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***N*-Demethylation Is a Major Route of 2-Amino-3-Methylimidazo[4,5-*f*]quinoline Metabolism in Mouse**

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Abstract

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) metabolism was evaluated in mouse to better understand its tumorigenicity. Urinary metabolites from mice orally administered 40 mg/kg [¹⁴C]IQ were compared with those from similarly treated rats. The recovery of radioactivity was significantly greater in mouse urine. The relative proportion of metabolites was significantly different, and a new rodent metabolite was detected. For rat, the proportion of previously identified metabolites excreted was 5-*O*-glucuronide > sulfamate > 5-sulfate > *N*-glucuronide. In mouse urine, a new metabolite, demethyl-IQ, represented approximately 26% of IQ metabolism with the proportion of metabolites as follows: 5-*O*-glucuronide > demethyl-IQ > sulfamate > *N*-glucuronide > 5-sulfate. Mouse metabolites were identified by electrospray ionization mass spectrometry. Demethyl-IQ was shown to be 2-aminoimidazo[4,5-*f*]quinoline. *N*-Acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline was not detected with mice. Mouse liver slices produced 5-*O*-glucuronide, demethyl-IQ, and sulfamate with the former two being significantly reduced by ellipticine. Liver microsomes only produced demethyl-IQ. Ellipticine, a cytochrome P450 1A inhibitor, but not furafylline, an 1A2 selective inhibitor, prevented microsomal *N*-demethylation. Inhibitors had similar effects on 7-ethoxyresorufin *O*-deethylation activity. Demethyl-IQ was not further metabolized by an intact mouse or liver microsomes. Thus, mouse IQ metabolism is significantly different from that in rat, and these differences may affect IQ tumorigenicity. *N*-Demethylation of IQ-like heterocyclic amines occurs in mouse, monkey, and human but not in rat.

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a heterocyclic amine (HCA) formed by high-temperature cooking of proteinaceous food (Felton and Knize, 1990). More than 20 similarly formed HCAs have been identified. In the human diet, consumption of 400 g of cooked lean meat could result in exposure to several micrograms of mutagenic HCAs. These amines are detected in urine and their excretion, as unchanged amine, is increased by a cytochrome P450 (P450) inhibitor (Lynch et al., 1992; Boobis et al., 1994), indicating their absorption from cooked foods and P450 metabolism.

IQ is a potent carcinogen inducing tumors in multiple tissues and in different species (National Toxicology Program Report on Carcinogens: Background Document for Heterocyclic Amines: MeIQ, MeIQx, IQ, and PhIP, 2005, <http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s092vhca.pdf>). In mouse, oral administration of IQ caused forestomach, liver, and lung tumors in both sexes. Although IQ also caused tumors in several organs in both sexes of rats, mammary and clitoral gland tumors were only detected in females and colon and skin tumors only in males. IQ administered orally

to monkeys or mice caused liver tumors. Using a colitis-induced carcinogenicity model with $Apc^{Min/+}$ mice (Cooper et al., 2001), we have preliminary data indicating that induction of colitis by treatment with 4% dextran sulfate sodium (DSS) in drinking water followed by oral doses of 40 mg/kg IQ resulted in a 100% incidence of invasive colorectal tumors, whereas mice treated with only DSS had no tumors (Clapper et al., 2006). The strong association of intake of high-temperature cooked meat and colorectal cancer risk (Giovannucci et al., 1994), the presence of HCAs in cooked meat (Felton and Knize, 1990), and the initiation of colon cancer by HCAs (Kristiansen et al., 1997) suggest that HCAs present in high-temperature cooked meat may be responsible for the increased risk of colon cancer associated with this dietary component.

Activation of IQ is thought to involve *N*-oxidation by P450, followed by *O*-acetylation with subsequent formation of a reactive intermediate, nitrenium ion, that binds DNA (Turesky et al., 1991). These DNA adducts are thought to cause mutations that initiate carcinogenesis. After IQ administration, DNA adducts are found in a variety of rodent and nonhuman primate tissues (Nerurkar et al., 1995; Turesky et al., 1996). Whereas *N*-oxidation of IQ has been demonstrated with rat microsomes (Turesky et al., 1998), excretion of *N*-hydroxy-IQ has not been detected in rat. There is a considerable amount of evidence indicating that P450s play an important role in HCA genotoxicity.

The major pathway for IQ inactivation involves P450-catalyzed ring oxidation at the 5 position followed by conjugation with sulfate or glucuronic acid (Fig. 1) (Inamasu et al., 1989; Luks et al., 1989). The other major route of metabolism is direct conjugation of the exocyclic amine to form N^2 -glucuronide or sulfamate (Turesky et al., 1986; Inamasu et al., 1989; Luks et al., 1989). The 5-*O*-glucuronide and 5-sulfate were detected in urine and bile but not in feces (Inamasu et al., 1989; Luks et al., 1989). In contrast, sulfamate excretion was observed in urine, bile, and feces (Turesky et al., 1986; Inamasu et al., 1989; Luks et al., 1989). This finding is consistent with the conjugates being hydrolyzed by bacterial enzymes in the intestine and reabsorbed from the intestinal tract. Similar pathways for activation and inactivation have been reported in different species and for other aminoimidazole HCAs (Snyderwine et al., 1992; Langouët et al., 2001). Rat P450 oxidation appears to favor the 5-position, whereas in humans *N*-oxidation may be favored (Turesky et al., 1998). Metabolism by the monkey is different from that in rat in that 2-aminoimidazo[4,5-*f*]quinoline (demethyl-IQ) is formed as a major product (Snyderwine et al., 1992). Human hepatocytes metabolize 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, a structurally similar HCA, to an *N*-demethyl derivative, whereas rat hepatocytes do not (Langouët et al., 2001). Although *N*-acetylation plays a major role in metabolism of aromatic amines (Lakshmi et al., 1995a), the *N*-acetyltransferase pathway plays only a minor role in IQ rat metabolism (Inamasu et al., 1989).

