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Species Difference among Experimental Rodents in the Activity and Induction of Cytochrome P-450 Isozymes for Mutagenic Activation of Carcinogenic Aromatic Amines

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The expressions of hepatic microsomal cytochrome P-450 isozymes in male rats, mice, hamsters and guinea pigs were studied comparatively with or without an ip injection of a cytochrome P-450 inducer. The activity and quantity of microsomal cytochrome P-450 isozymes were determined respectively by a bacterial mutation assay with *Salmonella typhimurium* TA98 and immunochemical assays using monoclonal antibodies against rat cytochrome P-450 isozymes. 3-Methoxy-4-aminoazobenzene (3-MeO-AAB), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole acetate (MeAαC) and 3-methylcholanthrene were used as cytochrome P-450 inducers, and 7 carcinogenic aromatic amines including 3-MeO-AAB and MeAαC were used as substrates for the mutation assay. By means of these assays, we examined the species differences among rodents in the activity and induction rate of hepatic cytochrome P-450 isozymes responsible for the mutagenic activation of carcinogenic aromatic amines.

Key words: Cytochrome P-450 — Hepatocarcinogens — Mutagenicity — Aromatic amines — Rodents

Many hepatocarcinogenic aromatic amines display carcinogenic and mutagenic activities after undergoing metabolic activation catalyzed by cytochrome P-450 (P-450[2]). The amount or activity of P-450 in the liver is influenced by xenobiotics, and a carcinogen itself may induce a certain type of P-450 responsible for its metabolism, as has been demonstrated with hepatocarcinogenic aromatic amines such as 4-aminoazobenzene derivatives[1-4] and amino acid-pyrolysate components.[5-9] The relationship between the activity of a certain type of P-450 (P450IA2 in the rat) responsible for the activation of carcinogens and the carcinogenic susceptibility of mice and rats has also been reported.[1,5-7] Further investigations on the P-450 isozymes in normal and induced states may contribute to our understanding of the susceptibility of various species of animals to chemical carcinogens.

In this work we determined the properties of hepatic microsomal P-450 isozymes induced in the rat, mouse, hamster and guinea pig by carcinogenic aromatic amines, and found that there is species difference in the activity and induction of P-450 isozyme(s) responsible for the mutagenic activation of hepatocarcinogenic aromatic amines.

MATERIALS AND METHODS

Chemicals Trp P-1, Glu P-1, Glu P-2, MeAαC, IQ and MeIQx were gifts from the Biochemistry Division, National Cancer Center Research Institute, Tokyo. MC was purchased from Tokyo Kasei Kogyo Co., Osaka, and 3-MeO-AAB was synthesized in our laboratory according to the method of Miller *et al.*[10]

Treatment of animals with cytochrome P-450 inducers and preparation of hepatic microsomes F344 rats, (BALB/c × DBA/2)F_1 (CDF_1) mice, golden hamsters and Hartley guinea pigs were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu. All animals were male and were used at 7 to 9 weeks of age after being kept in an air-conditioned room with free access to the basal diet, CE-2 (CLEA Japan) for rats, mice and hamster or RC4 (Oriental Yeast Co.) for guinea pigs.

Animals were treated with an ip injection of 3-MeO-AAB (0.22 mmol/kg), MeAαC (0.22 mmol/kg) or MC (0.11 mmol/kg) in corn oil and were killed 24 h after the treatment. Control animals received corn oil alone. The liver of animals was perfused *in situ* with 1.15% KCl solution and homogenized in 1.15% KCl solution to obtain 25% (w/v) liver homogenate. The supernatant was obtained from the liver homogenate by centrifugation at 9,000*g* for 20 min and recentrifuged at 105,000*g* for 60

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[2] Abbreviations: P-450, cytochrome P-450; MC, 3-methylcholanthrene; 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; Glu P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole hydrochloride; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole acetate; IQ, 2-amino-3-methylimidazo[4,3-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline.

min. The resultant precipitate was resuspended in 1.15% KCl solution and used as a microsomal preparation.

Amounts of protein and P-450 in microsomal preparations were measured by the methods of Lowry *et al.*[11] and Omura and Sato,[12] respectively.

