Sudan I Is A Potential Carcinogen for Humans: Evidence for Its Metabolic Activation and Detoxification by Human Recombinant Cytochrome P450 1A1 and Liver Microsomes

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ABSTRACT

1-Phenylazo-2-hydroxynaphthol (Sudan I, C.I. Solvent Yellow 14) is a liver and urinary bladder carcinogen in mammals. We compared the ability of hepatic microsomal samples from different species including human to metabolize Sudan I. Comparison between experimental animals and human cytochromes P450 (CYP) is essential for the extrapolation of animal carcinogenicity data to assess human health risk. Human microsomes generated the pattern of Sudan I metabolites reproducing that formed by hepatic microsomes of rats. Using hepatic microsomes of rats pretreated with specific CYP inducers, microsomes from Baculovirus-transfected insect cells expressing recombinant human CYP enzymes, purified CYP enzymes, and selective CYP inhibitors, we found that rat CYP1A1 and recombinant human CYP1A1 are the most efficient enzymes metabolizing Sudan I. Microsomes from livers (the target of Sudan I carcinogenicity) of different human donors were used to estimate whether authentic human CYPs oxidize Sudan I. Using Western blot analysis and NH₂-terminal sequencing, we were able to detect and quantify CYP1A1 in human hepatic microsomes. The sequence of nine amino acids of the protein band cross-reacting with antirat CYP1A1 in human microsomes, LFPISMSAT, matched the sequence of human CYP1A1 perfectly (residues 2–10). CYP1A1 expression levels varied significantly among the different human microsomes (0.04–2.4 pmol/mg protein), and constituted <0.6% of the total hepatic CYP complement. All of the human hepatic microsomal samples oxidized Sudan I to C-hydroxymetabolites. Moreover, using the nuclease P1-enhanced version of the 32P-postlabeling assay, we found that human microsomes were competent in activating Sudan I to form adducts with DNA. The role of specific CYP enzymes in the human hepatic microsomal metabolism was investigated by correlating the CYP-catalytic activities (or CYP contents) in each microsomal sample with the levels of individual metabolites and/or Sudan I-DNA adducts formed by the same microsomes, and by examining the effects of agents that can inhibit specific CYP in Sudan I metabolism. On the basis of these studies, we attribute most of Sudan I metabolism in human microsomes to CYP1A1, but participation of CYP3A4 cannot be ruled out. These results, the first report on the metabolism of Sudan I by human CYP enzymes, strongly suggest a carcinogenic potency of this rodent carcinogen for humans.

INTRODUCTION

Sudan I¹ was used as a food coloring in several countries (1), but it has been recommended as unsafe, because it causes tumors in the liver or urinary bladder in rats, mice, and rabbits, and is considered a possible carcinogen and mutagen for humans (1–5). Besides its carcinogenicity, Sudan I is a potent contact allergen and sensitizer, eliciting pigmented contact dermatitis in human (6). Nevertheless, it is widely used to color 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, but because it is still widely used, but because it is the simplest in a series of dyes and pigments that are used in very great quantities and occur everywhere in red- and-orange colored consumer products, foods, and printed matter. Such a wide use of these azo dyes could result in a considerable exposure.

Sudan I gives positive results in Salmonella typhimurium mutagenicity tests with S-9 activation (7, 8) and is mutagenic to mouse lymphoma L5178Y TK⁻/⁻ cells in vitro, with S-9 activation (8). It is clastogenic compound, inducing micronuclei in the bone marrow of rats (3). Whereas the metabolism of Sudan I in rat is not understood in humans, its metabolism has been characterized in rabbits (9), where it is metabolized primarily in the liver by oxidative or reductive reactions (9). C-Hydroxylated metabolites 4'-OH-Sudan I and 6-OH-Sudan I were found to be the major products of Sudan I oxidation in vivo and excreted in urine (1, 9), and of its oxidation by rat hepatic microsomes in vitro (10). Besides the C-hydroxylated metabolites, which are considered detoxication products, the BDI formed by microsome-dependent enzymatic splitting of the azo group of Sudan I was found to react with DNA in vitro (10–12). The major DNA adduct formed in this reaction has been characterized and identified as the 8-(phenylationo)guanine adduct (12). In addition to microsomal enzymes, Sudan I and its C-hydroxylated metabolites are also oxidized by peroxidas, as a consequence DNA, RNA, and protein adducts are formed (13–15).

