

# Cytochrome P450-Mediated Metabolism and DNA Binding of 2-Amino-1,7-dimethylimidazo[4,5-g]quinoxaline and Its Carcinogenic Isomer 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline in Mice

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**Supporting Information** 



ABSTRACT: 2-Amino-1,7-dimethylimidazo[4,5-g]quinoxaline (MeIgQx) is a recently discovered heterocyclic aromatic amine (HAA) that is formed during the cooking of meats. MeIgQx is an isomer of 2-amino-3,8-dimethylmidazo [4,5-f] quinoxaline (MeIQx), a rodent carcinogen and possible human carcinogen that also occurs in cooked meats. MeIgQx is a bacterial mutagen, but knowledge about its metabolism and carcinogenic potential is lacking. Metabolism studies on MeIgOx and MeIOx were conducted with human and mouse liver microsomes, and recombinant human P450s. DNA binding studies were also investigated in mice to ascertain the genotoxic potential of MeIgOx in comparison to MeIOx. Both HAAs underwent comparable rates of N-oxidation to form genotoxic N-hydroxylated metabolites with mouse liver microsomes (0.2–0.3 nmol/min/mg protein). The rate of N-oxidation of MeIQx was 4-fold greater than the rate of N-oxidation of MeIgQx with human liver microsomes (1.7 vs 0.4 nmol/min/mg protein). The rate of N-oxidation, by recombinant human P450 1A2, was comparable for both substrates (6 pmol/min/pmol P450 1A2). MeIgQx also underwent N-oxidation by human P450s 1A1 and 1B1 at appreciable rates, whereas MeIQx was poorly metabolized by these P450s. The potential of MeIgQx and MeIQx to form DNA adducts was assessed in female C57BL/6 mice given  $[^{14}C]$ -MeIgQx (10  $\mu$ Ci, 9.68 mg/kg body wt) or  $[^{14}C]$ -MeIQx (10  $\mu$ Ci, 2.13 mg/kg body wt). DNA adduct formation in the liver, pancreas, and colorectum was measured by accelerator mass spectrometry at 4, 24, or 48 h post-treatment. Variable levels of adducts were detected in all organs. The adduct levels were similar for both HAAs, when adjusted for dose, and ranged from 1 to 600 adducts per 10<sup>7</sup> nucleotides per mg/kg dose. Thus, MeIgQx undergoes metabolic activation and binds to DNA at levels that are comparable to MeIQx. Given the high amounts of MeIgQx formed in cooked meats, further investigations are warranted to assess the carcinogenic potential of this HAA.

# INTRODUCTION

More than 20 heterocyclic aromatic amines (HAAs) have been identified in cooked beef, poultry, and fish.<sup>1,2</sup> The formation of HAAs is dependent upon the type of meat and method of cooking: the concentrations of HAAs can range from less than 1 part-per-billion (ppb), up to 500 ppb in meat or poultry that is cooked well done.<sup>2–4</sup> The HAAs thus far assayed have been shown to induce tumors at multiple sites in rodents.<sup>1</sup> Several prevalent HAAs are classified as probable or possible human carcinogens (Group 2A and 2B), based on toxicity data reviewed by the International Agency for Research on Cancer.<sup>5,6</sup> The *Report on Carcinogens*, 11th edition, of the National Toxicology Program, also concluded that prevalent HAAs are "reasonably anticipated"

to be human carcinogens.<sup>7</sup> Thus, there is much concern about the health risk associated with the exposure to these chemicals.

2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) is one of the most abundant HAAs formed in cooked meats. MeIQx is a powerful bacterial mutagen and induces tumors at multiple sites in rodents during long-term feeding studies.<sup>1</sup> It is noteworthy that MeIQx and other known HAAs account for less than 30% of the mutagenicity attributed to this class of genotoxicants in well-done grilled meats and other uncharacterized HAAs are undoubtedly present.<sup>8</sup> We recently discovered

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2-amino-1,7-dimethylimidazo[4,5-g]quinoxaline (MeIgQx), a linear tricyclic ring isomer of MeIQx in the urine of meateaters,<sup>9</sup> and subsequently identified MeIgQx in cooked beef.<sup>9–11</sup> MeIgQx is the most mass-abundant HAA formed in cooked ground beef, steaks, and gravies;<sup>4</sup> it occurs at 5-fold or greater levels than the amounts of MeIQx or 2-amino-1-methyl-6phenylimidazo[4,5-*b*]pyridine (PhIP). Both MeIQx and PhIP are believed to contribute to diet related cancers.<sup>1</sup> MeIgQx is a less potent bacterial mutagen than MeIQx and PhIP; however, studies have not been conducted on the metabolism of MeIgQx, and the carcinogenic potential of MeIgQx is unknown.<sup>11</sup>

In this investigation, we have examined the capacities of human and mouse liver microsomes, and recombinant human cytochrome P450s to metabolize MeIgQx and MeIQx to electrophilic metabolites that are capable of binding to DNA. We also compared the potential of  $[^{14}C]$ -MeIgQx and  $[^{14}C]$ -MeIQx to bind to DNA in the liver, pancreas and colorectum, potential target organs of HAA carcinogenicity, in C57BL/6 mice. These data provide a preliminary estimate of the genotoxic potential of MeIgQx in vivo.

