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Changes in the Quantity and Activity of Cytochrome P-450 Isozymes in Primary Cultured Rat Hepatocytes

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Hepatocytes from male Sprague-Dawley rats pretreated with a cytochrome P-450 inducer, 3-methoxy-4-aminoazobenzene (3-MeO-AAB), 3-methylcholanthrene (MC) or phenobarbital (PB), were cultured *in vitro*, and changes in the quantity and activity of microsomal cytochrome P-450 isozymes in the cells were determined by means of immunochemical methods and a bacterial mutation test, respectively. The results of enzyme-linked immunosorbent assay using monoclonal antibodies against rat P-450 isozymes revealed that the amount of cytochrome P-450d induced by 3-MeO-AAB or MC declined rapidly during culture and fell to 10 to 15% of the initial value after 24 h. A similar tendency was observed with PB-induced cytochrome P-450b/e. By contrast, cytochrome P-450c in MC-induced hepatocytes declined more slowly than cytochrome P-450d and remained at 45 to 60% of the initial value after 24 h. Similar quantitative changes of the individual cytochrome P-450 isozymes in culture were also observed by immunoblotting using the anti-cytochrome P-450 monoclonal antibodies. Changes in the activities of individual cytochrome P-450 isozymes in hepatocytes by culture were in accordance with the quantitative changes of the cytochromes, as determined by a mutation test using *Salmonella typhimurium* TA 98 and carcinogenic aromatic amines. These results indicate that microsomal cytochrome P-450c in primary cultured rat hepatocytes is more stable in culture, in terms of both quantity and activity, than cytochrome P-450d and P-450b/e.

Key words: Rat hepatocytes — Cytochrome P-450 — Anti-P-450 monoclonal antibodies — Cytochrome P-450 inducers

Many hepatocarcinogenic chemicals show their carcinogenic activity after undergoing metabolic activation catalyzed by cytochrome P-450 (P-450)[2] present in hepatocytes. We have demonstrated previously[1-4] that the activity or amount of P-450 isozymes in hepatocytes regulates the susceptibility of mice and rats to carcinogenic aromatic amines. Since P-450 consists of various isozymes and since each isozyme shows a different substrate preference, it is important to determine the form and amount of P-450 isozyme(s) responsible for the metabolism of carcinogens. For such studies, *in vitro* tests using hepatocytes have been frequently employed. However, *in vitro* culture of hepatocytes tends to alter the activities or amounts of hepatic enzymes including P-450. In this respect, when hepatocytes are used for studies on the metabolism of drugs or chemical carcinogens, it is necessary to clarify the effect of *in vitro* culture on the quantity and activity of individual P-450 isozymes in the hepatocytes.

In this work, we induced P-450 isozymes in rat liver with drug-metabolizing enzyme inducers selective to each P-450 isozyme and assayed the quantitative change of each P-450 isozyme in primary cultured rat hepatocytes by means of immunochemical methods using monoclonal antibodies against rat P-450 isozymes and by a bacterial mutation assay. It was found that the content of total P-450 in primary cultured rat hepatocytes was decreased by culture, and the extents of decrease in the amounts (and activities) of individual P-450 isozymes were different.

MATERIALS AND METHODS

Chemicals Amino acid pyrolysate components, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp P-2) and 2-amino-6-methyldipyrido [1, 2-*a* : 3′,2′-*d*]imidazole hydrochloride (Glu P-1), were donated by the National Cancer Center Research Institute, Tokyo. Sodium phenobarbital (PB) was purchased from Tokyo Kasei Kogyo Co., Tokyo. 3-Methoxy-4-aminoazobenzene (3-MeO-AAB) (mp. 113–114°C) were synthesized in our laboratory according to the method of Miller *et al.*[5]

Induction of cytochrome P-450 and preparation of rat hepatocytes Male Sprague-Dawley rats were obtained from Shizuoka Experimental Animal Farm Co., Hamamatsu, and used at 8–10 weeks of age. They were treated with a single intraperitoneal injection of 3-MeO-

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[2] Abbreviations used: P-450, cytochrome P-450; P-450b/d, P-450b and P-450d; 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; Trp P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate; Glu P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole hydrochloride; MC, 3-methylcholanthrene; MoAb, monoclonal antibody; PB, sodium phenobarbiturate; PBS, phosphate-buffered saline, pH 7.4; protein A-ELISA, protein A-enzyme-linked immunosorbent assay.