Because CYPs are abundant in the liver where much of the metabolism of Sudan I in experimental animals occurs (9), CYPs were assumed to play a role in the oxidative metabolism of this carcinogen (9–12), but as yet no data are available on the participation of human CYP enzymes in its metabolism. Comparison between experimental animals and human CYPs is essential for the extrapolation of animal carcinogenicity data to assess human health risk, and consideration of species differences in catalytic activities of CYPs is important. In contrast to many experimental animal models, humans show large interindividual variations in the expression of CYP enzymes and catalytic activities, which may lead to different susceptibilities to carcinogens and must be considered in risk assessment (16). To assess the human health risk of Sudan I, we have compared the capacity of livers from humans, rats, and rabbits to metabolize Sudan I. In addition, the present study was undertaken to understand which human CYP enzymes are involved in Sudan I metabolic activation and/or detoxication. This knowledge will be useful in evaluating individual susceptibility to this carcinogen.
MATERIALS AND METHODS

Chemicals. α-NF, β-NF, NADPH, troleandomycin, ketocnazole, glucose 6-phosphate, chlorozoxazone, calf thymus DNA, coumarin, sulfaphenazole, and quinidine were from Sigma Chemical Co. (St. Louis, MO); furafylline from New England Biolabs (Beverly, MA); 6β-hydroxytestosterone from Merck (Darmstadt, Germany); glucose 6-phosphate dehydrogenase from Serva (Heidelberg, Germany); bufuralol and its 1'-hydroxyderivative from Gentest Corp. (Woburn, MA); bicionichinic acid from Pierce (Rockford, IL); and Sudan I and Sudan IV from British Drug Houses (Poole, United Kingdom). 3-IPMDIA was synthesized according to Olah et al. (17). The derivatives 4'-OH-Sudan I, 6-OH-Sudan I, 4'-6-dihydroxy-Sudan I and 3',4'-dihydroxy-Sudan I were synthesized as described (10). Enzymes and chemicals for the 32P-postlabeling assay were obtained from sources described previously (12).

Preparation of Microsomes and Assays. Microsomes from livers of untreated rats and rabbits were prepared as described previously (12). Microsomes from the livers of rats pretreated with β-NF (12) and Sudan I (18) were isolated as described (12), those pretreated with PB, PC, and ethanol as reported (19). Microsomes from human liver of eight human donors who died in a traffic accidents were isolated as described (20) and were a gift of B. Szotakova (Faculty of Pharmacy, Charles University, Hradec Kralove, The Czech Republic). The donors ranged in age from 24 to 70 years, and included five men and three women. All of the donors had no known drug history and none had a history of alcohol abuse. Microsomes from the liver of a male minipig were a gift from P. Anzenbacher (Palacky University, Olomouc, The Czech Republic) and isolated as described (20). Supersomes, microsomes isolated from insect cells transfected with Baculovirus constructs containing cDNA of one of the following human CYPs: CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, with cytochrome b6 and expressing NADPH:cytochrome c reductase were from Gentest Corp. Protein concentrations were assessed using the bicionichinic acid protein assay (21). The concentration of CYP was estimated according to Omura and Sato (22). Rat, rabbit, and minipig liver microsomes contained 0.62, 1.82, and 0.89 nmol CYP/mg protein, respectively. Microsomes of rats treated with β-NF, PB, PC, and ethanol contained 1.30, 2.74, 1.55, and 1.80 nmol CYP/mg protein, respectively. The content of CYP in human hepatic microsomes is shown in Table 1. Each human microsomal sample was analyzed for specific CYP activities by modification of the methods as described previously (12).

