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EVIDENCE FOR THE LACK OF HEPATIC *N*-ACETYLTRANSFERASE IN SUNCUS (*SUNCUS MURINUS*)

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Abstract—The abilities of liver cytosol fractions from the suncus and Sprague-Dawley (SD) rats to *N*-acetylate aniline, *p*-aminobenzoic acid, *p*-aminosalicylic acid and 2-aminofluorene (AF) were compared. The cytosol from rats *N*-acetylated these substrates at efficient rates, whereas the cytosol from the suncus did not *N*-acetylate these substrates at detectable rates. When AF was given to the suncus, 2-acetylaminofluorene (AAF), a metabolite of AF formed by *N*-acetyltransferase (NAT), was not detectable in serum, whereas the metabolite was seen clearly in rats. Northern blot and Southern blot analyses, using cDNAs coding for human NATs as probes, indicated that not only the transcripts but also the genes of the enzymes were undetectable in suncus. These results suggest that the suncus is among the few species known to lack NATs.

Key words: 2-acetylaminofluorene; *N*-acetyltransferase; 2-aminofluorene; arylamine; liver; suncus

The *Suncus murinus* (suncus), which belongs to the family of *Soricidae* of *Insectivora*, lives in Southeast Asia and looks like a ground mole (Fig. 1). This experimental animal has become increasingly useful for emesis research, since vomiting can be readily induced in the suncus [1, 2]. However, not much information is available on drug-metabolizing enzymes in this animal [3, 4]. In a previous study [5], we found that, in contrast to rats, the content of cytochrome P450IIE (CYP2E) in the suncus liver is decreased by starvation.

NAT[¶] is the only phase II drug-metabolizing enzyme that catalyzes the *N*-acetylation of arylamines and hydrazines. NAT localizes in the cytosol fraction of the liver and other organs and requires AcCoA for its activity. Hepatic cytosols of several animal species also catalyze the transfer of the acetyl moiety in arylhydroxamic acid into arylamines and *N*-hydroxyarylamines to form *N*-acetamides and *N*-acetoxyarylamines, respectively [6, 7]. Additionally, hepatic cytosols have been shown to activate *N*-hydroxyarylamines through AcCoA-dependent *O*-acetylation. These active metabolites bind covalently with endogenous macromolecules. Therefore, the above reactions are considered as major intoxication pathways for carcinogenic and mutagenic arylamines [8–13].

Recently, cDNA and genomic DNA clones coding for NAT were isolated from the livers of chickens [14], rabbits [15, 16], mice [17], hamsters [18] and humans [19–23]. In humans, two types of NAT cDNAs (NAT1 and NAT2) were found [20, 21]. Genomic analysis suggested that NAT1 and NAT2 are expressed in humans monomorphically and polymorphically, respectively [22–25]. Mammals such as rabbits, Syrian hamsters, rhesus monkeys, baboons, deer mice, laboratory mice and rats are also known as species that show *N*-acetylation polymorphism [26]. In contrast to the above animals, dogs and foxes have been shown to lack NATs [27].

We report herein that the suncus is an additional unique animal species that lacks NAT.

MATERIALS AND METHODS

Animals

Male suncus (7 weeks old), weighing 35–45 g, were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). Male Sprague–Dawley rats (7 weeks old), weighing 104–118 g, were obtained from the Nippon SLC Co. (Shizuoka, Japan). They were maintained in air-conditioned quarters with 12-hr light–dark cycles. Suncus and rats were given laboratory chow (Clea Japan, Tokyo, Japan) and water *ad lib.*

Preparation of liver cytosols

The animals were stunned by a blow on the head and decapitated. Livers were removed rapidly, washed,

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¶ Abbreviations: AAF, 2-acetylaminofluorene; AcCoA, acetyl coenzyme A; AF, 2-aminofluorene; DTE, dithioerythritol; DTT, dithiothreitol; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MOPS, morpholinopropanesulfonic acid (pH 7.0); NAT, *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; PAS, *P*-aminosalicylic acid; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SSC, standard saline citrate; and Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

Fig. 1. Male suncus (7 weeks old).

minced and homogenized with 3 vol. of 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM DTE and 1 mM EDTA. The homogenate was centrifuged at 9000 *g* for 20 min. The supernatant fraction was recentrifuged at 105,000 *g* for 60 min. The resultant supernatant fraction was defined as the cytosol. The concentration of cytosolic protein was determined by the method of Lowry *et al.* [28].

