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Androgen hydroxylation catalysed by a cell line (SD1) that stably expresses rat hepatic cytochrome *P*-450 PB-4 (IIB1)

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Androgen hydroxylation catalysed by Chinese hamster fibroblast SD1 cells, which stably express cytochrome *P*-450 form PB-4, the rat *P450IIB1* gene product, was assessed and compared to that catalysed by purified cytochrome *P*-450 PB-4 isolated from rat liver. SD1 cell homogenates catalysed the NADPH-dependent hydroxylation of androstenedione and testosterone with a regioselectivity very similar to that purified by *P*-450 PB-4 (16β-hydroxylation/16α-hydroxylation = 6.0–6.8 for androstenedione; 16β/16α = 0.9 for testosterone). Homogenates prepared from the parental cell line V79, which does not express detectable levels of *P*-450 PB-4 or any other cytochrome *P*-450, exhibited no androgen 16β- or 16α-hydroxylase activity. The hydroxylase activities catalysed by the SD1 cell homogenate were selectively and quantitatively inhibited (> 90%) by a monoclonal antibody to *P*-450 PB-4 at a level of antibody (40 pmol of antibody binding sites/mg of SD1 homogenate) that closely corresponds to the *P*-450 PB-4 content of the cells (48 pmol of PB-4/mg of SD1 homogenate). Fractionation of cell homogenates into cytosol and microsomes revealed that the *P*-450 PB-4-mediated activities are associated with the membrane fraction. Although the *P*-450 PB-4-specific content of the SD1 microsomes was 15% of that present in phenobarbital-induced rat liver microsomes, the *P*-450 PB-4-dependent androstenedione 16β-hydroxylase activity of the SD1 membrane fraction was only 2–3% of that present in the liver microsomes. This activity could be stimulated several-fold, however, by supplementation of SD1 microsomes with purified rat NADPH *P*-450 reductase. These studies establish that a single *P*-450 gene product (IIB1) can account for the hydroxylation of androgen substrates at multiple sites, and suggest that SD1 cells can be used to assess the catalytic specificity of *P*-450 PB-4 with other substrates as well.

INTRODUCTION

Mammalian cytochrome *P*-450 cDNAs have been studied using a variety of expression systems, including transformed yeast cells and Chinese hamster fibroblasts [1,2], which provide for stable expression of the *P*-450s, in addition to transfected *cos*-1 cells [3] and vaccinia-virus-infected mouse and human cells [4], where transient *P*-450 expression is achieved. These expression systems have been useful for (1) confirming the identities of cloned cDNAs (e.g. [5–7]), (2) identification of functional domains and essential amino acids by site-directed mutagenesis and construction of chimaeric *P*-450s [8, 9], (3) characterization of steroidogenic pathways requiring the participation of multiple *P*-450 enzymes [10] and (4) development of *P*-450-containing cell lines that can be used for bioactivation and mutagenicity testing [2]. *P*-450 expression systems also provide the opportunity to characterize the enzymic activities of individual *P*-450 enzymes that may otherwise be difficult to study by conventional methods, owing to, for example, their low endogenous expression in available tissues, their co-purification with closely-related *P*-450 forms or their denaturation during the course of purification.

Rat hepatic cytochrome *P*-450 form PB-4 (gene *IIB1*) is a phenobarbital-inducible *P*-450 enzyme that exhibits high activity and broad specificity for the oxidative metabolism of drugs, carcinogens and other xenobiotics [11–13]. (Other commonly used designations for *P*-450 PB-4 include *P*-450b [12], *P*-450 PB-B [13] and PBRLM5 [17], among others.) When assayed with steroid hormone substrates, this *P*-450 form exhibits a high selectivity for hydroxylation of the steroid D-ring [14,15]. Structure–activity analyses have established that the nature of the steroid's C_{17} substituent can markedly affect the stereoselectivity of the cytochrome for hydroxylation at C_{16}. Thus, with testosterone as substrate (C_{17} substituent = β-hydroxyl group) a 16β/16α ratio of $\sim$ 0.9 is observed, whereas with androstenedione (C_{17} substituent = oxo group) a 16β/16α ratio of $\sim$ 13–15 is seen (for review see [16]). The consistency of these hydroxylation patterns in equivalent preparations of this cytochrome subsequently described by other laboratories (e.g. [17–19]) and the ability of monoclonal antibodies to inhibit to a similar extent each of the multiple hydroxylation reactions catalysed by purified *P*-450 PB-4 [20] argues that these multiple hydroxylation activities are inherent to the *P*-450 PB-4 polypeptide. However, multiple *P*-450 PB-4-related proteins, including allozymic variants of the cytochrome, are known to be expressed in rat liver [21], and the extent to which these related forms contribute to the steroid hydroxylase profiles exhibited by isolated *P*-

Abbreviation used: *P*-450, cytochrome *P*-450.