Isolation of Individual CYPs. The CYP1A2, 2B4, 2C3, and 2E1 were isolated from insect cells transfected with Baculovirus constructs containing cDNA of one of the following CYPs: CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, with cytochrome b6 and expressing NADPH:cytochrome c reductase were from Gentest Corp. Protein concentrations were assessed using the bicionichinic acid protein assay (21). The concentration of CYP was estimated according to Omura and Sato (22). Rat, rabbit, and minipig liver microsomes contained 0.62, 1.82, and 0.89 nmol CYP/mg protein, respectively. Microsomes of rats treated with β-NF, PB, PC, and ethanol contained 1.30, 2.74, 1.55, and 1.80 nmol CYP/mg protein, respectively. The content of CYP in human hepatic microsomes is shown in Table 1. Each human microsomal sample was analyzed for specific CYP activities by modification of the methods as described previously (12).

Preparation of Antirat CYP1A1, Antirabbit CYP2E1, and Antihuman CYP3A4 Polyclonal Antibodies. Lethorn chickens were immunized s.c. three times a week by CYP antigens (rat recombinant CYP1A1, rabbit CYP2E1, and human recombinant CYP3A4; 0.1 mg/animal) emulsified in complete Freund’s adjuvant for the first injection and in incomplete adjuvant for boosters. The immunoglobulin fraction was purified from pooled egg yolks as described (32, 33).

Incubations. Incubation mixtures contained the following in a final volume of 20 μl: 50 mM sodium phosphate buffer (pH 7.4), 1 mM NADPH, 10 mM d-glucose 6-phosphate, 1 unit/ml d-glucose 6-phosphate dehydrogenase, 10 mM MgCl2, microsomal fraction containing 0.05–2.4 nmol CYP, and 0.1–100 μM Sudan I dissolved in 7.5 μl methanol. Incubation mixtures, in which the efficiencies of Supersomes expressing human CYPs were tested, were the same except that 100 μM of Sudan I and only 10–50 pmol of CYP were used. Incubations using purified CYP reconstituted with NADPH:CYP reductase and cytochrome b6 (34) contained 50–250 pmol of each CYP. After incubation (37°C, 5–140 min) the mixtures were extracted with ethyl acetate. The extracts were evaporated, dissolved in methanol, and chromatographed on a thin layer of silica gel (10). The BDI was detected by autoradiography with 1-phenyl-3-methyl-5-pyrazolone (10–12). Alternatively, the products were separated by HPLC on a MN Nucleosil 100–5 C18 column (Macherey-Nagel; 4.0 × 250 mm). An isotopic flow of methanol: 0.1 M NH4HCO3 (pH 8.5; 9:1, v/v) with flow rate of 0.8 ml/min was used to elute the metabolites, and no quantitation of human liver microsomal CYP 1A1, 1A2, 2E1, and 3A4 was conducted.