## MATERIALS AND METHODS

**Chemicals.** MeIQx and  $[2^{-14}C]$ -MeIQx (50 mCi/mmol, >98% radiochemical purity) were purchased from Toronto Research Chemicals (Ontario, Canada). KCN and Br<sub>2</sub> were was purchased from Sigma (St. Louis, MO). K[<sup>14</sup>C]N (54 mCi/mmol) was purchased from Moravek Chemicals (Brea, CA). Human liver samples were from Tennessee Donor Services, Nashville, TN, and kindly provided by Dr. F. P. Guengerich, Vanderbilt University. Recombinant human P450s 1A1, 1A2, and 1B1 contained in supersomes from baculovirus-infected insect cells and furafylline were purchased from Becton Dickinson (San Jose, CA).

**General Methods.** Mass spectra of synthetic derivatives and metabolites were acquired on a Finnigan TSQ Quantum Ultra triple stage quadrupole mass spectrometer (Thermo Fisher, San Jose, CA). The MS analyses were conducted in the positive ionization mode and employed an Advance CaptiveSpray source from Michrom Bioresource Inc. (Auburn, CA). Typical instrument tune parameters used were as follows: capillary temperature, 200 °C; source spray voltage, 1.4 kV; tube lens offset, 95 V; capillary offset, 35 V; and source fragmentation, 5 V. Argon, set at 1.5 mTorr, was used as the collision gas, and the collision energy was set between 15 to 30 eV; no sheath gas, sweep gas, or auxiliary gas was employed.

Synthesis. The synthesis of [2-14C]-MeIgQx was done as previously described for the synthesis of the unlabeled compound with minor modifications.<sup>11</sup> K[<sup>14</sup>C]N (0.093 mmol, 54 mCi/mmol) was diluted with KCN (0.4 mmol) in CH<sub>3</sub>OH (0.6 mL), sonicated for 10 min, and then slowly added to Br<sub>2</sub> (0.50 mmol) in CH<sub>3</sub>OH (0.5 mL) on ice. The reaction was allowed to proceed for 20 min. Thereafter, 4-fluoro-5-nitrobenzene-1,2-diamine (41 mg, 0.24 mmol) in C2H5OH (0.5 mL) was added to the newly formed  $[{\rm ^{14}C}]NBr,$  and the reagents were mixed overnight at room temperature. The  $2-[^{14}C]$ -5-fluoro-6-nitro-1H-benzimidazole-2-amine intermediate was reacted with NH4OH to displace the fluorine moiety, followed by reduction with NaBH4, ring-closure with methylglyoxal, and last by methylation with CH<sub>3</sub>I, as previously described.<sup>11</sup> [2-14C]-MeIgQx and its 6-methylated homologue were resolved by semipreparative HPLC employing a Waters XBridge phenyl column (10  $\times$  250 mm, 5  $\mu$ m particle size). An isocratic solvent composed of 23% CH<sub>3</sub>OH in 5 mM NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> (pH 8.7) was used for chromatography. The flow rate was 3 mL/min, and the run time was 70 min. The chemical and radiochemical purities of MeIgQx exceeded 98.5% as judged by HPLC with UV diode array detection and liquid scintillation counting. The specific activity of MeIgQx was determined by ESI-MS, by measuring the ratio of the  ${}^{14}C/{}^{12}C$  isotopes of the protonated molecules  $[M+H]^+$  at m/z 216.1 and 214.1: the specific activity was estimated at 11 mCi/mmol.

2-Hydroxyamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (HONH-MeIQx) was prepared by the reduction of 2-nitro-3,8-dimethylimidazo[4,5-*f*]quinoxaline with hydrazine and Pd/C serving as a catalyst.<sup>12</sup> 2-Amino-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]-quinoxaline (8-CH<sub>2</sub>OH-IQx) was prepared by the oxidation of MeIQx with SeO<sub>2</sub> as previously described.<sup>13</sup> N-(Deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx), [<sup>13</sup>C<sub>10</sub>]-dG-C8-MeIQx, and 5-(deoxyguanosin-N<sup>2</sup>-yl)-MeIQx (dG-N<sup>2</sup>-MeIQx) were synthesized by reaction of the N-acetoxy derivative of MeIQx with either dG or [<sup>13</sup>C<sub>10</sub>]-dG as previously reported.<sup>14</sup>

Microsomal Metabolism Studies. The metabolism studies were conducted with liver microsomes prepared from female C57BL/6J mice,<sup>15</sup> the mouse strain used for in vivo DNA binding studies, or with human liver microsomes, or recombinant human P450s 1A1, 1A2, and 1B1. Liver microsomal protein (1 mg/mL) or recombinant P450 (100 pmol/mL), in 100 mM potassium phosphate (pH 7.6) containing 0.5 mM EDTA, 5 mM glucose 6-phosphate, glucose 6-phosphate dehydrogenase (1 unit/mL), 1 mM NADPH, and 1 mM  $NAD^+$  were incubated with MeIgQx or MeIQx (200  $\mu$ M); the N-oxidation rates are at  $V_{\rm max}$  for MeIQx at this substrate concentration.<sup>15</sup> The enzymes plus cofactors were incubated for 3 min prior to the addition of HAA substrates. The metabolism was done for 5 or 10 min, and metabolite formation was a linear function with time. Some metabolism studies with human liver microsomes were conducted for time periods up to 30 min, to produce large amounts of metabolites for characterization by mass spectrometry. The enzyme reactions were terminated by the addition of 2 vol CH<sub>3</sub>OH. The precipitated proteins were removed by centrifugation, and the supernatants were assayed by HPLC with an Agilent 1100 Chemstation equipped with an UV photodiode array detector. The metabolites were resolved with an Aquasil C18 column (4.6  $\times$  250 mm, 5  $\mu$ m particle size, Thermo Scientific), employing a linear gradient starting from 10% CH<sub>3</sub>CN in 50 mM NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> (pH 6.8) and reaching 100% CH<sub>3</sub>CN at 20 min at a flow rate of 1 mL/min.

