METABOLISM OF CARCINOGENIC AZO DYE SUDAN I BY RAT, RABBIT, MINIPIG AND HUMAN HEPATIC MICROSOMES

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We investigated the ability of hepatic microsomal samples from different species including human to metabolize rodent carcinogen Sudan I (C.I. Solvent Yellow 14, 1-(phenylazo)-2-naphthol). A comparison between experimental animals and the human microsomal enzymatic system is essential for the extrapolation of animal carcinogenicity data to assess human health risk. Major metabolites produced from Sudan I by microsomes of all species were C-hydroxylated derivatives identified as 1-[(4-hydroxyphenyl)azo]-2-naphthol and 1-(phenylazo)naphthalene-2,6-diol. Additional minor C-hydroxylated products of Sudan I oxidation were 1-[(4-hydroxyphenyl)azo]naphthalene-2,6-diol and 1-[(3,4-dihydroxyphenyl)-azo]-2-naphthol. Human microsomes generated the pattern of Sudan I metabolites reproducing that formed by hepatic microsomes of rats. While microsomes of rabbit and minipig favored the production of the metabolite hydroxylated in position 6 of the naphthol ring of the Sudan I molecule, those of human and rat predominantly produced 1-[(4-hydroxyphenyl)azo]-2-naphthol. Therefore, rat microsomes are a suitable *in vitro* system mimicking the metabolism of Sudan I in humans. To define the role of specific cytochromes P450 in the Sudan I metabolism by rat microsomes, we investigated the modulation of Sudan I oxidation by specific inducers and selective inhibitors of these enzymes. The results suggest that cytochromes P450 1A1 and 3A are responsible for Sudan I metabolism by rat microsomes. Using purified enzymes, their ability to oxidize Sudan I was confirmed. The data clearly demonstrate the predominant role of cytochrome P450 1A1 in the Sudan I metabolism and suggest a carcinogenic potency of this rodent carcinogen for humans.

Keywords: 1-(Phenylazo)-2-naphthol; Azo compounds; Risk assessment; Metabolism; Cytochromes P450; Mechanism of action; Carcinogenesis; Mutagenesis; Toxicology.

Sudan I [C.I. Solvent Yellow 14, 1-(phenylazo)-2-naphthol] was used as a food coloring substance in several countries[1], but it has been recommended as unsafe, because it causes tumors in liver or urinary bladder of rats, mice, and rabbits[1–5]. In spite of its carcinogenicity for rodents[1–5], Sudan I was evaluated to be still unclassifiable as carcinogenic for humans by the International Agency for Research on Cancer (IARC)[5]. In addition, Sudan I is a potent contact allergen and sensitizer, eliciting pigmented contact dermati-

tis in human[6,7]. Nevertheless, this dye is widely used in coloring materials such as hydrocarbon solvents, oils, fats, waxes, plastics, printing inks, and shoe and floor polishes[1,5]. Moreover, Sudan I is an important compound being the simplest in the series of azo dyes and pigments that are used in large quantities and occur everywhere in red- and orange-colored consumer goods, food and printed materials. Such a wide use of these azo dyes could result in an occupational exposure.

Sudan I gives positive results in *Salmonella typhimurium* mutagenicity tests with S-9 activation[8,9] and is mutagenic to mouse lymphoma L5178Y $TK^{+/-}$ cells *in vitro*, with S-9 activation[9]. It is a clastogenic compound, inducing formation of micronuclei in the bone marrow of rats[3]. *In vivo* studies on the metabolism of Sudan I in rabbits revealed that this compound is metabolized primarily in the liver by oxidative or reductive reactions[10,11]. C-Hydroxylated metabolites 1-[(4-hydroxyphenyl)azo]-2-naphthol (4′-OH-Sudan I) and 1-(phenylazo)naphthalene-2,6-diol (6-OH-Sudan I) were found to be the major products of Sudan I oxidation *in vivo*[1,10]. These derivatives were found as major excreted products in urine[10]. Besides the C-hydroxylated metabolites, which are considered to be detoxication products, the benzenediazonium ion (BDI) formed by microsome-dependent enzymatic splitting of the azo group of Sudan I was found to react with DNA *in vitro*[12–14]. The major DNA adduct formed in this reaction has been characterized and identified as an 8-(phenylazo)guanine adduct[14]. In addition to microsomal enzymes, Sudan I and its C-hydroxylated metabolites are also oxidized by peroxidases[15–19]. In these reactions, DNA, RNA and protein adducts are also formed[15–19].

Since cytochromes P450 (CYP) are abundant in liver[20] where much of the metabolism of Sudan I in experimental animals is reported to occur[10], CYPs were assumed to play a role in the oxidative metabolism of this carcinogen[10,12–14]. Recently we found that most of Sudan I metabolism in human hepatic microsomes is mediated by CYP1A1 and that participation of CYP3A4 should be taken into account[21].