Spectrophotometric assay for NAT

Spectrophotometric assays for the *N*-acetylation of aniline, PABA and PAS were carried out according to the method of Andres *et al.* [29]. A typical incubation mixture consisted of 64.2 mM Tris–HCl (pH 7.5), 1.3 mM DTE, 1.3 mM EDTA, 6.4 mM acetyl phosphate, 0.64 U/mL phosphotransacetylase, a 0.14 mM concentration of each substrate and 2 mg/ml of cytosol in a final volume of 70 μL. After preincubation at 37° for 5 min, the *N*-acetylation reaction was initiated by the addition of 0.22 mM AcCoA in a final volume of 90 μL. The reaction was terminated by the addition of 50 μL of 20% (w/v) trichloroacetic acid. The mixture was centrifuged for 1 min, followed by incubation with 0.5 mL of 5% (w/v) dimethylaminobenzaldehyde (dissolved in acetonitrile) as a reagent for forming the chromogenic Schiff's base at room temperature (25°) for 10 min. The *N*-acetylated products formed in the reaction mixture were measured spectrophotometrically at 450 nm. Blank values were obtained by substituting water for AcCoA solution.

In vitro *assay for* N-*acetylation of AF*

The *N*-acetylation of AF was measured as described previously by Shinohara *et al.* [30]. Briefly, 100 mM Tris–HCl buffer (pH 7.4), containing 2 mg/mL of cytosol, 0.2 mM AcCoA, 0.2 mM DTT and 0.4 mM AF in a final volume of 100 μL, was added to a 1.5-mL tube. After preincubation at 37° for 3 min, the reaction was initiated by the addition of AcCoA. Incubations were carried out at 37° for 10 min. The reactions were terminated by the addition of 100 μL of acetonitrile. The mixture was centrifuged to precipitate protein. The supernatant was subjected to HPLC (Tosoh, Tokyo, Japan) using a Unisil NQ C18 packed column (4.6 × 250 mm) (GL Sciences, Tokyo, Japan). A mobile phase of acetonitrile-20 mM KH_2PO_4 (50:50, v/v) was used at a flow rate of 1.0 mL/min. The amount of AAF was quantified from its peak area of absorbance at 254 nm. The calibration curve generated from the peak area of standard AAF showed a good linearity in the range of 30 pmol to 60 nmol.

In vivo *acetylation of AF*

AF (50 mg/kg), dissolved in 10% dimethyl sulfoxide, was given intraperitoneally to male suncus and rats. Blood was collected from the heart periodically 1, 2, 4 and 8 hr after the injection. The blood samples were kept at 4° for 24 hr. Subsequently, the samples were centrifuged at 2,500 *g* for 15 min. The supernatants were analyzed by HPLC as described above.

Northern blot analysis

Total RNAs from livers of male suncus and rats were prepared by the guanidinium isothiocyanate method [31]. Poly(A)$^+$ RNAs were enriched by oligo (dT)-cellulose column chromatography [32]. Each poly (A)$^+$ RNA was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and 20 mM MOPS buffer (pH 7.0), and then transferred to a nylon membrane (Nytran NY13, Schleicher & Schuell, Dassel, Germany), and hy-

bridized with the [α-^{32}P]dCTP-labeled human NAT1 and NAT2 cDNAs [21] at 42° for 12 hr. Subsequently, the membrane was washed twice at 42° for 15 min with a solution of 1× SSC and 0.1% SDS. An X-ray film was exposed for 5 days.

Southern blot analysis

Genomic DNAs were prepared from the tail of the male suncus and from human blood [32]. Each genomic DNA (10 μg) digested with *Eco*RI or *Kpn*I was electrophoresed in a 0.8% agarose gel, and then transferred to a nylon membrane, and hybridized with the [α-^{32}P]dCTP-labeled human NAT1 or NAT2 cDNA at 65° for 12 hr. Subsequently, the membrane was washed twice at 65° for 30 min with a solution of 3× SSC and 0.1% SDS. An X-ray film was exposed for 3 days.