<table>
<thead>
<tr>
<th>Human hepatic microsomal samples</th>
<th>pmol CYP per mg protein</th>
<th>pmol CYP1A1/CYP protein</th>
<th>EROD (CYP1A1/2)</th>
<th>Coumarin 7-hydroxylation (CYP2A6)*</th>
<th>Tolbutamide methyl hydroxylation (CYP2D6)*</th>
<th>Bufuralol 1'-hydroxylation (CYP2D6)*</th>
<th>Chlorozoxazone 6-hydroxylation (CYP3A4)*</th>
<th>Estosterone 6β-hydroxylation (CYP3A4)*</th>
<th>Sudan I metabolites</th>
<th>Sudan I-DNA adducts</th>
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<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>0.080</td>
<td>5.65</td>
<td>0.93</td>
<td>0.76</td>
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<td>5</td>
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<td>10.82</td>
<td>0.84</td>
<td>0.12</td>
<td>2.53</td>
<td>2.45</td>
<td>10.19</td>
<td>0.32</td>
<td>2.29</td>
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<td>6</td>
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<td>0.600</td>
<td>11.73</td>
<td>0.98</td>
<td>0.26</td>
<td>0.92</td>
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<td>6.94</td>
<td>0.40</td>
<td>3.28</td>
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<tr>
<td>7</td>
<td>460</td>
<td>0.040</td>
<td>6.86</td>
<td>0.43</td>
<td>0.11</td>
<td>4.11</td>
<td>1.37</td>
<td>3.96</td>
<td>0.21</td>
<td>1.07</td>
</tr>
<tr>
<td>8</td>
<td>400</td>
<td>2.400</td>
<td>12.08</td>
<td>0.58</td>
<td>0.15</td>
<td>3.13</td>
<td>1.51</td>
<td>6.86</td>
<td>0.25</td>
<td>3.29</td>
</tr>
</tbody>
</table>

*CYP activities in nmol/min/nmol CYP, except for EROD activity, which is in pmol/min/nmol CYP.

**mM total C-hydroxylated metabolites/min/nmol CYP.

*RAL1/10 nucleotides per pmol CYP.

Table 1 CYP-dependent catalytic activities, CYP1A1 levels, amounts of ring-hydroxylated Sudan I metabolites, and DNA adducts formed by Sudan I in human hepatic microsomal samples

All results are presented as means of duplicate experiments. CYP1A1 content was determined by Western blot as described in “Materials and Methods.” Assays for CYP activities were carried out as described elsewhere (23).

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RESULTS

Metabolism of Sudan I by Rat, Rabbit, Minipig, and Human Hepatic Microsomes. When Sudan I was incubated with rat, rabbit, minipig, or human hepatic microsomes in the presence of NADPH, several product peaks were observed by HPLC analysis (Fig. 1). On the basis of cochromatography with the synthetic standards, the major metabolites produced from Sudan I by all of the tested 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 a colorless product, which was identified previously as BDI (Refs. 10, 12; not shown in the chromatogram of HPLC in Fig. 1). Whereas in microsomes of rabbit and minipig 100 μM Sudan I was preferentially oxidized to the 6-hydroxy-naphthol derivative of Sudan I, those of human and rat predominantly produced 4′-OH-Sudan I (Fig. 2). The ratios of metabolites were the same at lower Sudan I concentrations down to 10 μM.

To resolve which CYPs are able to oxidize Sudan I, five experimental approaches were used: (a) induction of specific CYPs;
(b) selective inhibition of CYPs; (c) utilization of the purified CYPs reconstituted with NADPH:CYP reductase; (d) heterologous expression systems (Supersomes); and (e) correlation of the efficiencies of microsomal samples to oxidize Sudan I with known marker activities of CYPs or with amounts of expressed CYP proteins.

**Involvement of Rat CYP Enzymes in Oxidation of Sudan I.** Individual CYP enzymes were induced in rats. Incubations of Sudan I with microsomes from β-NF- or Sudan I-treated rats led to a 10-fold increase in Sudan I metabolism, although induction with PB resulted in a 2-fold increase (Fig. 3). An inhibitor of CYP1A1/2, α-NF, was highly effective in inhibiting Sudan I oxidation; an equimolar concentration of α-NF and Sudan I inhibited its oxidation by 70%. Inhibitors of other CYP enzymes caused either weak (ketoconazole, troleandomycin, and 3-IPMDIA) or no inhibition (furafylline, sulfaphenazole, quinidine, and DDTC). The formation of Sudan I metabolites with β-NF microsomes was time-dependent and linear up to 20 min. Not only Sudan I, but its first hydroxylated products are substrates for additional oxidation by CYP. The values of maximum velocity and apparent Michaelis constant for the oxidation of these three substrates, Sudan I, 4′-OH-Sudan I, and 6-OH-Sudan I, in β-NF microsomes are 1.7, 4.6, and 2.8 nmol/min per nmol total CYP and 21, 79, and 40 μM, respectively.

