utilized to provide metabolism. The rat liver S9 fraction contains multiple CYPs and have been used with many target cell types in genotoxicity testing. However, this metabolic activation system suffers from certain limitations; (1) generation of reactive metabolites outside of the

target cell, (2) requirement of high exposure concentration to compensate for short exposure times and (3) differences in metabolism compared to intact tissues. To overcome these limitations, the use of genetically engineered stable cell lines expressing CYPs has studied. The liver is the tissue containing the greatest concentrations of drug-metabolizing enzymes, such as the CYP enzyme family, among many others. In human liver, about 70% of the total CYP could be accounted for by CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1 and 3A proteins (Rendic and Guengerich, 1997). In the extrahepatic organs such as lungs and kidneys, CYP1A1 is present. CYPs catalyze to form toxic reactive intermediates from many chemicals. As it is well known that there are significant quantitative and qualitative differences between laboratory animals and humans in their CYP subtypes, it is necessary to use human CYP isoforms to predict the metabolism and toxicity of chemicals in humans.

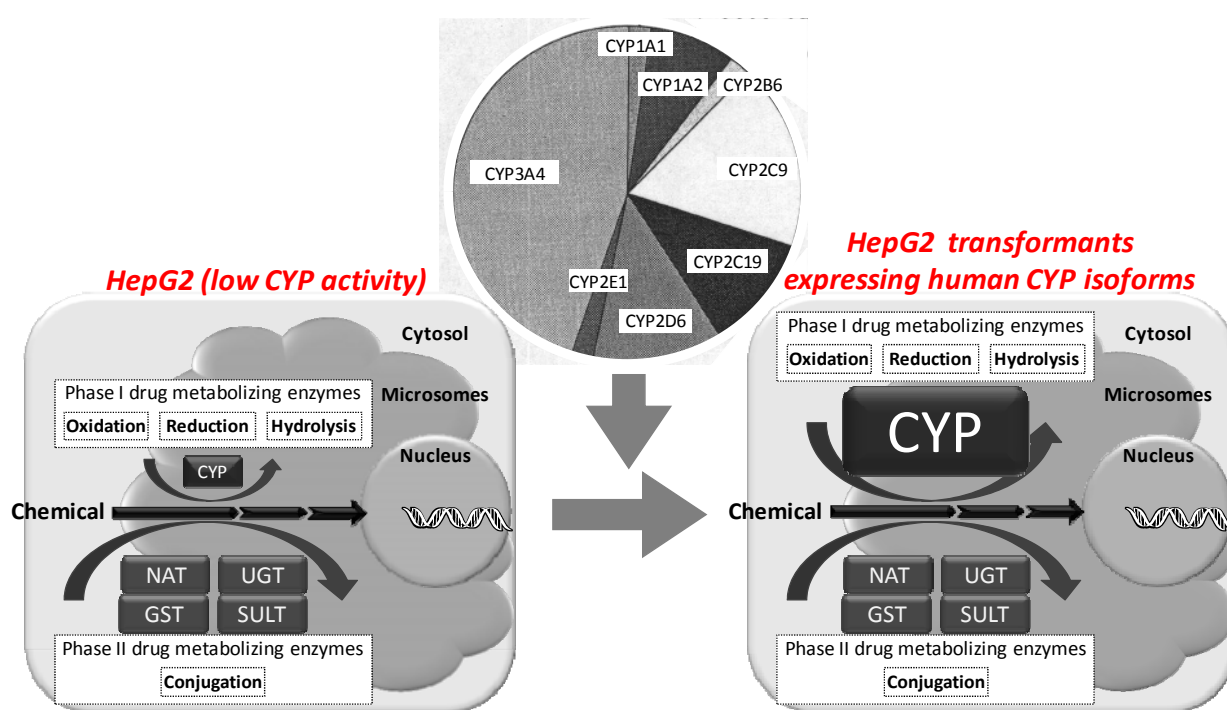


Fig. 3. HepG2 transformants expressing human CYP isoforms relating drug metabolism. The pie chart shows the contribution of each CYP isoform to the human drug metabolism (Lewis, 2004). It has been concerned about the low CYP activities in HepG2 cells, so we established the HepG2 transformant system expressing a series of human CYP isoforms.

In vitro systems, particularly those derived from liver, are a commonly applied tool to gain a better understanding of the metabolism of drugs and other xenobiotics. Also in genotoxicity, a number of publications are discussed which are relevant for the use of human derived liver cell lines. One of the most promising lines is the human HepG2 cell line, originally isolated by Aden et al. in 1972 from a primary hepatoblastoma of an 11-year-old Argentine boy. This cell line retains many of the specialized functions normally lost by primary hepatocytes in culture such as secretion of the major plasma proteins. Since several publications alerted that HepG2 lacks a few drug-metabolizing enzymes such as CYP2E1 and 1A2, transfectants constitutively expressing these enzymes have been constructed. Cederbaum and coworkers developed a line, which possesses CYP2E1 activity and used it in a number of mechanistic studies (for review see Kessova and Cederbaum, 2003).



In our previous study, Yoshitomi et al. has established a series of HepG2 transformants expressing the CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (Yoshitomi et al., 2001). Since most human drug metabolism is catalyzed by CYP1A2, 2C8, 2C9, 2C19, 2D6, 2E1 and P3A4, this HepG2 transformant system would be more suitable for the genotoxic assessment of chemicals than the induced rat liver S9 fraction in the routine screening when considering human hepatic metabolism in the future.

Therefore, we examined the advantages of HepG2 transformants expressing a series of human CYP isoforms as a better alternative for metabolic activation system in the in vitro genotoxicity testing. In section 2.1, the sensitivity of this system to detect genotoxicity requiring CYP activation was confirmed in the in vitro micronucleus (MN) tests using well-studied model chemicals. In section 2.2, this system allowed us to investigate the genotoxicity of model chemicals for which the contributing CYP isoforms, especially those mediated by CYP1A2 or 3A4 which is known to metabolize many drugs in humans, have not yet been identified. In section 2.3, the relevance of the interaction between phase I and phase II drug-metabolizing enzymes, e.g., UGT, GST, and SULT, in the test system was demonstrated in a MN test of tamoxifen or safrole, which has been reported to be metabolized by enzymes of both phases.

## **2.1 Basic characteristics of the HepG2 transformants on genotoxic assessment and confirmation of their sensitivity with model chemicals requiring CYP activation**

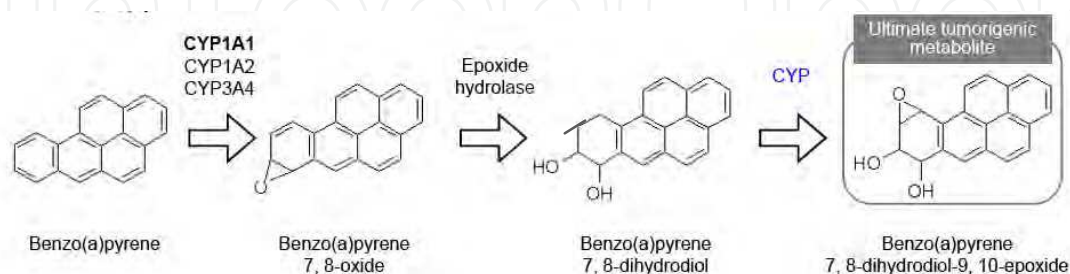
HepG2 transformants were checked for their response to known chemicals in which the CYP isoforms responsible for which genotoxicity has been reported. As model chemicals, we selected BP, DMBA, CP and ifosfamide. In BP metabolism, CYP1A1 showed clearly the highest activity among the hepatic CYP isoforms reported (Fig. 4 A). Significant formation of some metabolites was also observed with CYP1A2 and 3A4 (Bauer et al., 1995). In the DMBA metabolism (Fig. 4 B), it had been shown that CYP1A1 had clearly the highest activity among the hepatic CYP isoforms (Shimada and Fujii-Kuriyama, 2004; Shou et al., 1996) and that significant formation of some metabolites was also observed with CYP1A2, 2B6 and 2C9 (Shou et al., 1996). CP is efficiently metabolized by CYP2B6, 2C9 and 3A4 (Jing et al., 2006, Chang et al., 1993)(Fig. 4 C). Ifosfamide had been demonstrated to be efficiently metabolized by CYP2B6 and 3A4 (Chang et al., 1993; Jing et al., 2006)(Fig. 4 D).