RESULTS

N-Acetylation activity of cytosols from suncus and rat livers

During the study to characterize drug-metabolizing enzymes in suncus liver, we found that aniline, PABA, PAS and AF were not *N*-acetylated by the cytosolic fraction from the suncus (Fig. 2). The detection limit of the spectrophotometric assay for the NAT was <0.01 nmol/min/mg protein. The NAT activities were seen using the same substrates when the liver cytosolic fraction from rats was added to the incubation mixture. The activities seen in rats were 0.66 ± 0.01, 1.01 ± 0.05, 1.46 ± 0.09 and 1.74 ± 0.10 (nmol/min/mg protein) for the *N*-acetylations of aniline, PABA, PAS and AF, respectively. These results suggest that the suncus lacks NAT in liver cytosol.

N-Acetylation of AF by liver cytosols from suncus and rats

To further support the idea that the suncus lacks NAT, *N*-acetylation of AF was measured with liver cytosols from the suncus and rats. For this experiment, AF and AcCoA were incubated with other necessary components in the presence of liver cytosol from either the suncus or rats. After incubations, the formation of AAF was determined by HPLC. The HPLC elution patterns of AF and AAF, the *N*-acetylated metabolite of AF, are shown in Fig. 3. The AAF peak was detected when AF was incubated with rat liver cytosol in the presence of acetyl CoA, whereas no apparent peak indicating the presence of AAF was detectable when suncus liver cytosol was added to the incubation mixture (Fig. 3). These results lend support to the idea that the suncus lacks NAT in liver cytosols. The activity of NAT was not detectable even when the reaction mixture was incubated for longer periods (up to 30 min), when the concentration of AF was increased up to 2 mM, and when the cytosol fraction was incubated with up to 8 mg protein/mL of the incubation mixture (Fig. 4).

Serum concentration of AAF after administration of AF

As shown above, the cytosol from suncus liver did not show detectable *N*-acetylation. Since NAT is known to be present in extrahepatic organs such as the lung, kidney, gut, spleen and blood in rats [33–36], it was necessary to examine the possibility of whether the enzyme was present in extrahepatic tissues of the suncus. To measure the NAT activity in whole extrahepatic organs, AF was given to the suncus, and AAF in serum was determined. The representative HPLC elution pattern of AF and its metabolites in serum 1 hr after administration of AF is shown in Fig. 5. Although AF was not *N*-acetylated in the suncus, AF was eliminated from the serum of the suncus at a rate faster than that of rats. The apparent discrepancy between the fast elimination of AF and the apparent lack of AAF formation in the suncus may be accounted for by the alternative formation of the unknown metabolite of AF designated as C in Fig. 5. AAF appeared in the serum of rats, and its concentration reached a plateau level within 1 hr after AF administration. The concentration of AAF in rat serum decreased with time and was not detectable 8 hr after administration, in contrast to the formation of the unknown metabolite B. On the other hand, the content of AAF in suncus serum could not be detected with HPLC, which probably resulted from the formation of unknown metabolite C produced in the suncus. These results strongly support the idea that the suncus lacks NAT in all organs (Fig. 6, a and b).

Northern blot and Southern blot analyses of NAT

The causes involved in the apparent lack of NAT in the suncus may be studied with reference to the reported

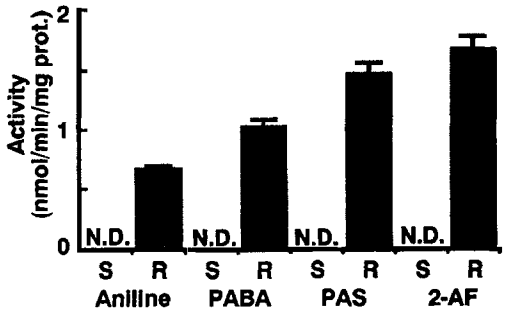

Fig. 2. Arylamine *N*-acetyltransferase activities in liver cytosols from suncus and rats. *N*-Acetylation of aniline, PABA, PAS and AF was determined spectrophotometrically, as described under Materials and Methods. Abbreviations: S, suncus cytosol; R, rat cytosol. ND = not detectable. Each value is the mean ± SEM from five animals. Almost the same results were obtained in two independent experiments.