Although metabolism can influence tumorigenicity, only balance studies of IQ excretion (Sjodin and Jagerstad, 1984) and its organ distribution (Bergman, 1985; Alldrick and Rowland, 1988) have been assessed in mouse. Because the mouse is an attractive model for testing tumorigenicity and its use in this area is likely to continue and even increase, we have evaluated pathways for mouse IQ metabolism. Results indicate that mouse IQ metabolism is similar to that for monkey and human but different from that for rat. Thus, parameters affecting IQ tumorigenicity in rat and mouse may be different.

Materials and Methods

Materials

[2- 14 C]IQ (10 mCi/mmol, >98% radiochemical purity) and IQ were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Diethylenetriaminepentaacetic acid, acetyl CoA, esterase from porcine liver, dithiothreitol, NADPH, ellipticine, ethoxyresorufin, resorufin, α -

naphthoflavone, furafylline, SKF-525A, ketoconazole, sulfaphenazole, *Escherichia coli* β -glucuronidase (type VII-A), and abalone sulfatase (type VIII) were purchased from Sigma-Aldrich (St. Louis, MO). Ultima-Flo AP was purchased from PerkinElmer Life and Analytical Sciences (Shelton, CT).

Animals and Dosing

Six-week-old C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were fed AIN-76A from Bio-Serv (Frenchtown, NJ) for 8 weeks before use. Female F344 rats at 13 weeks of age were purchased from Harlan (Indianapolis, IN) and fed the AIN-76A diet for 8 weeks before use. Experiments were performed according to the National Institutes of Health standards for care and use of experimental animals and the St. Louis VA Medical Center Animal Care and Use Committee. Animals were group-housed, maintained on a 12-h light/dark cycle, and had access to food and water ad libitum. Mice were administered 40 mg/kg [^{14}C]IQ (20 μCi ; 0.2 ml/20 g oral dose; 20% DMSO-80% 0.05 N HCl) and placed in metabolic cages, and a 24-h urine sample was collected. Rats were administered 40 mg/kg [^{14}C]IQ (20 μCi ; 2.5 ml/250 g oral dose; 20% DMSO-80% 0.05 N HCl) and placed in metabolic cages, and a 24-h urine sample was collected. The recoveries of radioactivity in the 24-h urine sample from rats and mice were 42 ± 2 and $55 \pm 3\%$ of the total radioactivity administered, respectively ($p < 0.05$). The 24- to 48-h urinary excretions of radioactivity from rats and mice were 4.2 ± 0.3 and $3.4 \pm 0.5\%$, respectively. To assess a different route for administering IQ, F344 rats were also administered 20 mg/kg [^{14}C]IQ (20 μCi ; 1.25 ml/250 g injected i.p.; 20% DMSO-80% 0.1 N HCl) and placed in metabolic cages, and a 24-h urine sample was collected. The recovery of radioactivity in the 24-h urine sample was $55 \pm 7\%$ of the total radioactivity administered.

HPLC Analysis of Metabolites

Urine was analyzed for IQ metabolites by HPLC. Metabolites were assessed using a Beckman HPLC with System Gold software and a C18 ultrasphere column ($5\mu\text{m}$, $4.6 \times 150\text{ mm}$) attached to a guard column. Analysis and purification of metabolites required the use of the following solvent systems at a flow rate of 1 ml/min: solvent system 1, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 2% acetonitrile for 0 to 5 min; 2 to 5% acetonitrile for 5 to 23 min; 5 to 10% acetonitrile for 24 to 29 min; 10 to 40% acetonitrile for 35 to 40 min; and 40 to 2% acetonitrile for 40 to 45 min; solvent system 2, the mobile phase contained 20 mM ammonium formate (pH 3.1) in 0% acetonitrile for 0 to 2 min; 0 to 4% acetonitrile for 2 to 22 min; 4 to 40% acetonitrile for 22 to 27 min; and 40 to 0% acetonitrile for 27 to 32 min; solvent system 3, the mobile phase was the same as solvent system 1, except the aqueous phase contained 20 mM ammonium formate (pH 3.1); solvent system 4, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 5% methanol for 0 to 3 min; 5 to 10% for 3 to 13 min; 10 to 90% for 18 to 23 min; and 90 to 5% for 25 to 30 min; solvent system 5, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 10% methanol for 0 to 15 min; 10 to 20% for 15 to 20 min; 20 to 90% for 20 to 25 min; and 90 to 10% for 30 to 35 min; solvent system 6, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 10% methanol for 0 to 2 min; 10 to 20% for 2 to 7 min; 20 to 30% for 7 to 17 min; 30 to 90% for 20 to 25 min; and 90 to 10% for 25 to 30 min; solvent system 7, the mobile phase contained 20 mM ammonium acetate (pH 5.0) in 6% acetonitrile with 6 to 12% acetonitrile for 0 to 6 min; 12 to 35% acetonitrile for 14 to 19 min; and 35 to 6% acetonitrile for 20 to 25 min; and solvent system 8, the mobile phase contained 20 mM ammonium formate (pH 3.1) in 6% acetonitrile with 6 to 7% acetonitrile for 0 to 10 min; 7 to 35% acetonitrile for 10 to 15 min; and 35 to 6% acetonitrile for 15 to 20 min. Radioactivity in HPLC eluents was continuously measured using a FLO-ONE (PerkinElmer Life and Analytical Sciences) radioactive flow detector. Data are expressed as a percentage of total radioactivity or nanomoles recovered after HPLC.