AAB (0.22 mmol/kg) or MC (0.11 mmol/kg) in corn oil or PB (0.22 mmol/kg) in physiological saline and were killed 24 h (3-MeO-AAB) or 48 h (MC or PB) after the drug treatment. The hepatocytes were obtained from the liver by an *in situ* collagenase perfusion method as reported previously,[6] and the isolated fresh hepatocytes were suspended in Eagle's minimum essential medium (MEM; Nissui Seiyaku Co., Tokyo), supplemented with 5% fetal calf serum. The viability of the cells was assessed by a trypan blue dye-exclusion method, and the cell preparations containing more than 90% viable hepatocytes were used. Aliquots (10 ml) of the cell suspension containing 7×10^5 cells/ml were inoculated into tissue culture dishes (Corning No. 25020) and cultured for 6 or 24 h at 37°C in a humidified CO_2 incubator.

Immunochemical analysis of microsomal cytochrome P-450 isozymes Monoclonal antibodies (MoAbs) used were APH-3, APH-8, APL-1, APL-2 and APF-3, which recognize different epitopes on rat P-450 isozymes. Derivation and antigen specificities of these MoAbs have been reported in our previous papers.[7-9]

After culturing for 6 or 24 h, primary cultured hepatocytes were collected by scraping with a rubber policeman and washed twice with phosphate-buffered saline, pH 7.4 (PBS). The cells were resuspended in PBS and homogenized with a Polytron homogenizer, and the microsomal fractions were collected by differential centrifugation.

Protein A-enzyme-linked immunosorbent assay (Protein A-ELISA) was performed as described in our previous paper.[10] Briefly, microsomal preparations (0.1 mg protein/well) were fixed to wells of Costar No. 2590 polyvinyl chloride strips by incubating at 4°C overnight and then successively treated with 100 μl of each of the following solutions; 1) 1% bovine serum albumin (BSA) in PBS, 2) MoAb (APH-3, APL-1 or APF-3) in hybridoma supernatant, 3) rabbit anti-mouse immunoglobulin (Zymed Laboratory Inc., San Francisco, CA) in 1% BSA-PBS, 4) horseradish-conjugated protein A in 1% BSA-PBS, and 5) 0.05% 2,2′-azino-di(3-ethylbenthiazole)-6-sulfonic acid (Sigma) and 0.01% H_2O_2 in citrate buffer solution, pH 4.0. Absorbance of the developed color was measured at 414 nm using an Inter-Med NJ-200 automatic ELISA reader.

The procedures for immunoblotting of microsomal preparations have been described in our previous report.[10] Briefly, microsomal preparations were solubilized with sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred to a nitrocellulose sheet and then immunostained by means of protein A-ELISA with APH-8, APL-2 or APF-3 MoAb using 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma) as a color developer.

Contents of microsomal proteins and P-450 were assayed by the methods of Lowry *et al.*,[11] and Omura and Sato,[12] respectively.

Mutagenicity test Substrates used were Trp P-2, Glu P-1, and 3-MeO-AAB, which were dissolved in dimethylsulfoxide and added to a suspension of bacteria. Activities of microsomes for mutagenic activations of Trp P-2 (2 nmol/plate), Glu P-1 (2 nmol/plate) and 3-MeO-AAB (100 nmol/plate) were assessed by the use of Ames' bacterial mutation test using S-9 (9,000*g* supernatant of liver homogenate), and *Salmonella typhimurium* TA98 as a tester strain.[2] Throughout the present experiments, the number of spontaneous revertant colonies (with vehicle alone) was in the range of 10–40. Data shown are the values obtained by subtracting the number of spontaneous revertant colonies from the total number of colonies.