Assay for microsomal ability to mutagenically activate aromatic amines 3-MeO-AAB, MeAαC or Glu P-2 (100 nmol) or Trp P-2, Glu P-1, IQ or MeIQx (2 nmol) was dissolved in 50 μl of dimethylsulfoxide and used as a substrate. Mutagenic activities of these substrates after activation by hepatic microsomal preparations were assessed by a bacterial mutation test using *Salmonella typhimurium* TA98 as described previously.[9] In brief, a substrate was added to a mixture containing the bacteria (100 μl of suspension containing 10^8 cells) and 500 μl of microsome suspension containing co-factors. The mixture was preincubated for 20 min at 37°C and then assayed for the induction of revertant colonies in an agar plate by incubation for 2 days at 37°C. Throughout the present experiments, the number of spontaneous revertant colonies was in the range of 20 to 40. Data shown are the values obtained by subtracting the number of spontaneous revertant colonies from the total number of colonies developed by addition of a sample. Microsomal activity for each sample was determined from a linear dose-response curve obtained by using 4 different amounts (25–200 μg of protein) of a microsome preparation.

Immunochemical analyses Anti-rat P-450 MoAbs, APH-3, APH-8, APL-1 and APL-2, prepared in our laboratory,[13,14] were used for immunochemical assays of P-450 isozymes. APL-1 and APH-3 react selectively with cytochrome P-448L (P450IA1[3]) and cytochrome P-448H (P450IA2), respectively.[15] APL-2 and APH-8 are reactive with both P450IA1 and P450IA2 but APL-2 is more reactive with P450IA1 than P450IA2, and APH-8 shows the opposite selectivity.[13,14] These MoAbs show no inhibitory effect on P-450 isozyme activity. The anti-rat P-450 MoAbs except APH-3 are found to be cross-reactive with the P-450 components of animals other than the rat, although the type of P-450 isozyme recognized by each of these MoAbs is unknown in animals other than the rat.

Protein A-ELISA was performed as described in our previous report.[7] Briefly, aliquots (50 μl) of microsomal preparations (0.1 mg protein/well) were fixed to wells of a Costar No. 2590 polyvinyl chloride strip by incubating at 4°C overnight and then successively treated with 50 μl of each of the following solutions; 1) 1% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.2 (PBS), 2) MoAb, 3) rabbit anti-mouse immunoglobulins (Zymed Lab., San Francisco, AC) in 1% BSA-PBS, 4) horseradish peroxidase-conjugated protein A, and 5) citrate buffer solution, pH 4.0, containing 0.05% 2,2′-azino-di(3-ethylbenzthiazole)-6-sulfonic acid (Sigma) and 0.01% hydrogen peroxide solution. The absorbance of the developed color was measured at 414 nm, using an InterMed NJ-200 automatic ELISA reader.

Immunoblotting of microsomal preparations was carried out according to the method described previously.[7] Briefly, microsomal preparations solubilized in sodium dodecyl sulfate (SDS) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at a dose of 40 μg protein/lane. The separated components in a gel sheet were transferred to a nitrocellulose sheet and immunostained by means of protein A-ELISA using MoAb and 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma).

RESULTS

Species difference in hepatic microsomal activity for mutagenic activation of aromatic amines The activity and induction of hepatic microsomal enzymes responsible for mutagenic activation of carcinogenic aromatic amines were assayed by use of 7 hepatocarcinogenic aromatic amines as substrates. These substrates are classified into the following 3 groups based on their sensitivity to different rat P-450 isozymes in mutagenic activation[16]: 1) Glu P-1, Glu P-2, IQ and MeIQ, which are sensitive to P450IA2; 2) Trp P-2 and MeAαC, sensitive to both P450IA1 and P450IA2; and 3) 3-MeO-AAB, which is more sensitive to PB-inducible P-450 isozymes than to P450IA1 and P450IA2.