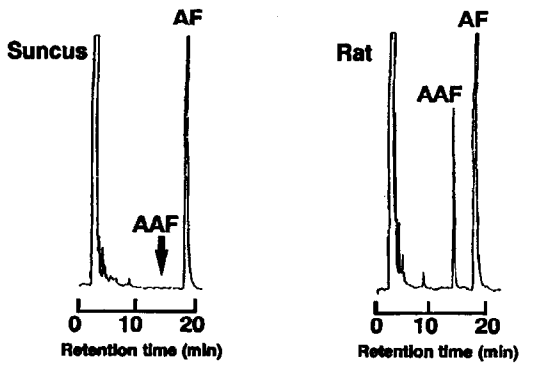

Fig. 3. HPLC elution patterns of AF and AAF extracted from reaction mixtures after incubation of AF in the presence of AcCoA and liver cytosol of suncus or rats.

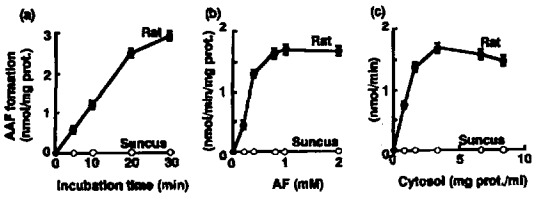

Fig. 4. *N*-Acetylation of AF by liver cytosols from suncus and rats. The *N*-acetylation of AF by liver cytosol from suncus (○) or rats (●) was measured as a function of incubation time (a), AF concentration (b), or the amount of liver cytosol added to the incubation mixture (c). Each point is the mean ± SEM for five animals.

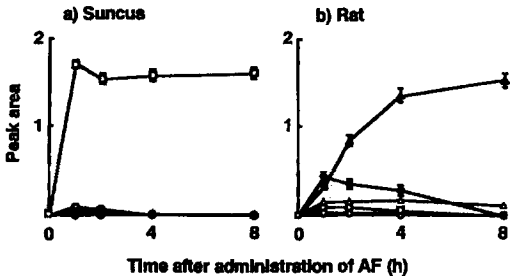

Fig. 6. Peak areas of AF, AAF and the unknown metabolites (A, B and C) in (a) suncus and (b) rat serum after administration of AF at a dose of 50 mg/kg. Key: (○) AF; (●) AAF; (△) metabolite A; (▲) metabolite B; (□) metabolite C. Each point is the mean ± SEM from five animals. Almost the same results were obtained in two independent experiments.

genetic defect of NAT in humans [25]. As a preliminary approach, we examined the expression of NAT mRNA. Northern blot analysis was performed using a mixed probe containing human monomorphic (NAT1) (D-24) and polymorphic (NAT2) (O-7) NAT cDNAs [21]. The results is presented in Fig. 7. When poly(A)$^+$ RNA (5 μg) from human liver was applied, as well as a positive control, a clear band was seen. On the contrary, no positive band appeared even when increased amounts (5–30 μg) of poly(A)$^+$ RNA from suncus liver were applied.

Since NAT mRNAs were not detected in suncus liver, we examined whether or not suncus lacked an NAT gene. Suncus genome digested with *Eco*RI or *Kpn*I was electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane. After hybridization with human NAT1 or NAT2 cDNA, the membrane was washed under low stringency. No bands were seen in suncus genome in contrast to human genome (Fig. 8).

DISCUSSION

The suncus is phylogenetically situated between primates and rodents [37]. This animal is small in body size, and is susceptible to motion sickness. In recent years, the suncus has been evaluated frequently as an animal model for research in the development of antiemetic drugs [1, 2]. Despite increasing interest in this animal species as a useful experimental animal, only a few studies on the properties of drug-metabolizing enzymes have been performed.