Processing Urine and Purification of Metabolites

The 24-h urine samples were treated with an equal volume of methanol and spun at 1500g for 20 min to precipitate protein and debris. After evaporation of methanol in the supernatant, the urine was applied to a C18 solid-phase extraction column (1 g, PrepSep), previously prepared with methanol-water, washed twice with 6 ml of water, and eluted with 6 ml of 100% methanol. The latter was concentrated by evaporation and dissolved in a small volume of methanol. Urine from all animals was compared and analyzed using solvent system 1 (Fig. 2; Table 1). Rat metabolites were purified as described previously (Armbrecht et al., 2007).

For purification of mouse urine, metabolites were first separated using solvent system 1, individual fractions were collected, and samples were concentrated and applied to the next solvent system for further purification. *N*-Glucuronide was further purified with solvent systems 2 and 4, 5-*O*-glucuronide with systems 2 and 4, sulfamate with systems 2 and 5, 5-sulfate with systems 3 and 5, and demethyl-IQ and IQ with systems 3 and 6.

Qualitative Identification of Urinary and Liver Slice Metabolites

Metabolites were identified by their HPLC elution times relative to those of authentic products identified previously by us and by susceptibility to specific treatments (Armbrecht et al., 2007). Urine metabolites were purified before their susceptibility was assessed. Slice media was partially purified by extraction with ethyl acetate and metabolites in the aqueous fraction collected from a C18 Sep-Pak column before susceptibility was assessed. The 5-*O*-glucuronide was susceptible to *E. coli* β -glucuronidase (type VII-A) with 10 units of enzyme, 50 mM sodium acetate buffer at pH 6.8, and 0.3 μ g of substrate/0.1 ml at 37°C for 4 h. 5-Sulfate was susceptible to abalone sulfatase (type VIII) with 200 units, 50 mM sodium acetate buffer at pH 5.0, and 0.3 μ g of substrate/0.1 ml at 37°C for 72 h. *N*²-Glucuronide and sulfamate were hydrolyzed with 1 N HCl at 60°C for 2 h.

To determine whether *N*-acetyl-IQ was present in mouse urine samples, we synthesized this compound (see below) and then determined its elution profile with different HPLC solvent systems. Although it coeluted with IQ in our standard solvent system 1, *N*-acetyl-IQ was separated from IQ in system 3. Under this condition, a peak, which coelutes with *N*-acetyl-IQ (approximately 1% of recovered radioactivity), was observed in mouse urine. Urine samples were treated with carboxyesterase, which converts *N*-acetyl-IQ to IQ. When urine was incubated with esterase from porcine liver (25 units) in sodium phosphate buffer (pH 8.0) for 7 h at 37°C, no loss of the peak, which coelutes with *N*-acetyl-IQ, or increase in IQ was detected. Under these conditions, synthetic *N*-acetyl-IQ (0.025 mM) and *N*-acetylbenzidine (0.03 mM) were completely hydrolyzed to their corresponding free amine. Furthermore, whereas adding synthetic *N*-acetyl-IQ to urine resulted in complete hydrolysis of synthetic standard to IQ, residual radioactivity (approximately 1%) previously present remained unhydrolyzed. These results suggest that *N*-acetyl-IQ is not present in mouse urine. Similar studies with rat urine were unsuccessful. Subsequent studies determined that *N*-acetyl-IQ was not synthesized from IQ by mouse liver cytosol fractions containing *N*-acetyl-transferase activity for benzidine (see below).

Liver Slice IQ Incubation

Mouse liver slices were prepared with a Stadie-Riggs microtome as described previously (Lakshmi et al., 1995b) and incubated with Dulbecco's modified Eagle's media with high glucose containing 3.7 μ g/ml NaHCO₃ and 6 μ g/ml Hepes. Approximately 150 to 200 mg of liver were placed in a 20-ml scintillation vial with 1 ml of media, gassed with 5% O₂/95% CO₂ for 1 min, and incubated at 37°C for 60 min. Media contained 0.06 mM IQ and where indicated 0.5 mM ellipticine or SKF-525A. To stop incubations, 1 ml of cold methanol was added, and samples were frozen. Thawed samples were sonicated three times for 15 s, samples

were spun to remove protein and cell debris, and the supernatant was analyzed by HPLC using solvent system 1. Activity is expressed as a percentage of radioactivity recovered by HPLC.

Microsomal IQ Incubation

To identify specific pathways for IQ metabolism, liver microsomes were prepared using a procedure described previously (Zenser et al., 1978). Mice were sacrificed by cervical dislocation and open thoracotomy. Samples were stored in small aliquots at -70°C for the assays; unused material was discarded after one freeze-thaw cycle. Microsomes (1 mg/ml) were incubated in sodium phosphate buffer (pH 7.4), containing 0.1 mM diethylenetriaminepentaacetic acid, 1 mM NADPH, 5 mM MgCl_2 , and 0.06 mM $[^{14}\text{C}]\text{IQ}$ for 30 min at 37°C . Microsomes were preincubated with furafylline for 10 min before $[^{14}\text{C}]\text{IQ}$ addition. The reaction was linear with respect to protein concentration and time and was stopped by addition of an equal volume of methanol with 1 mM vitamin C. After 30 min on ice, the mixture was centrifuged to remove the precipitated protein. Supernatants were analyzed by HPLC, using solvent system 7. Activity is expressed as nanomoles or percentage of radioactivity recovered by HPLC as product. 7-Ethoxyresorufin *O*-deethylation (EROD) was determined by measuring the fluorescence of resorufin with 0.002 mM ethoxyresorufin (Pohl and Fouts, 1980).