Comparison between experimental animals and human CYPs is essential for the extrapolation of animal carcinogenicity data to assess human health risk[22]. To assess the human health hazard of Sudan I, we have compared the capacity to metabolize Sudan I of livers from humans and species, which succumb to tumors in exposition studies with this carcinogen, rats and rabbits[1–5].

EXPERIMENTAL

Abbreviations used: Ah receptor, aryl hydrocarbon receptor; α-NF, α-naphthoflavone; β-NF, β-naphthoflavone; BDI, benzenediazonium ion; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propane-1-sulfonate; CYP, cytochrome P450; DDTC, sodium diethyldithiocarbamate; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IARC, International Agency for Research on Cancer; 3-IPMDIA, 3-isopropenyl-3-methyldiamantane; 4′-OH-Sudan I, 1-[(4-hydroxyphenyl)azo]-2-naphthol; 6-OH-Sudan I, 1-(phenylazo)naphthalene-2,6-diol; 4′,6-di(OH)-Sudan I, 1-[(4-hydroxyphenyl)azo]naphthalene-2,6-diol; 3′,4′-di(OH)-Sudan I, 1-[(3,4-dihydroxyphenyl)azo]-2-naphthol; PB, phenobarbital (5-ethyl-5-phenylpyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione; PCN, pregnenolone-16α-carbonitrile (3β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile); R_F, retention factor (relative mobility); TLC, thin layer chromatography.

Chemicals

Chemicals were obtained from the following sources: α-naphthoflavone (α-NF), β-naphthoflavone (β-NF), pregnenolone-16-carbonitrile (PCN), NADPH, testosterone, troleandomycin, ketoconazole ((±)-*cis*-1-acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1*H*-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine), sodium diethyldithiocarbamate (DDTC), 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulfonate (CHAPS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine, glucose 6-phosphate, chlorzoxazone (5-chloro-3*H*-benzooxazol-2-one) and quinidine from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 7-pentoxy- and 7-ethoxyresorufin from Fluka Chemie AG (Buchs, Switzerland), glutathione from Roche Diagnostics Mannheim (Germany), furafylline from New England Biolabs (Beverley, MA, U.S.A.), 6β-hydroxytestosterone from Merck (Darmstadt, Germany), glucose 6-phosphate dehydrogenase from Serva (Heidelberg, Germany), bufuralol and its 1′-hydroxy derivative were from Gentest Corp. (Woburn, MA, U.S.A.), bicinchoninic acid from Pierce (Rockford, IL, U.S.A.) and Sudan I (1-(phenylazo)-2-naphthol) from British Drug Houses (Pool, U.K.). Sulfaphenazole (4-amino-*N*-(1-phenyl-1*H*-pyrazol-5-yl)benzenesulfonamide) was kindly provided by P. Anzenbacher (Palacký University, Olomouc). 3-Isopropenyl-3-methyldiamantane (3-IPMDIA) was synthesized according to Olah and collaborators[23]. Its purity was >99.5% (estimated by HPLC, methanol–water, 95:5, v/v). 1-[(4-Hydroxyphenyl)azo]-2-naphthol (4′-OH-Sudan I), 1-(phenylazo)naphthalene-2,6-diol (6-OH-Sudan I), 1-[(4-hydroxyphenyl)azo]naphthalene-2,6-diol [4′,6-di(OH)-Sudan I] and 1-[(3,4-dihydroxyphenyl)azo]-2-naphthol [3′,4′-di(OH)-Sudan I] were synthesized as described[12,24]. They were purified by column chromatography on basic alumina and by TLC on silica gel[12,24] (Merck, Darmstadt, Germany). Their purity was >99% (estimated by HPLC, methanol–0.1 M NH_4HCO_3, pH 7.5; 9:1, v/v). All commercial and other chemicals were reagent grade or better.

Preparation of Microsomes and Assays

Microsomes from livers of ten untreated rats and three rabbits were prepared by the procedure described previously[14]. Microsomes from the livers of ten male Wistar rats pretreated with β-NF [14] and Sudan I [25] were isolated as described[14,26]; those pretreated with phenobarbital (PB) as reported by Hodek *et al.*[27], those pretreated with PCN as reported by Gut *et al.*[28]