**Oxidation of Sudan I by Purified CYP Enzymes.** To identify the role of individual CYPs in oxidation of Sudan I, several CYP enzymes were purified, reconstituted with NADPH:CYP reductase and cytochrome b₅ (34), and used as the oxidation system. All of the CYPs reconstituted with reductase were active with their typical substrates. Rat recombinant CYP1A1 was the most efficient enzyme oxidizing 100 μM Sudan I (Fig. 4). α-NF inhibited Sudan I oxidation in as in microsomes (Fig. 4).

**Oxidation of Sudan I by Recombinant Human CYP Enzymes.** To investigate whether human recombinant CYPs oxidize Sudan I, we used microsomes of Baculovirus-transfected insect cells containing recombinantly expressed human CYPs and NADPH:CYP reductase. The recombinant human CYPs used in the experiments efficiently oxidized their typical substrates. Human CYP1A1, and to a much lesser extent, CYP3A4, metabolized Sudan I. Other CYPs were almost ineffective (Fig. 5). All of the above results indicate that rat CYP1A1 and human recombinant CYP1A1 are the most efficient enzymes oxidizing Sudan I.

**Estimation of CYP1A1 in Human Hepatic Microsomes and Its Involvement in Sudan I Oxidation.** To identify authentic human CYPs capable of oxidizing Sudan I, microsomal samples from livers of eight different human donors were used in additional experiments. All of the human microsomal preparations metabolized Sudan I (Table 1). Correlations between the CYP catalytic activities (Table 1) and the amounts of each of the C-hydroxylated Sudan I metabolites in each microsomal sample were used to examine the role of specific human CYP enzymes in the metabolism of Sudan I. The formation of all of the Sudan I C-hydroxylated metabolites was highly correlated with EROD activity, a marker for CYP1A1/2 (Table 2).

Whereas CYP1A2 protein is constitutively expressed in the human
liver, the content of CYP1A1 enzyme in this organ is low; so low that it has been discussed whether it is expressed in this organ at all or only in extrahepatic tissues (38, 39), and readily induced by ligands of the aryl hydrocarbon receptor (40). In contrast, composite results obtained with mRNA, protein, and activity measurements indicates that low expression levels of CYP1A1 occur in human livers (41–43) at <1% of total hepatic CYP (42, 43).

Using two independent methods, we were able to detect and quantify CYP1A1 in human hepatic microsomes. A polyclonal antibody raised against rat recombinant CYP1A1, which highly cross-reacts with recombinant human CYP1A1 and only poorly with CYP1A2, was used in the first method (Fig. 6A). The detection sensitivity was as low as 0.005 pmol CYP1A1 per lane. In immunoblots (Fig. 6B), this polyclonal antibody reacted with one and/or two immunoreactive bands in most analyzed human hepatic microsomes. The high and low mobility bands (Fig. 6B) were assumed to be CYP1A1 and 1A2, respectively, based on the reported electrophoretic mobilities of these proteins in microsomes from human tissues (44, 45). To confirm that the band with lower molecular weight really corresponds to human CYP1A1, NH2-terminal sequencing was carried out with this protein band. The bands of microsomal samples 5 and 6 were excised from a PVDF membrane and subjected to automated Edman degradation. The sequence of nine amino acids, LPISMSAT, was identical to the residues 2–10 of the NH2-terminal sequence of CYP1A1 (MLFHPISMSAT; Ref. 46). NH2-terminal methionine was not found in the CYP1A1 protein band by NH2-terminal sequencing.

