W-4

IN VITRO DRUG METABOLISM BY A HUMAN P450 EXPRESSION SYSTEM USING VACCINIA VIRUS.

S. Ono[1,4], T. Hatanaka[1], H. Hotta[1], M. Tsutsui[1], F. J. Gonzalez[2], T. Aoyama[3] and T. Satoh[4].

[1]Research and Development Laboratory, Amersham K.K., Chiba, Japan; [2]Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; [3]Department of Biochemistry, School of Medicine, Shinshu University, Nagano, Japan; [4]Laboratory of Biochemical Pharmacology and Biotoxicology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

P450s are a superfamily of heme-containing monooxygenases and important in the metabolism of numerous physiological substrates and foreign compounds. It has been established that there are at least 30 distinct human isoforms of P450. Four families containing numerous individual P450s are mainly responsible for metabolizing foreign compounds. A cDNA expression system in which individual human P450s are synthesized in cultured human hepatoma (Hep G2) cells infected with a recombinant vaccinia virus containing human P450 cDNA has been constructed.

To select drugs that have more effect and less side-effect is an important in developing a new drug. Most of drugs are metabolized by a variety of enzymes, and these metabolic processes generally produce metabolites that are less toxic than the parent compound, in some cases the metabolites may be more reactive, producing toxic effects. The metabolic profiling of drugs undergoing development is, therefore, necessary to assess the effect and toxicity of the proposed drug. These metabolism studies are carried out in an *in vivo* study using experimental animals and in an *in vitro* study using liver from various animal species and humans. However, we are facing increased pressure to minimize the use of animals and ethical problem to use human liver. Furthermore, there are species

interactions as well as assessing their effects and the toxicity. It is also helpful in assessing whether or not drugs are in fact metabolized by these isoforms in relation to polymorphism.

P450s are a superfamily of heme-containing monooxygenases and important in the metabolism of numerous physiological substrates and foreign compounds. However, P450s alone are enzymatically inactive in the absence of an electron transport systems. Microsomal P450s use the flavoprotein, NADPH P450 reductase and, in some cases, cytochrome b_5 as a conduit to collect electrons from NADPH.

It has been established that there are at least 30 distinct human isoforms of P450 (Nelson et al., 1993). Four families containing numerous individual P450s are mainly responsible for metabolizing foreign compounds (Gonzalez, 1992). A cDNA expression system in which individual human P450s are synthesized in cultured human hepatoma (Hep G2) cells infected with a recombinant vaccinia virus containing human P450 cDNA has been constructed. The Hep G2 cells used for P450 expression contain much these electron transport enzymes, which cause the assay system much more reliable and useful. Several investigations using this system have been carried out to elucidate the identity of specific P450 isoforms which are involved in carcinogen and drug metabolism (Aoyama et al., 1990; Rettie et al., 1992; Flammang et al., 1992). These approaches can use to predict human P450 isoforms reflecting on drug metabolism and the profile of drug metabolism in humans.

Experimental procedure

Hep G2 cells were infected with a recombinant vaccinia virus containing the cDNA for one of the following; CYP1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A3, 3A4, and 3A5. Detailed information concerning

the construction of these recombinant viruses has been published elsewhere (Gonzalez et al., 1991). Control Hep G2 cells were infected with wild-type vaccinia virus. The cells were maintained for 20 hrs in Dulbecco's Modified Eagle's medium (high glucose) supplemented with 10 % fetal calf serum (FCS) at 37°C in 5 % CO_2 in air. For metabolism studies, the medium was exchanged for that without FCS and phenol red, containing various substrates. One to 24 hrs later, the metabolites in the medium and cells were analyzed. Within the period of metabolism, P450 expression levels in the vaccinia-infected cell lysates ranged from 10 to 20 pmol/mg protein. The level of NADPH P450 reductase in Hep G2 cells was 0.59 nmol/min/mg cell lysate protein, and the amount of cytochrome b_5 was 10.5 pmol/mg cell lysate protein.

Substrate specificity in human P450 isoforms (Table 1)

We studied the substrate specificity in ten human P450 isoforms expressed in Hep G2 cells using a recombinant vaccinia virus. Caffeine N3-demethylation was found to be suitable markers for the CYP1A2 isoform, and the CYP3A subfamily mediated testosterone 6β-hydroxylation and diazepam 3-hydroxylation. Other assays included coumarin 7-hydroxylation with CYP2A6, 7-benzyloxyresorfin O-debenzylation and diazepam N-demethylation with CYP2B6, tolbutamide methyl-hydroxylation with CYP2C8 and 2C9, bufuralol 1'-hydroxylation and dextromethorphan O-demethylation with CYP2D6, and aniline 4-hydroxylation as well as chlorzoxazone 6-hydroxylation with CYP2E1. However, some of their substrates were metabolized by multiple P450 subfamilies at high concentrations. As catalysts of foreign compound metabolism, P450s are quite unique; a single P450 can oxidize a large number of different substrates. Some chemicals can also be metabolized at different positions by various P450 isoforms, although the rates of