Synthesis of *N*-Acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline and *N*-Acetyl-2-aminoimidazo[4,5-*f*]quinoline

To prepare synthetic *N*-acetyl-IQ, 26 μg of $[^{14}\text{C}]\text{IQ}$ was added to 1 mM acetic anhydride in 0.1 ml of pyridine for 15 min at 60°C . The reaction was stopped by the addition of distilled water (0.2 ml). This mixture was later diluted with 10 ml of water, applied to a C18 Sep-Pak column and washed with 10 ml of water, and the *N*-acetylated product was eluted with 3 ml of methanol. The product was purified by solvent systems 8, 7, and 6 and then identified by electrospray ionization (ESI) mass spectrometry (MS) in the positive-ion mode. The product exhibited $[\text{M} + \text{H}]^+$, $[\text{M} + \text{Na}]^+$, and $[\text{M} + \text{K}]^+$ ions at m/z 241, 263, and 279, respectively. The $[\text{M} + \text{H}]^+$ at m/z 241 gave rise to prominent ions at m/z 199, 184, and 157, representing IQ and consecutive losses of CH_3 and HCN. This fragmentation pattern is consistent with the product being *N*-acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline.

N-Acetyl-2-aminoimidazo[4,5-*f*]quinoline was synthesized from demethyl-IQ as described above for *N*-acetyl-IQ, except for being only partially purified by immediate methylene chloride extraction because of its lability in methanol and aqueous solutions. The *N*-acetylated product was identified by ESI MS in the positive-ion mode. The product exhibited an $[\text{M} + \text{H}]^+$ ion at m/z 227, which gave rise to prominent ions at m/z 185, 158, and 143 representing demethyl-IQ and consecutive losses of HCN and $\text{NH}_2\text{-CN}$. *N*-Acetyl-2-aminoimidazo[4,5-*f*]quinoline was not stable in aqueous solution and hydrolyzed to demethyl-IQ.

We also attempted to prepare synthetic *N*-acetyl-IQ from mouse liver supernatant. The supernatant was saved from the microsomal preparation described above and used to assess *N*-acetyltransferase activity. Samples were stored in small aliquots at -70°C for the assays; unused material was discarded after one freeze-thaw cycle. Supernatant (1 mg/ml) was incubated (total volume 0.1 ml) in sodium phosphate buffer (pH 7.4), containing 1 mM acetyl CoA, 1 mM dithiothreitol, and 0.06 mM $[^{14}\text{C}]\text{IQ}$ or ^3H -*N*-acetylbenzidine for 60 min at 37°C . The reaction was stopped by addition of 0.1 ml of methanol. Supernatants were analyzed by HPLC, using solvent system 8. Under these conditions, 70% of *N*-acetylbenzidine is converted to *N,N'*-diacetylbenzidine. However, no formation of *N*-acetyl-IQ could be detected.

Mass Spectral Analysis

Metabolites were identified by ESI MS. Analyses were performed with a Finnigan TSQ-7000 triple-stage quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with a Finnigan ESI source and controlled by Finnigan ICIS software operated on a DECα workstation. Samples were loop injected onto the ESI source with a Harvard syringe pump, which was continuously infused with methanol at a flow rate of 5 μ l/min. The skimmer was at ground potential, and the electrospray needle was at 4.5 kV. The heated capillary temperature was 250°C. To obtain collisionally activated dissociation tandem mass spectra, the collision energy was set at 22 eV, and argon (2.3 mTorr) was used as target gas. The product ion spectra were acquired in the profile mode at the scan rate of one scan per 3 s.

Statistical Analysis

Data are expressed as mean $\pm$ S.E., and significant differences were evaluated using a Student's unpaired *t* test with *p* < 0.05.

Results

HPLC Analysis of IQ Metabolites in Mouse and Rat Urine

To determine whether metabolism of IQ was different in mouse than in rat, 24-h urine samples from each species were examined after dosing with IQ. In Fig. 2 (top), the HPLC elution profile of rat metabolites of IQ previously identified by us and others is illustrated (Turesky et al., 1986; Inamasu et al., 1989; Luks et al., 1989; Armbrrecht et al., 2007). These include *N*-glucuronide, 5-*O*-glucuronide, sulfamate, and 5-sulfate. Although these same metabolites were also present in mouse urine, there was an additional metabolite observed, demethyl-IQ (Fig. 2, bottom). Table 1 reports the relative abundance of excreted metabolites using HPLC data illustrated in Fig. 2. The relative abundance of metabolites in these species was different. For rat, the proportion of metabolites excreted was 5-*O*-glucuronide > sulfamate > 5-sulfate > *N*-glucuronide. The presence of demethyl-IQ in rat urine was not confirmed by mass spectrometry. For mouse, the proportion of metabolites excreted was 5-*O*-glucuronide > demethyl-IQ > sulfamate > *N*-glucuronide > 5-sulfate. With HPLC solvent system 3, the amount of unmetabolized IQ in mouse urine was $2.3 \pm 0.4\%$ of recovered radioactivity. *N*-Acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline was not detected. The urinary IQ metabolite profile in mouse and rat is different, and demethyl-IQ is a major new metabolite in mouse.