Firstly, BP treatment produced MN induction in the transformants expressing CYP1A1, CYP1A2 and CYP3A4 (Fig. 5 A). These CYP isoforms were reported to be responsible for BP activation (Bauer et al., 1995). Secondly, MN induction by DMBA in a HepG2 transformant expressing CYP1A1 was significantly higher than those in HepG2 and Hepc-Mock cells (Fig. 5 B). CYP1A1 is known to be the most active among the CYP isoforms to metabolize DMBA (Shou et al., 1996). Thirdly, CP treatment caused MN induction in the transformants expressing CYP1A2, 2B6, 2C9 and 3A4 (Fig. 5 C). CYP2B6, 2C9 and 3A4 are reported to be involved the metabolic activation of CP (Jing et al., 2006; Chang et al., 1993). Finally, in the treatment with ifosfamide, significant MN inductions were found in the transformants expressing CYP1A1, 2C9, 2C19, 2D6 and 3A4 (Fig. 5 D). Ifosfamide had been demonstrated to be efficiently metabolized by CYP2B6 and 3A4 (Chang et al., 1993; Jing et al., 2006). These results showed HepG2 transformants system have the appropriate sensitivity to detect genotoxicity requiring CYP activation tests using well-studied model chemicals.

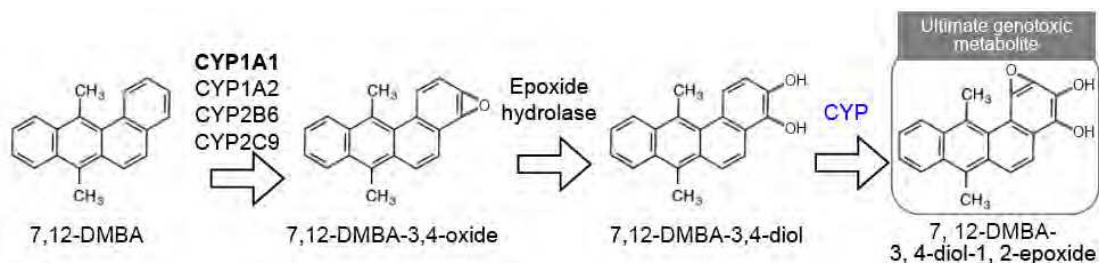
In addition, DMBA treatment unexpectedly produced MN induction in some transformants expressing CYP2C9, 2D6 and 3A4 (Fig. 4 B). However, it was reported that CYP2C9 was

capable of metabolizing DMBA while CYP2D6 and 3A4 exhibited relatively low metabolic activity to DMBA (Shou et al., 1996). Similarly, significant MN inductions by ifosfamide were found in the transformants expressing CYP1A1, 2C9, 2C19, 2D6 and 3A4 (Fig. 4 D). Ifosfamide is mainly metabolized by CYP2B6 and 3A4 (Chang et al., 1993; Jing et al., 2006). CYP1A1 and 2C19 are relatively minor CYP isoforms to DMBA metabolic activation, but the involvement in the genotoxicity of ifosfamide metabolite of these CYP isoforms were demonstrated in the present study (Fig. 4 D).

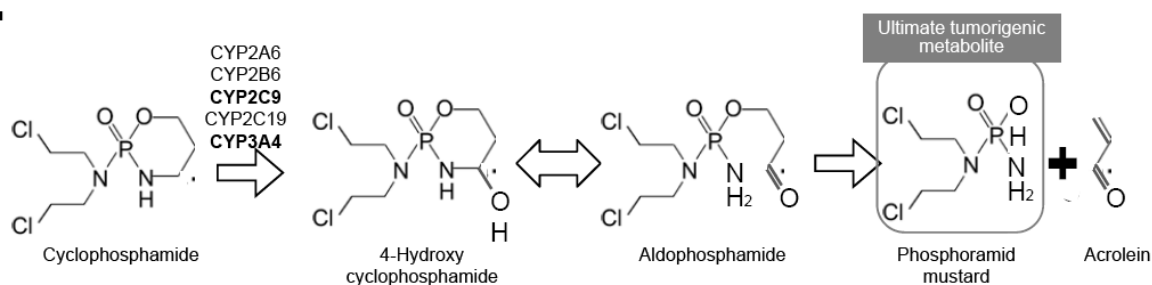
A



B



C



D

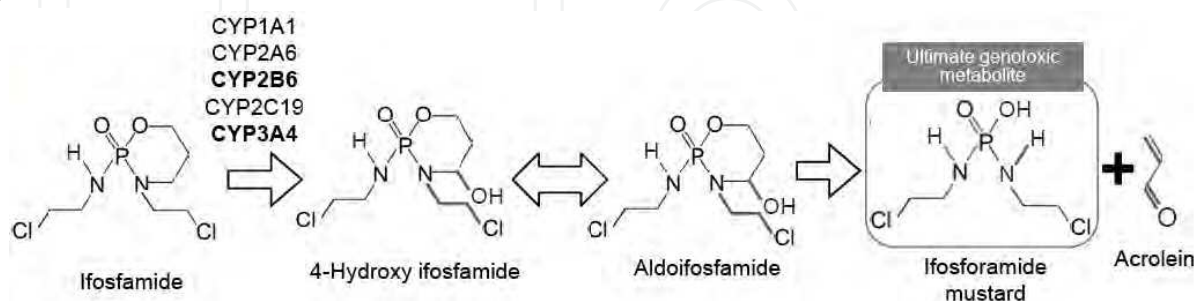


Fig. 4. Metabolic activation pathway of BP (A), DMBA (B), CP (C) and ifosfamide (D). A). BP is mainly metabolized by CYP1A1 to produce the benzo(a)pyrene 7, 8-oxide. B). DMBA (7, 12-DMBA) is mainly metabolized by CYP1A1 to produce the 7, 12-DMBA-3, 4-oxide. C). Cyclophosphamide (CP) is mainly metabolized by CYP2C9 and 3A4 to produce the 4-hydroxy cyclophosphamide. D). Ifosfamide is mainly metabolized by CYP2B6 and 3A4 to produce the 4-hydroxy ifosfamide.

