

## Metabolism of phenacetin in V79 Chinese hamster cell cultures expressing rat liver cytochrome P4501A2 compared to isolated rat hepatocytes

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**Abstract**—In a genetically engineered V79 cell line (XEMd-MZ) expressing rat cytochrome P4501A2 the activity of phenacetin-*O*-deethylase was determined and compared with freshly isolated rat hepatocytes. In the V79 cells the apparent  $K_m$  was 0.99  $\mu\text{M}$  ( $N = 4$ ), compared to the high affinity  $K_m$  (0.23  $\mu\text{M}$ ,  $N = 4$ ) found in freshly isolated rat hepatocytes, where as the  $V_{\text{max}}$  found in the XEMd-MZ cells (14.90 pmol/min/ $10^6$  cells) corresponded to the  $V_{\text{max}}$  for the high affinity site of the hepatocytes (18.2 pmol/min/ $10^6$  cells). The metabolic capacity of phenacetin was quantitatively comparable to that of freshly isolated hepatocytes on a cell to cell basis.

Cultivation of cells almost inevitably leads to a loss of tissue-specific functions, in particular the expression of cytochromes P450, which are key enzymes in the metabolism and toxicity of many xenobiotics. Construction of cell lines expressing cytochromes P450 by means of genetic engineering is therefore an important tool in the search for detailed information on the oxidative metabolism of xenobiotics by individual forms of cytochrome P450. Stable expression of several cDNA-encoding rat cytochromes P450 in V79 Chinese hamster cells has been achieved by Doehmer *et al.* [1], Dogra *et al.* [2] and Wölfel *et al.* [3].

*In vitro* the cytochrome P4501A2 isoenzyme (CYP1A2\*) activates aromatic amines to proximate carcinogens as well as paracetamol to its hepatotoxic metabolite. Thus, CYP1A2 is of major toxicological interest. CYP1A2 is also responsible for the high affinity deethylation of phenacetin, which has been used as an activity measure of the enzyme in liver microsomes from humans and rats [4–6].

In the present study we have investigated the kinetics of phenacetin-*O*-deethylation (POD) in a genetically engineered V79 cell line (XEMd-MZ) expressing rat CYP1A2 in comparison with freshly isolated rat hepatocytes.

### Materials and Methods

**Genetic engineering of V79 Chinese hamster cells.** The construction of the cell line used here has been described in detail elsewhere [3]. Briefly, full length cDNA was recombined with the expression vector pSV2 and placed under the control of the simian virus 40 early promoter. These pSV450 plasmids were cotransfected with plasmid pdBPV-MTneo (342-2) which carries the neomycin phosphotransferase and thus confers resistance to the drug geneticin (G418).

**Cells.** V79 Chinese hamster cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Uxbridge, U.K.) supplemented with 7.5% heat-inactivated mycoplasma- and virus-screened fetal calf serum (Gibco), penicillin (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ). Selection of plasmid-containing cells was maintained by addition of G418 (400  $\mu\text{g}/\text{mL}$ , Gibco). Cells were kept from reaching confluence at any time. All incubations were performed at 37° in 5%  $\text{CO}_2$  in air. Cells (400,000) were seeded in plastic petri dishes (9.6  $\text{cm}^2$ ) and incubated for 24 hr giving asynchronously growing populations. Cultures were rinsed twice with 2.5 mL Hanks balanced salt solution at 37° and then incubated with phenacetin at nine

concentrations from 0.25 to 20  $\mu\text{M}$  in 2.5 mL of Dulbecco's modified Eagle's medium (–G418) pH 7.2–7.4 for 4 hr at 37°. The supernatant was removed and frozen at –20° for later analysis.

Hepatocytes were isolated from three laboratory breed male (225–275 g) Wistar rats by liver perfusion as described previously [7]. The cell viability was in excess of 90%. The hepatocytes were incubated in 50 mL round bottom flasks, each containing 4 mL cell suspension  $3 \times 10^5$  viable cells/mL (three rats) or  $2 \times 10^6$  viable cells/mL (one rat). The flasks were mounted on a rotary evaporator adapter with humidified carbogen gas introduced via plastic tubing through the vacuum exit and rotated at 20 rpm and at a 45° angle through a water bath (37°) [7]. The cells from each rat were incubated with 12 (two rats), six (one rat) or eight (one rat) concentrations of phenacetin from 0.1 to 50  $\mu\text{M}$ . Samples (750  $\mu\text{L}$ ) were collected after 15, 30 and 60 min of incubation and immediately chilled and centrifuged. The supernatant was stored at –20° until analysis. In a few cases the pellet (cells) was ultrasonicated and shown to contain concentrations of phenacetin and paracetamol identical to those of the corresponding supernatants. In one experiment hepatocytes were incubated with 1  $\mu\text{M}$  paracetamol which was metabolized on average 6% during 15 min.