RESULTS

Hepatocytes from untreated rats or rats pretreated with a P-450 inducer were cultured for 6 or 24 h and assayed for total P-450 and each P-450 isozyme. As P-450 inducers, we selected MC, 3-MeO-AAB and PB, because they induce different forms of P-450 iso-

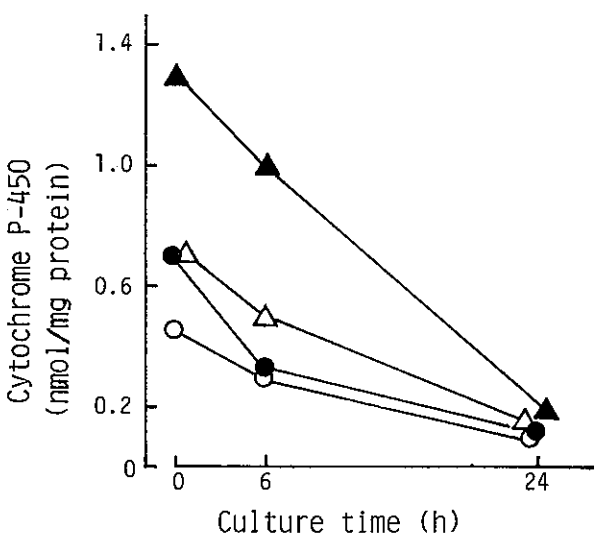

Fig. 1. Change in total microsomal cytochrome P-450 content in primary cultured hepatocytes. Freshly isolated hepatocytes were prepared from male Sprague-Dawley rats as described in "Materials and Methods." Before or after culture, the total microsomal P-450 contents in hepatocytes were assayed as dscribed in "Materials and Methods." Symbols on lines represent the means of triplicate samples. The values of standard errors were less than 10% of the means. Hepatocytes from; ○, an untreated rat; ●, a 3-MeO-AAB-treated rat; △, an MC-treated rat; ▲, a PB-treated rat.