Table 1
Specific Substrate Metabolism by Human P450 Isoforms Expressed in Hep G2 cells Using Vaccinia Virus

P450 isoform	Substrate	Reaction	Activity (μM / hr)
CYP1A2	Caffeine	N3-Demethylation	5.97
	Theophylline	8-Hydroxylation	1.65
CYP2A6	Coumarin	7-Hydroxylation	4.17
CYP2B6	7-Benzyloxyresorfin	O-debenzylation	0.09
	Diazepam	N-demethylation	4.44
CYP2C8	Tolbutamide	Methyl-hydroxylation	0.05
CYP2C9	Tolbutamide	Methyl-hydroxylation	0.18
	Phenytoin	4-Hydroxylation	0.08
CYP2D6	Dextromethorphan	O-demethylation	0.39
	Bufuralol	1'-Hydroxylation	1.03
CYP2E1	Aniline	4-Hydroxylation	9.33
	Chlorzoxazone	6-Hydroxylation	10.46
CYP3A3	Testosterone	6β-Hydroxylation	15.1
	Diazepam	3-Hydroxylation	3.71
CYP3A4	Testosterone	6β-Hydroxylation	25.3
	Diazepam	3-Hydroxylation	5.27
CYP3A5	Testosterone	6β-Hydroxylation	8.50
	Diazepam	3-Hydroxylation	14.0

catalysis can vary, particularly at low substrate concentrations. At high concentrations of a chemical, more than one P450 may be involved in its metabolism. At low concentrations of a chemical, a single P450 isoform might predominate. These results suggest that the metabolic activity of P450 isoforms should be measured at low substrate concentrations so that their substrate specificity can be defined in human liver microsomes as well as in purified or cDNA-expressed human P450s.

Table 2
The contribution of demethylation and hydroxylation pathways to caffeine metabolism in different systems

		Primary metabolites formed (%)				
System	Caffeine	TB	PX	TP	TMU	Reference
In vivo (Human)	Unknown	20	73	8	2	Fuhr et al., 1992
Human CYP1A2	0.5mM	10	85	5	—	
Human CYP1A2	10 mM	13	83	4	—	
Human liver Microsomes	0.5mM	10	66	9	15	
Human liver Microsomes	10 mM	9	32	8	51	
Mouse CYP1A1	10 mM	10	84	4	2	
Mouse CYP1A2	10 mM	33	63	4	1	

— : Below detection limit.

Table 3
Contribution of demethylation and hydroxylation pathways to theophylline metabolism in different systems

		Primary metabolites formed (%)			
System	Theophylline	3X	1X	DMU	Reference
In vivo (Human)	Unknown	15	24	61	Fuhr et al., 1992
Human CYP1A2	1 mM	28	32	40	
Human CYP1A2	10 mM	21	23	56	
Human liver Microsomes	1 mM	27	10	63	
Human liver Microsomes	10 mM	15	12	73	
Mouse CYP1A1	10 mM	10	29	61	
Mouse CYP1A2	10 mM	2	16	83	

Caffeine and theophylline metabolism by human P450 isoforms (Tables 2 and 3)

We investigated caffeine metabolism, which has been used as an index of CYP1A activity, using a vaccinia virus cDNA expression system in which cDNAs for ten human P450 isoforms, and mouse CYP1A1 and

1A2 were respectively expressed. Additionally, the ability of these P450s to metabolize theophylline was studied. The metabolism of caffeine and theophylline was also investigated using human liver microsomes. The demethylation of these drugs was both mediated by human 1A2, as well as by mouse 1A1 and 1A2, and at high caffeine concentration (10 mM), 2D6, 2E1, 3A3, and 3A4 were also involved. The 8-hydroxylation of theophylline was mediated by human 2E1 as well as by human 1A2, and mouse 1A1 and 1A2. Whereas, the 8-hydroxylation of caffeine was not mediated by any of human P450 isoforms tested, and only by mouse 1A1 and 1A2 at very low levels of activity. The major metabolites of caffeine and theophylline by P450 were paraxanthine and 1,3-dimethylurate, respectively. These results reflect the metabolism in humans (*in vivo*), whereas the predominant metabolic pathways of 0.5 and 10 mM caffeine in human liver microsomes were 3-demethylation and 8-hydroxylation, respectively. Furthermore, the metabolic profiles of caffeine and theophylline by human 1A2 were similar to those by mouse 1A1, but not by mouse 1A2. These suggest that human P450 expression system using vaccinia virus in this study, but not the system using human liver microsomes, will be especially applicable to predict the metabolic behaviour of drugs metabolized by P450 isoforms in humans.