and those pretreated with ethanol were isolated using a procedure described by Yang *et al.*[29] Human hepatic microsomes used in experiments were a pooled sample prepared by mixing microsomes from human liver of eight donors who died after a traffic accident, isolated as described[30], which were a gift of B. Szotáková (Faculty of Pharmacy, Charles University, Hradec Králové). The age of the donors ranged from 24 to 70 years and included five males and three females. All the samples had no known drug history. None of the donors had a history of alcohol abuse. The activities of individual CYP enzymes in human hepatic microsomes used in the experiments were in nmol/min/nmol total CYP as follows: 0.08 for 7-ethoxyresorufin O-deethylation (CYP1A1/2), 1.05 for coumarin 7-hydroxylation (CYP2A6), 0.19 for tolbutamide methyl hydroxylation (CYP2C9), 2.12 for bufurarol 1′-hydroxylation (CYP2D6), 1.69 for chlorzoxazone 6-hydroxylation (CYP2E1) and 7.13 for testosterone 6β-hydroxylation (CYP3A4). Microsomes from the liver of a male minipig were a gift from P. Anzenbacher (see above) and isolated as described[30]. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard[31]. The concentration of CYP was estimated according to Omura and Sato[32] by measuring the absorption of the complex of reduced CYP with carbon monoxide. Rat, rabbit, minipig and human liver microsomes contained 0.62, 1.82, 0.89 and 0.22 nmol CYP/mg protein, respectively. Microsomes of rats induced with β-NF, PB, PCN and ethanol contained 1.30, 2.74, 1.55 and 1.80 nmol CYP/mg protein, respectively.

Isolation of Individual CYPs

The CYP1A2, 2B4, 2C3 and 2E1 were isolated from liver microsomes of rabbits induced with β-naphthoflavone (CYP1A2), phenobarbital, (CYP2B4) and ethanol (CYP2E1, 2C3), by procedures described by Haugen and Coon[33] and Yang *et al.*[34]. The CYP3A1 and 3A6 were isolated from rat and rabbit hepatic microsomes of animals induced with PCN [28] and rifampicin[35], respectively. The procedure was analogous to that used for isolation of CYP2B4. Recombinant rat CYP1A1 protein was purified to homogeneity by the procedure described previously[36] from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA, in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, TX, U.S.A.) by P. Hodek (Charles University, Prague). Recombinant human CYP1A2 was from Oxford Biomedical Research, Inc. and human recombinant CYP3A4 was a gift of P. Anzenbacher (see above). Rabbit liver NADPH:CYP reductase was purified as described[37]. Rabbit liver cytochrome b_5 was prepared as described elsewhere[38,39].

Incubations

Unless stated otherwise, final concentrations in incubation mixtures used for study of the Sudan I metabolism were: 50 mM sodium phosphate buffer (pH 7.4), 1 mM NADPH, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase, 10 mM $MgCl_2$, and 0.1–100 μM Sudan I. The final volume of this mixture (750 μl) contained also 7.5 μl of methanol (used as solvent for Sudan I) and microsomal fraction with an amount of CYP 0.05–2.4 nmol. The reaction was initiated by adding the substrate. Incubations with purified CYP reconstituted with NADPH:CYP reductase and cytochrome b_5 contained 50–250 pmol of each CYP (instead of microsomal fraction). Briefly, CYP was reconstituted as follows (0.5 μM CYP, 0.5 μM NADPH:CYP reductase, 0.5 μM cytochrome b_5, 0.5 μg/μl CHAPS, 2.0 μg/μl liposomes [dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine (1:1:1)], 3 mM reduced glutathione and 50 mM HEPES/KOH, pH 7.4)[40,41].

An aliquot of the reconstitution mixture was then added to incubation medium. Another aliquot of the reconstitution mixture was used for estimation of CYP activities with typical substrates. The reconstitution mixture was analyzed for specific CYP activities by monitoring the following reactions: 7-ethoxyresorufin O-deethylation (CYP1A1/2), 7-pentoxyresorufin O-dependentylation (CYP2B4)[42], tolbutamide methyl hydroxylation (CYP2C3), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A)[43]. In the control incubation, the CYP was omitted from the reconstitution mixture. After incubation in open glass tubes (37 °C, 5–60 min), the incubation mixtures were extracted twice with ethyl acetate (2 × 0.75 ml). The extracts were evaporated under nitrogen, dissolved in a minimum volume of methanol, chromatographed on a thin layer of silica gel and developed in hexane–diethyl ether–acetone (1:0.7:0.3, v/v). The same TLC was performed with standards. The R_F values of 3′,4′-di(OH)-Sudan I, 4′,6-di(OH)-Sudan I, 6-OH-Sudan I, 4′-OH-Sudan I and Sudan I were 0.18, 0.23, 0.47, 0.53 and 0.87, respectively. The benzenediazonium ion was detected by azo coupling with 3-methyl-1-phenylpyrazol-5(4*H*)-one (1-phenyl-3-methyl-5-pyrazolone) as described[12–14]. Alternatively, the products, dissolved in methanol, were separated by HPLC on a MN Nucleosil 100-5 C18 column (Macherey–Nagel, 4.0 × 250 mm) preceded by a C-18 guard column. A mixture of methanol:0.1 M NH_4HCO_3, (pH 7.5; 9:1, v/v) with flow rate of 0.8 ml/min, was used to elute the metabolites, detection was at 254, 333 and 480 nm. The Sudan I metabolites were identified by co-chromatography with authentic standards, 3′,4′-di(OH)-Sudan I, 4′,6-di(OH)-Sudan I, 6-OH-Sudan I and 4′-OH-Sudan I, having the retention times 3.4, 3.8, 5.2 and 5.7 min, respectively, and by mass and UV/VIS spectroscopy[12,24]. Recoveries of products were around 95% in the presence of enzymes without a CYP cofactor (NADPH).