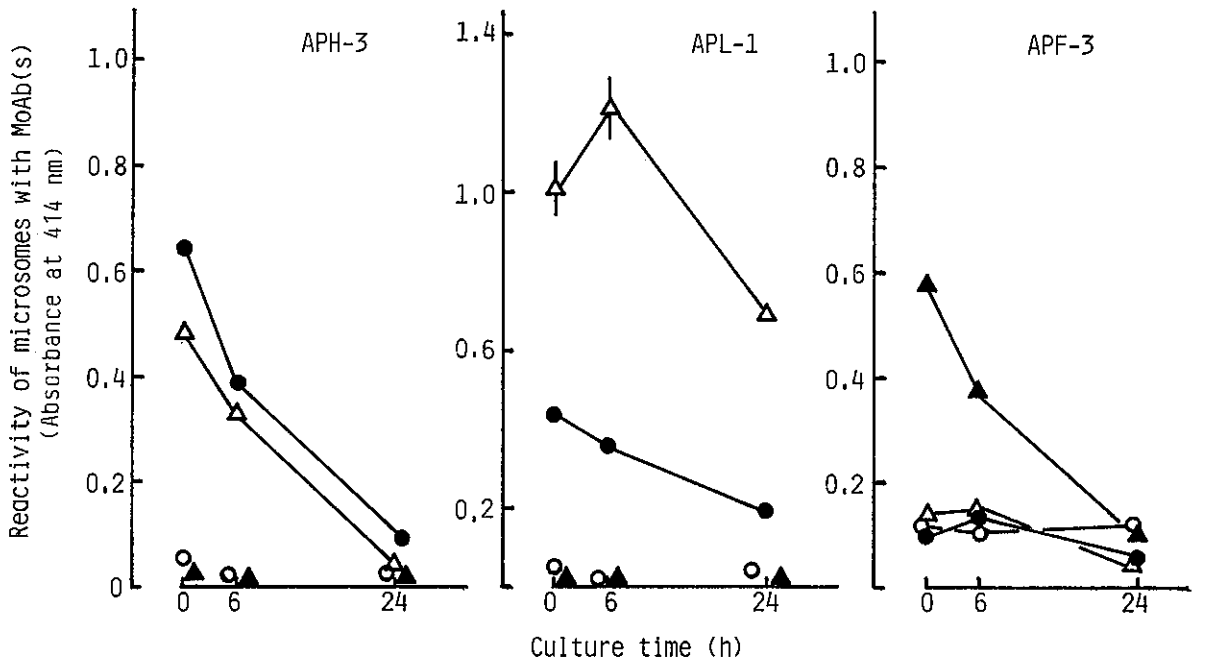

Fig. 2. Changes in microsomal P-450 components reactive with anti-P-450 MoAbs in primary cultured hepatocytes. Hepatocytes used were the same as those in Fig. 1. Before or after culture, the reactivities of hepatic microsomes with MoAbs (APH-3, APL-1 or APF-3) were assayed by protein A-ELISA as described in "Materials and Methods." Symbols on lines and vertical lines on symbols represent the means of triplicate samples and the standard errors of the mean, respectively. Hepatocytes from; ○, an untreated rat; ●, a 3-MeO-AAB-treated rat; △, an MC-treated rat; ▲, a PB-treated rat.

zyme[13-16]; MC is a potent inducer for both P-450c and P-450d (but is preferential for P-450c), 3-MeO-AAB predominantly induces P-450d,[13] and PB induces P-450b and P-450e.

Total microsomal P-450 content in hepatocytes from either untreated or inducer-treated rats declined during *in vitro* culture; it had fallen to 50–70% of the initial value after 6 h and to 15– 20% after 24 h, as determined spectrophotometrically (Fig. 1).

The amounts of microsomal P-450 isozymes in hepatocytes were assessed by means of a protein A-ELISA using anti-rat P-450 MoAbs. APL-1 is selective to P-450c, APH-3 to P-450d, and APF-3 to P-450b/e, and APH-8 and APL-2 are reactive with both P-450c and P-450d.[7] Treatment of rats with MC or 3-MeO-ABB induced microsomal components reactive with both APH-3 (P-450d) and APL-1 (P-450c) in the liver (Fig. 2). When the MC-induced hepatocytes were cultured, a P-450 component recognized by APH-3 (P-450d) declined to about 15% of the initial value after 24 h, but an APL-1-defined component (P-450c) remained in the hepatocytes at about 65% of the initial content. A P-450d component in the 3-MeO-AAB-induced hepatocytes also declined during culture as observed with the MC-induced hepatocytes. As to P-450 components recognized by APF-3 (P-450b/e) in the PB-induced hepatocytes, they declined as rapidly as the P-450d component in MC- or 3-MeO-AAB-induced hepatocytes. These results obtained by ELISA indicate that the stabilities of microsomal P-450 isozymes in primary cultured hepatocytes are different, and that P-450c is more stable than P-450d and P-450b/e.

To assess further the quantitative changes of individual P-450 isozymes in hepatocytes during *in vitro* culture, microsomes from noninduced or induced hepatocytes were subjected to immunoblotting (Fig. 3) and examined for the level of each P-450 isozyme. Both MC-induced and 3-MeO-AAB-induced microsomes revealed two distinct protein bands on staining with APH-8 or APL-2 MoAb at positions corresponding to molecular weights of 56,000 and 54,000 daltons, which can be assigned to P-450c and P-450d, respectively. The microsomal P-450d component in the hepatocytes tended to decrease during culture and only a small amount of the component was detectable after 24 h. However, culture of MC- or 3-MeO-AAB-induced hepatocytes resulted in only a small

decrease in the P-450c component. Immunoblots of PB-induced microsomes with APF-3 MoAb revealed a protein of 52,000–53,000 daltons, which corresponded to P-450b/e. The decrease of this component during culture was as rapid as that of P-450d in the MC- or 3-MeO-AAB-induced hepatocytes. These results on the decrease of P-450 isozymes during culture are consistent with those obtained by ELISA.