The estimates of formation of 2-hydroxyamino-1,7-dimethylimidazo[4,5-g]quinoxaline (HONH-MeIgQx) and 2-hydroxyamino-3,8-dimethylimidazo[4,5-f]quinoxaline (HONH-MeIQx), and other oxidation products were determined by HPLC with UV detection. The metabolites of MeIgQx were monitored at 370 or 390 nm, and metabolites of MeIQX were monitored at 274 nm. We assumed that the molar extinction coefficients of *N*-hydroxylated and ring-oxidized metabolites were the same values as the molar extinction coefficients of the parent compounds at their respective maximal absorbance wavelengths. The UV responses at these wavelengths were used to estimate rates of metabolite formation.

Liquid Chromatography–Electrospray lonization/Mass Spectrometry (LC-ESI/MS) Characterization of Metabolites. The *N*-oxidized and ring-oxidized metabolites were collected from the HPLC effluent and directly characterized by electrospray ionization tandem mass spectrometry (ESI/MS<sup>2</sup>) with the triple stage quadrupole mass spectrometer. The metabolites were also characterized by ESI/MS<sup>2</sup> following the reaction with nitrosobenzene (10  $\mu$ g, in 0.1% HCO<sub>2</sub>H in CH<sub>3</sub>OH) at 50 °C for 1 h.

In Vivo DNA Binding Studies. DNA binding studies were conducted with female C57BL/6Ntac mice (Taconic Farm, Albany, NY). Animals were housed in polycarbonate cages and allowed to acclimate for 1 week prior to the commencement of the study.

Animal Dosing. Mice (20 g, N = 5 animals per time point) were dosed by gavage with <sup>14</sup>C-MeIgQx (10  $\mu$ Ci, 9.68 mg/kg body wt) or <sup>14</sup>C-MeIQx (10  $\mu$ Ci, 2.13 mg/kg body wt) in a volume of 0.25 mL water. At 4, 24, or 48 h, animals were sacrificed by asphyxiation with CO<sub>2</sub> gas, followed by cervical dislocation; blood was retrieved via the inferior vena cava. The liver and pancreas were harvested, rinsed with chilled PBS, and snap-frozen in liquid nitrogen. The colorectal tissue (ileo-cecal junction to the rectum) was retrieved, the fecal contents were removed, and the tissue segment was incubated with cold, freshly prepared EDTA buffer (phosphate-buffered saline containing 1.5 mM Na<sub>2</sub>-EDTA, 6000 U sodium heparin, DTT (8 mg), and phenylmethanesufonyl fluoride (4 mg, 100  $\mu$ L of 40 mg/mL DMSO, per 100 mL of buffer). After 20 min, the mucosal layer containing the colonocytes was retrieved by placing the intestine segment in a Petri dish, and the loosened mucosal layer was displaced by gently scraping

the outer intestinal segment with the edge of a glass slide to eject the colonocytes. The colonocytes were centrifuged at 3,000g at 4  $^\circ$ C. Pelleted colonocytes were resuspended in EDTA buffer (2 mL) and centrifuged again. This washing procedure was repeated a third time, and the colonocytes were quick-frozen in liquid nitrogen.

**Radioactivity in Plasma.** Radioactivity was measured with a Beckman Coulter liquid scintillation counter (Fullerton, CA) with external calibration.

**Isolation of DNA.** The entire liver, pancreas, or colonocytes were homogenized in 2 mL of TE buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) and centrifuged for 10 min at 3,000g. DNA was isolated from the nucelar pellets of the liver and kidney (equivalent of 200 mg of tissue) and from the entire nuclear pellet of the colorectum, by solvent extraction as described by Gupta.<sup>16</sup> The purified DNA underwent a second solvent extraction procedure and ethanol precipitation step, to further remove noncovalently bound radioactivity.

The DNA was dissolved in distilled-deionized  $H_2O$ , and the concentration was determined by UV spectroscopy, assuming a concentration of DNA (50  $\mu$ g/mL) is equal to the 1.0 absorbance unit at 260 nm. The A260:280 ratios were on average greater than 1.75.

Accelerator Mass Spectrometry. AMS analyses were conducted at the MIT BEAMS Lab using procedures described elsewhere in detail.<sup>17</sup> Sample aliquots (1.50  $\mu$ L) were applied directly to a CuO matrix and introduced into a laser-induced combustion interface for subsequent AMS analysis. Each run contained two quantitation standards ([<sup>14</sup>C-methyl] bovine serum albumin, 0.003 dpm/ $\mu$ L), followed by two blanks (1  $\mu$ g/ $\mu$ L human serum albumin), 10 samples, and another two standards. Quantitation was performed by integrating peaks produced in the continuous trace of <sup>14</sup>C detector count rate versus time generated during operation of the combustion interface, which produces and delivers the CO<sub>2</sub> of combustion to the AMS ion source, and taking the product of the sample/standard peak area ratio multiplied by the standard concentration as the concentration of the sample. Blank values, if measurable, were subtracted from the sample peak areas prior to calculating sample concentration.