The CYP1A1 expression levels varied greatly among the different human microsomal samples (Table 1), being present at <0.6% of total hepatic CYP. With the same antibody, we also estimated the expression levels of CYP1A2 in all of the human microsomal samples. The CYP1A2 content ranged from 5 to 35 pmol per mg of microsomal protein (data not shown).

To resolve which of these two CYPs is the predominant enzyme oxidizing Sudan I, correlations between the CYP1A1 or 1A2 protein levels and Sudan I oxidation were used. A significant correlation was seen between hepatic CYP1A1 content and Sudan I oxidation (r = 0.810; P = 0.010), but not between the content of CYP1A2 and oxidation of this carcinogen (r = 0.405; P = 0.320). Because the EROD activity highly correlated with CYP1A1 content (r = 0.762; P < 0.05) but not with the content of CYP1A2 protein (r = −0.309; P = 0.456), O-deethylation of ethoxyresorufin seems to be catalyzed mainly by CYP1A1 in human hepatic microsomes used in the study.

Whereas catalytic activities of CYP2A6, 2C9, 2D6, and 3A4 did not exhibit significant correlation with the levels of Sudan I metabolites formed by the same human hepatic samples, a significant correlation was seen with the CYP2E1 activity (Table 2). However, there is a cross-correlation between EROD and chlorozoxazone 6-hydroxylation activity (r = 0.783; P = 0.038) within these liver samples. To additionally clarify this correlation, multivariate analysis was used to investigate the dependence of the Sudan I oxidation on these two isofrom activities. The two activities (CYP1A and 2E1) in each microsomal sample were combined in pairs to see if a combination of two activities gave an improvement in the correlation with Sudan I oxidation, i.e., an increase in the correlation coefficient when compared with the correlation with the individual activities. The inclusion of the CYP2E1 activity produced no improvement in the correlation coefficient. Multivariate analysis was also used to examine the dependence of the Sudan I oxidation on activities of CYP3A4 and 2C9. Although the activities of these CYPs did not exhibit significant correlations with Sudan I oxidation, these activities showed certain correlation tendencies (Table 2) and recombinant CYP3A4 was active with Sudan I (Fig. 5). The inclusion of the CYP3A4 or 2C9 activities with CYP1A in multivariate analysis produced no improvement in the correlation coefficient.

To confirm the role of individual human hepatic CYP enzymes in metabolism of Sudan I, two human microsomal samples with high CYP1A, 2E1, and 3A4 activities, samples 5 and 8, were selected, and incubations were carried out in the absence and presence of specific inhibitors of CYP1A1/2, 1A2, 2E1, and 3A4, α-NF, furafylline, DDTc, and ketoconazole, respectively. A substrate of CYP2E1, chlorozoxazone, was used as additional inhibitor. α-NF inhibited Sudan I metabolism to 50%, whereas no effect of furafylline, DDTc, or chlorozoxazone was observed. Ketoconazole weakly inhibited the oxidation of Sudan I by these human microsome samples by 15%.

All of these results strongly suggest that Sudan I oxidation in human hepatic microsomes is mediated mainly by CYP1A1, as in the systems using the isolated rat recombinant and human CYP1A1 enzymes (see Figs. 4 and 5). Nevertheless, although CYP3A4 activities showed poor correlation with Sudan I oxidation (Table 2), the inhibition of Sudan I oxidation by ketoconazole indicated that the participation of CYP3A4 in Sudan I oxidation in human hepatic microsomes cannot be excluded.