To determine whether the route of IQ administration is responsible for the lack of demethyl-IQ formation in rat, rats were administered IQ by i.p. injection. In these experiments, the proportion of metabolites excreted was sulfamate ($48 \pm 1\%$) > 5-*O*-glucuronide ($15 \pm 0.5\%$) = 5-sulfate ($15 \pm 0.5\%$) > *N*-glucuronide ($4.8 \pm 0.4\%$). The amount of radioactivity that coeluted with demethyl-IQ was $1.9 \pm 0.2\%$ of the total radioactivity after HPLC and not further identified.

Identification of Urinary IQ Metabolites

Rat metabolites were identified by their HPLC elution profile relative to authentic products previously identified by us (Armbrrecht et al., 2007) and their susceptibility to specific treatments. The *N*-glucuronide and sulfamate were susceptible to treatment with 1 N HCl at 60°C, whereas 5-*O*-glucuronide and 5-sulfate were susceptible to β -glucuronidase and sulfatase, respectively. Rat metabolites were only susceptible to the treatments indicated.

Mouse metabolites were further identified by their elution profile relative to rat metabolite standards (Armbrrecht et al., 2007), susceptibility to specific treatments described above for rat, and ESI MS spectra in Fig. 3. In the negative-ion mode, the 12-min peak exhibited an $[M - H]^-$ at *m/z* 373 and gave prominent ions at *m/z* 239 and 197, representing *N*-acetyl-IQ and

IQ. The 12-min metabolite is IQ-*N*²-glucuronide (Fig. 3A). The 17-min peak, in the negative-ion mode, yielded deprotonated $[M - H]^-$ molecular ion at m/z 389 and gave prominent ions at m/z 213 and 198, representing 5-OH-IQ and loss of CH_3 . This is consistent with the 17-min product being IQ-5-*O*-glucuronide (Fig. 3B). The 19.5-min peak in the negative-ion mode exhibited an $[M - H]^-$ ion at m/z 277 and gave rise to prominent ions at m/z 197 and 182, representing IQ and loss of CH_3 . The ion at m/z 80 corresponded to SO_3 . This fragmentation pattern is consistent with the 19.5-min peak being IQ-sulfamate (Fig. 3C). For the 26-min peak, the negative-ion mode displayed an $[M - H]^-$ ion at m/z 293 and gave prominent ions at m/z 213 and 198, representing 5-OH-IQ and loss of CH_3 . The ion at m/z 80 corresponds to SO_3 . In the positive-ion mode, the product yielded $[M + Na]^+$ at m/z 317. The 26-min metabolite is IQ-5-sulfate (Fig. 3D). Urinary IQ was identified by its coelution with authentic commercial material and by its mass spectra (Fig. 3E). In the positive-ion mode, the $[M + H]^+$ at m/z 199 gave prominent ions at m/z 184, 157, and 131 representing consecutive losses of CH_3 , HCN, and C_2H_2 , respectively. The spectra of the commercial starting material were identical to that of urinary IQ. The ESI MS fragmentation patterns of all the metabolites matched their corresponding metabolite in rat. This is consistent with the mouse and rat metabolites being identical to one another and not isomers.

The ESI MS spectra of the new metabolite eluting at 31.5 min is illustrated in Fig. 4. In the positive mode, this urinary metabolite exhibited $[M + H]^+$ at m/z 185. The product ion spectra of m/z 185 along with its fragmentation pattern is consistent with it being 2-aminoimidazo[4,5-*f*]quinoline (demethyl-IQ).

Analysis of IQ Metabolites Produced by Mouse Liver Slices

To further evaluate IQ metabolism in the mouse, liver slices were assessed (Fig. 5). The HPLC elution profile indicates that the following metabolites were present: 5-*O*-glucuronide, sulfamate, and demethyl-IQ. 5-*O*-Glucuronide and sulfamate were further identified by their susceptibility to β -glucuronidase and acid treatment, respectively. Table 2 reports the percentage of total radioactivity recovered after HPLC for each metabolite. P450 inhibitors, 0.5 mM ellipticine or SKF-525A, were incubated with slices to determine the role of these enzymes in IQ metabolism. 5-*O*-Glucuronide synthesis was significantly inhibited by ellipticine (19% of control radioactivity). SKF-525A did not prevent glucuronide formation. Sulfamate synthesis was not altered by either inhibitor, but demethyl-IQ production was significantly decreased by ellipticine (44% of control radioactivity) and SKF-525A (56%). These results demonstrate mouse liver slice synthesis of three IQ metabolites with 5-*O*-glucuronide and demethyl-IQ being produced by P450.