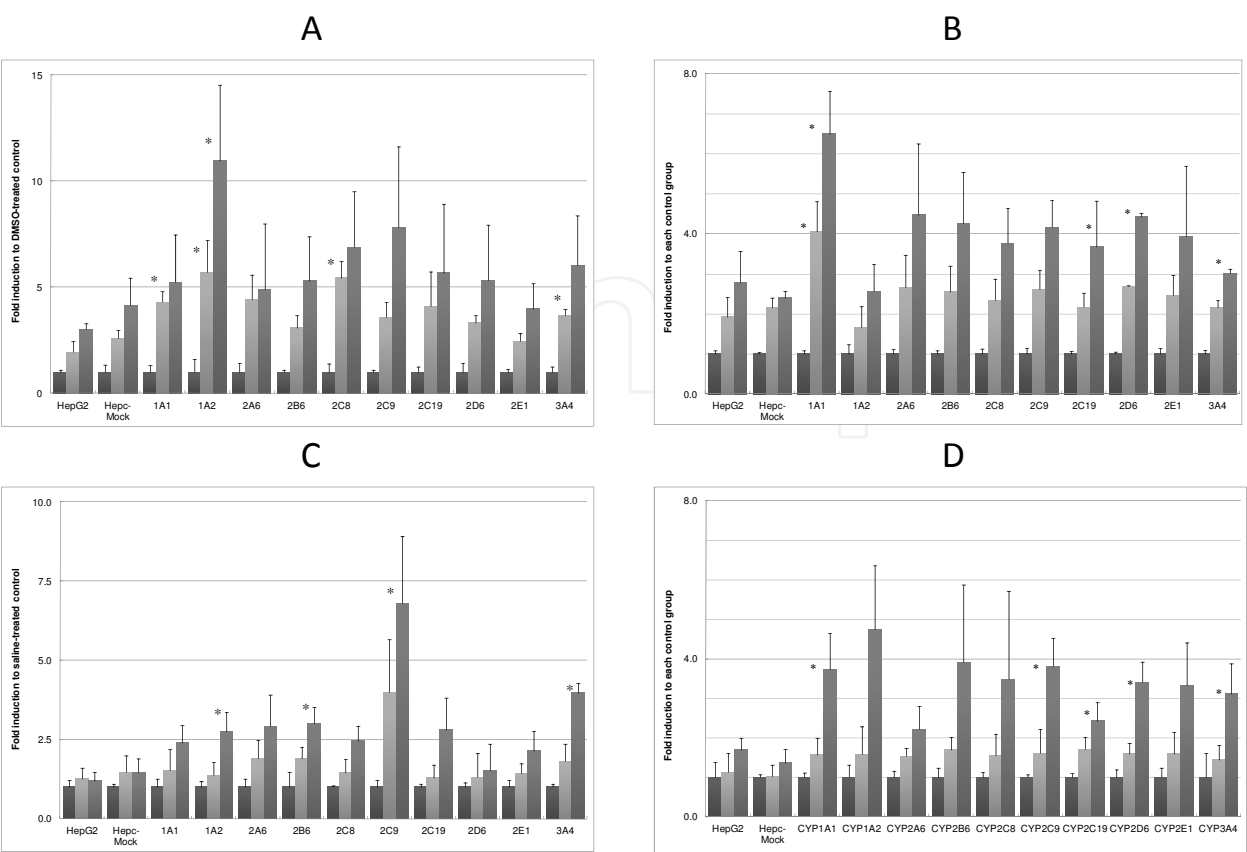


Fig. 5. Micronucleus induction of BP (A), DMBA (B), CP (C) and ifosfamide (D) by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. A). The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (heavy gray bars), 200 ng/ml (light gray bars) or 400 ng/ml (medium gray bars) BP. B). The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (heavy gray bars), 78 ng/ml (light gray bars) or 156 ng/ml (medium bars) DMBA. C). The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% saline (heavy gray bars), 1 mg/ml (light gray bars) or 2 mg/ml (medium gray bars) CP. D). The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% saline (heavy gray bars), 500  $\mu\text{g}/\text{ml}$  (light gray bars) or 1000  $\mu\text{g}/\text{ml}$  (medium gray bars) ifosfamide. Values were normalized with the mean DMSO- or saline-treated control value of 3 experiments for each transformant. Each bar represents the mean  $\pm$  S.D. Data were tested using Student's t-test when the variance was homogeneous or Aspin & Welch t test when the variance was heterogeneous (\* $P<0.05$ , compared with Hepc-Mock).

Based on the results in section 2.1, it was showed that genotoxic metabolites could be produced by not only the most active CYP isoform but also by other less active CYPs and that this transformant system could detect the genotoxic potential of chemicals requiring CYP activation not tested routinely in the early stage of drug development. One of major advantages of our system is the variety of human CYP isoforms. When considering replacement of the rat induced liver S9 fraction, increasing the number of the principal CYP isoforms would be desirable in order to cover the diverse CYP activities. As mentioned in the general introduction, our HepG2 system includes CYP1A1, 1A2, 2A6, 2B6,

2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. Seven CYP isoforms (1A1, 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4) account for 95% of this activity and with 3A4 responsible for over 65% of the metabolism of current therapeutic agents. During phase I metabolism in humans, 90% of all drugs are oxidized by CYP isoforms with different substrate selectivities. Thus, our HepG2 transformant system seems to cover most human drug metabolism mediated by human CYP isoforms but not by rat ones. Based on the results obtained in section 2.1, it was demonstrated that our HepG2 transformant system has an appropriate sensitivity for well-studied chemicals which requires CYP activation for their genotoxicity.

For assessing the genotoxicity of chemicals with human metabolism, our HepG2 transformant system has more appropriate characteristics than other established cell lines used in toxicological testing and reviewed by Sawada and Kamataki (Sawada and Kamataki, 1998). Our HepG2 transformants are derived from hepatocyte that possesses other factors necessary for the function of CYP. Generally, the reactions catalyzed by CYP molecules require the presence of NADPH-CYP reductase and cytochrome b5 to support some CYP-mediated reactions. HepG2 has been shown to have NADPH-CYP reductase activity and cytochrome b5, although the levels are lower than those of human liver (Waxman et al., 1991; Patten et al., 1992). Therefore, our HepG2 transformant system does not need co-expression of reductase and/or cytochrome b5 with CYP enzymes.

## 2.2 An exploration using HepG2 transformant to identify the CYP isoforms contributing to the genotoxicity by novel chemicals

Given the multiplicity of CYP isoforms and the importance of other enzymes (hydrolases, transferase, etc.) in the metabolism of chemicals, there are two possible approaches to engineering cell lines. The introduction of single enzymes allows simple controlled mechanistic studies of the role of an individual enzyme in the metabolic activation of chemicals. Such system can also be viewed as the replacement of the laborious CYP purification/reconstitution analyses with a panel of engineered cells. However, to specify CYP isoform(s) involved in the activation of a certain chemical of unknown metabolism, a set of cell lines individually expressing the different CYP isoforms is needed. A series of HepG2 transformants expressing major 10 human CYP isoforms is a valuable tool, since most human hepatic drug metabolism is catalyzed by these expressing CYP isoforms.

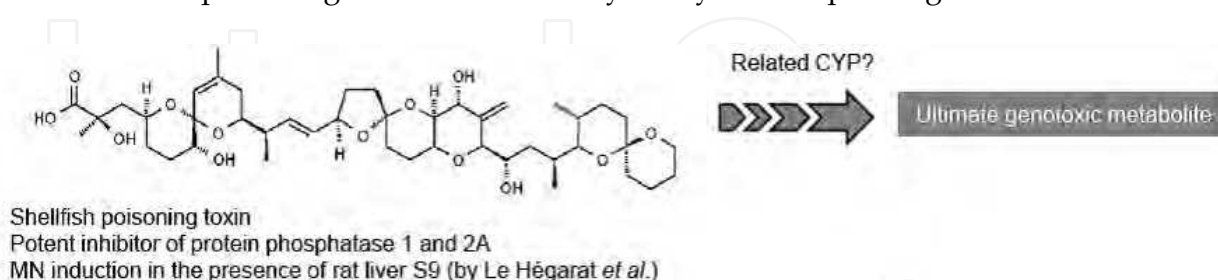


Fig. 6. Chemical structure and significant biological features of okadaic acid. This chemical is a shellfish poisoning toxin and known as a potent phosphatase 1 and 2A. This chemical was reported to be induced micronucleus in the presence of rat liver S9 mix (Hégarat et al., 2004).

In this section, the following possibility was elucidated that the set of transformants can be used for screening for the genotoxicity of newly developed pharmaceutical candidates of unknown metabolism in human *in vivo*. As model chemicals, we selected okadaic acid (OA) and  $\beta$ -endosulfan.



Hégarat et al. found that OA enhanced formation of MN in the presence of a metabolic activation system (Hégarat et al., 2004), although the CYP isoforms involved in the MN induction were not reported. Thus we selected OA as a model chemical to evaluate the ability of our system to investigate which CYP isoform is involved in producing unknown genotoxic metabolites.