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450 PB-4 preparations remains unknown. These and related questions are addressed in the present study, which characterizes the oxidative metabolism of androstenedione and testosterone catalysed by the Chinese hamster fibroblast cell line SD1 [2], which stably expresses the *P450IIB1* gene product *P*-450 PB-4.

MATERIALS AND METHODS

Cells

V79 Chinese hamster fibroblast (parental) cells and SD1 cells that were transfected with *P450IIB1* cDNA and stably express *P*-450 PB-4 protein were prepared and grown as described by Doehmer *et al.* [2]. Cell pellets were harvested and frozen at −80 °C until use. Cells were disrupted by sonication in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 20% (v/v) glycerol (2×10^8 cells/ml) on ice for 4×5 s using a Branson sonicator (Model 200; B15 microtip with pulse sonication at an output control of 5 at 40% duty cycle). Particulate matter was removed by centrifugation for 7 min at 12000 ***g*** and a membrane fraction ('microsomes') was pelleted by centrifugation for 45 min at 100000 ***g***. The resultant supernatant was designated as a cytosolic fraction.

Enzyme assays

Hydroxylation of [4-^{14}C]androstenedione (6.3 mCi/mmol) and [4-^{14}C]testosterone (35 mCi/mmol) (Amersham) catalysed by cell homogenates (0.5 mg of protein) or isolated membrane fractions (0.25 mg) was assayed for 30 min at 37 °C in 0.1 M-Hepes buffer, pH 7.4/0.1 mM-EDTA containing 50 μM steroid substrate/1 mM-NADPH (0.4 ml total volume). Reactions were linear for at least 45 min under these assay conditions (see Fig. 2*a*). Product analysis by t.l.c. and comparison with authentic hydroxysteroid standards was carried out as described previously [14,22]. Where indicated, complete assay mixtures minus NADPH were preincubated (30 min at 20–22 °C) in the presence of inhibitory monoclonal antibody to *P*-450 PB-4 (MAb B4, clone 4-29-5; [20,23]). The antibody was purified from ascites fluid by the method of McKinney & Parkinson [24] prior to use to minimize the non-specific effects that ascites fluid can have on *P*-450-catalysed reactions. Where indicated, assays of cell fractions were supplemented with NADPH *P*-450 reductase, which had been purified to apparent homogeneity from phenobarbital-induced rabbit liver as described elsewhere [11].

Other methods

Western blotting with polyclonal anti-(*P*-450 PB-4) antibodies [22] and immunoquantification of *P*-450 PB-4 levels in SD1 cell fractions were carried out using methods detailed elsewhere [25]. NADPH *P*-450 reductase activity was assayed at 30 °C in 0.3 M-potassium phosphate buffer, pH 7.7, by the reduction of cytochrome *c* (monitored at 550 nm; ϵ 21 $mM^{-1} \cdot cm^{-1}$) (1 unit = 1 μmol of cytochrome *c* reduced/min). Protein was assayed using the Bio-Rad protein assay reagent with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Cell homogenates prepared from SD1 and V79 cells were analysed for expression of rat hepatic *P*-450 PB-4 by Western blotting (Fig. 1). A single polypeptide, corresponding to *P*-450 PB-4 in its electrophoretic mobility, was detected in the SD1 cells using anti-(*P*-450 PB-4) antibodies, but was absent in the parental cell line V79 (lane 2 versus lane 3). Quantitative Western blotting with comparisons to purified PB-4 standards revealed a PB-4 level of 48 pmol/mg of protein in the SD1 cell homogenate. This level of expression is similar to that obtained for other *P*-450s in yeast expression systems (e.g. [5]).