To establish whether the quantitative changes of P-450 isozymes during culture reflect the changes in their activity, S-9 fractions from primary cultured hepatocytes were examined for their catalytic activities (S-9 activities) in the mutagenic conversion of carcinogenic aromatic amines. 3-MeO-AAB, Trp P-2 and Glu P-1 were used as the substrates, because they show different susceptibilities to each of the P-450 isozymes in terms of their mutagenic conversion; Glu P-1 is susceptible to P-450d, Trp P-2 to both P-450c and P-450d, and 3-MeO-AAB to

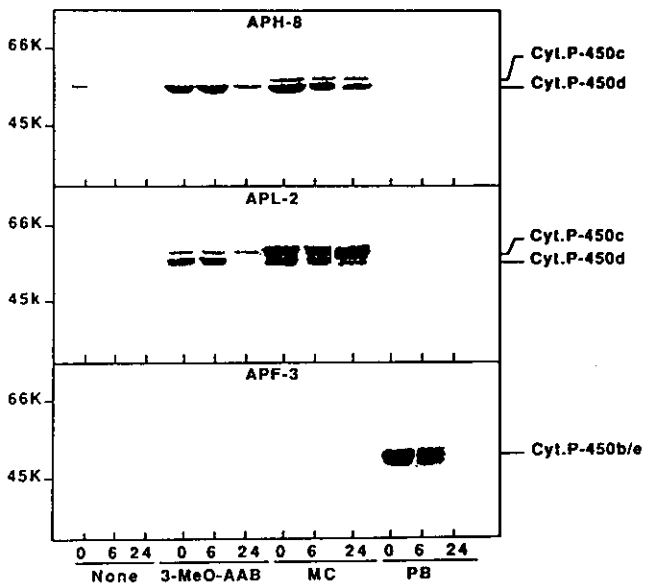

Fig. 3. Immunoblots of the hepatic microsomes from the untreated and inducer-treated rats. Hepatocytes used were the same as those in Fig. 1. Immunoblots were carried out with an aliquot (about 40 μg protein/lane) of each microsome preparation and anti-P-450 MoAb (APH-8, APL-2 or APF-3) as described in "Materials and Methods." The ordinate shows the molecular weight of immunostained components (K, kilodaltons) and the abscissa shows the culture time (h) of hepatocytes and the compounds used for P-450 induction.

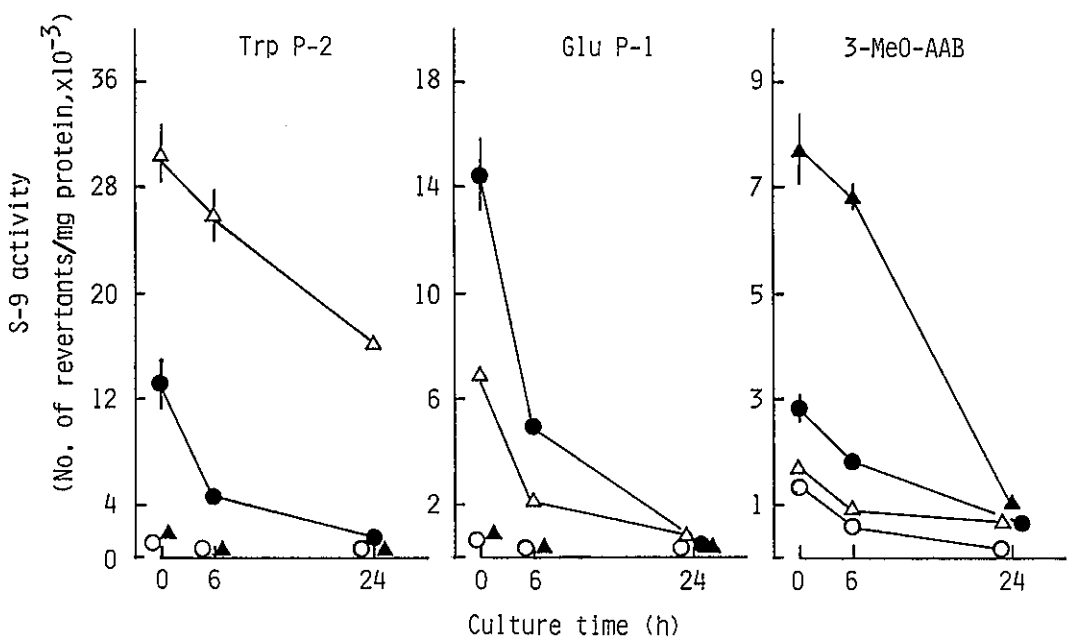

Fig. 4. Changes in S-9 activity in hepatocytes for mutagenic activations of carcinogenic aromatic amines. Hepatocytes used were the same as those in Fig. 1. The S-9 activities for mutagenic activations of the indicated compounds were assayed as described in "Materials and Methods." Symbols on lines and vertical lines on symbols represent the means of triplicate samples and the standard errors of the mean, respectively. Hepatocytes from; ○, an untreated rat; ●, a 3-MeO-AAB-treated rat; △, an MC-treated rat; ▲, a PB-treated rat.