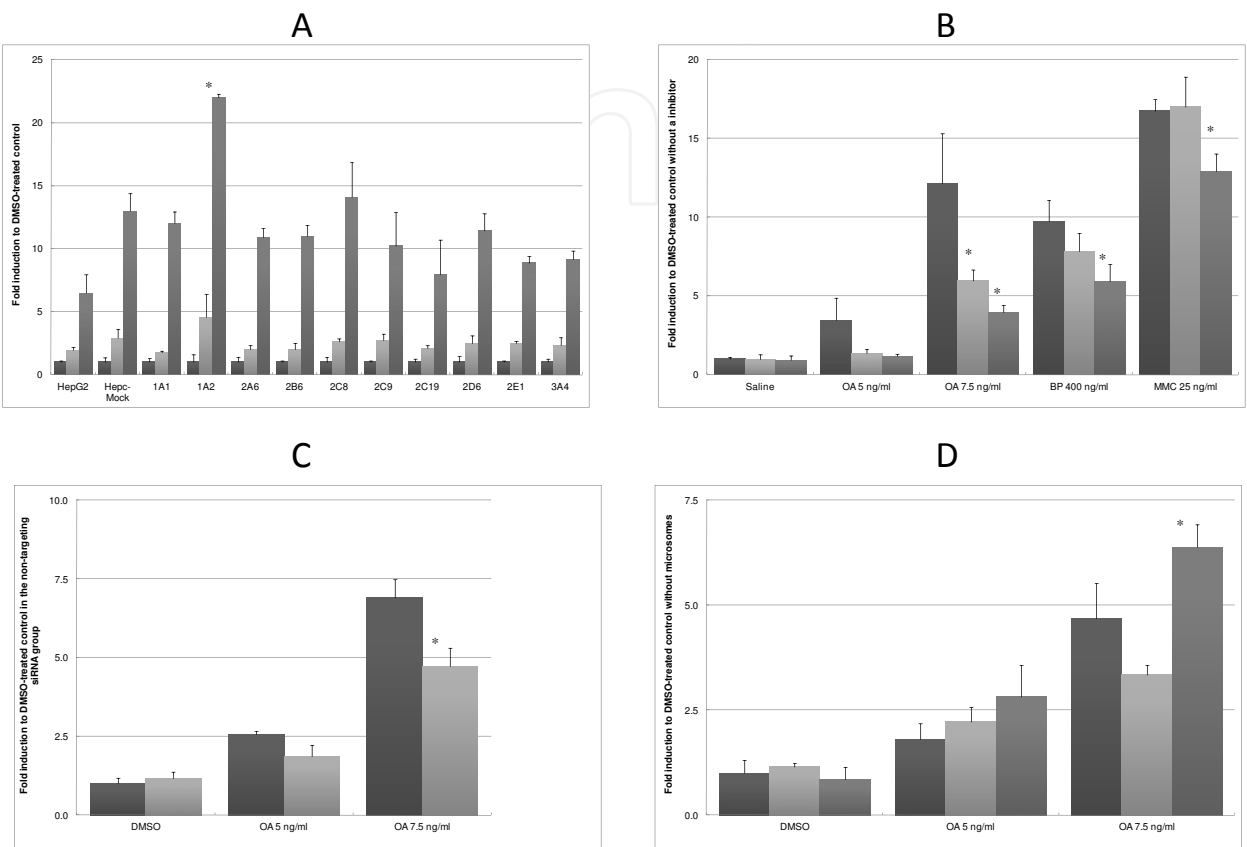


Fig. 7. Micronucleus induction of OA. A). Micronucleus induction of OA by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (heavy gray bars), 5 ng/ml (light gray bars) or 10 ng/ml (medium gray bars) OA. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value of 3 experiments in each transformant. Each bar represents the mean  $\pm$  S.D. of 3 experiments. Data were tested using Student's t-test (\* $P<0.05$ , compared with Hepc-Mock). B). Effects of furafylline, a CYP1A2 specific inhibitor for micronucleus induction by various chemicals in the transformant expressing CYP1A2. The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO, 5 or 7.5 ng/ml OA, 400 ng/ml BP and 25 ng/ml MMC in the absence (heavy gray bars) or presence of 5  $\mu$ M (light gray bars) or 50  $\mu$ M (medium gray bars) furafylline. Values were normalized with the mean DMSO-treated control value without an inhibition of three experiments in each transformant. Each bar represents the mean  $\pm$  S.D. of 3 experiments. Data were tested using Student's t-test (\* $P<0.05$ , compared with no inhibition). C). Effects of siRNA to CYP1A2 on micronucleus induction by OA in the transformant expressing CYP1A2. The cells ( $1\times 10^5$  cells) were seeded onto a 24-well plate for 24 h in the presence of 50 nM siRNA for non-targeting (heavy gray bars) or CYP1A2 (light gray bars). Medium was



changed with a fresh one containing 0.5% DMSO, 5 and 7.5 ng/ml OA. After 48 h, the cells were collected and sampled. Values were normalized with the mean DMSO-treated control value without siRNA of three experiments in each transformant. Each bar represents the mean  $\pm$  S.D. of 3 experiments. Data were tested using Student's t-test (\* $P < 0.05$ , compared with non-targeting siRNA). D). Effects of external metabolic activation system for CYP1A2 on micronucleus induction by okadaic acid in Hepc. The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO, 5 or 7.5 ng/ml OA in the absence (heavy gray bars) or presence of Insect Cell Control Supersomes<sup>TM</sup> (light gray bars) or Human CYP1A2 Supersomes<sup>TM</sup> (medium gray bars). Values were normalized with the mean DMSO-treated control value without microsomes of 3 experiments in each transformant. Each bar represents the mean  $\pm$  S.D. of 3 experiments. Data were tested using Student's t-test (\* $P < 0.05$ , compared with the control microsomes).

OA significantly increased the fold induction of MN in the transformant expressing CYP1A2 compared with that obtained in Hepc-Mock (Fig. 7 A). Furthermore, inhibitory effects of a specific inhibitor of CYP1A2 and siRNA to CYP1A2 on MN induction by OA were shown (Figs. 7 B and 7 C, respectively). Moreover, co-treatment with OA and microsomes expressing CYP1A2 showed MN induction in Hepc-Mock cells (Fig. 7 D). These results indicated that MN induction by OA could be associated with the presence of CYP1A2 activity, suggesting that CYP1A2 is involved in the genotoxic activation of OA.

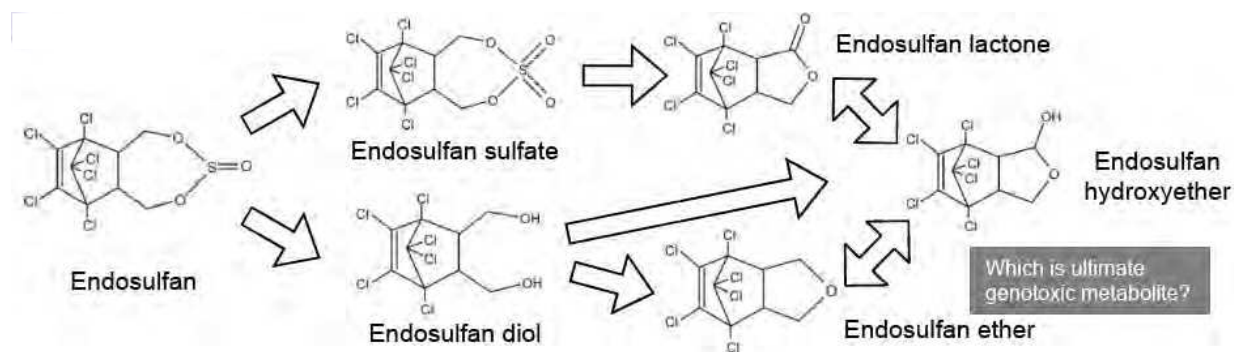


Fig. 8. Proposed metabolic pathway of  $\beta$ -endosulfan. In mammalian systems,  $\beta$ -endosulfan is metabolized to endosulfan sulfate, which is the most persistent metabolite, and also to endosulfan diol, which is further metabolized to endosulfan ether, hydroxyether, and lactone [WHO, 1999].

$\beta$ -Endosulfan, shown in Fig. 8, is also reported to induce MN in HepG2 cells, suggesting that CYP activation might be involved in the MN induction (Lu et al., 2000); however the contributing CYP isoform to induce MN has not yet been investigated, to the best of our knowledge. Therefore we examined whether a series of HepG2 transformants could identify the CYP isoform contributing to the MN induction by  $\beta$ -endosulfan as a model chemical.

$\beta$ -Endosulfan significantly increased the fold induction of MN in the transformant expressing CYP3A4 compared with that obtained with the transformant Mepc-Mock (Fig. 8 A). Furthermore, inhibitory effects of a specific inhibitor of CYP3A4 and of siRNA to CYP3A4 on MN induction by  $\beta$ -endosulfan were shown (Figs. 8 B and 8 C, respectively). The activity of CYP3A4 in the transformant using the luminogenic substrate demonstrated that these inhibitory conditions decreased the activity to approximately 10% compared to control level. These results indicated that MN induction by  $\beta$ -endosulfan could be

associated with the presence of CYP3A4 activity and suggested that CYP3A4 is involved in producing the genotoxic metabolites of  $\beta$ -endosulfan.