Inhibition Studies

The following chemicals (CYP inhibitors)[20] were used to inhibit the metabolism of Sudan I in hepatic microsomes and in the reconstitution experiments with purified CYPs (the inhibited CYPs are given in parenthesis): α-NF (1A1 and 1A2), furafylline (1A2), 3-IPMDIA (2B)[44,45], DDTC (2A6 and 2E1), sulfaphenazole (2C), quinidine (2D), troleandomycin and ketoconazole (3A). Inhibitors were dissolved in 7.5 μl of methanol to give final concentrations of 1–100 μM in the incubation mixtures. The mixtures containing the inhibitors were then incubated at 37 °C for 5 min with NADPH prior to adding Sudan I and then for another 30 min at 37 °C. An equal volume of methanol alone was added to the control incubations.

RESULTS

Comparison of Sudan I Metabolism by Rat, Rabbit, Minipig and Human Hepatic Microsomes

When Sudan I was incubated with rat, rabbit and human hepatic microsomes in the presence of NADPH, several product peaks were observed by HPLC analysis (Fig. 1). On the basis of co-chromatography with synthetic standards, and mass and UV/VIS spectroscopy[12,24], major metabolites pro-

duced from Sudan I by all microsomes were identified as 4′-OH-Sudan I and 6-OH-Sudan I. Additional minor products were 4′,6-di(OH)-Sudan I and 3′,4′di(OH)-Sudan I (Fig. 1). Another metabolite was the colorless product previously identified as the benzenediazonium ion (BDI)[12,14] (not shown in the chromatogram in Fig. 1). No products were observed when NADPH was omitted from the incubation mixtures. The formation of Sudan I metabolites with microsomal systems was time-dependent, being linear early in the incubation (20 min) but appeared to significantly deviate from linearity later on (data not shown).

In addition to rat and rabbit, minipig was found to be another animal model suitable to mimic metabolism of several xenobiotics in humans[30]. Hence, the liver microsomal system of this species was also tested to investigate whether it is an appropriate enzyme model for Sudan I metabolism. While microsomes of rabbit and minipig favored the production of the metabolite hydroxylated on carbon 6 of the naphthol ring of the Sudan I molecule, those of human and rat predominantly produced 4′-OH-Sudan I (Fig. 2). The species difference in catalytic activities of CYPs might be the cause of these metabolic differences.

To confirm this suggestion, it was necessary to identify the most efficient CYP enzymes metabolizing Sudan I in microsomes of all the used animal species and characterize the products of reactions. Three experimental ap-

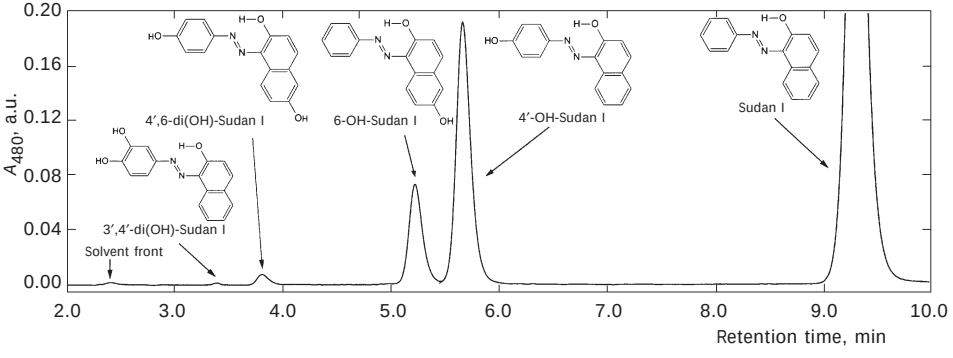


FIG. 1

HPLC chromatogram (at 480 nm) of Sudan I metabolites formed by human microsomes. Incubations (1 mM NADPH, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase, mixed human microsomal sample containing 0.1 nmol CYP, 100 μM Sudan I dissolved in 7.5 μl methanol in 50 mM potassium phosphate buffer, pH 7.4, final volume 750 μl) were stopped after 20 min by extraction with ethyl acetate and the extracts were analyzed by HPLC (see Experimental)

proaches were employed for such a study: (i) selective inhibition of CYPs, (ii) induction of specific CYPs, and (iii) utilization of the purified CYPs reconstituted with NADPH:CYP reductase.