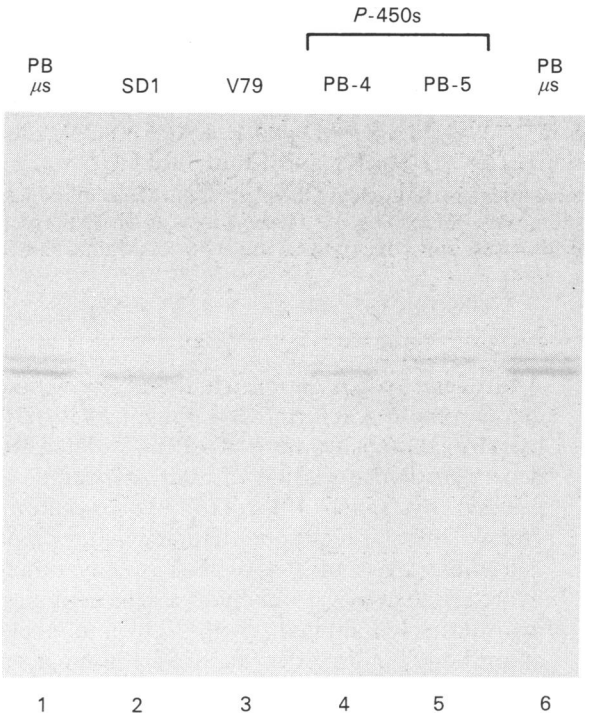

Fig. 1. Western blot of SD1 and V79 cell homogenates probed with anti-(*P*-450 PB-4) antibodies

Homogenates prepared from SD1 cells (lane 2) and V79 cells (lane 3) (20μg/lane) were electrophoresed on an SDS/polyacrylamide gel, transferred to nitrocellulose then probed with polyclonal anti-(*P*-450 PB-4) antibodies. Purified *P*-450 PB-4 and *P*-450 PB-5 standards [11] (lanes 4 and 5) (1 pmol/lane) and phenobarbital-induced rat liver microsomes (PB μs, lanes 1 and 6) (2 μg/lane) were analysed in the adjacent lanes. Independent analysis of these same fractions over a range of protein loadings revealed a *P*-450 PB-4 content of 48 pmol/mg for the SD1 homogenate and 850 pmol/mg for the phenobarbital-induced liver microsomes (results not shown).

NADPH-dependent metabolism of androstenedione catalysed by the SD1 cell homogenate yielded two detectable hydroxylated metabolites, 16β-hydroxyandrostenedione and 16α-hydroxyandrostenedione, with a 16β/16α product ratio of 6.3. In addition, reduction of androstenedione to testosterone, presumably via an NADPH-dependent 17-ketosteroid oxidoreductase reaction, was also observed (Fig. 2*a*). No significant metabolism of androstenedione was observed in the absence of NADPH (Table 1). Negligible formation of the hydroxylated metabolites was observed when using a cytosolic fraction in place of the cell homogenate, or when using a cell homogenate prepared from the parental cell line V79 (Table 1). In contrast, reduction of androstenedione to testosterone was efficiently catalysed by