**Analytical procedures.** Phenacetin-*O*-deethylase activity by P4501A2 was determined by the appearance of the metabolite paracetamol in the incubation medium. Phenacetin and paracetamol were analysed by HPLC [8]. Aliquots (100  $\mu\text{L}$ ) of the incubation medium were mixed with an equal volume of 2 N perchloric acid. Ten to twenty microliters of the supernatant after centrifugation were injected onto the HPLC column. For phenacetin analysis a 15 cm Nucleosil ODS 5  $\mu\text{m}$  column was eluted with methanol/water 45/55 (v/v) and the effluent was monitored at 254 nm. For measurement of paracetamol a Spherisorb ODS 5  $\mu\text{m}$  15 cm column was eluted with phosphate buffer pH 4.4/methanol 92/8 (v/v) and the conductivity of the effluent was monitored with a ESA Coluchem II electrochemical detector equipped with a 5010 analytical cell set at 150 (electrode 1) and 350 (electrode 2) mV and a 0.5  $\mu\text{amp}$  full range deflection. The assay limit of paracetamol was 2 nM and the inter-day coefficient of variation 5%.

**Calculations.** The Michaelis–Menten constants  $V_{\text{max}}$  and  $K_m$  were determined for the formation of metabolite in the cells by weighted least-square non-linear regression analysis. The rate was weighted by the square of the reciprocal value. In the hepatocytes a low affinity site was included in the equation:

$$V = \frac{V_{\text{max}_1} \times S}{K_{m_1} + S} + \frac{V_{\text{max}_2}}{K_{m_2}} \times S$$

\* Abbreviations: POD, phenacetin-*O*-deethylation; CYP1A2, cytochrome P4501A2; G418, geneticin; XEMd-MZ, V79 Chinese hamster cell line expressing P4501A2 activity.

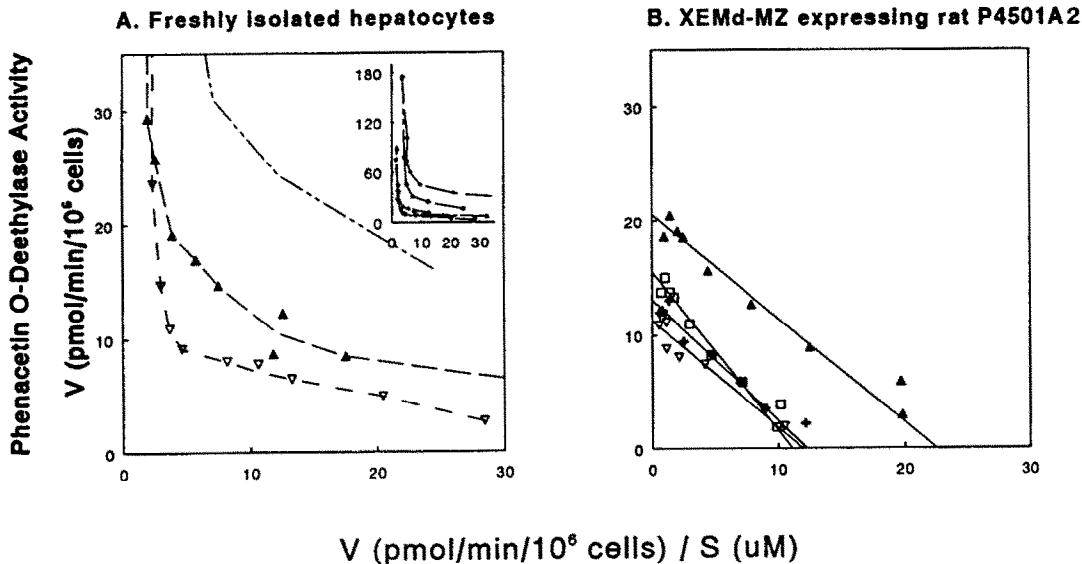


Fig. 1. Eadie-Hofstee plots of the kinetics of phenacetin O-deethylation. (A) Freshly isolated hepatocytes from four different rats. (B) XEMd-MZ expressing rat P4501A2, four separate experiments.