An inhibitor of CYP1A1/2, α-NF, was highly effective in inhibiting Sudan I oxidation by rat hepatic microsomes; the concentration of α-NF equimolar to that of Sudan I inhibited its oxidation by ≈53%. Inhibitors of CYP3A enzymes, ketoconazole and troleandomycin, were also highly effective in inhibiting Sudan I oxidation (Fig. 3a). Inhibitors of CYP2C (sulfaphenazole) and 2D (quinidine) were much less effective and those of other CYPs (furafylline, 3-IPMDIA, DDTC) were without effect (Fig. 3a). These results suggest a major role of CYP1A1 and 3A in Sudan I oxidation by hepatic microsomes of uninduced rats. It should be noted that the interpretation of the results of inhibitors is sometimes difficult, because one inhibitor may be more effective with one substrate than another. Therefore, to confirm the role of these rat CYPs in Sudan I oxidation, the induction of individual CYP enzymes was performed with this animal model.

Microsomes isolated from livers of rats pretreated with β-NF or Sudan I (enriched with CYP1A1/2), PB (enriched with CYP2B1/2), PCN (enriched with CYP3A1/2) and ethanol (enriched with CYP2E1) were used in the experiments. Incubations of Sudan I with β-NF, and Sudan I-microsomes led to ten-fold increase in formation of Sudan I metabolites (Fig. 4). An inhibitor of CYP1A1/2, α-NF, strongly inhibited Sudan I oxidation in β-NF micro-

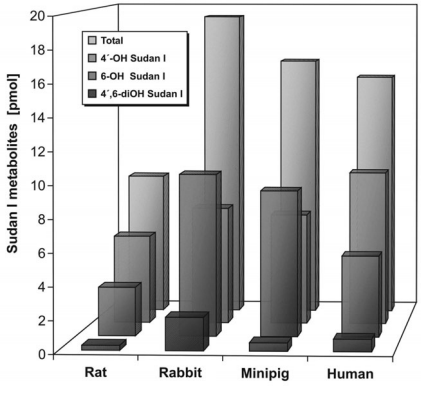


FIG. 2

Oxidation of Sudan I to ring-hydroxylated metabolites by rat, rabbit, minipig and human hepatic microsomes. Microsomes containing 1 nmol CYP and 100 μM Sudan I were used in all experiments. Other conditions were the same as in Fig. 1. Amounts of Sudan I metabolites are averages of triplicate incubations. Standard deviations were equal to or less than 10%

somes, but a selective inhibitor of CYP1A2, furafylline, was without effect (Fig. 3b). Inhibitors of other CYPs were ineffective (not shown). These results support the former suggestion (see above) that CYP1A1 is the enzyme oxidizing Sudan I in the rat microsomal system.

An inhibitor of CYP2B, 3-IPMDIA [45], slightly inhibited Sudan I oxidation by microsomes enriched with CYP2B1/2 (PB microsomes) (Fig. 3b), suggesting a minor role of CYP2B enzymes in this oxidation and explaining a

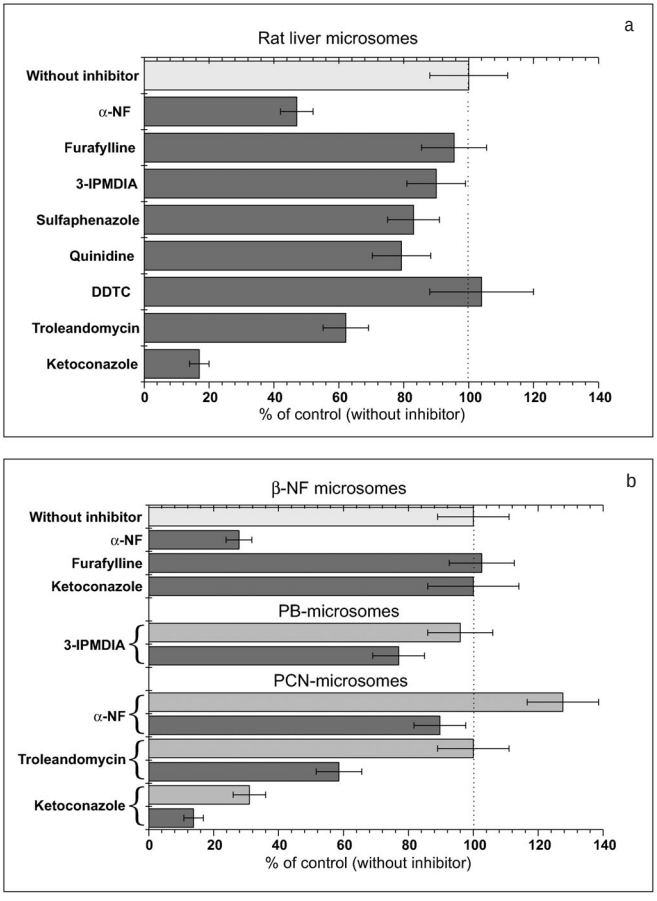


FIG. 3

Effect of CYP inhibitors on Sudan I oxidation by rat (a and b), rabbit (c) and minipig (d) microsomes. 100 μM Sudan I, 10 μM (light columns) and 100 μM (dark columns) inhibitors were used in experiments. Other conditions were the same as in Fig. 1. Amounts of Sudan I metabolites are averages and standard deviations of triplicate incubations

two-fold increase in formation of Sudan I metabolites in these microsomes (Fig. 4).

