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[1]Department of Pharmacology, University of Copenhagen, Denmark, [2]Institut für Toxicologie und Umwelthygiene, Technische Universität, München, Germany

Kinetic Studies in Engineered V79 Cells in Comparison with Primary Hepatocytes and Human Liver Microsomes

STEFFEN LOFT[1], KLAUS GJERVIG JENSEN[1], LISE-LOTTE RINGBY[1], HENRIK ENGHUSEN POULSEN[1], JOHANNES DOEHMER[2]

Address for correspondence: Prof. S. LOFT, Department of Pharmacology, University of Copenhagen, Panum Institute, Blegdamsvej 3, 2200 Copenhagen, Denmark.

Key words: Cytochrome P450, Genetically engineered cell lines, Enzyme kinetics, Foreign compound metabolism, Liver microsomes, Hepatocytes, mutagenicity, aneuploidy.

Abbreviations

CYP	cytochrome P450	PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine
K_m	Michaelis-Menten constant		
V_{max}	maximal velocity	MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline
K_i	inhibitor constant	POD	phenacetin O-dethylation
MeIQ	2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline	MROD	methoxyresorufin O-demethylation
		EROD	ethoxyresorufin O-deethylation
ABP	aminobiphenyl	B[a]P	benzo[a]pyrene

Summary

Cell lines genetically engineered for expression of xenobiotic metabolising enzymes, particularly cytochrome P450'ies (CYP's), are increasingly important tools for the study of metabolism and toxicity of foreign compounds. For metabolism and (geno)toxicity studies V79 cell lines expressing human CYP1A1, 1A2, 2A6, 2D6, 2E1, 3A4, 3A5, 11B1 and rat 1A1, 1A2 and 2B1 have been developed. However, for validation as model or test systems the kinetic performance of the intact cells should be compared with relevant targets.

As examples the metabolic performance of rat and human CYP1A2 expressed in V79 cells has been compared with freshly isolated rat hepatocytes and human liver microsomes, respectively. The activity of CYP1A2 was assessed in intact cells by the specific phenacetin O-deethylation to paracetamol measured in the incubation medium. In the V79 cells expressing rat CYP1A2 the apparent K_m was 0.99 μM, compared with the high affinity K_m (0.23 μM) found in the rat hepatocytes whereas the apparent V_{max} of the reaction was similar in the two systems on a cell to cell basis. In V79 cells expressing human CYP1A2 the K_m of phenacetin-O-deethylation and the K_i of the specific inhibitor fluvoxamine were similar to the constants found in human liver microsomes.

V79 cells transfected with relevant enzymes, e.g. CYP1A1 and 2E1, have shown the expected pattern of metabolism and genotoxic effects of classic mutagens, such as benzo[a]pyrene and nitrosamines, requiring only oxidative steps for activation, respectively. Similarly, cytotoxicity and genotoxicity in terms of disturbances of the spindle apparatus of dividing cells induced by paracetamol was augmented in V79 cells expressing human CYP1A2 which is also involved in the formation of the hepatotoxic metabolite NAPQI. However, the V79 cell lines lack phase II enzymes, including N-

acetyltransferase and sulfotransferases. Heterocyclic and aromatic amines, such as 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 4-aminobiphenyl (ABP), require N-hydroxylation by CYP1A2 followed by O-acetylation, sulfation or acidic pH for mutagenicity as shown in *Salmonella* assays. Thus, MeIQ and ABP were not specifically mutagenic in V79 cells expressing human CYP1A2 although they were potent competetive inhibitors of the enzyme activity as assessed by the O-demethylation of methoxyresorufin. The development of V79 cells coexpressing the relevant phase I and phase II enzymes should solve this problem.
In conclusion, V79 cells genetically engineered for expression of CYP's have considerable potential for the study of metabolism, interactions and genotoxicity of compounds as long as requirement of secondary phase II activation is considered.

Introduction

Cell lines genetically engineered for expression of xenobiotic metabolizing enzymes, particularly cytochrome P450'ies (CYP's), are increasingly important tools for the study of metabolism and toxicity of foreign compounds. For metabolism and (geno)toxicity studies V79 cell lines expressing human CYP1A1, 1A2, 2A6, 2D6, 2E1, 3A4, 3A5, 11B1 and rat 1A1, 1A2 and 2B1 have been developed (DOEHMER et al. 1992, 1995b; WÖLFEL et al. 1992). However, for validation as model or test systems the kinetic performance of the intact cells should be compared with relevant targets.