Representative data in one of three repeated mutation assays are depicted in Fig. 1. The other two experiments gave similar results. As for the activity of hepatic microsomes in the uninduced state, the microsomes obtained from different species of animals showed different spectra in mutagenic activation of the aromatic amines. For example, IQ was most sensitive to activation in the rat, whereas in other species of animals, Trp P-2 was most sensitive as compared with the other aromatic amine substrates. The microsomal activity towards 3-MeO-AAB was much stronger in the guinea pig than in the other species.

Treatment of animals with a P-450 inducer such as MC, MeAαC or 3-MeO-AAB did not significantly alter the total microsomal P-450 content (average nmol/mg protein: rat, 0.58; mouse 0.62; hamster, 1.00; guinea pig, 0.91) in any species of animals. However, the microsomal activities for mutagenic activations of the heterocyclic amines were induced by these P-450 inducers in all species of animals but in different patterns towards the

[3] Nomenclature for cytochrome P-450 isozymes: Although there are a variety of nomenclatures for P-450 isozymes, we adopted that proposed by Nebert *et al.* (reference 15). P450IA1 and P450IA2 correspond to P-448L (P-450c) and P448H (P-450d) in the rat and to P_1-450 and P_3-450 in the mouse, respectively.

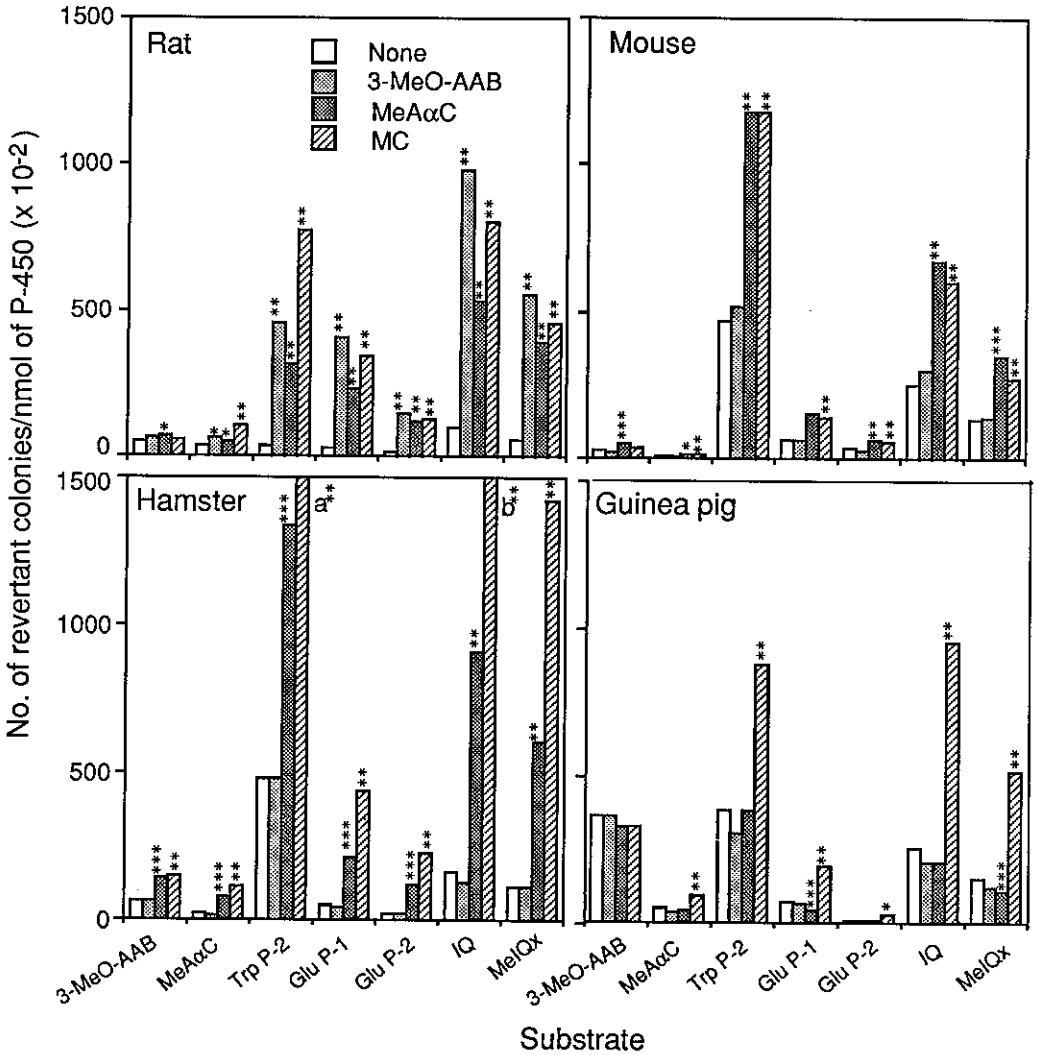

Fig. 1. Changes in hepatic microsomal P-450 isozyme(s) responsible for mutagenic activation of carcinogenic aromatic amines by the induction with 3-MeO-AAB, MeAαC and MC in the rat, mouse, hamster and guinea pig. Hepatic microsomes were prepared from pooled livers of 5 animals in each group and were used as enzyme sources. The data shown represent the means with triplicate samples. The standard errors of the means were less than 20% of the corresponding means. The letters a and b represent the microsomal activities (No. of revertant colonies/nmol of P-450): a, 360,000; b, 220,000. *, **, *** Statistically significant differences from the corresponding controls in Student's t test: *, $P<0.05$; **, $P<0.001$; ***, $P<0.01$.

individual substrates. MC strongly induced the activities in all species for all substrates, with the exception of 3-MeO-AAB (substrate) in the rat, mouse and guinea pig. The MC-induced rat microsomes showed more than 8 times greater activity for the mutagenic activations of Trp P-2, Glu P-1, Glu P-2, IQ and MeIQx than microsomes from untreated rats. MeAαC also induced the activity in the rat, mouse and hamster but not in the guinea pig. By contrast, the induction by 3-MeO-AAB was apparent only in the rat.