Diazepam metabolism by human P450 isoforms

Diazepam is a minor tranquilizer that undergoes N-demethylation and 3-hydroxylation to form nordiazepam and temazepam, respectively, in the liver. Studies using human liver microsomes have suggested that the CYP2C and CYP3A subfamilies are involved in diazepam metabolism which is also correlated with that of S-mephenytoin. However, the contribution of these P450s to diazepam metabolism in humans remains

unclear. Among the tested isoforms, diazepam was significantly demethylated by 2B6, 3A3, 3A4 and 3A5, with 2B6 exhibiting the highest rate, as well as hydroxylated by the later three, of which 3A5 was the most active. Under our conditions, however, 2C8 and 2C9 did not metabolize diazepam. Since genetic polymorphism occurs in the human liver CYP2C subfamily, it is possible that other members of this group participate in diazepam metabolism. However, this remains to be elucidated. Our results suggest that in the hufman liver, the metabolism of diazepam to nordiazepam is mediated by 2B6 and 3A subfamily and to temazepam, by 3A subfamily.

Drug metabolism in relation to CYP2D6 polymorphism

In human, polymorphisms in drug metabolizing enzymes are important in drug therapy. Some drugs exert their actions over a narrow range of concentrations or therapeutic index. Thus, individuals lacking an enzyme that inactivates a drug could experience hyperresponses or toxicity due to high serum concentrations of the active drug. The debrisoquine/sparteine drug oxidation polymorphism is due to mutant alleles of the enzyme CYP2D6 (Gonzalez and Meyer, 1991). The CYP2D6 polymorphism is associated with metabolism of over 30 clinically used drugs (Eichelbaum and Gross, 1990). Among these, we studied bufuralol and dextromethorphan metabolism by human P450 isoforms expressed in Hep G2 cells using a recombinant vaccinia virus. The 1'-hydroxylation of bufuralol was mediated not only by CYP2D6, but also by CYP1A2. The Km values of the reaction were 3.97 and 64.3 μM, and those of the Vmax were 2.90 and 1.38 nmol/hr/plate of CYP2D6 and CYP1A2, respectively. These results suggest that the reaction is catalyzed by the CYP2D6 at a low substrate concentration, but

by both CYP2D6 and CYP1A2 at a high substrate concentration. As the CYP1A2 content in human liver is fairly higher than the CYP2D6 content, the index of CYP2D6 using human liver microsomes should be measured at a low bufuralol concentration. The O-demethylation of dextromethorphan was mediated by CYP2C9 as well as by CYP2D6. The Km values of the reaction were 2.20 and 149 μM, and those of the Vmax were 1.60 and 2.99 nmol/hr/plate of CYP2D6 and CYP2C9, respectively. Furthermore, the level of CYP2C9 in the human liver is also higher than that of CYP2D6. These suggest that, at a low concentration of dextromethorphan, CYP2D6 mainly mediates the O-demethylation, whereas at a high concentration, CYP2C9 predominates.

Chlorzoxazone metabolism by human P450 isoforms

Chlorzoxazone has been suggested as an *in vivo* probe for CYP2E1 (Peter et al., 1990). However, population studies of this muscle-relaxing drug have not yet been carried out. We studied the specificity of chlorzoxazone using vaccinia virus expressed human P450 isoforms. The 6-hydroxylation of chlorzoxazone was mediated by CYP1A2 as well as by CYP2E1. The Km values of CYP1A2 and CYP2E1 for the reaction were 5.69 and 232 μM, respectively. However, the Vmax value of CYP2E1 for the reaction was about 8.3-fold higher than that of CYP1A2. These results raise questions about the suitability of chlorzoxazone as an *in vivo* prove of hepatic CYP2E1 activity. Furthermore, in hepatic microsomes, the Km (40 μM) was different from that of either CYP1A2 and CYP2E1. This is explained by the fact that the expression levels of CYP1A2 and CYP2E1 in the human liver are similar, and both P450s catalyze the 6-hydroxylation of chlorzoxazone. Furthermore, it is suggested that the role of CYP2E1 in 6-hydroxychlorzoxazone formation

at the physiological chlorzoxazone concentration of 30-60 μM is similar to that of CYP1A2.