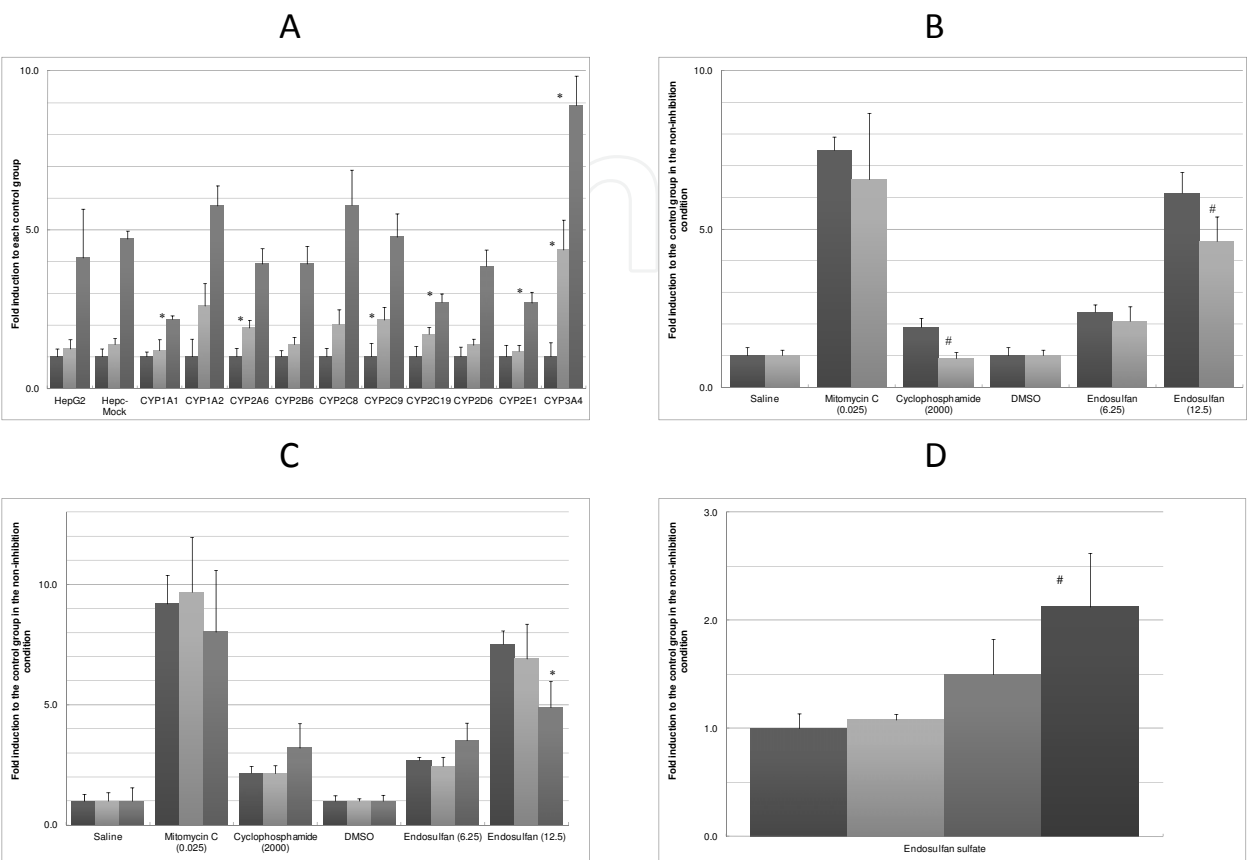


Fig. 9. Micronucleus induction of  $\beta$ -endosulfan by CYP3A4-mediated activation. a). A micronucleus induction of  $\beta$ -endosulfan by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (heavy gray bars), 6.25  $\mu\text{g}/\text{ml}$  (light gray bars) or 12.5  $\mu\text{g}/\text{ml}$  (medium gray bars)  $\beta$ -endosulfan. b). Effects of ketoconazole, a CYP3A4 specific inhibitor for micronucleus induction by various chemicals in the transformant expressing CYP3A4. The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% saline, 100 ng/ml mitomycin C, 1000  $\mu\text{g}/\text{ml}$  cyclophosphamide, 0.5% DMSO, 6.25 or 12.5  $\mu\text{g}/\text{ml}$   $\beta$ -endosulfan in the absence (heavy gray bars) or presence (light gray bars) of 1  $\mu\text{M}$  ketoconazole. c). Effects of siRNA to CYP3A4 on micronucleus induction by various chemicals in the transformant expressing CYP3A4. The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 8 h in absence (heavy gray bars) or the presence of 50 nM siRNA for Negative control (light gray bars) or CYP3A4 (medium gray bars). d). Micronucleus induction of endosulfan sulfate in Hepc-Mock, the transformant expressing an empty vector only. The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (heavy gray bars), 8  $\mu\text{g}/\text{ml}$ , (light gray bars), 10  $\mu\text{g}/\text{ml}$  (medium gray bars) or 12.5  $\mu\text{g}/\text{ml}$  (solid bars) endosulfan sulfate. Statistical analysis was done in the same procedure as Fig. E, except for the Student's t- test ( $\#P < 0.05$ , compared with the DMSO-treated control group) in Fig. F d).

Lee et al. reported that  $\beta$ -endosulfan is metabolized by CYP3A4 based on the results in the study with CYP isoform-selective inhibitor in human liver microsomes and with the incubation study of cDNA-expressed enzymes (Lee et al., 2006). They have also reported that human liver microsome incubation of  $\beta$ -endosulfan in the presence of NADPH resulted in the formation of endosulfan sulfate (Lee et al., 2006). Based on these reports, we examined the genotoxicity of endosulfan sulfate in the Hepc-Mock cells in order to investigate whether this sulfate is the metabolite that induces MN in the  $\beta$ -endosulfan-treated transformant expressing CYP3A4. As shown in Fig. Fd, endosulfan sulfate induced MN with statistical significance at 12.5  $\mu$ g/ml. This result demonstrated that endosulfan sulfate was the genotoxic metabolite and that this metabolite was formed by CYP3A4 in the transformant treated with  $\beta$ -endosulfan.

Based on the results obtained in the OA and  $\beta$ -endosulfan treatments, it was clearly demonstrated that the HepG2 transformant system was able to identify the CYP isoform relating to the genotoxicity of chemical metabolite(s) and was useful to elucidate the genotoxicity of a new chemical or a drug candidate in the presence of the metabolic activation system.

More effort as for CYP induction is necessary, but the results obtained in the present study demonstrated the availability of these transformants expressing human CYP to elucidate the genotoxic potential of the chemicals that require metabolic activation to create risk to humans. In order to validate these transformants, an additional study is in progress with more chemicals that have been well studied in the metabolic activation or inactivation by CYP enzymes.

These results clearly demonstrated that the HepG2 transformant system was able to identify the CYP isoform related to the genotoxicity of chemical metabolite(s) and was useful to elucidate the genotoxicity of a new chemical or a drug candidate in the presence of the metabolic activation system.

### **2.3 Genotoxic assessment of chemicals metabolized by phase I and phase II drug-metabolizing enzymes**

As mentioned in the Introduction section, to detect chemicals which require bioactivation to electrophiles to exhibit a genotoxic and carcinogenic response, the standard in vitro genotoxicity testing also include incubation of the test chemicals with liver microsomal or S9 fractions, as activation systems so that chemically stable xenobiotics can be converted to reactive electrophiles (Malling, 1971; Ames et al., 1973; Levin et al., 1984). However, it is possible that the Aroclor induced rat liver S9 system is not the most appropriate metabolite generation system for detecting drugs that may pose a carcinogenic hazard to humans. This is because the system is set up to favor CYP-mediated metabolism and the other enzymes present in the system that could be responsible for detoxication of reactive intermediates are not supplemented with the appropriate cofactors (e.g., UGT, GST, methyl transferases, etc), thus potentially providing an unrealistic metabolic profile. In a recent work by Obach and Dobo, it was revealed that many human in vivo metabolites arise via conjugation reactions with the limited 16 drugs (Obach and Dobo, 2008). This can be important not only for reducing potential false positives (e.g., reactive electrophiles that would be rapidly quenched by conjugation in vivo before being able to cause mutation) but can also be important for false negatives because some conjugation reactions can yield metabolites that are more reactive than their substrate (e.g., sulfation of aliphatic alcohols or glucuronidation of carboxylic acids; [Glatt et al., 1998; Sallustio et al., 2006]).