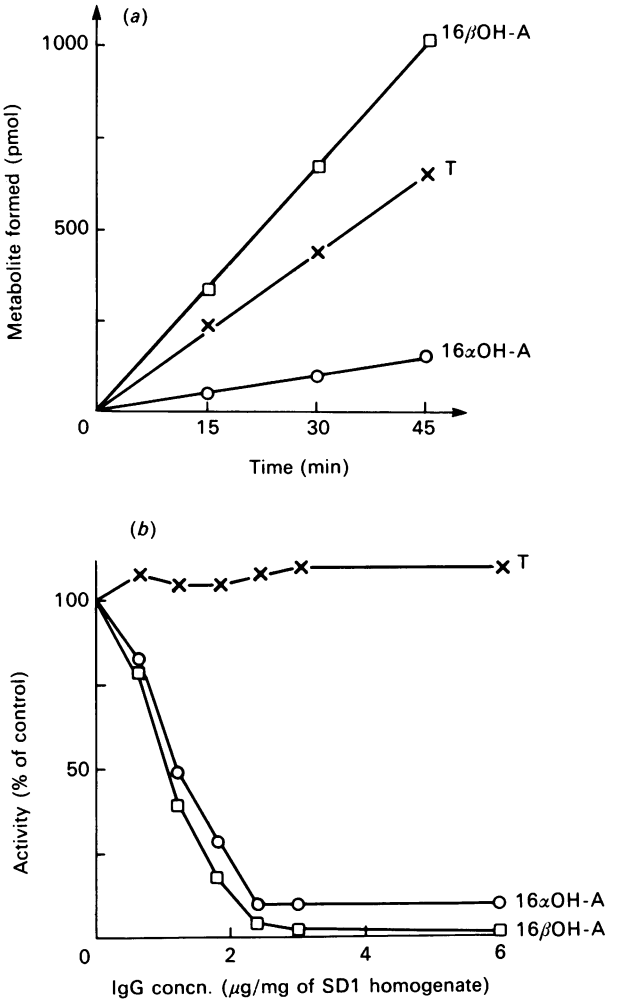

Fig. 2. NADPH-dependent metabolism of androstenedione: (*a*) time course and (*b*) inhibition by monoclonal antibody to *P*-450 PB-4

Incubation of androstenedione with an SD1 cell homogenate at 37 °C was carried out as described in the Materials and methods section. (*a*) Time-dependence for formation of the three metabolites detected: 16β-hydroxyandrostenedione (16βOH-A), testosterone (T) and 16α-hydroxyandrostenedione (16αOH-A). 1 pmol = 12.5 c.p.m. in the experiment shown in (*a*). (*b*) Specific inhibitory effect of monoclonal antibody to *P*-450 PB-4 on the 16β- and 16α-hydroxylase activities of the SD1 cell homogenate (0.5 mg) assayed in the presence of 2 units of purified NADPH *P*-450 reductase (see Fig. 3). Uninhibited rates correspond to 130 (16βOH-A), 20 (16αOH-A) and 39 (T) pmol/min per mg.

both the SD1 cytosolic fraction and the V79 cell homogenate, indicating that the reduction of androstenedione to testosterone is an activity that is independent of the expression of *P*-450 PB-4 in the SD1 cells.

Monoclonal antibody to *P*-450 PB-4 inhibited SD1-catalysed androstenedione 16β- and 16α-hydroxylation by $\geqslant 90\%$ without affecting the rate of testosterone formation (Fig. 2*b*). This further supports the conclusion that the hydroxylase activities but not the oxidoreductase activity is *P*-450 PB-4-dependent. The amount of purified monoclonal antibody required for complete inhibition of the 16β- and 16α-hydroxylase activities [3μg, or ~ 40 pmol of antibody binding sites/mg of SD1 homogenate (calculation based on two antibody binding sites per IgG molecule; M_r 150000)] closely corresponds to the *P*-450 PB-4 content of the SD1 homogenate determined by Western blotting (48 pmol of *P*-450/mg of protein).

The *P*-450 PB-4-specific content and androstenedione hydroxylase activities of the SD1 cells were both enriched about 2-fold upon preparation of a microsomal fraction from the cell homogenate (Table 2); this is consistent with a localization of the *P*-450 enzyme to the endoplasmic reticulum of these cells. The 16β/16α hydroxy product ratio was similar for both cell fractions (ratio = 6.0–6.8; Table 2) but was ~ 2-fold lower than that exhibited by purified *P*-450 PB-4 when assayed in a reconstituted system containing dilauroylphosphatidylcholine (16β/16α = 13–15 [14–16]). The pattern of testosterone hydroxylation exhibited by the SD1 microsomes [hydroxylation at the 16β and the 16α positions at a ratio of 0.9:1, in addition to some oxidation of testosterone to androstenedione (Table 2)], which resembles that found by Platt *et al.* [26], is essentially the same as that reported for the purified cytochrome (16β/16α = 0.8–0.9 [14,15]). These observations establish that the previously-described androgen hydroxylase profiles of isolated preparations of *P*-450 PB-4 accurately reflect the catalytic specificity of a single *P*-450 form.

Table 2 also expresses the *P*-450 PB-4-dependent hydroxylase activities of the SD1 cell fractions as apparent turnover numbers (mol of activity/min per mol of *P*-450 PB-4). These activity values (1.7–2.0 min^{-1} for androstenedione as substrate and 0.2 min^{-1} for testosterone as substrate) are lower than those exhibited by rat liver microsomal *P*-450 PB-4 (7.6 min^{-1} for *P*-450 PB-4-dependent androstenedione 16β-hydroxylation catalysed by phenobarbital-induced rat liver microsomes [16]), and substantially lower than the activities exhibited by the purified cytochrome (apparent turnover = 6 min^{-1} for testosterone and 34 min^{-1} for androstenedione [16]).