In rat microsomes enriched with CYP3A1/2 (PCN microsomes), selective inhibitors of CYP3A, ketoconazole and troleandomycin at equimolar concentrations with Sudan I, decreased its oxidation significantly, by 86 and 42%, respectively (Fig. 3b). Contrary to uninduced or β-NF microsomes, α-NF caused a different effect on Sudan I oxidation by PCN microsomes. α-NF at 10 μM, which is ten-fold less that the Sudan I substrate concentration, increased the Sudan I oxidation, by ≈30% (Fig. 3b). This is in accordance with data published previously[35], showing stimulation effects of

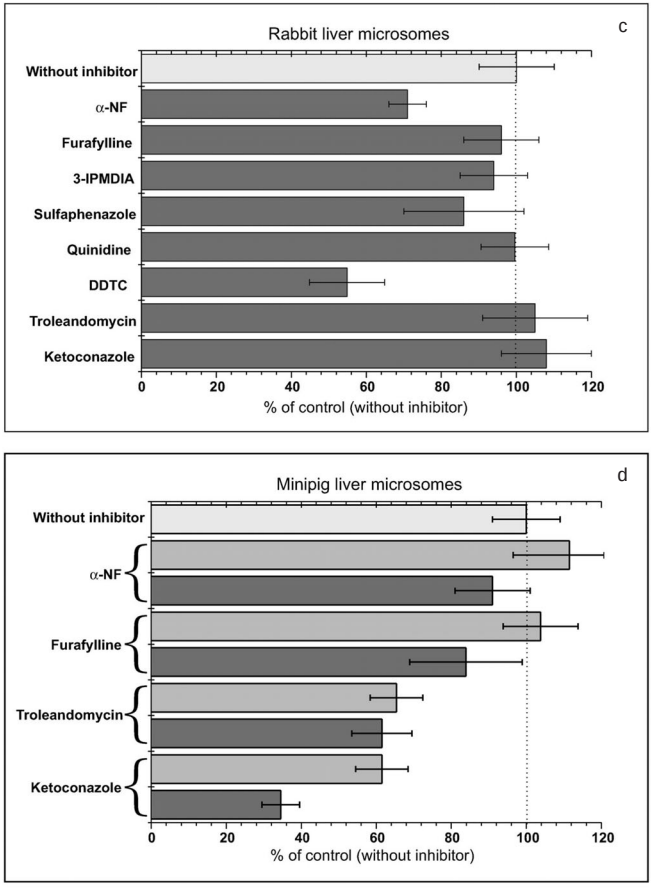


FIG. 3
(*Continued*)

α-NF on oxidation of several compounds by CYP3A under certain conditions. On the basis of these data, we attribute most of the Sudan I metabolism by PCN microsomes to CYP3A. However, CYP3A seem to be less effective enzymes than CYP1A1, because their induction by PCN in rats did not increase the efficiency of hepatic microsomes to oxidize Sudan I (Fig. 4).

The pretreatment of rats with ethanol decreased the Sudan I metabolism (Fig. 4), indicating no participation of rat CYP2E1 in Sudan I oxidation.

The Sudan I oxidation catalyzed by hepatic microsomes of another animal species susceptible to carcinogenic effects of Sudan I, rabbit, significantly decreased by addition of inhibitors of CYP2E1 and 1A1/2, DDTC and α-NF, respectively (Fig. 3c). The inhibition effects of inhibitors of other CYPs were insignificant. Therefore, besides CYP1A1, the Sudan I oxidation in rabbit microsomes might also be mediated by CYP2E1. Rabbits show larger interindividual variation in the expression of CYP enzymes and catalytic activities than rats[35]. Hence, rabbits are less suitable models for experiments, in which specific CYP inducers are utilized in the CYP enrichment. The effects of specific CYP inducers on the Sudan I oxidation by rabbit enzyme systems were, therefore, not studied herein.