**Sudan I Is Activated by Human Hepatic Microsomes to Form DNA Adducts.** Using the nuclease P1 version of the 32P-postlabeling assay we found that during oxidation of Sudan I by human hepatic microsomes DNA adducts are formed. One major (the closed circle in Fig. 7D) and two minor adduct spots, overlapping the major adduct,

<table>
<thead>
<tr>
<th>CYP content</th>
<th>CYP1A content</th>
<th>EROD (CYP1A1/2)</th>
<th>Coumarin 7-hydroxylation (CYP2A6)</th>
<th>Tolbutamide methyl hydroxylation (CYP2C9)</th>
<th>Bufuralol 1'-hydroxylation (CYP2D6)</th>
<th>Chlorozoxone 6-hydroxylation (CYP2E1)</th>
<th>Testosterone 6β-hydroxylation (CYP3A4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total C-hydroxymetabolites</td>
<td>0.286</td>
<td>0.810&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.903&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−0.024</td>
<td>0.405</td>
<td>0.095</td>
<td>0.810&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4'-OH-Sudan I</td>
<td>0.286</td>
<td>0.810&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.903&lt;sup&gt;b&lt;/sup&gt;</td>
<td>−0.024</td>
<td>0.405</td>
<td>0.095</td>
<td>0.810&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-OH-Sudan I</td>
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<td>0.810&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.752&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.667</td>
<td>−0.071</td>
<td>0.833&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4',6-dihydroxy-Sudan I</td>
<td>0.5</td>
<td>0.714&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.813&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0.214</td>
<td>0.119</td>
<td>0.643</td>
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<td>DNA adducts</td>
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<td>−0.143</td>
<td>0.405</td>
<td>−0.024</td>
<td>0.738&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> P < 0.001.<nlop><sup>b</sup> P < 0.01.<nlop><sup>c</sup> P < 0.05.
were detected in autoradiographs of DNA reacted with Sudan I activated by human microsomes (Fig. 7A). The major adduct spot exhibited similar chromatographic properties as the major adduct formed in DNA by Sudan I activated with rat microsomes (Fig. 7B), which corresponds to the 3′,5′-bisphospho-derivative of an 8-(phenylazo)deoxyguanosine adduct identified previously (12). The identity of the major adduct in human and rat microsomes was confirmed by cochromatography on PEI-cellulose plates in two different solvent systems (not shown).

The adducts were quantified and expressed as RALs (Table 1). A highly significant correlation was found between the EROD activity and the formation of Sudan I-DNA adducts (r = 0.905; P = 0.002) in human microsomes (Table 2). In addition, Sudan I-DNA adduct formation highly correlated with levels of the CYPIA1 protein determined in microsomes by Western blot analysis (Table 2). A weaker but significant correlation was determined between CYP2E1 activities and formation of Sudan I-DNA adducts (Table 2). Again, a cross-correlation between the CYPIA1 and 2E1 activities in these liver samples might explain these results (see above). The binding of Sudan I to DNA catalyzed by one microsomal sample with high CYPIA1 and 2E1 activities (sample 8) was inhibited by α-NF, but not by furafylline, a selective inhibitor of human CYPIA2, or DDTC, an inhibitor of CYP2E1.

DISCUSSION

We present for the first time data that show that human hepatic microsomes metabolize carcinogenic Sudan I. Human microsomes oxidize Sudan I to ring hydroxylated metabolites and are capable of activating this carcinogen to species binding to DNA. The major DNA adduct generated by Sudan I activated by human microsomes exhibits the same chromatographic properties as the 8-(phenylazo)deoxyguanosine adduct identified in rat microsomal systems. One of the most important results of our study is the finding that metabolism of Sudan I by the human enzymatic system is analogous to that observed in rats. Human microsomes generated the same pattern of Sudan I metabolites as hepatic microsomes of rats.