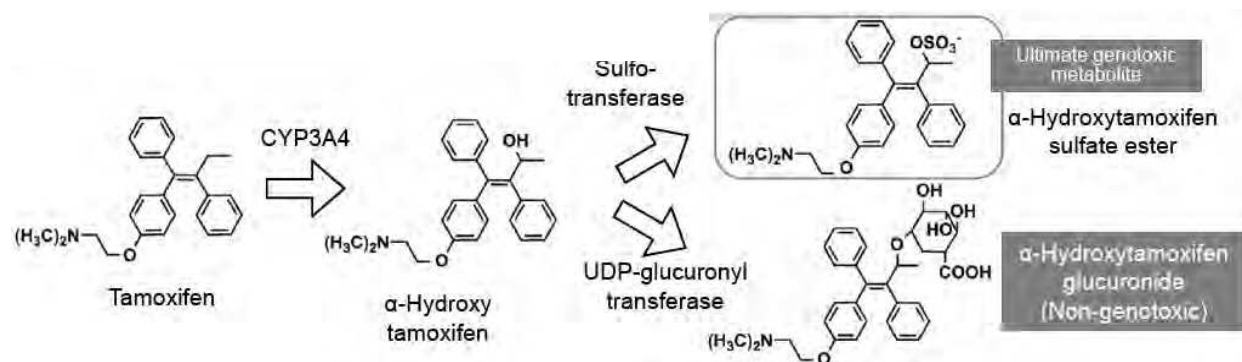


Fig. 10. Metabolic activation and inactivation pathways of tamoxifen.

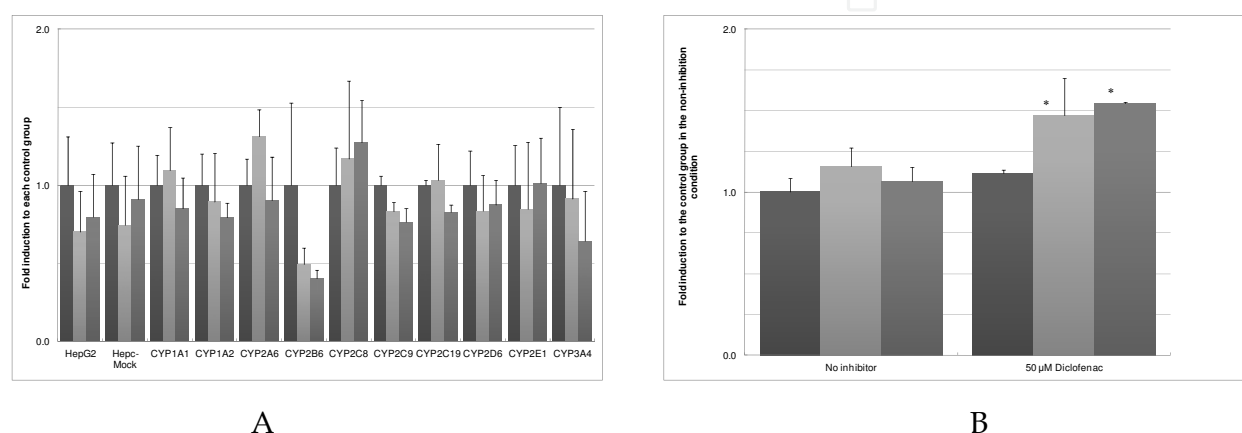


Fig. 11. Micronucleus induction of tamoxifen by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (A), and effects of a phase II inhibitor on micronucleus induction by tamoxifen (B). A). The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (heavy gray bars), 1  $\mu\text{g}/\text{ml}$  (light gray bars) or 2  $\mu\text{g}/\text{ml}$  (medium gray bars) tamoxifen. No statistically significant increase was observed when compared with both HepC-Mock and HepG2. B). The transformant cells expressing CYP3A4 ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (heavy gray bars), 2  $\mu\text{g}/\text{ml}$  (light gray bars) or 3  $\mu\text{g}/\text{ml}$  (medium gray bars) tamoxifen in the absence or presence of 50  $\mu\text{M}$  diclofenac, a UGT inhibitor.

In order to evaluate the relevance of the interaction between phase I and phase II drug-metabolizing enzymes in the test system, the transformants were treated with tamoxifen as shown in Fig. 10. Tamoxifen is reported to be metabolized by CYP3A4 to  $\alpha$ -hydroxytamoxifen and further metabolized by SULT to  $\alpha$ -hydroxytamoxifen sulfate ester as the putative reactive intermediate (Brown, 2009; White, 2003; Zhao et al., 2009). This intermediate reacts with the exocyclic amino group of guanines (the major reaction) and adenines (a minor reaction) in DNA (Osborne et al., 1996). UGT plays a detoxification role through the glucuronidation of  $\alpha$ -hydroxytamoxifen (Brown, 2009; White, 2003; Zhao et al., 2009).

As shown in Fig. 11 A, tamoxifen did not significantly induce MN at any concentration tested in any transformant. At much higher concentrations of each chemical, the frequencies of the micronuclei were decreased (data not shown), suggesting that the tested concentrations were appropriate to evaluate MN induction. Then to investigate the involvement of the detoxification pathway by UGT in the metabolism of tamoxifen, we



tested the effect of UGT inhibitor on the MN induction by tamoxifen. A small but significant increase in MN by tamoxifen was observed in the presence of the UGT inhibitor, diclofenac, in the transformants expressing CYP3A4 which contribute to the metabolic activation of tamoxifen to  $\alpha$ -hydroxytamoxifen (Fig. 11 B). This result indicated that the CYP3A4-metabolite,  $\alpha$ -hydroxy-tamoxifen, was further metabolized by UGT to a genotoxically inactive substance.

Another example is safrole. Safrole is reported to be hydroxylated predominantly by CYP2A6, 2C9, 2D6 or 2E1 and further metabolized by SULT to 1'-sulfooxysafrole (Andrew and Brian, 2007; Rietjens et al., 2005)(Fig. 12). This intermediate forms the electrophilic carbocation of safrole, suggesting the production of DNA adduct (Rietjens et al., 2005). On the other hand, the safrole-2', 3'-oxide formed from the parent safrole by epoxide hydrolases or 1'-hydroxysafrole-2', 3'-oxide from 1'-hydroxysafrole are reported to be detoxified by GST (Rietjens et al., 2005).

As shown in Fig. 13 A, safrole did not significantly induce MN at any concentration tested in any transformant. At much higher concentrations of each chemical, the frequencies of the micronuclei were decreased (data not shown), suggesting that the tested concentrations were appropriate to evaluate MN induction. Then to investigate the involvement of the detoxification pathway by GST in the metabolism of safrole, we tested the effect of GST inhibitor on the MN induction by safrole. Significant increases were seen in the presence of the GST inhibitor, ethacrynic acid, in the transformants expressing CYP2D6 responsible for the genotoxic activation of safrole to 1'-hydroxysafrole (Fig. 13 B). This result suggested that CYP2D6-mediated metabolite, 1'-hydroxysafrole, was further metabolized by GST not exerting its genotoxicity in the metabolic pathway.

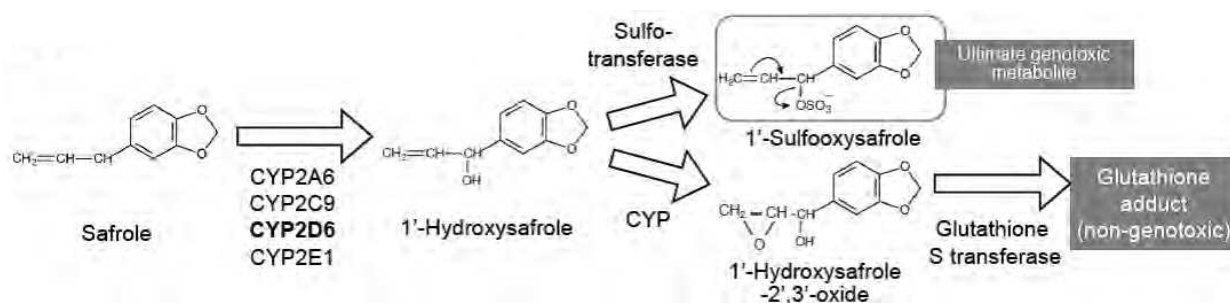


Fig. 12. Metabolic activation and inactivation pathways of safrole.