**DNA Adduct Measurements by LC-ESI/MS<sup>3</sup>.** DNA (100  $\mu$ g) in 5 mM Bis-Tris-HCl buffer (pH 7.1, 50  $\mu$ L) underwent enzymatic digestion with DNase I for 1.5 h, followed by incubation with nuclease P1 for 3 h, and then digested with alkaline phosphatase and phosphodiesterase for 18 h.<sup>18</sup> These enzyme digestion conditions were shown to be highly efficient in the recovery of the dG-C8-MeIQx from calf thymus DNA modified with *N*-acetoxy-MeIQx.<sup>14,19</sup> The digest was added to 2 vol of chilled C<sub>2</sub>H<sub>5</sub>OH and placed on ice for 1 h, followed by centrifugation at 15,000g to pellet salt and protein. The supernatants were transferred to silylated capillary LC vials and concentrated to dryness by vacuum centrifugation. The extracts were dissolved in 1:1 H<sub>2</sub>O/DMSO (20  $\mu$ L).

The adducts were separated from nonmodified nucleosides with a NanoAcquity UPLC system (Waters Corporation, Milford, MA) interfaced with a linear quadrupole ion trap mass spectrometer (LTQ MS, Thermo Fisher, San Jose, CA). A Waters Symmetry trap column (180  $\mu$ m × 20 mm, 5  $\mu$ m particle size) was employed for online solid phase enrichment of the DNA adducts. The analytical column was a Michrom C18 AQ (0.3 × 150 mm, 3  $\mu$ m particle size, Michrom Bioresources Inc., Auburn, CA). The DNA digests were injected onto the trap column and washed with 0.2% HCO<sub>2</sub>H in 5% CH<sub>3</sub>CN at a flow rate of 12  $\mu$ L/min for 5 min. Thereafter, the DNA adducts were back-flushed onto the C18 AQ Michrom AQ column. A linear gradient was employed to resolve the DNA adducts, starting at 0.01% HCO<sub>2</sub>H containing 5% CH<sub>3</sub>CN and arriving at 0.01% HCO<sub>2</sub>H in 95% CH<sub>3</sub>CN at 20 min. The flow rate was set at 5  $\mu$ L/min.

Mass spectrometric measurements were performed at the MS<sup>3</sup> scan stage in the positive ionization mode. The ion source was an Advance CaptiveSpray source from Michrom Bioresource Inc. (Auburn, CA). Representative optimized instrument tuning parameters were as follows: capillary temperature, 200 °C; source spray voltage, 2.0 kV; no sheath gas, sweep gas or auxiliary gas was employed; capillary voltage, 40 V; tube lens voltage, 110 V; and in-source fragmentation, 10 V. The automatic gain control settings were full MS target 30000 and MS<sup>n</sup> target 10000, and the maximum injection time was 10 ms. The MS/MS scan mode produced the aglycone ion BH<sub>2</sub><sup>+</sup> adducts [M+H-116]<sup>+</sup> or [M+H-121]<sup>+</sup> from the protonated dG-C8-MeIQx and dG-C8-[<sup>14</sup>C]-MeIQx adducts or [<sup>13</sup>C<sub>10</sub>]dG-C8-MeIQx adduct, respectively. The ions monitored were as follows: dG-C8-MeIQx (m/z 479.1 > 363.1 >); dG-C8-[<sup>14</sup>C]-MeIQx (m/z 481.1 > 365.1 >); and [<sup>13</sup>C<sub>10</sub>]-dG-C8-C8-MeIQx (m/z 489.1 > 368.1 >). The product ion spectra of the aglycone adducts at the MS<sup>3</sup> scan stage were acquired from m/z 125 to 400. The normalized collision energies were set at 24 and 34 eV, and the isolation widths were set at an m/z = 4.0 and 1.0, respectively, for the MS<sup>2</sup> and MS<sup>3</sup> scan modes. The activation Q was set at 0.35, and the activation time was 10 ms for both transitions. Helium was used as the collision damping gas in the ion trap and was set at a pressure of 1 mTorr. One  $\mu$ scan was used for data acquisition. The same mass spectral parameters were employed to screen for putative dG-C8 adducts of MeIgQx.

# RESULTS

Metabolism of MelQx and MelgQx with Human and Mouse Liver Microsomes and Recombinant Human P450s. HAAs undergo bioactivation by cytochrome P450mediated N-oxidation of the exocyclic amine group, to form electrophilic N-hydroxy-HAA metabolites that covalently bind to DNA.<sup>20-22</sup> The liver is the most active organ in the meta-bolism of HAAs,<sup>20-22</sup> and P450 1A2 is the major hepatic P450 involved in the N-oxidation of most HAAs.<sup>15,23,24</sup> We previously characterized the principal oxidation products of MeIQx formed by rat and human liver microsomes, rat P450 1A2, and recombinant human P450 1A2.15 In this study, we have examined the pathways of P450-mediated metabolism and compared the rates of N-oxidation of MeIQx and MeIgQx, by employing microsomes prepared from liver of female C57BL/6 mice, the species used for in vivo DNA adduct binding studies. The capacities of human liver microsomes, recombinant human P450 1A2, P450 1A1, and P450 1B1 to bioactivate both compounds were also investigated. Both P450 1A1 and P450 1B1 are prominently expressed in extrahepatic tissues<sup>25,26</sup> and catalyze the N-oxidation of several HAAs.<sup>27,28</sup> The chemical structures of MeIQx and MeIgQx and their proposed pathways of metabolism by human P450 1A2 (vide infra) are depicted in Figure 1.