In contrast to rat and rabbit microsomal systems, α-NF did not inhibit the Sudan I oxidation catalyzed by minipig microsomes. A slight stimula-

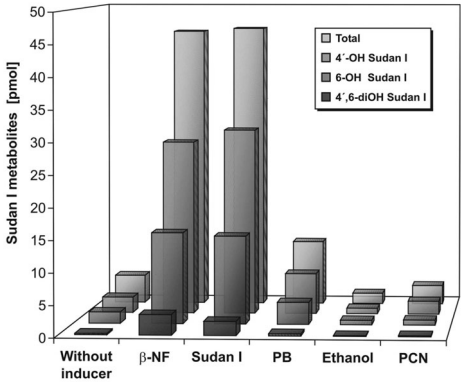


FIG. 4

Oxidation of Sudan I to ring-hydroxylated metabolites by hepatic microsomes from rats pretreated with selective CYP inducers. Microsomes containing 1 nmol CYP and 100 μM Sudan I were used in all experiments. Other conditions were the same as in Fig. 1. Amounts of Sudan I metabolites are averages of triplicate incubations. Standard deviations were equal to or less than 10%

tion of the reaction by α-NF was even detected (Fig. 3d). The strongest inhibitors in this system were selective inhibitors of CYP3A, ketoconazole and troleandomycin (Fig. 3d). Inhibitors of other CYPs were ineffective (not shown). All these results suggest that CYP3A might be the major CYP enzyme oxidizing Sudan I in minipig microsomes.

Oxidation of Sudan I by Purified CYP Enzymes

To confirm the role of individual CYPs in Sudan I oxidation, several CYP enzymes were purified, reconstituted with NADPH:CYP reductase and cytochrome b_5 (ref.[41]) and used as the oxidation system. All used CYPs reconstituted with reductase were active with their typical substrates (data not shown). Among the CYP enzymes tested, rat recombinant CYP1A1 was the most efficient enzyme oxidizing Sudan I followed by human CY3A4 and rabbit CYP3A6 (Fig. 5). Other CYPs were much less effective (rabbit CYP2E1, rat CYP3A1, human and rabbit CYP1A2) or ineffective under the conditions used (rabbit CYP2B4 and 2C3) (Fig. 5). An inhibitor of CYP1A, α-NF at 10 μM, which is ten-fold less than the Sudan I concentration, inhibited its CYP1A1-mediated oxidation, by 45%, while the concentration equimolar to that of Sudan I inhibited its oxidation by 72% (Fig. 5).

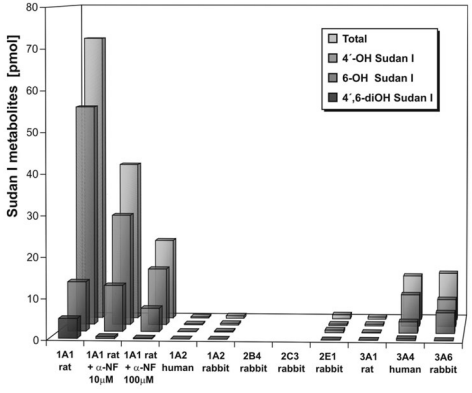
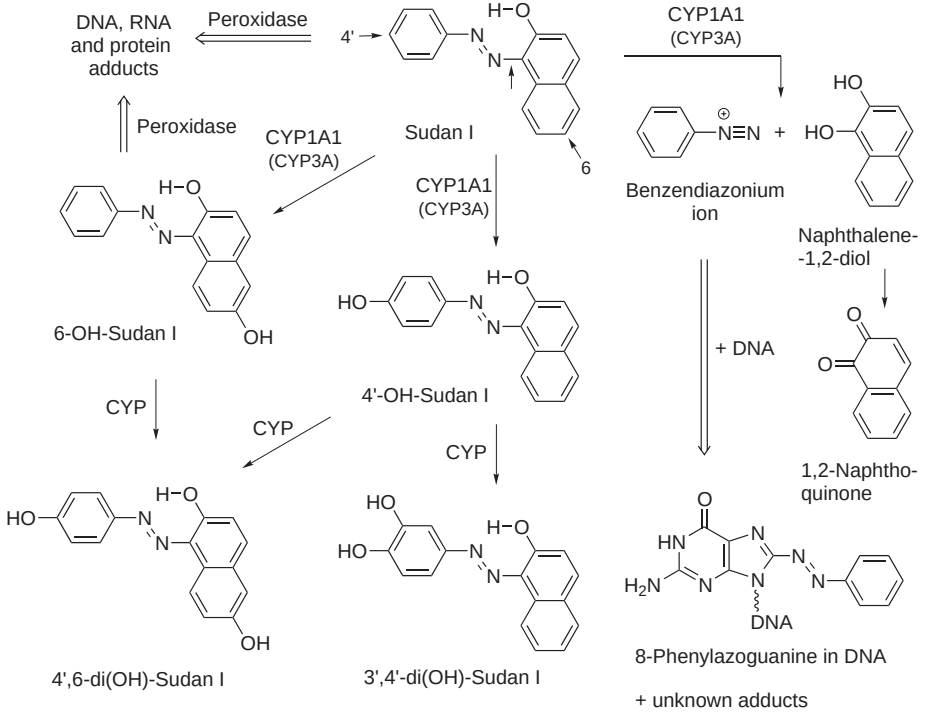