Immunochemical analyses for microsomal carcinogen-activating enzymes To characterize microsomal components induced by P-450 inducers, immunochemical analyses (protein A-ELISA and immuno-Western blots) were performed using anti-rat P-450 MoAbs such as APH-3, APH-8, APL-1 and APL-2 (for their antigen specificity, see "Materials and Methods"). The results of protein A-ELISA are summarized in Table I. The component reactive with the MoAbs was hardly detected from the hepatic microsomes of any of the species in the

Table I. Effects of 3-MeO-AAB, MeAαC and MC on the Amounts of Hepatic Microsomal Components Reactive with Anti-rat P-450 MoAbs in the Rat, Mouse, Hamster and Guinea Pig

Species	Chemical treatment	Reactivity of microsomes with a MoAb (absorbance at 414 nm)			
		APH-3	APL-1	APH-8	APL-2
Rat	None	<0.05	<0.05	<0.05	<0.05
	3-MeO-AAB	0.71	0.14	0.18	0.06
	MeAαC	0.22	0.67	0.31	0.34
	MC	0.74	1.22	0.51	0.87
Mouse	None	<0.05	<0.05	0.10	0.10
	3-MeO-AAB	<0.05	<0.05	0.11	0.12
	MeAαC	<0.05	<0.05	0.32	0.16
	MC	<0.05	<0.05	0.30	0.17
Hamster	None	<0.05	<0.05	<0.05	<0.05
	3-MeO-AAB	<0.05	<0.05	<0.05	<0.05
	MeAαC	<0.05	<0.05	0.45	0.12
	MC	<0.05	<0.05	0.35	0.10
Guinea pig	None	<0.05	<0.05	<0.05	<0.05
	3-MeO-AAB	<0.05	<0.05	<0.05	<0.05
	MeAαC	<0.05	<0.05	<0.05	<0.05
	MC	<0.05	0.58	0.27	0.05

Hepatic microsomes were prepared from 3 untreated animals or 3 animals treated with 3-MeO-AAB, MeAαC or MC. The reactivities of each microsome preparation to MoAbs (APH-3, APL-1, APH-8 and APL-2) were examined by means of protein A-ELISA as described in "Materials and Methods." The values represent the means obtained on triplicate samples.

normal (uninduced) state. When animals were treated with MC, APH-8- and APL-2-reactive microsomal components were detected in all species of animals. However, an APH-3-reactive component was found only in the rat and an APL-1-reactive component in the rat and guinea pig. MeAαC treatment induced APH-8- and APL-2-reactive components of hepatic microsomes in the animals except the guinea pig, whereas a component(s) reactive with APH-3 or APL-1 was detected only in the rat. 3-MeO-AAB induced the components reactive with APH-3, APH-8, APL-1 or APL-2 MoAb specifically in the rat.