Kinetic performance of CYP1A2 expressed in V79 cells

CYP1A2 plays a role in the elimination of a number of drugs as well as the generation of toxic and carcinogenic metabolites from paracetamol and aromatic amines, respectively (Butler et al. 1989a; Raucy et al. 1989; Kadlubar et al. 1990). Accordingly, the metabolic performance of this important enzyme expressed in V79 cells and the potentially use for toxicity studies have received particular attention. V79 cell lines transfected with CYP1A2 have shown catalytic activity toward the substrates, caffeine and theophylline (FUHR et al. 1992; WÖLFEL et al. 1992). For kinetic characterization of CYP1A2 activity *in vitro* the specific O-deethylation of phenacetin is well suited (BUTLER et al. 1989b). The reaction rate can be measured by the appearance of the product, paracetamol, in the incubation medium, allowing study of whole cells. Alternatively, the O-demethylation of methoxyresorufin (MROD) can be used as a marker of CYP1A2 activity in subcellular as well as whole cell incubations (WORTELBOER et al. 1990; JENSEN et al. 1993b). The catalytic activity is linear with time for at least 4 hours and with the number of plated cells up to 600.000 per 9.6 cm^2 dish. However, maximal catalytic activity is only achieved if the cells are kept from reaching confluency (JENSEN et al. 1993a, b).
V79MZr1A2 cells with expression of rat CYP1A2 were compared with freshly isolated rat hepatocytes (JENSEN et al. 1993a). The apparent K_m of phenacetin-O-deethylation was 0.99 µM in V79MZr1A2 cells compared with the high affinity K_m (0.23 µM) found in the rat hepatocytes whereas the apparent V_{max} of the reaction was similar in the two systems on a cell to cell basis (Table 1). The kinetics of phenacetin-O-deethylase was investigated in V79MZh1A2 cells expressing human CYP1A2 (Fig. 1; JENSEN et al. 1995). The apparent K_m of the reaction ranged from 27–66 µM. This value compares well with previously estimated K_m values of 14-57 µM for the high affinity site of phenacetin O-deethylation in human microsomes, attributed to CYP1A2 (BRØSEN et al. 1993). Similarly, the V_{max} in V79MZh1A2 is in the same order of magnitude as estimated for human liver microsomes, with a conservative extrapolation assumption of 0.175 mg microsomal protein per 10^6 hepatocytes (Table 1; WORTELBOER et al. 1990) Moreover, the systems have also been compared with respect to the effect of fluvoxamine, a specific and potent CYP1A2 inhibitor (BRØSEN et al. 1993;

Table 1. Kinetics of phenacetin O-deethylase and inhibition by fluvoxamine in V79MZ cells with expression of human (V79MZh1A2) and rat (V79r1A2) CYP1A2 in comparison with human liver microsomes and freshly isolated rat hepatocytes (high affinity site).

	V_{max} (pmol/min/10^6 cells)	K_m (μM)	K_i (for fluvoxamine) (nM)
Rat hepatocytes	18.1 ± 9.6	0.23 ± 0.07	–
V79MZr1A2	14.9 ± 3.4	0.99 ± 0.16	>1000
V79MZh1A2	45 ± 12	54 ± 24	9.7 ± 4.4
Human liver microsomes	315 ± 209*	39 ± 18	180 ± 49

values are means ± SD of 3-4 experimental sets; *assuming 0.175 mg microsomal protein per 10^6 hepatocytes (WORTELBOER et al. 1990). Data are from BRØSEN et al. 1993; JENSEN et al. 1993a, 1995.

RASMUSSEN et al. 1995). Indeed, the extent of inhibition of phenacetin-O-deethylase activity was similar in the V79MZh1A2 cells and for the high affinity reaction in human liver microsomes, i.e. the K_i values were 3-15 nM and 120-240 nM, respectively (Fig. 1; Table 1). The substrate specificity of the CYP1A2 expressed in V79 cells appears from Fig. 2 which shows that the reaction rate toward CYP1A1 and 2B1 substrates is minimal. Accordingly, the human or rat CYP1A2 enzymes transfected into V79MZ cells perfoms kinetically very similar to the CYP1A2 found in human liver microsomes or rat hepatocytes, respectively.