Evaluation of Demethyl-IQ Formation by Mouse Liver Microsomes

To examine P450 *N*-demethylase catalyzed metabolism of IQ in more detail, liver microsomal metabolism was examined. Consistent with liver slice data, Demethyl-IQ was a product of liver microsomal metabolism and its formation was inhibited by ellipticine (Fig. 6). Demethyl-IQ was the only metabolite of IQ observed. In similar studies with rat microsomes, Demethyl-IQ was not detected (not shown). The ESI MS spectrum of the microsomal product was identical to that illustrated in Fig. 4. To determine the P450(s) responsible for demethyl-IQ formation, inhibitors of different P450s previously used to assess *N*-demethylation were examined (Iribarne et al., 1996; Yamagata et al., 1998). In Table 3, ellipticine, a P450 1A inhibitor, was the only inhibitor to completely prevent demethyl-IQ formation at 0.02 mM. At this concentration, inhibition was also observed with α -naphthoflavone (27% of total nanomoles; 1A1/1A2 inhibitor), SKF-525A (74%; nonspecific inhibitor), and ketoconazole (64%; 3A inhibitor). No inhibition was detected with 0.02 mM furafylline (1A2 inhibitor) or 0.1 mM sulfaphenazole (2C inhibitor). To assess the efficiency of ellipticine inhibition of *N*-demethylation, a range of concentrations was used and a Dixon plot determined the K_i to be

0.001 mM. EROD is thought to represent 1A1 activity and was used to further evaluate ellipticine inhibition. By using Kinetic plots EROD K_i values for ellipticine and furafylline were determined to be 0.0008 and 0.011 mM, respectively. These results are consistent with P450 1A1 mediating *N*-demethylation of IQ in mouse liver.

Analysis of Mouse Urine for Demethyl-IQ Metabolites

To determine whether demethyl-IQ is further metabolized, purified demethyl-IQ (10 μ g) was injected i.p. into a mouse, and a 24-h urine sample was collected. No metabolism of demethyl-IQ was detected (not shown). We have synthesized *N*-acetyl-demethyl-IQ and determined that it elutes at 41.5 min (Fig. 2). Thus, this *N*-acetylated product was not detected in urine from a mouse injected with either IQ or demethyl-IQ. Additional studies demonstrated that demethyl-IQ was not metabolized by liver microsomes or *N*-acetylated by liver cytosol (not shown). Thus, demethyl-IQ does not further contribute to mouse metabolites derived from IQ.

Discussion

In this study we examined mouse IQ metabolism and discovered a previously unidentified rodent metabolite. All of the major urinary metabolites previously identified in rats were also found in mice. Although the 5-*O*-glucuronide represented a similar percentage of total radioactivity excreted in both species, the rat excretion of sulfamate and 5-sulfate exceed that in mouse by 3- and 4-fold, respectively. The major difference between these species was the large amount of demethyl-IQ synthesized by mouse (26% of the total radioactivity excreted) but not by rat. In mouse, excretion of demethyl-IQ exceed that of sulfamate by more than 2-fold. The presence of demethyl-IQ in urine was consistent with its synthesis by mouse liver slices and microsomes. No further metabolism of demethyl-IQ could be demonstrated by an intact mouse or liver microsomes. However, incubations demonstrating mutagenicity of demethyl-IQ were supplemented with the S9 fraction of liver from Aroclor 1254-treated rats, suggesting that metabolic activation of demethyl-IQ occurs (Barnes et al., 1985). Demethyl-IQ is a major metabolite in monkey (Snyderwine et al., 1992). Mice have a high capacity for metabolizing IQ as little IQ was found unmetabolized ($1.2 \pm 0.3\%$) in urine after administration of a high dose of IQ (40 mg/kg). Results are consistent with mouse IQ metabolism being significantly different from that in rat and with demethyl-IQ being a major new rodent metabolite unique to the mouse.

It is not clear whether rats *N*-demethylate IQ. Although we reported demethyl-IQ in rat urine (Table 1), this finding was not confirmed by ESI MS. In a prior study in which rats were given an oral dose of IQ (40 mg/kg), the 24-h urine was thought to contain demethyl-IQ along with some other nonpolar metabolites, including IQ, *N*-acetyl-IQ, and 7-OH-IQ (Inamasu et al., 1989). This nonpolar urine fraction represented less than 1% of the dose administered. The metabolites were only identified by their coelution with synthetic standards. Another study by this group provided MS and nuclear magnetic resonance structural identification for the sulfamate, 5-*O*-glucuronide, 5-sulfate, and *N*-glucuronide conjugates of IQ, but no further mention of demethyl-IQ or the other nonpolar metabolites (Luks et al., 1989). Studies of rat IQ metabolism by other investigators have not demonstrated the formation of demethyl-IQ (Alexander et al., 1989). However, rat liver microsomes *N*-demethylate dacarbazine by 1A enzymes (Yamagata et al., 1998). Thus, structural proof for the presence of demethyl-IQ in rat urine has not been demonstrated and, if present, *N*-demethylation would only be a minor pathway compared with that in mouse.

Results suggest that the route of IQ administration does not affect the formation of demethyl-IQ in the rat. With either oral or i.p. administration of IQ, less than 2% of radioactivity recovered after HPLC coeluted with demethyl-IQ compared with $26 \pm 1.6\%$ in mouse. Because this could not be confirmed by ESI MS, it is not clear whether rats *N*-demethylate IQ. Although excretion

of sulfamate and 5-*O*-glucuronide was similar after oral IQ administration in the rat, the excretion of sulfamate was 3-fold greater than that of 5-*O*-glucuronide after i.p. injection. This change in metabolite distribution is probably due to higher plasma concentrations of IQ after i.p. injection with higher concentrations of IQ within hepatocytes saturating P450 metabolism and allowing more sulfamate formation.

Studies with monkey identified three demethyl-IQ metabolites in urine (Snyderwine et al., 1992): demethyl-IQ, demethyl-IQ *N*-glucuronide, and demethyl-7-oxo-IQ. The latter was thought to be derived from intestinal bacterial metabolism of demethyl-IQ. Approximately, 16 to 22% of the dose given at 2.2 $\mu\text{mol/kg}$ IQ in two monkeys was derived from *N*-demethylation. In contrast to monkey, we observed no further metabolism of demethyl-IQ. Human hepatocytes *N*-demethylate a HCA structurally similar to IQ (Langouët et al., 2001). Demethyl-IQ is mutagenic (Barnes et al., 1985). Thus, human, monkey, and mouse *N*-demethylate HCAs.