The HPLC-UV profiles of the metabolites of MeIQx and MelgOx formed by human liver microsomes are shown in Figure 2, along with the UV spectra of the parent amines and their principal N-oxidized metabolites. The major pathway of metabolism of MeIQx occurs by N-oxidation of the exocyclic amine group, to form HONH-MeIQx. The UV spectrum of HONH-MeIQx is identical to the spectra of the metabolite and synthetic derivative previously reported;<sup>29</sup> the absorbance maximum (272 nm) displays a slight hypsochromic shift relative to the absorbance maximum of MeIQx (274 nm) (Figure 2).<sup>29</sup> The dihydroxylated product, 2-(hydroxyamino)-8-hydroxymethyl-3-methylimidazo[4,5-*f*]quinoxaline (HONH-8-CH<sub>2</sub>OH-IQx) is the second most abundant metabolite, followed by the alcohol, 8-CH<sub>2</sub>OH-IQx, which is a minor metabolite.<sup>30</sup> Human liver microsomes also produced three oxidation products (M-1, M-2, and M-3) of MeIgQx (Figure 2). Their UV and mass spectral data are discussed below.

The metabolites of MeIQx and MeIgQx were collected from the HPLC effluent and directly infused into the triple stage quadrupole mass spectrometer. The product ion spectra of HONH-MeIQx and its azoxy derivative, obtained by reaction of HONH-MeIQx with nitrosobenzene, are shown in Figure 3. The product ion spectrum of HONH-MeIQx ( $[M+H]^+$  at m/z230.1) showed prominent fragment ions at m/z 213.1 and 212.1, indicative of the loss of 17 and 18 Da, which are



Figure 1. Chemical structures of MeIQx and MeIgQx and proposed pathways of P450-mediated metabolism. The asterisk depicts the site of incorporation of the  ${}^{14}C$  isotope.

attributed to the loss of HO<sup>•</sup> and H<sub>2</sub>O. These fragment ions are typically observed in product ion spectra of HONH-HAA metabolites, but the product ion spectra of ring-oxidized HAA metabolites often display a neutral loss of 18 Da (H<sub>2</sub>O) or 28 Da (CO).<sup>30,31</sup> The product ion spectrum of the azoxy derivative confirms the structure as HONH-MeIQx.<sup>29,32</sup> The product ion spectra of HONH-8-CH<sub>2</sub>OH-IQx and 8-CH<sub>2</sub>OH-IQx (Figure 4) are also in excellent agreement to the spectra of the reference compounds previously reported.<sup>30</sup>

The mass spectrum of the principal metabolite of MeIgQx (M-3) displayed a protonated ion  $[M+H]^+$  at m/z 230.1, a mass which is 16 Da greater than MeIgQx. The ESI product ion spectra of M-3 and its adduction product with nitrosobenzene were very similar to the product ion spectra of HONH-MeIQx and its azoxy adduct (Figure 3). M-3 underwent oxidation with time, as did HONH-MeIQx, to form the presumed nitroso derivative: a prominent ion  $[M+H]^+$  was observed at m/z 228.1. The product ion spectra of these intermediates displayed prominent fragment ions at m/z 213.1 and 198.1, which are ascribed, respectively, to the loss of CH<sub>3</sub><sup>•</sup> and NO moieties (data not shown). Thus, the structure of M-3 is assigned as 2-(hydroxyamino)-1,7-dimethylimidazo[4,5-f]quinoxaline (HONH-MeIgQx).

A large bathochromic shift was observed in the UV spectrum of HONH-MeIgQx compared to that in the spectrum of MeIgQx. The maximum of absorbance of M-3 was at 385 nm, whereas the maximum absorbance of MeIgQx occurred at 365 nm (Figure 2). This large bathochromic shift is not observed in the UV spectrum of HONH-MeIQx, nor is this bathochromic shift observed in the UV spectra of the structurally related N-hydroxy-HAA derivatives of 2-amino-3-methylimidazo[4,5-f]quinoline or PhIP. However, the UV spectrum of the proposed N3-glucuronide conjugate of 2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-b]pyridine (HONH-PhIP), which exists as an oxime, also displays a bathochromic shift of ~20 nm in its maximum absorbance in comparison to HOHN-PhIP.<sup>31,33</sup> We surmise that HONH-MeIgQx exists preferentially as the oxime and not as the N-hydroxylamine tautomer. The carcinogen 4-hydroxyamino quinoline 1-oxide was also reported to preferentially exist in its 4-hydroxyimino tautomeric form.<sup>34</sup>

The second most abundant metabolite of MeIgQx (M-2) displayed a UV spectrum similar to that of MeIgQx. The mass spectrum of M-2 displayed a protonated ion at  $[M+H]^+$  at m/z 230.0, indicative of another mono-oxidation metabolite. Prominent ions were observed in the product ion spectrum at m/z 201.0, 200.0, and 186.1 (Figure 4). These fragment ions



Figure 2. HPLC UV profiles of MeIQx and MeIgQx metabolites formed with human liver microsomes, following a 30 min incubation. The UV spectra of the parent compounds and major *N*-oxidized metabolites of each HAA are presented.



Figure 3. Product ion spectra of HONH-MeIQx and HONH-MeIgQx (M-3) (right panel) and product ion spectra of the azoxy conjugates formed with nitrosobenzene (left panel).

are proposed to arise through losses of HCO<sup>•</sup>, H<sub>2</sub>CO, and HCO<sup>•</sup> + CH<sub>3</sub><sup>•</sup>. The product ion spectrum of M-2 is consistent with a ring-hydroxylated metabolite of MeIgQx: the site of oxidation may have occurred at the C-4 or C-9 position of the quinoxaline skeleton (Figure 1). If oxidation had occurred at the 7-CH<sub>3</sub>-group, a prominent fragment ion would have been expected at m/z 212.1 [M+H-18]<sup>+</sup>, due to the loss of H<sub>2</sub>O, as is observed in the product ion spectrum of 8-CH<sub>2</sub>OH-IQx (Figure 4).