In addition, the present study documents the role of specific human CYP enzymes in metabolic pathways of Sudan I. CYPIA1 seems to be the principal enzyme responsible for the metabolism of Sudan I. There is still conflicting evidence for the expression or inducibility of CYPIA1 protein in human livers (39–43). Using a highly efficient chicken polyclonal antibody raised against rat CYPIA1, strongly cross-reacting with human recombinant CYPIA1, we were able to detect and quantify the CYPIA1 protein content in human hepatic samples used in the study by Western blot analysis with a detection sensitivity of 0.005 pmol CYPIA1 per lane. Moreover, we sequenced for the first time the nine NH2-terminal amino acids of the CYPIA1 protein band, separated from other human hepatic microsomal proteins by SDS-PAGE. This amino acid sequence was identical with that of CYPIA1 cDNA (46). The successful immunodetection of CYPIA1 shown in our study may be explained by the use of a highly sensitive and selective anti-CYP1A1 antibody. The range of CYPIA1 expression levels in our eight human livers (see Table 2) is comparable with values reported recently (42, 43). The role of CYPIA1 in Sudan I oxidation was supported by strong correlation coefficients between the levels of CYPIA1 protein expression (or the rates of EROD), and the levels of Sudan I metabolites and/or Sudan I-derived DNA adducts in the eight human hepatic microsomal samples. The participation of CYPIA1 in Sudan I metabolism was confirmed also by inhibition of Sudan I oxidation by α-NF, an inhibitor of CYPIA1/2, whereas furafylline, a specific inhibitor of CYPIA2, did not inhibit Sudan I oxidation. 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. Nevertheless, the utilization of pure CYPIA1 as well as microsomes containing human recombinant CYPIA1 fully corroborated the major role of CYPIA1 in the metabolism of Sudan I. Interestingly, the highly homologous human CYPIA1 and 1A2 with 73% amino acid sequence identity exhibit extremely different potency to oxidize Sudan I. CYPIA2 is almost ineffective in Sudan I oxidation. Besides the CYPIA1, the CYP3A4 enzyme might also participate in Sudan I oxidation in human hepatic microsomes, because human recombinant CYP3A4 oxidizes Sudan I. The efficiency of this CYP to oxidize Sudan I is ~10-fold lower than that of CYPIA1. However, because of high expression levels of CYP3A4 in human livers, its contribution to Sudan I metabolism might be relevant, although the correlation studies showed only correlation tendencies with levels of Sudan I metabolites and DNA adducts.

Human CYPIA1 seems to be induced by planar aromatic compounds binding to the aryl hydrocarbon receptor, e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (42) and/or by polycyclic hydrocarbons present in cigarette smoke (40). The CYPIA1 enzyme is strongly induced by Sudan I itself in rats by this mechanism (47). Hence, long-term occupational exposure of humans to Sudan I might be an important risk factor for individuals, improving Sudan I metabolism and binding to DNA, thereby increasing its toxicological relevance.

Our results suggest that rats may predict human susceptibility to Sudan I. This is highly significant in view of the prediction of Sudan I carcinogenicity to humans. Whereas Sudan I is carcinogenic to rats (1–5), its carcinogenicity to humans has not yet been proven. Sudan I was evaluated to be still unclassifiable as carcinogenic to humans by IARC (5). In a meeting March 3–5, 1999, a European Union commission working group for classification, packaging, and labeling of dangerous substances recommended that Sudan I should be consid-

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eroded of “concern for man owing to possible carcinogenic effects” (Cat. Carc. 3; Ref. 5) and of “concern for man because of possible mutagenic effects” (Mutat. Cat. 3; Ref. 5). We fully support the recommendation of this working group. Our results, showing for the first time an analogy in the Sudan I metabolism by human and rat enzymes, strongly suggest a carcinogenic potential of this rat carcino gen for humans. An increased cancer risk should be taken into account mainly for individuals working in the dye industry and exposed to Sudan I, its derivatives, and to other compounds inducing CYP1A1. Furthermore, caution is highly recommended in using this dye and its derivatives to color materials, which are used by humans in their daily use.

REFERENCES

Sudan I Is a Potential Carcinogen for Humans: Evidence for Its Metabolic Activation and Detoxication by Human Recombinant Cytochrome P450 1A1 and Liver Microsomes

Marie Stiborová, Václav Martinek, Helena Rýdlová, et al.


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