The results for tamoxifen and safrole clearly demonstrated that interaction between the phase I and II drug-metabolizing enzymes was crucial to assess the genotoxicity of chemicals in the presence of a metabolic activation system. The interplay between the phase I and II enzymes is lacking in the NADPH-supplemented rat liver S9 system due to an absence of co-factor necessary for several phase II enzymes such as UGT or GST. Furthermore, the reactive intermediates have to be formed in the target cell because some conjugates have poor membrane permeability. These results raise the possibility that the induced rat liver S9 system may generate mutagenic metabolites of no relevance, or worse even may not generate a mutagenic metabolite that would be generated by human enzymes. Therefore, a set of HepG2 transformants is a superior test system for mimicking the metabolism occurring in the human liver and the use of this system can potentially provide more relevant data than current genotoxicity tests.



Drug metabolism is generally regarded as proceeding via 2 stages, phase I and phase II. The induced rat liver S9 fraction as an exogenous metabolic activation system is supplemented with only NADPH for CYP-mediated metabolism. The appropriate cofactors for phase II drug-metabolizing enzymes (e.g. UGT, GST, SULT and NAT) are absent. This means these phase II enzymes are not active in the rat liver S9 fraction and that this leads not only for potential false positives (e.g., reactive electrophiles that would be rapidly quenched by conjugation *in vivo* before being able to cause mutation) but also for false negatives because some conjugation reactions can yield metabolites that are more reactive than their substrate (Dashwood, 2002; Ku et al., 2007). In other words, the use of an S9 system with NADPH may represent an incomplete picture of the metabolism that can occur *in vivo*. In particular, it is well-studied that SULTs are able to sequester some proximate mutagens through the

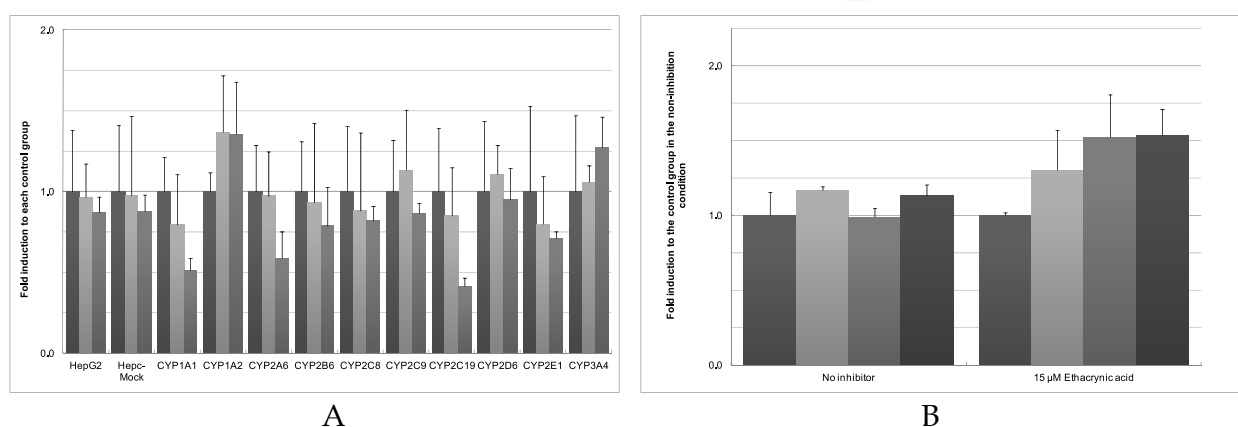


Fig. 13. Micronucleus induction of safrole by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (a), and effects of a phase II inhibitor on micronucleus induction by safrole (b). a). The cells ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (heavy gray bars), 83.3 µg/ml (light gray bars) or 125 µg/ml (medium gray bars) safrole. No statistically significant increase was observed when compared with both Hepc-Mock and HepG2. b). The transformant cells expressing CYP2D6 ( $1 \times 10^5$  cells) were seeded onto a 24-well plate for 24 h and then treated with 0.5% DMSO (heavy gray bars), 41.7 µg/ml (light gray bars), 62.5 µg/ml (medium gray bars) or 83.3 µg/ml (solid bars) safrole in the absence or presence of 15 µM ethacrynic acid, a GST inhibitor.

transfer of a sulfonyl group. However, these are also not active in the standard testing system because the necessary cofactor, 3'-phosphoadenosine-5'-phosphosulfate, is not added (Glatt, 2000; Glatt, 2005). From the study by Obach and Dobo using 16 drugs commonly used, not all metabolites observed as significant in humans *in vivo* are generated in the system using rat or human S9 fractions (Obach and Dobo, 2008). Furthermore, they reported that a metabolite observed in humans *in vivo* was only seen in the rat S9 system and not the human S9 system in a few cases, (Obach and Dobo, 2008). Many human *in vivo* metabolites arise via conjugation reactions, which will not be observed in the *in vitro* S9 system as presently supplemented in standard *in vitro* genotoxicity tests. In addition, with regard to similarity to *in vivo* metabolite profiles, the results of the *in vitro* testing presented in the literature by Obach and Dobo clearly demonstrate a limitation of both systems, in that both human and rat S9 predominantly generate metabolites that are the result of one to two metabolic reactions (>90%).

The induced rat liver S9 fraction has another limitation. To be detected as mutagens, some genotoxic metabolites have to be formed within the target cell by enzymes that are not represented in standard in vitro test systems. SULT-dependent activations are not uncommon. Using genetically modified target cells, activation by SULTs has been demonstrated for more than 100 chemicals, including various carcinogens (such as tamoxifen, cyproterone acetate, safrole, nitrofen and some nitrotoluenes) that are missed in conventional test systems (Glatt 2000, Glatt 2005, Glatt and Meinel, 2005). Depending on the compound, varying SULT forms were required for the activation. Like N-sulfooxy-2-acetylaminofluorene, several other sulfo conjugates [e.g. furfuryl sulfate and 1-(L-sulfooxyethyl) pyrene] had to be formed within the target for a positive test result. Other reactive sulfo conjugates undergo spontaneous substitution reactions with components of the culture medium, such as chloride anions, leading to the formation of secondary, membrane-penetrating active species (Glatt et al., 1990). Moreover, cDNA-mediated expression of organic anion transporters in target cells enhanced the genotoxic effects of some reactive sulfuric acid esters externally added (Bakhiya et al., 2006). Such uptake mechanisms might play a role in the organotropism of reactive species, but should not be relied on when testing new compounds.

Other conjugating enzymes (some UGTs, GSTs and NATs) have also been expressed in target cells. The activation of promutagens by UGTs in such models has not yet been reported (and not been studied). However, co-expression of human UGT1A1 provided protection against the mutagenicity and cytotoxicity of PhIP in CHO-derived cells engineered for expression of CYP1A2 (Malfatti et al., 2005). Human GST T1, expressed in *Salmonella typhimurium*, strongly enhanced the mutagenicity of various dihalogenated alkanes as well as diepoxybutane (Thier et al., 1996). The activation of some of these agents could also be demonstrated using external GSH-conjugating systems (Rannug et al., 1978), but the extent of the uptake and its dependence on the structures of the reactive GSH conjugates are largely unexplored. Heterologous expression of GSTs in mammalian cells conferred resistance against various alkylating agents; in some cases, this protection was enhanced by, or was even strictly dependent on, the co-expression of an export pump (MRP-1 or MRP-2) (Smitherman et al., 2004). The expression of endogenous acetyltransferases in *Salmonella* may be a reason for the high mutagenic activity observed in the Ames test with many amino- and nitro-arenes, whose final activation step is often an O-acetylation. *Salmonella* strains are available in which O-acetyltransferase has been replaced by a mammalian NAT (Glatt and Meinel 2005, Grant et al., 1992), which differ in substrate specificity. Thus, various aromatic hydroxamic acids are activated to mutagens by human NATs, but not by OAT. Such differences may often lead to misleading results when the standard bacterial strains are used. Unlike typical phase II metabolites, acetyl conjugates are uncharged. Nevertheless the site of their formation can strongly affect the outcome of mutagenicity experiments. Thus, PhIP shows much higher mutagenicity in *S. typhimurium* TA98 compared to an O-acetyltransferase-deficient variant of this strain; however, purified O-acetyltransferase in the presence of its cofactor acetyl-CoA had drastically reduced its bacterial mutagenicity (although it strongly enhanced the covalent binding to naked DNA) (Saito et al., 1985). Various standard mammalian target cells, including most sublines of V79 cells, do not express any endogenous NAT. Heterologous expression of human NATs in these cells strongly enhanced the genotoxic effects of many amino- and nitro-arenes (Glatt, 2005; Glatt, 2006). For example, induction of gene mutations by 3-nitrobenzanthrone required 100 times lower substrate concentrations in NAT2-expressing compared to control V79 cells. The isomer, 2-nitrobenzanthrone, was mutagenic in cells engineered for

expression of human SULT1A1, but not in control cells. 2-Amino-3-methylimidazo[4,5-f]-quinoline induced gene mutations in V79 cells co-expressing human NAT2 or NAT1 together with human CYP1A2, even at a concentration of 0.01 and 1  $\mu$ M, respectively, but was inactive (even at 30  $\mu$ M) in cells expressing only CYP1A2 (Glatt, 2005; Glatt, 2006).