It is known from previous studies that a suboptimal

Table 1. Androstenedione metabolism catalysed by SD1 and V79 cell fractions

Fraction	Androstenedione metabolism (pmol/min per mg of protein)		
Metabolite...	16βOH-A*	16αOH-A	Testosterone
SD1 cell homogenate	46	8	33
SD1 cell homogenate, −NADPH	1	1	1
SD1 cell cytosol	< 1	2	77
V79 cell homogenate	< 1	2	47

* A, androstenedione.

Table 2. *P*-450 PB-4 content, androstenedione and testosterone hydroxylase activities and NADPH *P*-450 reductase activity in an SD1 cell homogenate and a microsomal fraction

Immunoquantification of *P*-450 PB-4 levels and enzymic activity assays were carried out as described in the Materials and methods section. Hydroxylation at several other potential sites (e.g. 6β-hydroxylation, 7α-hydroxylation) was shown to proceed at < 2 pmol/min per mg of SD1 microsomes for both steroid substrates. Rates of androstenedione reduction to testosterone (50 and 17 pmol/min per mg for the homogenate and microsomal fraction, respectively) are not included since this activity is not *P*-450 PB-4-dependent and is primarily cytosolic (see Table 1). Although 80–95% of the testosterone 16β and 16α hydroxylase activities of the SD1 microsomal fraction were inhibited by antibody to *P*-450 PB-4, only 30% of the androstenedione formation activity was inhibitible by the antibody (results not shown). This suggests that a portion of this activity is *P*-450 PB-4-independent.

	SD1 homogenate	SD1 microsomal fraction
P-450 PB-4 content (pmol/mg)	48	123
Androstenedione metabolism:		
16βOH-Androstenedione produced (pmol/min per mg)	82	155
16αOH-Androstenedione produced (pmol/min per mg)	12	26
Total activity ($min^{-1} \cdot P\text{-}450^{-1}$)*	2.0	1.7
16β:16α ratio	6.8	6.0
Testosterone metabolism:		
16βOH-Testosterone produced (pmol/min per mg)	–†	11
16αOH-Testosterone produced (pmol/min per mg)	–	12
Androstenedione produced (pmol/min per mg)	–	10
Total activity ($min^{-1} \cdot P\text{-}450^{-1}$)*	–	0.3
NADPH *P*-450 reductase (nmol/min per mg)	8.8	17.3

* Apparent turnover numbers at 50 μM steroid substrate were calculated by summing the rates of formation of the individual metabolites, then dividing by the *P*-450 PB-4 content of the cell fraction [e.g. (82 + 12)/48 = 2.0, for androstenedione metabolism catalysed by the SD1 homogenate].

† Not determined.

content of NADPH *P*-450 reductase in liver microsomes is a major contributing factor to the lower turnover numbers exhibited by microsomal *P*-450s as compared to purified *P*-450 forms assayed in reconstituted systems (e.g. [27]). The NADPH *P*-450 reductase activity in the SD1 microsomal fraction was therefore assayed and compared to that of liver microsomes. *P*-450 reductase-catalysed cytochrome *c* reduction rates were found to be very low for the SD1 microsomes, i.e. 17.3 nmol of cytochrome *c* reduced/min per mg of protein. This can be compared with a rate of 520 nmol of cytochrome *c* reduced/min per mg of protein determined for phenobarbital-induced rat liver microsomes (results not shown). These activities correspond to a *P*-450 reductase/*P*-450 PB-4 molar ratio of 1:21 for the SD1 microsomes versus 1:4.9 for the induced liver microsomes. {A cytochrome *c* reduction rate of 17.3 nmol/min per mg for the SD1 microsomes corresponds to a *P*-450 reductase-specific content of 5.8 pmol of *P*-450 reductase protein/mg of SD1 microsomal protein (calculation based on a specific activity of 40 μmol of cytochrome *c* reduced/min per mg of purified *P*-450 reductase under these assay conditions [11] and a theoretical specific content of 13.3 nmol of *P*-450 reductase/mg of protein, based on an M_r of 75000 for the purified *P*-450 reductase). The ratio (5.8 pmol of *P*-450 reductase/mg)/(123 pmol of *P*-450 PB-4/mg) = 1:21. Similarly 520 nmol of cytochrome *c*/min per mg for the phenobarbital-induced liver microsomes corresponds to 174 pmol of *P*-450 reductase protein/mg of liver microsomes, which were found to contain 850 pmol of *P*-450 PB-4 (results not shown). The ratio (174 pmol of *P*-450 reductase/mg)/(850 pmol of *P*-450 PB-4/mg) = 1:4.9.} This suggests that the low turnover numbers exhibited by the SD1 microsomes reflect, in part, a suboptimal level of NADPH *P*-450 reductase in the SD1 cells. In accord with this suggestion, at least a 3-fold increase in turnover