Mexazolam metabolism by human P450 isoforms

Mexazolam, a benzodiazepinooxazole-anxiolytic, was first N-dealkylated to 7-chloro-5-(2-chlorophenyl)-1-hydro-3-dihydro-2H-1,4-benzodiazepin-2-one (M1), then hydroxylated to lorazepam in rat liver microsomes (Ikeda et al., 1998). Studies using rat liver microsomes have suggested that each metabolic step is mediated by the P450 induced by phenobarbital. However, the P450 isoform(s) contributing to mexazolam metabolism remains unclear. Mexazolam was not metabolized in Hep G2 cells infected with wild type vaccinia virus. In Hep G2 cells expressing CYP2B6, 3A3, 3A4, or 3A5, mexazolam was metabolized to M1, and furthermore, to lorazepam by CYP3A subfamily. However, mexazolam was not metabolized by CYP1A2, 2A6, 2C8, 2C9, 2D6 and 2E1. Furthermore, the chemical structure of the major metabolite of mexazolam by cDNA-expressed human CYP3A4 was analyzed by mass spectrometry, which confirmed that it was M1.

Conclusion

The increasing availability of cDNA-expressed P450s, will allow predictive tests to determine how drugs or other chemicals are metabolized by individual P450 isoform(s) before being administered to humans. If a drug is processed to toxic metabolites, or by polymorphic P450s, it might allow some structural modification of the parent compound. P450s have been produced using vaccinia virus, retrovirus, baculovirus, COS cells, V79 cells, yeast and bacteria. P450 system using vaccinia virus in the present study constantly expresses high level of

individual P450 isoforms. P450 system used here has a high level of the P450 holoenzyme, and the host cells contain sufficient amounts of mammalian NADPH P450 reductase and cytochrome b_5. It means that our system expresses higher contents of P450 and has higher turnover rate for drugs than other system. This suggests that our system can accurately mirror the metabolic profile of drugs and, furthermore, can identify which P450 isoforms are involved in metabolism of a particular drug. This system will be especially applicable to drugs metabolized by human P450 isoforms in relation to polymorphisms. Furthermore, this system will be able to produce tailor-made metabolites.

We need to derive a more knowledge of the structure-activities relating to substrate specificity for drug metabolism by various human P450 isoforms. If possible, we should build up a chemical database about drug metabolism. Therefore, we are necessary to study the metabolism of more compounds using cDNA-expressed human P450s. Furthermore, this strategy is applicable to develop the co-expression of human drug metabolizing enzymes, allowing the prediction of complete metabolic pathways in humans.

References

Aoyama, T., Yamano, S., Guzelian, P.S., Gelboin, H.V. and Gonzalez, F.J.: Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically active aflatoxin B_1. Proc. Natl. Acad. Sci. USA 87: 4790-4793, 1990.

Eichekbaum, M. and Gross, A.S.: The genetic polymorphism of debrisoquine/sparteine metabolism-clinical aspects. Phamacol. Ther. 46: 377-394, 1990.

Flammang, A.M., Gelboin, H.V., Aoyama, T., Gonzalez, F.J. and McCoy, G.D.: Nicotine metabolism by cDNA-expressed human cytochrome P-450s. Biochem. Arch. 8: 1-8, 1992.

Fuhr, U., Doehmer, J., Battula, N., Wölfel, C., Kudla, C., Keita, Y. and Staib, A.H.: Biotransformation of caffeine and theophylline in mammalian cell lines genetically

engineered for expression of single cytochrome P450 isoforms. Biochem. Pharmacol. 43: 225-235, 1992.

Gonzalez, F.J., Aoyama, T. and Gelboin, H.V.: Expression of mammalian cytochrome P450 using vaccinia virus. Methods Enzymol. 206: 85-92, 1991.

Gonzalez, F.J. and Meyer, U.A.: Molecular genetics of the debrisoquin-sparteine polymorphism. Clin. Pharm. Ther. 50: 233-238, 1991.

Gonzalez, F.J.: Human cytochromes P450: problems and prospects. Trends Pharmacol. Sciences 13: 346-352, 1992.

Ikeda, T., Mori, I., Kamai, T. and Tanaka, M.: Interaction of cimetidine and ranitidine, the H_2-receptor blockers, with mexazolam, a benzodiazepinooxazole-anxiolic in rats. Xenobiotic Metab. Dispos. 3: 395-405, 1988.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereinsen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W.: The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol. 12: 1-51, 1993.

Peter, R., Bocker, R., Beaune, P.H., Iwasaki, M., Guengerich, F.P. and Yang, C.S.: Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. Chem. Res. Toxicol. 3: 566-573, 1990.

Rettie, A.E., Korzekwa, K.R., Kunze, K.L., Lawrence, R.F., Eddy, A.C., Aoyama, T., Gelboin, H.V., Gonzalez, F.J. and Trager, W.F.: Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interractions. Chem. Res. Toxicol. 5: 54-59, 1992.