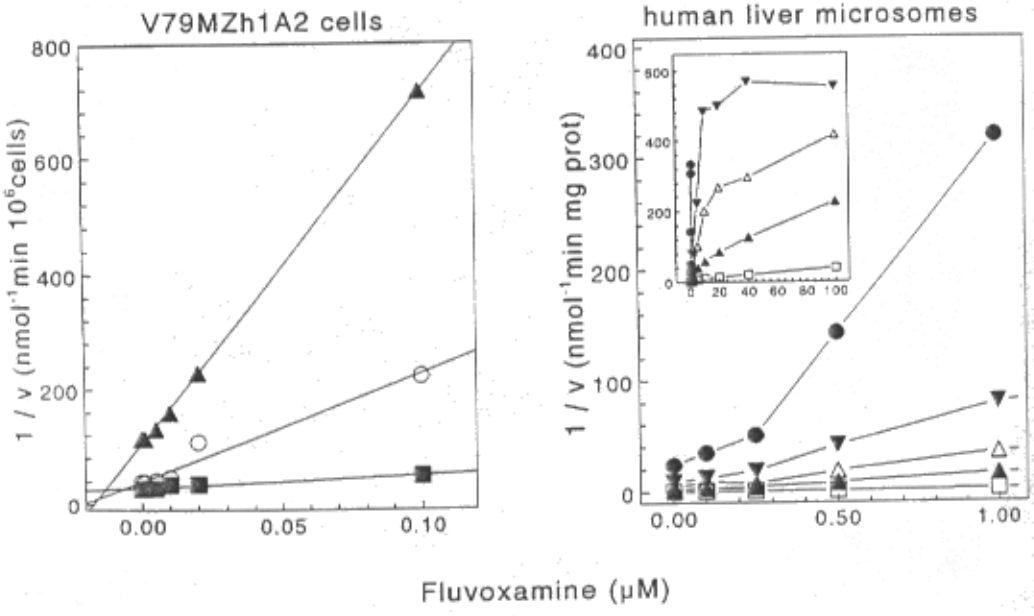

Fig. 1. Dixon plots of phenacetin O-deethylase with fluvoxamine as inhibitor in V79MZh1A2 cells genetically engineered for expression of human CYP1A2 and in human liver microsomes. Data from BRØSEN et al. 1993; JENSEN et al. 1995.

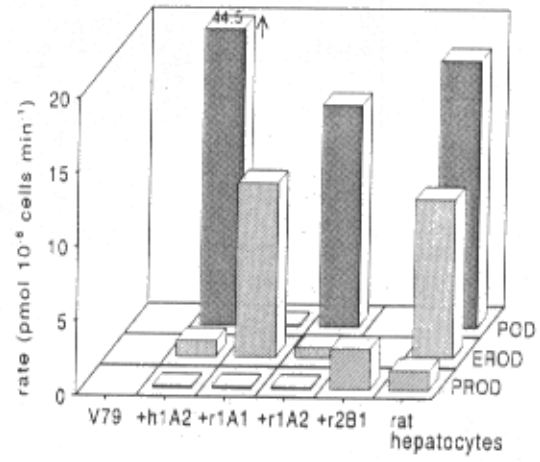

Fig. 2. Substrate specificity of rat (r) and human (h) CYP1A1 and 1A2 expressed in V79MZ cells in comparison with freshly isolated rat hepatocytes. Values are average (maximal) reaction rates of phenacetin O-deethylase (POD; 1A2), ethoxyresorufin deethylase (EROD; 1A1) and pentoxyresorufin O-deethylase (PROD; 2B1). Data are from WORTELBOER et al., 1990; JENSEN et al., 1993a+b, JENSEN et al. 1995.

CYP1A2 and paracetamol toxicity

Paracetamol in overdose cause severe hepatotoxicity, probably due to an arylating reactive intermediate, N-acetyl-benzoequiononeimine, NAPQI (HINSON 1980; HINSON et al. 1990; PUMFORD et al. 1990), produced by oxidative metabolism catalysed by CYP1A2, CYP2E1 and CYP3A4 (RAUCY et al. 1989; THUMMEL et al. 1993; THOMSEN et al. 1995). Although not shown carcinogenic in humans and animals or mutagenic in relevant test systems, paracetamol may induce DNA and/or chromosome damage as shown in vitro, in experimental animals treated with paracetamol and even in humans tak-