Genotoxicity is a branch of the field of toxicology that assesses the effects of chemicals on DNA or genetic processes of living cells. Such effects can be accessed directly by measuring the interaction of chemicals with DNA or more indirectly through the production of gene mutation or chromosome alterations. The observations of these consequences in the genotoxicity tests suggest the carcinogenic concern of a chemical. Thus it is important to improve the genotoxicity test system to evaluate accurately based on the *in vivo* situation in human as much as possible. In the present research, I tried to imitate human metabolism by using human hepatocyte cell line expressing human CYP enzymes. My results indicated that metabolism focused only on CYP was not sufficient to evaluate the genotoxicity of the chemicals such as tamoxifen and safrole. A comprehensive metabolic pathway not only by phase I drug-metabolizing enzymes but also by phase II enzymes would be needed for the accurate assessment of genotoxicity. Moreover, other cellular defense systems (i.e., antioxidant system, GSH or ascorbic acid, and DNA repair system) are involved in the expression of genotoxicity by a chemical. Despite the proof that most chemical carcinogens undergo metabolic conversion into DNA-reactive intermediates, some compounds do not bind to DNA and are not mutagenic, yet they are carcinogenic in animal models and possibly also in humans. These non-genotoxic mechanisms such as induction of inflammation, immunosuppression, formation of reactive oxygen species, activation of receptors such as arylhydrocarbon receptor or estrogen receptor, and epigenetic silencing. Therefore, another approach based on the non-genotoxic mechanism is necessary to predict the carcinogenic action from a certain chemical. Together, these genotoxic and non-genotoxic mechanisms can alter signal-transduction pathways that finally result in hypermutability, genomic instability, loss of proliferation control, and resistance to apoptosis – some of the characteristic features of cancer cells. In this regard, we need to learn much more about the role and interplay of susceptibility and resistance function targeted by human carcinogens or involved in modulating human responses to carcinogenic chemicals.

### 3. Future considerations

Genotoxicity is a branch of the field of toxicology that assesses the effects of chemicals on DNA or genetic processes of living cells. Such effects can be accessed directly by measuring the interaction of chemicals with DNA or more indirectly through the production of gene mutation or chromosome alterations. The observations of these consequences in the genotoxicity tests suggest the carcinogenic concern of a chemical. Thus it is important to improve the genotoxicity test system to evaluate accurately based on the *in vivo* situation in human as much as possible. In the present research, I tried to imitate human metabolism by using human hepatocyte cell line expressing human CYP enzymes. My results indicated that metabolism focused only on CYP was not sufficient to evaluate the genotoxicity of the chemicals such as tamoxifen and safrole. A comprehensive metabolic pathway not only by phase I drug-metabolizing enzymes but also by phase II enzymes would be needed for the accurate assessment of genotoxicity. Moreover, other cellular defense systems (i.e., antioxidant system, GSH or ascorbic acid, and DNA repair system) are involved in the

expression of genotoxicity by a chemical. Despite the proof that most chemical carcinogens undergo metabolic conversion into DNA-reactive intermediates, some compounds do not bind to DNA and are not mutagenic, yet they are carcinogenic in animal models and possibly also in humans. These non-genotoxic mechanisms such as induction of inflammation, immunosuppression, formation of reactive oxygen species, activation of receptors such as arylhydrocarbon receptor or estrogen receptor, and epigenetic silencing. Therefore, another approach based on the non-genotoxic mechanism is necessary to predict the carcinogenic action from a certain chemical. Together, these genotoxic and non-genotoxic mechanisms can alter signal-transduction pathways that finally result in hypermutability, genomic instability, loss of proliferation control, and resistance to apoptosis – some of the characteristic features of cancer cells. In this regard, we need to learn much more about the role and interplay of susceptibility and resistance function targeted by human carcinogens or involved in modulating human responses to carcinogenic chemicals.

#### 4. Summary and conclusion

Many carcinogens are known to be procarcinogens and require metabolic activation to exert their genotoxicity through the formation of reactive intermediates. Therefore, for hazard identification on the genotoxic potential of drug candidate and its metabolites, S9 fraction prepared from the livers of rats pretreated with phenobarbital and 5,6-benzoflavone or with Aroclor 1254 to induce drug-metabolizing enzyme activity must be used in the in vitro genotoxicity testing. However, it is frequently questioned as to whether such an in vitro metabolite generation system is the most relevant for human risk, or whether the assay would be better served by using a human-derived in vitro system. In the present study, we examined the advantages of HepG2 transformants expressing a series of human CYP isoforms as a better alternative for metabolic activation system in the in vitro genotoxicity testing.

In section 2.1, the sensitivity of this system to detect genotoxicity requiring CYP activation was confirmed in the in vitro micronucleus tests using well-studied model chemicals. These results showed HepG2 transformants system have the appropriate sensitivity to detect genotoxicity requiring CYP activation tests using well-studied model chemicals. In addition, based on results obtained in the DMBA and ifosfamide treatments, HepG2 transformant system showed that genotoxic metabolites would be produced by not only the most active CYP isoform but also by other less active CYPs.

In chapter 2.2, this system allowed us to investigate the genotoxicity of model chemicals for which the contributing CYP isoforms, especially those mediated by CYP1A2 or 3A4 which is known to metabolize many drugs in humans, have not yet been identified. Based on the results obtained in the okadaic acid and  $\beta$ -endosulfan treatments, it was clearly demonstrated that the HepG2 transformant system was able to identify the CYP isoform relating to the genotoxicity of chemical metabolite(s) and was useful to elucidate the genotoxicity of a new chemical or a drug candidate in the presence of the metabolic activation system.

In chapter 2.3, the relevance of the interaction between phase I and phase II drug-metabolizing enzymes, e.g., UGT, GST, and SULT, in the test system was demonstrated in a MN test of tamoxifen or safrole, which has been reported to be metabolized by enzymes of both phases. Based on the results for tamoxifen and safrole, it was clearly demonstrated that the interaction between the phase I and phase II drug-metabolizing enzymes was crucial to



assess the genotoxicity of chemicals in the presence of a metabolic activation system. Therefore, a set of HepG2 transformants is a superior test system for mimicking the metabolism occurring in the human liver and the use of this system can potentially provide more relevant data than current genotoxicity tests.

In conclusion, we have demonstrated the benefits of a newly established HepG2 transformants expressing a series of human CYP isoforms for in vitro genotoxicity testing that reflects the comprehensive metabolic pathways including not only human CYP isoforms but also the phase II drug-metabolizing enzymes.

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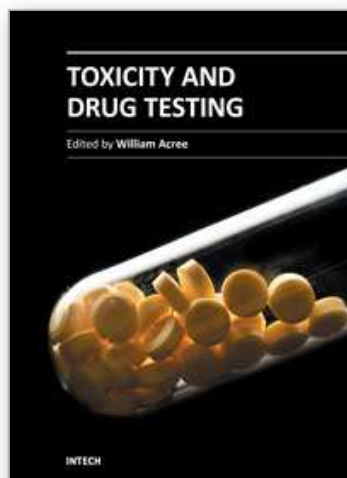


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Modern drug design and testing involves experimental in vivo and in vitro measurement of the drug candidate's ADMET (adsorption, distribution, metabolism, elimination and toxicity) properties in the early stages of drug discovery. Only a small percentage of the proposed drug candidates receive government approval and reach the market place. Unfavorable pharmacokinetic properties, poor bioavailability and efficacy, low solubility, adverse side effects and toxicity concerns account for many of the drug failures encountered in the pharmaceutical industry. Authors from several countries have contributed chapters detailing regulatory policies, pharmaceutical concerns and clinical practices in their respective countries with the expectation that the open exchange of scientific results and ideas presented in this book will lead to improved pharmaceutical products.

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