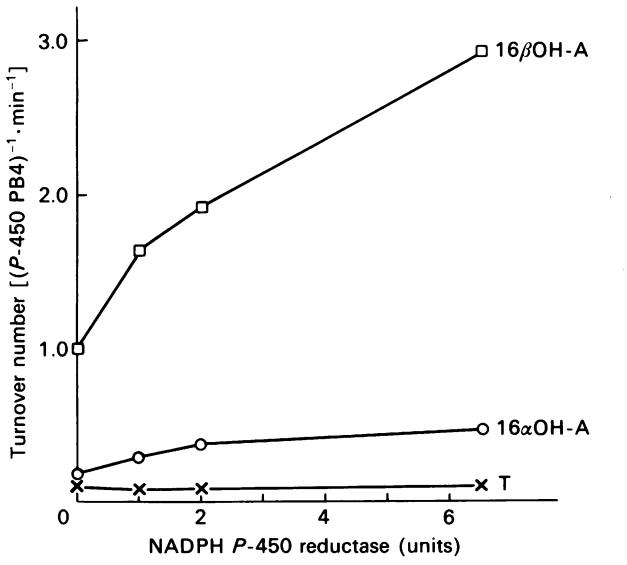

Fig. 3. NADPH *P*-450 reductase stimulation of androstenedione metabolism catalysed by SD1 microsomal fraction

Assay samples were supplemented with exogenous *P*-450 reductase as described in the Materials and methods section. Activities are expressed as apparent turnover numbers (pmol of metabolite/min per pmol of *P*-450 PB-4) calculated on the basis of the *P*-450 PB-4 content of the SD1 microsomes (123 pmol/mg of protein; Table 2).

number (with no significant change in the 16β/16α hydroxy product ratio) was achieved by carrying out the hydroxylase assays in the presence of exogenous purified rat liver NADPH *P*-450 reductase (Fig. 3). Although the highest level of exogenous *P*-450 reductase used in this experiment (6.6 units of *P*-450 reductase/mg of SD1 membrane fraction) corresponds to a *P*-450 reductase/*P*-450 PB-4 ratio of 18:1, the turnover number of the SD1 microsomal *P*-450 PB-4 still does not appear to be saturated (Fig. 3). This apparent requirement for even higher *P*-450 reductase levels may reflect a low efficiency of incorporation of the exogenous *P*-450 reductase into the SD1 membrane fraction.

In summary, the present studies establish that SD1 cells hydroxylate androstenedione and testosterone with a regioselectivity and stereospecificity close to that exhibited by purified preparations of rat hepatic *P*-450 form PB-4. This suggests that these cells can be utilized to reliably assess the catalytic specificity of *P*-450 PB-4 with other substrates as well. While NADPH *P*-450 reductase is expressed constitutively in the SD1 cells and is capable of electron transfer to the expressed *P*-450 cytochrome, the endogenous level of this flavoprotein reductase is a limiting factor for the expression of mono-oxygenase activity. Although this limitation can be partially overcome when assays of SD1 microsomes are carried out *in vitro* in the presence of exogenous purified *P*-450 reductase, an even more effective stimulation of *P*-450-dependent mono-oxygenase activity might be achieved by transfection of the SD1 cells with the cDNA for NADPH *P*-450 reductase [28]. This could lead to an increased sensitivity of these cells for metabolism and mutagenicity studies involving *P*-450 PB-4-dependent reactions.

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