Although *N*-acetylation is a characteristic feature of aromatic amine metabolism, this pathway does not appear to be active in heterocyclic amine metabolism. We were unable to detect *N*-acetyl-IQ in mouse urine or as a product of mouse liver cytosol *N*-acetyltransferase activity. The lack of *N*-acetyltransferase (NAT1 and NAT2) *N*-acetylation of IQ and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline has been attributed to their 3-methyl group. Results with *N*-acetylation of *ortho*-toluidine compared with those for *para*-toluidine suggested that there is steric hindrance of the exocyclic amine group resulting from methyl group substitution *ortho* to the amine (Hein et al., 1993). However, because *N*-acetylation of demethyl-IQ was not demonstrated either in vitro or in vivo, steric hindrance by the 3-methyl group does not appear to be the reason for the lack of *N*-acetylation of these heterocyclic amines. In aqueous solutions, chemically synthesized *N*-acetyl-IQ was stable, whereas *N*-acetyl-demethyl-IQ was not stable. In addition, the *N*-glucuronides of aromatic amines are quite acid-labile, i.e., the *N*-glucuronide of *N*-acetylbenzidine has a half-life at pH 5.5 of approximately 8 min (Babu et al., 1995), whereas *N*-glucuronides of these heterocyclic amines are relatively acid-stable (see *Materials and Methods*). Results suggest that the chemistry involving the exocyclic amine of these heterocyclic compounds is different from that of aromatic amines and may also contribute to the lack or low rate of *N*-acetylation of heterocyclic amines. For example, the electron withdrawing effects of the nitrogen substituents of the imidazole ring may reduce the reactivity of the exocyclic amine or the amine may preferentially exist as an imine tautomer, making it less likely to be *N*-acetylated.

A variety of inhibitors were used to determine the P450(s) responsible for *N*-demethylation of IQ. The 1A inhibitor ellipticine was effective in liver slices and also in microsomal incubations with a K_i for demethyl-IQ formation of 0.001 mM. At the concentration of 0.02 mM used for all inhibitors (Table 3), other compounds exhibited inhibition with α -naphthoflavone > ketoconazole > SKF-525A. The former, like ellipticine, is an inhibitor of both 1A1 and 1A2 and would be expected to be an effective inhibitor of 1A1-catalyzed reactions. Ketoconazole has been reported to be an effective inhibitor of 1A2 in rat (Kobayashi et al., 2003) and may exhibit some 1A1-like activity in mouse. SKF-525A is a nonspecific inhibitor. The lack of effect of furafylline, a 1A2-selective inhibitor, further demonstrates the apparent selectivity of 1A1 for IQ *N*-demethylation. Although P450 3A does not mediate mouse liver IQ *N*-demethylation, previous studies have shown 3A to be expressed in this tissue and to catalyze *N*-demethylation of cocaine (Pellinen et al., 1994; Martignoni et al., 2006). EROD activity demonstrated preferential inhibition by ellipticine compared with furafylline similar to that observed for IQ *N*-demethylation. In addition, mouse liver benzo[*a*]pyrene hydroxylase, an activity attributed to 1A1, was inhibited by 0.005 mM α -naphthoflavone but not by 0.02 mM furafylline (Tsyrov et al., 1993). That study also demonstrated preferential inhibition of mouse 1A2 by furafylline compared with 1A1. Thus, mouse IQ *N*-demethylation appears to be catalyzed by hepatic P450 1A1.

Because of our interest in the role of colitis in colon cancer (Cooper et al., 2000; Clapper et al., 2006), we assessed IQ metabolism in female C57BL/6 mice administered DSS in their drinking water. After three cycles of DSS to induce colitis, an oral dose of 40 mg/kg IQ was administered, and analysis was performed as described in this report. Although urinary 5-*O*-glucuronide excretion decreased from $30.8 \pm 2.5\%$ in controls to $17.2 \pm 2.1\%$ of total radioactivity for DSS-treated mice, the distribution of other excreted metabolites was not different. These results are consistent with a similar study in which rats were administered DSS at 3% in drinking water for 7 days. Decreases in several hepatic P450 enzyme activities, including P450 1A1 and A2 phenacetin *O*-deethylation activity, were reported (Masubuchi and Horie, 2004). Inflammation decreases P450-mediated metabolism (for review, see Morgan, 1997), and this is a likely mechanism by which DSS decreased 5-*O*-glucuronide formation.

In conclusion, this study demonstrated for the first time that *N*-demethylation is an important pathway for IQ metabolism in mouse, possibly mediated by P450 1A1. However, the constitutive expression of 1A1 in mouse liver is low (Dey et al., 1999), and additional studies may be needed to determine the P450 responsible for demethyl-IQ formation. Demethyl-IQ is mutagenic (Barnes et al., 1985). *N*-Demethylation is a prominent pathway in mouse, monkey, (Snyderwine et al., 1992), and human (Langouët et al., 2001) and may play a role in IQ tumorigenicity in these species. Differences in metabolism between mouse and rat, especially demethyl-IQ formation, suggests that there is more similarity of mouse to human metabolism of HCAs than to rat metabolism and that the mouse is a useful model for testing tumorigenicity of these chemicals.