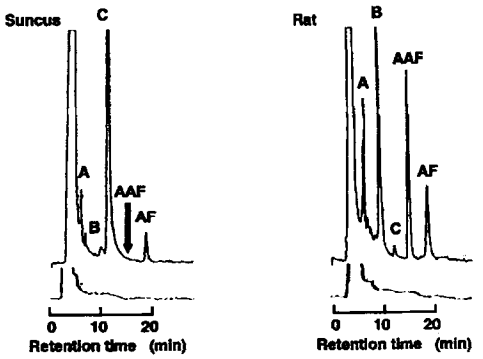

Fig. 5. Representative HPLC elution pattern of AF and its metabolites in serum from suncus and rats. Blood was collected 1 hr after intraperitoneal administration of AF at a dose of 50 mg/kg. The arrow indicates the retention time of AAF. A, B and C are unknown metabolites.

NAT polymorphism in humans was discovered as the result of studying the fate of isoniazid in tuberculous patients. This was later shown by observations with healthy families to be a genetically controlled matter. Numerous drugs and carcinogens, such as sulfamethazine, hydralazine, procainamide, dapsone, caffeine, benzidine, β-naphthylamine and AF, in addition to isoniazid, are acetylated polymorphically in humans *in vivo* and *in vitro*. On the other hand, PAS, PABA and sulfonamide are monomorphically acetylated *in vivo* [26, 28–41]. In hamsters, two forms of NAT, AT-I and AT-II, were purified from hamster livers. AT-II was a polymorphic NAT enzyme that *N*-acetylated AF and PABA [42]. The arylamine carcinogens, AF, α-naphthylamine, β-naphthylamine, benzidine and methylene bis-2-chloroaniline, are acetylated by polymorphic NAT as well as isoniazid and sulfamethazine in rabbits [41]. Although

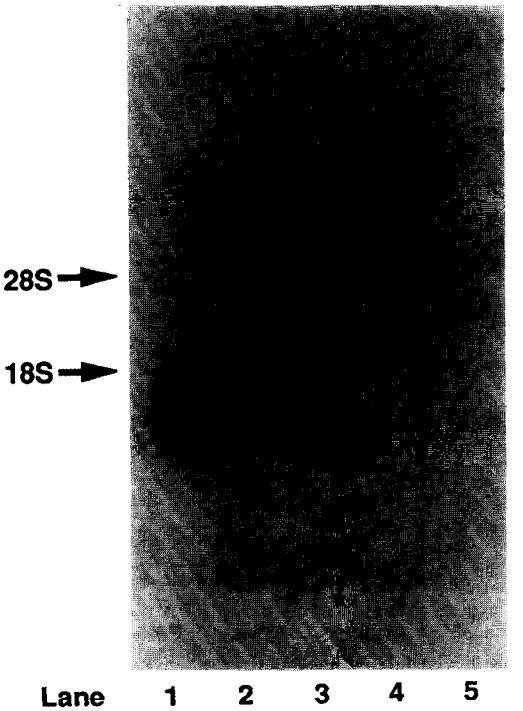

Fig. 7. Northern blot analysis for the expression of NAT mRNAs. Lane 1, poly(A)$^+$ RNA from human liver (5 μg); lanes 2–5, poly(A)$^+$ RNA from suncus liver (5, 10, 20 and 30 μg, respectively).