P-450b/e.[17-19] The results of the assay are depicted in Fig. 4. Before culture of hepatocytes, S-9 fractions from both MC- and 3-MeO-AAB-induced hepatocytes showed a high capacity for mutagenic conversion of Trp P-2 and Glu P-1, though the S-9 fractions from MC- and 3-MeO-AAB-induced hepatocytes showed preference for Trp P-2 and Glu P-1, respectively. When hepatocytes were cultured for 6 h, S-9 activity in the 3-MeO-AAB-induced hepatocytes toward Trp P-2 decreased to 35% of the initial value and the activity toward Glu P-1 to 2%. S-9 activity in the MC-induced hepatocytes was higher toward Trp P-2 than Glu P-1 at the beginning of culture, and the activity toward Glu P-1 decreased more rapidly than that toward Trp P-2. The S-9 from PB-induced hepatocytes showed strong activity toward 3-MeO-AAB but very weak activity toward both Trp P-2 and Glu P-1 at the beginning of culture. After 6 h of culture, the S-9 activity toward 3-MeO-AAB remained at 80% of the initial value, but it fell to 13% after 24 h. These results show that changes of the catalytic activities of individual P-450 isozymes in primary cultured hepatocytes reflect the quantitative changes of the isozymes that had been observed by ELISA and immunoblotting.

DISCUSSION

This work demonstrated that the amounts (and activities) of individual P-450 isozymes induced in primary cultured rat hepatocytes were decreased during culture with different kinetics. As indicated by immunochemical analyses (protein A-ELISA and immunoblotting using anti-P-450 MoAbs), the decreases in the amounts of the 3-MeO-AAB- or MC-induced P-450d and PB-induced P-450b/e were faster than that of the MC-induced P-450c. The catalytic activities of individual P-450 isozymes in the hepatocytes were also decreased during culture consistently with the quantitative changes, as determined by a bacterial mutation test using three hepatocarcinogenic aromatic amines.

It has been reported that monolayer culture of hepatocytes from rats resulted in a rapid decrease of total microsomal P-450 content,[20-26] as measured by the method of Omura and Sato,[12] and that the degradation rate of total P-450 is regulated by constituents (amino acids,[25,26] hormones[24] and others[25]) added to a culture medium. Quantitative changes of individual P-450 isozymes in primary cultured rat hepatocytes have already been determined by Steward *et al.*,[21,22] but with the use of polyclonal anti-P-450 antibodies. They reported that the contents of both $P\text{-}450_{\beta NF.B}$ and $P\text{-}450_{\beta NF/ISF.G}$ (corresponding to P-450c and P-450d, respectively) in MC-induced male Sprague-Dawley rat hepatocytes declined at similar rates in culture, and both remained at about 50% of the initial values after 24 h, whereas that of $P\text{-}450_{PB.B/D}$ (corresponding to P-450b/e) in PB-induced hepatocytes changed little, as determined by immunoblotting. These results are inconsistent with our present observation that the contents of P-450d and P-450b/e in hepatocytes declined rapidly during culture of the hepatocytes and fell to a very low or undetectable level after 24 h. A possible reason for this discrepancy is the differences in antibodies or culture media employed for the assays. Under our experimental conditions, the results obtained are unequivocal, because not only the results of two quantitative assays (ELISA and immunoblotting) but also the result of the mutagenicity assay for P-450 activity were consistent with each other.

Since an appropriate method for hepatocyte culture without loss of microsomal P-450 has not been established yet, examination of the changes of P-450 isozymes in the cultures of hepatocytes will be a prerequisite for *in vitro* studies of P-450-dependent metabolism of drugs, including chemical carcinogens. In this context, the present results obtained by both immunochemical methods using MoAbs and bacterial mutation tests may provide a useful basis for further studies on the metabolic conversion of drugs in primary cultured hepatocytes.

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