where  $V$  is the rate and  $S$  the substrate concentration. At low concentrations and high enzyme activity (large cell number) up to 20% of the substrate was metabolized during the incubation. Thus, the logarithmic average concentration was used for the calculations:

$$S = \frac{S_i - S_e}{\ln S_i - \ln S_e}$$

where  $S_i$  and  $S_e$  are the substrate concentrations initially and at the end of incubation, respectively. The rate was expressed as product formed per minute per  $10^6$  viable cells, calculated from the metabolite concentrations measured after 15 min or 4 hr incubation with phenacetin for hepatocytes and V79 cells, respectively.

#### Results

V79 cells expressing CYP1A2 deethylated phenacetin, whereas native V79 cells and lines expressing CYP2B1 and CYP1A1 were unable to do so at measurable rates. The

formation of paracetamol from phenacetin in XEMd-MZ was linear with time up to at least 4 hr and number of plated cells up to  $6 \times 10^5$  (data not shown). In isolated rat hepatocytes POD activity was linear with time up to 30–60 min and cell concentration up to  $2 \times 10^6/\text{mL}$  (data not shown).

The POD activity of XEMd-MZ and by freshly isolated rat hepatocytes adhered to Michaelis-Menten kinetics (Fig. 1). The apparent  $K_m$  values were similar in four series of experiments with the XEMd-MZ cells (Table 1). In the hepatocytes the high affinity  $K_m$  was slightly lower than in the XEMd-MZ (Table 1). However, the apparent average  $V_{\max}$  found with the XEMd-MZ cells ( $14.90 \text{ pmol/min}/10^6$  cells) corresponded to the apparent  $V_{\max}$  for the high affinity site of the hepatocytes ( $18.12 \text{ pmol/min}/10^6$  cells).

The kinetic parameters of the low affinity site of POD in hepatocytes could only be determined as a ratio of  $V_{\max}$  to  $K_m$ . At a substrate concentration of  $1 \mu\text{M}$  the low affinity site contributed  $20.2 \pm 5.9\%$  of the POD activity in the rat hepatocytes ( $N = 4$ ).

Table 1. Michaelis-Menten parameters of phenacetin-O-deethylation in XEMd-MZ and freshly isolated hepatocytes from male rats

	$V_{\max_1}$ ( $\text{pmol/min}/10^6$ cells)	$K_{m_1}$ ( $\mu\text{M}$ )	$\frac{V_{\max_2}}{K_{m_2}}$ ( $\mu\text{L/min}/10^6$ cells)
<b>Hepatocytes</b>			
Rat 1	$13.46 \pm 1.66$	$0.36 \pm 0.14$	1.36
Rat 2	$18.30 \pm 1.71$	$0.20 \pm 0.10$	3.27
Rat 3	$7.43 \pm 0.63$	$0.17 \pm 0.04$	1.61
Rat 4	$33.30 \pm 2.60$	$0.20 \pm 0.06$	3.03
Mean	18.12	0.23	
<b>XEMd-MZ</b>			
Series 1	$20.27 \pm 0.62$	$0.84 \pm 0.06$	
Series 2	$11.21 \pm 0.49$	$0.90 \pm 0.09$	
Series 3	$15.24 \pm 0.48$	$1.27 \pm 0.09$	
Series 4	$12.88 \pm 0.51$	$0.96 \pm 0.08$	
Mean	14.90	0.99	

Values are means  $\pm$  SD.

### Discussion

In this study the rat CYP1A2 transfected into V79 Chinese hamster cells grown in non-confluent cultures deethylated phenacetin to paracetamol at a rate that on a cell to cell basis was comparable to that of freshly isolated rat hepatocytes. The specificity of phenacetin as a probe for CYP1A2 was supported by the fact that CYP1A1 and CYP2B1 transfection did not yield any measurable phenacetin deethylation activity.