The molecular properties of microsomal components induced with 3-MeO-AAB, MeAαC or MC in the rat, mouse, hamster and guinea pig were examined by immunoblotting using the anti-P-450 MoAbs (Fig. 2). As for uninduced microsomes, the amount of the component reactive with APH-8 or APL-2 was very small in the rat, mouse and hamster and it was undetectable in the guinea pig. In the rat, 3-MeO-AAB, MeAαC and MC increased two components reactive with either APH-8 or APL-2 MoAb (mol. wt., 56,000 and 54,000 daltons, corresponding to P450IA1 and P450IA2, respectively). In the mouse and hamster, both MeAαC and MC induced a component (mol. wt., 55,000 daltons) reactive with APH-8 or APL-2 MoAb, whereas 3-MeO-AAB did not induce the component. In the guinea pig, a component (mol. wt., 55,000 daltons) reactive with either APH-8 or APL-2 was detected only from the MC-induced microsomes, but not from the other microsome preparations.

DISCUSSION

We have demonstrated herein that there are species differences in the activity and induction of hepatic microsomal enzymes responsible for the mutagenic activation of carcinogenic aromatic amines among the rat, mouse, hamster and guinea pig. In the normal (uninduced) state, the microsomes obtained from each species showed different capacities for the mutagenic activations of the carcinogenic aromatic amine substrates. For example, guinea pig liver microsomes showed more than 6 times greater ability to activate 3-MeO-AAB than microsomes of other species. The ability of rat microsomes to activate Trp P-2 mutagenesis was as weak as one-tenth of those in other species. Furthermore, there are clear differences in the substrate specificity of microsomal enzyme(s) among the rat and the other species. The rat enzyme(s) preferred IQ to the other amines as a substrate, while the enzyme(s) in other species of animals catalyzed predominantly the mutagenesis of Trp P-2, although the difference could also reflect the intrinsic mutagenic activities of the active metabolites of these substrates. As for the induction of microsomal enzyme activity, each species showed a different response to P-450 inducers. For example, MC showed the most potent activity in hamster and guinea pig, whereas in the mouse, the activities of MC and MeAαC were similar. Both 3-MeO-AAB and MeAαC were found to be potent inducers of the MC-inducible P-450 isozymes in the rat, but these chemicals did not induce the P-450 components in the guinea pig. In the mouse and hamster, MeAαC induced enzymes corresponding to an MC-inducible P-450 isozyme(s), but 3-MeO-AAB did not.

Although the existence of species differences in the induction rate of hepatic P-450 isozymes and in the mutagenic activation of carcinogenic aromatic amines has been reported in the case of polycyclic hydrocarbons as inducers,[17-19] we have demonstrated herein that the induction rates of microsomal enzyme(s) responsible for mutagenic activation of carcinogenic aromatic amines by these carcinogenic amines are also quite different among the different species of rodents. The rat is most sensitive to P-450 inducers such as 3-MeO-AAB and MeAαC, while the guinea pig is the most insensitive to these inducers. Since a cytosolic receptor protein plays a cen-