The third metabolite of MeIgQx (M-1) displayed a UV spectrum that was very similar to the spectrum of HONH-MeIgQx (M-3), with a maximum absorbance situated at 385 nm. The full scan mass spectrum of M-1 displayed a

protonated ion  $[M+H]^+$  at m/z 246.1, indicating that the metabolite is a dihydroxylated product of MeIgQx (Figure 4). Prominent ions are observed in the product ion spectrum at m/z 228.1 and 229.1, indicative of the loss of 17 and 18 Da and suggestive of the presence of the *N*-hydroxylamine moiety (Figure 4). The base peak, observed at m/z 200.1, is ascribed to the loss H<sub>2</sub>O, followed by the loss of CO. Thus, the product ion spectrum is consistent with the structure of a dihydroxylated metabolite of MeIgQx that contains the *N*-hydroxylamine moiety with the second site of hydroxylation possibly occurring at the C-4 or C-9 position of the quinoxaline ring.



Figure 4. ESI/MS/MS product ion spectra of 8-CH<sub>2</sub>OH-IQx, 8-CH<sub>2</sub>OH-HONH-IQx, and the monohydroxylated (M-2) and dihydroxylated (M-1) metabolites of MeIgQx.

Table 1. Estimated N-Oxidation and C-Oxidation Rates of MeIgQx and MeIQx with Liver Microsomes or Recombinant Human P450s<sup>a</sup>

	nmol/min/mg microsomal protein or 100 pmol P450					
enzyme	HONH-MeIgQx	HO-MeIgQx	HONH-(HO)-MeIgQx	HONH-MeIQx	HONH-8-CH <sub>2</sub> OH-IQx	8-CH <sub>2</sub> OH-IQx
mouse liver microsomes	$0.3 \pm 0.1$	$0.5 \pm 0.2$	ND	$0.2 \pm 0.01$	ND	ND
human liver microsomes	$0.4 \pm 0.02$	$0.1 \pm 0.01$	$ND^{b}$	$1.7 \pm 0.2$	$0.09 \pm 0.01$	ND
recombinant human P4501A1	14.1 ± 4.9	6.9 ± 2.4	ND	$0.3 \pm 0.1$	ND	ND
recombinant human P4501A2	$5.4 \pm 2.1$	ND	ND	$6.1 \pm 0.9$	$1.7 \pm 0.5$	ND
recombinant human P4501B1	$3.9 \pm 0.6$	$2.5 \pm 0.3$	ND	ND	ND	ND

<sup>*a*</sup>[Substrate] = 200  $\mu$ M; mean ± SD, N = 3 or 4 independent measurements. ND = not detected (<0.05 nmol/min/mg protein or <0.05 nmol/min/ 100 pmol P450), <sup>*b*</sup>only observed following 30 min incubation.

8-CH<sub>2</sub>OH-HONH-IQx and the dihydroxylated metabolite of MeIgQx (M-1) were reacted with nitrosobenzene to form azoxy adducts. Protonated molecules  $[M+H]^+$  were observed at m/z 335.2, a mass that is consistent with the molecular weight of the proposed azoxy conjugates (Figure S-1, Supporting Information). The product ion spectrum of the azoxy derivative of 8-CH<sub>2</sub>OH-HONH-IQx displays prominent ions at m/z 317.1 and 299.3, which are ascribed to the loss of one and two molecules of H<sub>2</sub>O, whereas the product ion spectrum of the azoxy derivative of X-1 displays prominent fragment ions at m/z 317.2 and 289.2, which are consistent with the loss of H<sub>2</sub>O and H<sub>2</sub>O+CO. These mass spectral data provide additional

support for the secondary site of oxidation proposed to occur at the quinoxaline ring and not the 7-methyl group of MeIgQx.

Estimated Rates of P450-Mediated Oxidation of MelQx and MelgQx. The estimated rates of N- and C-oxidation of MelQx and MelgQx, by mouse and human liver microsomes and recombinant human P450s, are summarized in Table 1. The N-oxidation rates of MelgQx and MelQx with mouse liver microsomes were comparable, whereas the rate of N-oxidation of MelQx was about 4-fold higher than that for MelgQx with human liver microsomes. MelgQx underwent N-oxidation by recombinant human P450 1A2 but also by extrahepatic P450s 1A1 and 1B1. Moreover, the rate of MelgQx N-oxidation by P450 1A1 was  $\sim$ 3-fold greater than the rate of N-oxidation

carried out by P450 1A2. Human P450 1A2 appears to be involved only in *N*-oxidation (bioactivation), whereas P450 1A1 and P450 1B1 catalyze both *N*-oxidation and ringoxidation (detoxication) of MeIgQx. In contrast to MeIgQx, the *N*-oxidation of MeIQx by P450s 1A1 and 1B1 was low to not detectable.

In Vivo DNA Binding Studies in Mice. *Plasma Clearance*. The study design was not intended to completely describe the rate of absorption and clearance profile of these chemicals. However, the data obtained from the 3 time points show that the kinetics of absorption and elimination of both <sup>14</sup>C-MeIgQx and <sup>14</sup>C-MeIQx were rapid. The peak plasma levels in radioactivity associated with both compounds occurred at 4 h, and greater than 95% of the radioactivity was cleared from plasma within 48 h (Figure 5).