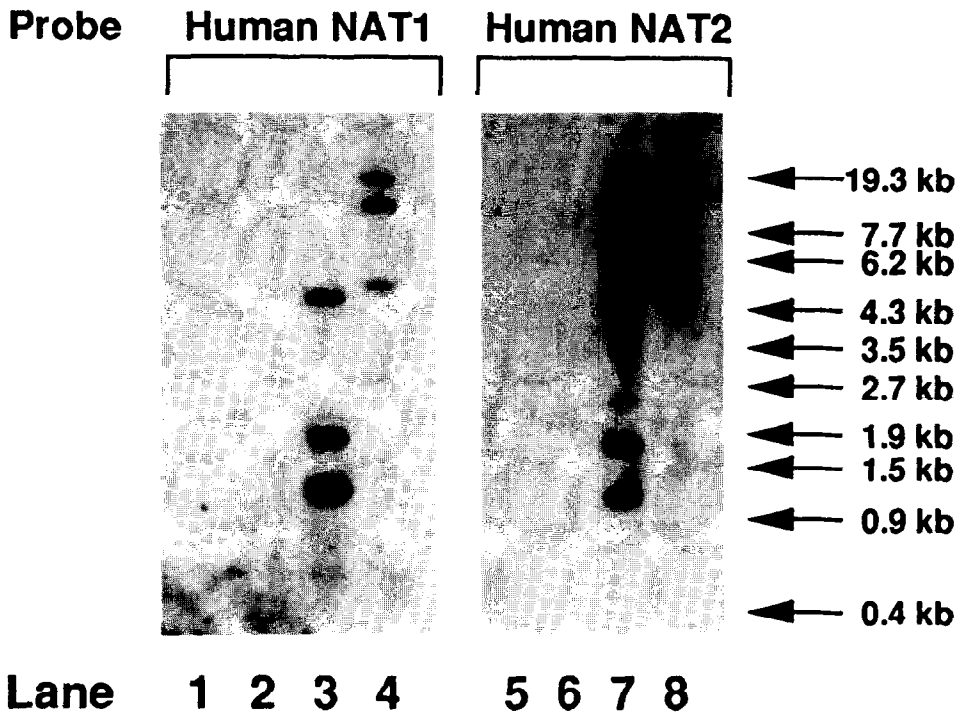

Fig. 8. Southern blot analysis for NAT genes. Lanes 1 and 5, suncus genome (10 μg) digested with *Eco*RI; lanes 2 and 6, suncus genome (10 μg) digested with *Kpn*I; lanes 3 and 7, human genome (10 μg) digested with *Eco*RI; lanes 4 and 8, human genome (10 μg) digested with *Kpn*I. Lanes 1–4, human NAT1 cDNA as a probe; lanes 5–8, human NAT2 cDNA as a probe.

we employed aniline, PAS, PABA and AF as substrates for NAT, we could not detect any NAT activities in suncus liver cytosols *in vivo* and *in vitro*, suggesting that this animal species lacks both monomorphic and polymorphic NATs.

Recently, it was reported that liver microsomes from dogs and rats possess NAT activities that are attributable to carboxyesterase [43, 44]. To elucidate whether or not microsomal fractions from suncus liver contain NAT and whether or not NAT is present in extrahepatic tissues, we measured the concentration of AAF in the blood after intraperitoneal administration of AF. The results indicated that AF was not *N*-acetylated in the suncus *in vivo*. Although we could detect neither mRNAs (Fig. 7) nor genes (Fig. 8) corresponding to suncus NATs, using human NAT1 and NAT2 cDNAs as probes, these results do not strictly indicate that suncus lacks NAT genes, since it is possible that the homology between suncus and human NATs is low. The following reasons may possibly account for the apparent lack of NAT in the suncus: (1) NAT gene is absent in the genome; (2) NAT gene is not transcribed; (3) NAT protein is expressed but is not active for *N*-acetylation. The purpose of our current study was to elucidate why suncus lacks NAT activities.

The *O*-acetylation of hydroxyarylamines is believed to be mediated also by AcCoA-dependent NAT. The *O*-acetylation reaction is known to participate in the metabolic activation of heterocyclic amines, such as Glu-P-1, Trp-P-2, IQ, MeIQ, MeIQx and PhIP, leading to the formation of highly reactive products capable of binding covalently to tissue macromolecules [45, 46]. Although it has not been directly tested, one would have every reason to expect that *O*-acetylation will also be impaired in this species. If the suncus does not possess the capacity to *O*-acetylate the carcinogenic amine compounds, the suncus is an additional useful animal that has no capacity to activate heterocyclic amines.

Williams [27] reported that dogs and foxes lack NAT. Accordingly, *N*-acetylated sulfanilamide was not detected in urine after oral administration of sulfanilamide. Since these animals are larger in size than the suncus, the latter may be more useful for drug metabolism and/or toxicological studies than those other animals.

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