Phenacetin is metabolized to paracetamol which in its turn is further metabolized by several routes, oxidative as well as conjugative. This does not hamper the use of formation of paracetamol as a measure of CYP1A2 activity since paracetamol was eliminated at a much slower rate, even in freshly isolated hepatocytes with maintained oxidative and conjugative metabolism.

The deethylation of phenacetin obeyed Michaelis-Menten kinetics in V79 cells expressing CYP1A2. In isolated rat hepatocytes biphasic kinetics suggest at least two active catalytic sites. The apparent  $V_{max}$  of the V79 cells was very similar to the apparent  $V_{max}$  values of the high affinity site of rat hepatocytes. The  $K_m$  values differed slightly between the two systems but were within the same order of magnitude and not far from those reported for the high affinity site of liver microsomes from rats and humans, i.e. approximately 4–5  $\mu\text{M}$  [4]. The  $K_m$  estimations, however, are determined with some variation due to a combination of substrate consumption, the low concentration of substrate required and, in isolated hepatocytes, because of contribution from the low affinity site. In addition, V79 cells and hepatocytes were incubated under different conditions, i.e. plated and in suspension, respectively.

Fuhr *et al.* [9] reported that XEMd-MZ cells metabolize caffeine and theophylline to the expected metabolites, in agreement with the involvement of P4501A2 in their formation [10]. Cytochrome P4501A2 has been reported to activate a series of aromatic amines, heterocyclic amines and aflatoxin B<sub>1</sub>. Some of these carcinogens have been identified as causative agents in occupational carcinogenesis, and are also constituents of cigarette smoke, agricultural chemicals and cooked foods. The use of cell lines with well-defined cytochrome activity(ies) is of considerable toxicological interest, since the various cytochromes show a preference for activation of a variety of xenobiotics. This warrants promising opportunities for the use of XEMd-MZ as an analytical tool in a variety of toxicological studies involving cytochrome P4501A2.

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### REFERENCES

1. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt H and Oesch F, Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B<sub>1</sub>. *Proc Natl Acad Sci USA* **85**: 5769–5773, 1988.
2. Dogra S, Doehmer J, Glatt H, Mölders M, Siegert P, Friedberg T, Seidel A and Oesch F, Stable expression of rat cytochrome P-450IA1 cDNA in V79 Chinese hamster cells and their use in mutagenicity testing. *Mol Pharmacol* **37**: 608–613, 1990.
3. Wölfel C, Platt K, Dogra S, Glatt H, Wächter F, Oesch F and Doehmer J, Stable expression of rat cytochrome P-450IA2 cDNA in V79 Chinese hamster cells and hydroxylation of 17 $\beta$ -estradiol and 2-aminofluorene. *Mol Carcinogen* **4**: 489–498, 1991.
4. Boobis AR, Brodie MJ, Kahn GC, Toverud EL, Blair IA, Murray S and Davies DS, Comparison of the *in vivo* and *in vitro* rates of formation of the three main metabolites of antipyrine in man. *Br J Clin Pharmacol* **12**: 771–777, 1981.
5. Distlerath LM, Reilly PEB, Martin MV, Davis GG, Wilkinson GR and Guengerich FP, Purification and characterisation of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin O-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **260**: 9057–9067, 1985.
6. Sesardic D, Boobis AT, Edwards RJ and Davies DS, A form of cytochrome P450 in man, orthologous to form *d* in the rat, catalyses the O-deethylation of phenacetin and is inducible by cigarette smoking. *Br J Clin Pharmacol* **26**: 363–372, 1988.
7. Loft S and Poulsen HE, Metabolism of metronidazole and antipyrine in isolated rat hepatocytes. *Biochem Pharmacol* **38**: 1125–1136, 1989.
8. Loft S, Otton V, Lennard MS, Tucker GT and Poulsen HE, Characterization of metronidazole metabolism by human liver microsomes. *Biochem Pharmacol* **38**: 1125–1136, 1989.
9. Fuhr U, Doehmer J, Battula N, Wölfel C, Kudla C, Keita Y and Staib AH, Biotransformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. *Biochem Pharmacol* **43**: 225–235, 1992.
10. Butler MA, Iwasaki M, Guengerich FP and Kadlubar FF, Human cytochrome P-450<sub>pa</sub> (P450IA<sub>2</sub>), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc Natl Acad Sci USA* **86**: 7696–7700, 1989.