Figure 5. Plasma clearance of  $[^{14}C]$ -MeIQx and  $[^{14}C]$ -MeIgQx. Data represent the means and the standard deviations of 5 animals per time point.

DNA Adducts. The apparent levels of [14C]-MeIgQx- and <sup>14</sup>C]-MeIQx-DNA adduct formation and removal in the liver, pancreas, and colorectal DNA are presented in Figure 6. The clearance of <sup>14</sup>C-isotope associated with DNA was less rapid than the clearance of <sup>14</sup>C-radioactivity associated with either carcinogen from plasma. The [14C]-MeIgQx isotope associated with liver DNA at 24 and 48 h was 33% and 27% of the <sup>14</sup>Cisotope detected at 4 h, whereas the isotope level associated with pancreas DNA was 44% and 17% of the level of <sup>14</sup>C-isotope bound to DNA at 4 h. The  $[^{14}C]$ -MeIQx isotope associated with liver and pancreas DNA also appeared to persist: up to 74% and 25% of the <sup>14</sup>C-isotope observed in DNA of these organs at 4 h remained, respectively, at 48 h. The [14C]-MeIgQx isotope in colorectal DNA reached its maximum at 24 h, while the highest level of the [<sup>14</sup>C]-MeIQx isotope in colorectal DNA was observed at 4 h. The disposition and elimination of MeIgQx in the mouse is not known. The fecal content may contain high levels of <sup>14</sup>C isotope, resulting in high levels of exposure to this carcinogen, perhaps at greater levels than most other tissues.

*LC-ESI/MS*<sup>3</sup> of *DNA* Adducts. The formation of DNA adducts of  $[^{14}C]$ -MeIQx and  $[^{14}C]$ -MeIQQx was examined by LC-ESI/MS<sup>3</sup> with a linear quadrupole ion trap mass spectrometer. The ion trap MS permits multistage tandem scanning for structural characterization and identification of the aglycone adducts  $[BH_2]^{+,18,35}$  Two isomeric dG adducts of MeIQx, dG-C8-MeIQx, and dG-N<sup>2</sup>-MeIQx are formed in vivo in the liver of rats<sup>19</sup> and in human hepatocytes. <sup>36</sup> In addition, a third adduct is formed with dA; the structure has been tentatively assigned as 5-(deoxyadenosin-N<sup>6</sup>-yl)-MeIQx (dA-N<sup>6</sup>-MeIQx) (Figure 7).<sup>37</sup>

The stable isotope dilution method was used to measure the levels of dG-C8-MeIQx formed in mouse liver at the 24 h time



**Figure 6.** Level of apparent [<sup>14</sup>C]-MeIQx and [<sup>14</sup>C]-MeIgQx DNA adduct formation in the liver, pancreas, and colon as a function of time. Data represent the means and the standard error of the means of 5 animals per time point. The values of adduct formation were normalized to adducts per nucleotide/mg HAA/kg body weight .

point. Reconstructed ion chromatograms for dG-C8-MeIQx, dG-C8-[<sup>14</sup>C]-MeIQx, [<sup>13</sup>C<sub>10</sub>]-dG-C8-MeIQx, and a putative isomeric dG-C8-MeIQx at the MS<sup>3</sup> scan stage are presented in Figure 8. Both the dG-C8-MeIQx and dG-C8-[<sup>14</sup>C]-MeIQx adducts are observed. The product ion spectra of the unlabeled and labeled adducts, at the MS<sup>3</sup> scan stage, confirmed the structure of the adducts.<sup>19,35</sup>

The estimate of total [<sup>14</sup>C]-MeIQx-DNA adduct formation in the liver 24 h post-treatment, based upon AMS measurements, was  $11.4 \pm 7.6$  adducts per  $10^8$  nucleotides versus  $3.8 \pm 0.6$  dG-C8-MeIOx adducts per  $10^8$  nucleotides, based on quantitative LC-ESI/MS<sup>3</sup> measurements. The contribution of dG-C8-MeIQx to total <sup>14</sup>C bound to DNA ranged from 13-72% of the radioactivity. The relative response of the signals for dG-N<sup>2</sup>-MeIQx and dA-N<sup>6</sup>-MeIQx adducts, by LC-ESI/MS<sup>3</sup>, are  $\sim 5\%$  of the response of the signal observed for dG-C8-MeIQx.<sup>37</sup> The dG- $N^2$ -MeIQx and dA- $N^6$ -MeIQx adducts are formed in rat liver at levels that are respectively  $\sim$ 30–50% and ~5% of the level of dG-C8-MeIQx, 24 h following oral dosing with MeIQx (10 mg/kg).<sup>19,37</sup> If the relative level of formation of isomeric dG-MeIQx and dA-MeIQx adducts are similar in rat and mouse liver, the weak response in signals of  $dG-N^2$ -MeIQx and dA-N<sup>6</sup>-MeIQx would preclude their detection, by LC-ESI/MS<sup>3</sup>, in mouse liver DNA. Thus, the contribution of



Figure 7. Chemical structures of dG-C8-MeIQx, dG-N<sup>2</sup>-MeIQx, and dA-N<sup>6</sup>-MeIQx.