FIG. 5
Oxidation of Sudan I to ring-hydroxylated metabolites by purified CYPs reconstituted with NADPH:CYP reductase and the effect of α-NF on Sudan I oxidation by CYP1A1. 100 pmol reconstituted CYP/incubation and 100 μM Sudan I were used in all experiments. Other conditions were the same as in Fig. 1. Amounts of Sudan I metabolites are averages of triplicate incubations. Standard deviations were equal to or less than 10%

DISCUSSION

The results of this study clearly demonstrate that hepatic microsomes of different species including humans oxidize Sudan I. While ring hydroxylation of Sudan I seems to be a detoxication pathway of this carcinogen, its oxidative splitting to BDI was considered to be an activation pathway[10,12–14]. However, one of the detoxicating products, 6-OH-Sudan I, is effectively activated by prostaglandin H synthase, a prominent enzyme present in the second target organ for Sudan I carcinogenicity, the urinary bladder, to form DNA adducts[19,46]. Hence, the oxidation of Sudan I to this product is a possible activation pathway of its metabolism (Scheme 1).

One of the most important results found in the present study is the finding that the metabolism of Sudan I by the rat enzymatic system is analogous to that observed in humans. Human microsomes generated a pattern of Sudan I metabolites reproducing that found to be formed by hepatic microsomes of rats. Human and rat hepatic microsomal samples preferred



SCHEME 1
Proposed pathways of Sudan I metabolism

formation of 4′-OH-Sudan I to 6-OH-Sudan I, which is in contrast to findings in rabbit and minipig, where the formation of 6-OH-Sudan I is prevalent. Furthermore, recently[21] we determined that human microsomes also formed the DNA adduct chromatographically indistinguishable from that formed by the rat microsomal system[21]. In addition, we found that analogous CYPs oxidize Sudan I in rat (present paper) and human microsomes[21]. Besides the major CYP enzyme oxidizing Sudan I in microsomes of both species (CYP1A1), the CYP enzymes of a 3A subfamily [CYP3A1 (present paper) and CYP3A4 [21]] also participate in Sudan I oxidation. The efficiency of purified CYP3A to oxidize Sudan I is much lower than that of CYP1A1. However, because of high expression levels of CYP3A4 and 3A1/2 in human and rat livers[20], respectively, their contribution in Sudan I metabolism should be considered as significant. While CYP1A1, a major enzyme oxidizing Sudan I, is not constitutively expressed in human livers, it seems to be induced by planar aromatic compounds binding to the aryl hydrocarbon (Ah) receptor, *e.g.* 2,3,7,8-tetrachlorodibenzodioxin[47] and/or by polycyclic aromatic hydrocarbons present in cigarette smoke[20,48–51]. The CYP1A1 enzyme is even strongly induced by Sudan I itself in rats by this mechanism[52]. Hence, long-term occupational exposure of humans to Sudan I might be an important risk factor for individuals, because of increasing Sudan I metabolism and toxicological relevance. Interestingly, highly homologous human CYP1A1 and 1A2, having 73% amino acid sequence identity, exhibit extremely different potency in oxidation of Sudan I. The CYP1A2 enzyme is almost ineffective in Sudan I oxidation.

In contrast to several CYP substrates whose metabolism in minipigs was analogous to that in humans[30], Sudan I is metabolized by minipig microsomes differently from human microsomes. Therefore, minipigs are not suitable animal models to predict metabolism and carcinogenicity of Sudan I for humans. Similarly, rabbit microsomes metabolized Sudan I distinguishably from the human system. Diverse pattern of Sudan I metabolites formed by rabbit and minipig microsomes might be caused not only by various CYP enzymes oxidizing this carcinogen, but also by different specific regioselectivity of individual CYPs of these species. Indeed, human CYP3A4 and rat CYP3A1 produced prevalently 4′-OH-Sudan I, while orthologous rabbit CYP3A6 generated more efficiently 6-OH-Sudan I (see Fig. 5). Likewise, distinct regioselectivity of CYPs of a human and minipig 3A subfamily should be considered, because minipig microsomes prevalently form 6-OH-Sudan I and CYP3A is the major enzyme oxidizing Sudan I in these microsomes. Unfortunately, isolated minipig CYP3A has not been available to confirm this suggestion.

CONCLUSIONS

The results presented in this work suggest that rats, but not rabbits or minipigs, may predict human susceptibility to Sudan I. This is important in view of estimation of Sudan I carcinogenicity for humans. The results of our study, showing an analogy in the Sudan I metabolism catalyzed by human and rat enzymes, suggest a certain carcinogenic potential of this rat carcinogen for humans. The analysis of Sudan I metabolites in urine of individuals working in dye industry and exposed to Sudan I as well as that of Sudan I-mediated adducts in DNA of their blood should confirm or exclude this suggestion.

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