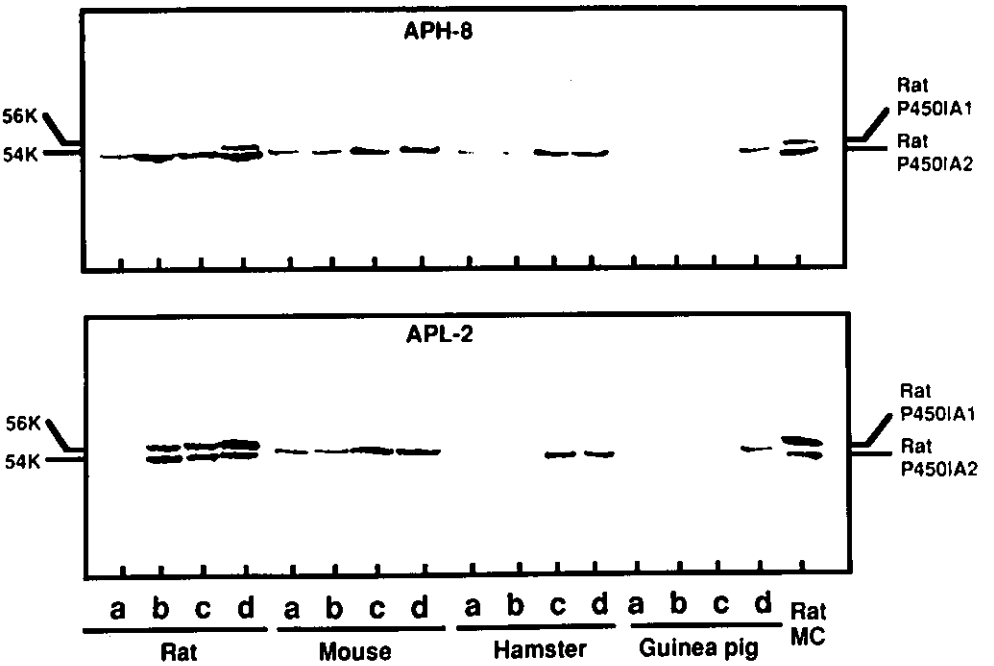

Fig. 2. Immuno-Western blots of hepatic microsomes from the rat, mouse, hamster and guinea pig. Each sample of microsomes was prepared from pooled livers of 5 animals. Immunoblots were carried out with an aliquot (40 μg protein/lane) of each microsome preparation and anti-rat P-450 MoAbs (APH-8 and APL-2). Lanes a, control (chemical-untreated); b, 3-MeO-AAB-treated; c, MeAαC-treated; d, MC-treated. k, kilodalton.

tral role in P-450 induction, as reported for the P-450 induction by MC, the species difference in enzyme induction may be attributable to the different properties of the cytosolic receptor protein(s) of a P-450 inducer among different species of animals.

As to P-450 isozymes induced by MC treatment, it has been reported that P450IA1 (P450c) and P450IA2 (P-450d) are induced in the rat, P450IA1 (P_1-450) and P450IA2 (P_3-450) in the mouse,[14] P-450-I, P-450-II and P450-III in the hamster,[20] and P-450_I, P-450_{IIA} and P-450_{IIB} in the guinea pig.[21] Among these MC-inducible P-450 isozymes, rat P450IA2 (mol. wt., 54,000),[16,22] hamster P-450-II (mol. wt., 58,500)[20] and guinea pig P-450_{IIA} (mol. wt., 54,000)[21] are of high spin form and show the greatest activity for the mutagenic activation and/or N-hydroxylation of carcinogenic aromatic amines. In our work too, it is quite probable that the enzymes mediating the mutagenic conversion of aromatic amines are certain P-450 isozymes of each species, since 1) the enzyme activity was induced by treatment of the animals with 3 kinds of P-450 inducers; 2) components reactive with the anti-rat P-450 MoAbs were detected from the inducer-treated microsomes by ELISA; 3) components having molecular weights similar to those of P-450 isozymes in each species of animals were detected by immunoblotting. In each species, the quantity of the P-450 component(s) detected by immunoblotting was closely correlated with the enzyme activity determined by the mutation assay. However, such a correlation was not always observed between different species. This is probably due to the different reactivity of our anti-rat P-450 MoAbs to P-450 components from different species.

We previously demonstrated that the activity and induction rate of a certain P-450 isozyme is correlated with the carcinogenic susceptibility of the mice and rat to aromatic amine carcinogens.[1,5-7] In this work, we have shown the existence of species differences in the activity and the indution rate of P-450 isozymes in rodents. However, in order to examine the correlation between these results and the carcinogenic susceptibility of the hamster and guinea pig, it will be necessary to carry out carcinogenicity experiments with the aromatic amines used in the present work.

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