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ABBREVIATIONS

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline
HCA, heterocyclic amine
P450, cytochrome P450
DSS, dextran sulfate sodium
demethyl-IQ, 2-aminoimidazo[4,5-*f*]quinoline
SKF-525A, 2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride
DMSO, dimethylsulfoxide
HPLC, high-performance liquid chromatography
N-acetyl-IQ, *N*-acetyl-2-amino-3-methylimidazo[4,5-*f*]quinoline
EROD, 7-ethoxyresorufin *O*-deethylation
ESI, electrospray ionization
MS, mass spectrometry

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Structures of IQ and Its Metabolites

Fig. 1.
Structures of IQ and its metabolites.

Fig. 2.

Urinary excretion of [^{14}C]IQ in rat and mouse. Mice and rats were administered an oral dose of 40 mg/kg [^{14}C]IQ, and a 24-h urine sample was collected. A representative HPLC profile from each treatment group is shown using solvent system 1. The peaks were tentatively identified by retention times and confirmed by mass spectrometry and susceptibility to specific treatments.

Fig. 3. ESI MS spectra of IQ urinary metabolites. A to E, purified urinary metabolite IQ- N^2 -glucuronide, IQ-5- O -glucuronide, IQ-sulfamate, IQ-5-sulfate, and IQ, respectively. All spectra were obtained in the negative ion mode, except for IQ in E, which is in the positive ion mode.

Fig. 4.

Positive-ion ESI MS of demethyl-IQ purified from mouse urine. The product ion spectrum of m/z 185 is illustrated along with its fragmentation pattern. Results confirm that demethyl-IQ is 2-aminoimidazo[4,5-*f*]quinoline.

Fig. 5. Mouse liver slice metabolism of IQ. Slices were incubated with 0.06 mM [^{14}C]IQ for 60 min at 37°C in the presence and absence of 0.5 mM ellipticine. A representative HPLC profile with solvent system 1 from each treatment group is shown. Peaks were identified by retention times and susceptibility to specific treatments.

Fig. 6. Mouse liver microsomal metabolism of IQ. Microsomes were incubated with 0.06 mM [^{14}C] IQ for 30 min at 37°C in the presence and absence of 0.01 mM ellipticine. A representative HPLC profile from each treatment group is shown using solvent system 7. The demethyl-IQ peak was tentatively identified by its retention time and confirmed by mass spectrometry.

TABLE 1

IQ metabolites in rat and mouse urine

Urine samples from rats and mice were analyzed by HPLC as illustrated in Fig. 2. The relative abundance of excreted metabolites is provide by table entries as the mean $\pm$ S.E. of four rats and four mice. Rodents were administered [^{14}C]IQ and placed in metabolic cages for a 24-h urine collection.

Condition	N-Glucuronide	5-O-Glucuronide	Sulfamate	5-Sulfate	Demethyl-IQ
	% of total urine radioactivity				
Rat	5.0 $\pm$ 0.6	35 $\pm$ 1	30 $\pm$ 2 *	15 $\pm$ 0.9 *	0.9 $\pm$ 0.2 *
Mouse	5.2 $\pm$ 0.4	36 $\pm$ 1	11 $\pm$ 1	3.8 $\pm$ 0.1	26 $\pm$ 2

* Significantly different from mouse ($P < 0.05$).

TABLE 2**IQ metabolites produced by mouse liver slices**

Slices were incubated for 60 min at 37°C with 0.06 mM IQ and where indicated 0.5 mM ellipticine or SKF-525A. Samples were analyzed by HPLC, and data are recorded as % of total radioactivity recovered. Entries represent mean $\pm$ S.E. of determinations from three mice.

Condition	5- <i>O</i> -Glucuronide	Sulfamate	Demethyl-IQ
% of total radioactivity			
Control	4.3 $\pm$ 0.2	5.7 $\pm$ 0.3	5.0 $\pm$ 0.6
+Ellipticine	0.8 $\pm$ 0.2 [*]	4.2 $\pm$ 0.9	2.2 $\pm$ 0.4 [*]
+SKF-525A	2.9 $\pm$ 0.5	5.5 $\pm$ 0.5	2.8 $\pm$ 0.4 [*]

^{*} Significantly different from control ($P < 0.05$).

TABLE 3

P450 inhibition of mouse microsomal demethyl-IQ formation

Samples were incubated for 30 min at 37°C with 0.06 mM IQ. Furafylline was preincubated for 10 min before addition of IQ. For those incubations, the microsomal activity was 0.80 ± 0.06 nmol, which is not different from the control value below. Samples were analyzed by HPLC, and data are expressed as mean nanomoles of demethyl-IQ $\pm$ S.E. with $n = 3$ to 5.

Test Agents	Type of P450 Inhibitor	Concentration	Activity	% Total
		μM	$nmol$	
Control			0.74 ± 0.04	100
Ellipticine	1A	10	$0.02 \pm 0.01^*$	3
		20	N.D.	0
Furafylline	1A2	20	0.70 ± 0.04	95
α -Naphthoflavone	1A	20	$0.20 \pm 0.02^*$	27
SKF-525A	Nonspecific	20	$0.55 \pm 0.06^*$	74
		100	$0.21 \pm 0.02^*$	28
Ketoconazole	3A	20	$0.47 \pm 0.06^*$	64
		100	$0.17 \pm 0.04^*$	23
Sulfaphenazole	2C	20	0.65 ± 0.03	88
		100	0.79 ± 0.05	107

N.D., not detected.

* $P < 0.05$ compared with control.