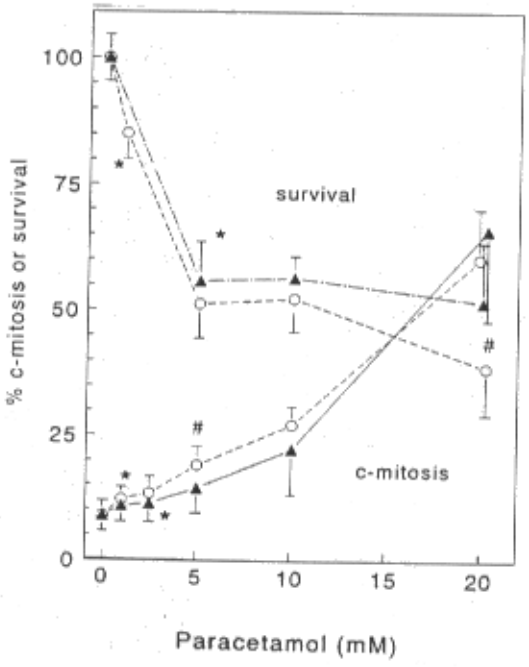

Fig. 3. Relationship between the concentration of paracetamol in 2 h exposure and genotoxic effects in terms of spindle disturbances measured by c-mitosis frequency in % and cytotoxicity in terms of survival (% of control) in a native V79MZ cell line (▲) and a V79MZh1A2 cell line expressing human CYP1A2 (○). Values are means with SD of 5–8 experiments. *denotes $p < 0.05$ vs control; #denotes p y 0.05 between the two cell lines. Data are from (JENSEN et al., 1996).

ing therapeutic doses (DYBING et al. 1984; KOCISOVA et al. 1988; TOPINKA et al. 1989; ANONYMOUS 1990; HONGSLO et al. 1991, 1994; GIRI et al. 1992). Indeed, NAPQI has the properties to bind covalently to proteins, including tubulin which could cause malfunction of the spindle apparatus during mitosis leading to aneuploid daughter cells. This possibility was addressed by studying the effect of paracetamol on spindle disturbances during cell division, scored by aberrant mitotic figures (partial and full c-mitosis - see ÖNFELT 1986) in V79 cells with and without expression of human CYP1A2 (JENSEN et al. 1996). As shown in Fig. 3 paracetamol exposure for 2 h induced c-mitosis in V79 cells in a concentration dependent manner. In V79h1A2 cells expressing human CYP1A2 the concentration effect curve was slightly shifted to the left suggesting that the oxidative metabolism of paracetamol to the reactive metabolite, NAPQI, only plays a minor role in the spindle disturbing effect. The increases in c-mitosis frequency were mirrored by reduced survival of the cells (Fig. 3). The indicated limited role of CYP1A2 in paracetamol toxification is in agreement with a recent study showing that, CYP1A2 inhibition by fluvoxamine had no effect on the hepatoxicity of a paracetamol overdose in mice whereas inhibition of CYP2E1 was very effective in this context (THOMSEN et al. 1995).

CYP1A2 and mutagenicity of aromatic amines

Prototype carcinogens activated by CYP1A2 catalysed N-hydroxylation include the aromatic amine 4-aminobiphenyl (ABP) present in cigarette smoke and a number of cooked food mutagens, such as MeIQ, 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (MeIQ$_x$) and 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine (PhIP). ABP is mutagenic in *Salmonella typhimurium* with a metabolic activation system with CYP1A2 (YOU et al. 1994). MeIQx and PhIP are extensively N-hydroxylated in human liver microsomes and in humans in vivo by CYP1A2 and the products are highly mutagenic in susceptible *Salmonella* species (RICH et al. 1992; BOOBIS et al. 1994; ZHAO et al. 1994). Accordingly, the mutagenicity and cytotoxicity of aromatic and heterocyclic amines in V79 cells expressing CYP1A2 are matters of interest. In V79MZ and V79MZh1A2 cells, however, MeIQ showed no toxicity or mutagenicity at concentrations up to 300 µM, although it was a potent inhibitor (K_i approximately 1 µM) of CYP1A2 activity measured by MROD (unpublished data). Similarly, ABP was a rather weak mutagen in both cell lines and also an inhibitor of MROD (K_i approximately 40 µM). Only PhIP has been shown to be mutagenic specifically related to expression of CYP1A2 in V79MZh1A2 cells (YADOLLAHI-FARSANI et al. 1996). The apparent lack of mutagenicity of MeIQ and ABP in CYP1A2 expressing V79 cells is probably related to lack of the second step enzymes, in the activation process, N-acetyltransferase and sulfotransferases which are present in liver cells. The ultimate DNA binding mutagens of such amines, nitrenium/carbonium ions, are formed from N-hydroxylamines by presence of hydrogen ions or via metabolism to N-sulfonyloxy or N-acetoxy esters (BUONARATI et al. 1990; KADLUBAR et al. 1990). V79 cells with expression of N-acetyltransferase have been engineered for expression of CYP1A2 (KIEFER et al. 1994), although the enzyme activity in terms of phenacetin O-deethylase capacity was undetectable in our laboratory (unpublished data). In studies with other genetically engineered cell lines, Chinese hamster lung fibroblasts, the combined expression of CYP1A2 and N-acetyltransferases was necessary for dose-dependent mutagenicity of IQ and MeIQ$_x$ whereas no effect was seen with expression of either or no enzyme (YANAGAWA et al. 1994) The development of V79 cells with sufficient coexpression of the relevant phase I and phase II enzymes should solve this problem.

Metabolic performance and genotoxicity of polycyclic hydrocarbons related to CYP1A1

CYP1A1 is involved in the rate limiting steps of the multiple pathways of metabolic activation of many polycyclic aromatic hydrocarbons to ultimate carcinogens and mutagens, as illustrated by stud-

ies on prototype compounds, such as benzo[a]pyrene (B[a]P). V79MZh1A1 expressing human CYP1A1 and human hepatoma HepG2 cells stimulated with benzanthracene had similar ethoxyresorufin O-deethylase activity (EROD; a specific CYP1A1 reaction) (SCHMALIX et al. 1993). Similarly, V79 cells expressing rat CYP1A1 and freshly isolated rat hepatocytes have the same EROD activity and the expressed enzyme showed substrate specificity with minimum reaction rates with respect to CYP1A2 and CYP2B1 reactions (Fig. 2). Moreover, in V79 cells expressing the human CYP1A1 more of the ultimate toxic species and cytotoxicity was produced from B[a]P than in the corresponding cells expressing rat CYP1A1 whereas minimum effects were seen in V79 cells without enzyme expression (DOEHMER et al. 1995a). In that study similar species related differences were seen for other polyaromatic hydrocarbons. Similarly, B[a]P and even more so the preultimate mutagen B[a]P-7,8-diol, produced and further activated by CYP1A1 to the 7,8-diol-9,10-oxide, were highly cytotoxic, mutagenic, and capable of inducing micronuclei in V79MZh1A2 cells (SCHMALIX et al. 1993). In V79MZr1A1 cells B[a]P induced c-mitosis, a more direct proof of mitotic spindle disturbances, at much lower concentration than in V79 without enzyme expression or with expression of CYP1A2 (JENSEN et al. 1993b). Interestingly, the B[a]P-7,8-diol induced c-mitosis at extremely low concentration (10 nM), irrespective of CYP expression, indicating a direct effect in contrast to the requirement for further activation by CYP1A1 to the 7,8-diol-9-10 oxide for maximum mutagenic activity (JENSEN et al. 1993b; SCHMALIX et al. 1993). Accordingly, genetically engineered V79 cell lines have been very useful for the elucidation of genotoxic effects of polycyclic hydrocarbons.

Conclusion

V79 cells genetically engineered for expression of CYP's have similar kinetic capabilities as the target or activating cells. The enzymes show the expected substrate specificities. The cell lines have been most valuable for a number of studies of metabolism, interactions at the enzyme levels and toxicity, in particular genotoxicity, of foreign compounds and drugs. Thus, the cell lines have considerable potential for study of metabolism, interactions and genotoxicity of compounds as long as requirement of secondary phase II activation is considered.

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Cytochrome P-450 dependent biotransformation following transplantation of fetal liver tissue suspensions into the spleens of adult syngenic fisher rats

A. Lupp, A.-K. Trautmann, T. Krauße, and W. Klinger

Address for correspondence: Dr. med. A. Lupp, Institute of Pharmacology and Toxicology, Friedrich-Schiller-University Jena, Löbderstr. 1, 07740 Jena, Germany

Key words: Fetal hepatocytes; spleen; cytochrome P-450; monooxygenase function; phenobarbital; β-naphthoflavone; dexamethasone.

Abbreviations: P450: cytochrome P-450; END: ethylmorphine N-demethylation; ECOD: ethoxycoumarin O-deethylation; EROD: ethoxyresorufin O-deethylation; PB: phenobarbital; β-NF: β-naphthoflavone; DEX: dexamethasone; DMSO: dimethylsulfoxide.

Summary

Fetal liver tissue suspensions were transplanted into the spleens of adult male syngenic Fisher inbred rats. Transplant recipients were compared with sham operated and age matched control rats.