**Figure 8.** Reconstructed ion chromatograms at the MS<sup>3</sup> scan stage for (A) dG-C8-MeIQx, dG-C8-[<sup>14</sup>C]-MeIQx, and [<sup>13</sup>C<sub>10</sub>]-dG-C8-MeIQx, and (B) putative dG-C8-MeIQQx and dG-C8-[<sup>14</sup>C]-MeIgQx with [<sup>13</sup>C<sub>10</sub>]-dG-C8-MeIQx employed as an internal standard. The product ion spectra at the MS<sup>3</sup> scan stage of dG-C8-MeIQx, dG-C8-[<sup>14</sup>C]-MeIQx, and [<sup>13</sup>C<sub>10</sub>]-dG-C8-MeIQx are depicted in panel C.

dG- $N^2$ -MeIQx and dA- $N^6$ -MeIQx adduct formation to the amount of [<sup>14</sup>C]-MeIQx bound to DNA has not been taken into account, and the <sup>14</sup>C-isotope associated with the DNA as covalently bound adducts likely exceeds the contribution made by the dG-C8-MeIQx adduct. In a previous AMS study conducted with male C57BL/6 mice treated with [<sup>14</sup>C]-MeIQx, the formation of liver adducts was shown to be linearly dependent on dose from an exposure of 5 mg down to 500 ng per kg of body weight).<sup>38</sup> The amount [<sup>14</sup>C]-MeIQx bound to liver DNA was estimated at about 20 adducts per 10<sup>8</sup> nucleotides at 24 h post-treatment with [<sup>14</sup>C]-MeIQx (5 mg/kg). This estimate in <sup>14</sup>C-adduct formation is remarkably similar to the level of [<sup>14</sup>C]-MeIQx bound to liver DNA, when adjusted per dose, in our study.

In contrast to MeIQx, a putative dG-C8-MeIgQx adduct was not detected, when scanning at the MS<sup>2</sup> or MS<sup>3</sup> scan stages (Figure 8B). The potential formation of a dA adduct of MeIgQx ([M+H]<sup>+</sup> at m/z 463.2) was also monitored, but no peaks were detected above background level. The data-dependent constant neutral loss-triple stage mass spectrometry scanning mode (CNL/MS<sup>3</sup>) was employed to monitor for the loss of the deoxyribose moiety, followed by MS<sup>3</sup> of the putative aglycone adducts [BH<sub>2</sub>]<sup>+.37</sup> However, the CNL/MS<sup>3</sup> mode also failed to detect any adducts of MeIgQx.

**Carcinogen Binding Index (CBI).** The CBI is a measure of the amount of adduct formed from a given dose; it is calculated by dividing the amount of adduct per unit of DNA by the dose per unit of body mass ( $\mu$ mol adduct bound per mole

nucleotide)/(mmol chemical administered per kg animal).<sup>39</sup> The CBI of MeIgQx and MeIQx in the liver, pancreas, and colorectum are presented in Table 2. The CBI data are reported

Table 2. Covalent Binding Index of MeIQx and MeIg	Qx"
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	liver: (µmol/mol dNp)/(mmol carcinogen/kg bw)				
time	MeIgQx	MeIQx			
4	$21.7 \pm 8.6$	$27.7 \pm 8.3$			
24	$7.0 \pm 3.2$	$11.5 \pm 7.7$			
48	$5.8 \pm 10.6$	$20.5 \pm 8.4$			
pancreas: $(\mu mol/mol \ dNp)/(mmol \ carcinogen/kg \ bw)$					
time	MeIgQx	MeIQx			
4	$2.4 \pm 2.4$	$0.8 \pm 0.2$			
24	$1.1 \pm 0.6$	$0.2 \pm 0.1$			
48	$0.2 \pm 0.2$	$0.1 \pm 0.1$			
colon: $(\mu mol/mol dNp)/(mmol carcinogen/kg bw)$					
time	MeIgQx	MeIQx			
4	$12.0 \pm 16.5$	$113 \pm 119$			
24	$138 \pm 114$	$10.4 \pm 11.9$			
48	$28.7 \pm 18.5$	$2.1 \pm 0.9$			
<sup><i>a</i></sup> The mean $\pm$ SI	D of 4 or 5 animals per tim	e point.			

at the 4, 24, and 48 h time points. The CBI values for liver DNA adduct formation of MeIgQx and MeIQx were not significantly different at 4 and 24 h (P > 0.4, Student's unpaired t test); however, the CBI values were significantly different at 48 h (P < 0.005). These data are suggestive of a faster rate of apparent MeIgQx-DNA adduct removal than for MeIQx-DNA adducts in the liver; additional time points are required to accurately determine the rate of adduct elimination. For extrahepatic tissues, there was a significant difference (P < 0.01) between MeIgQx and MeIQx CBI values of the pancreas at 24 h and colorectal tissue at 48 h.

## DISCUSSION

This study was designed to examine the capacity of MeIgQx to undergo metabolic activation to form DNA adducts in comparison with its isomer, MeIQx, a multisite carcinogen in rodents.<sup>1</sup> The metabolism and DNA binding data provide a preliminary assessment of the genotoxic potential of MeIgQx relative to MeIQx, which also induces *lacI* mutations in the liver and colon<sup>40</sup> and aberrant crypt foci in the colon of C57BL/6N mice,<sup>41</sup> the species employed for our DNA binding studies. The estimated rates of P450-mediated *N*-oxidation of MeIgQx with mouse liver microsomes and the in vivo <sup>14</sup>C-binding data of MeIgQx, when adjusted for dose, are comparable to those data obtained for MeIQx. We employed the highly sensitive AMS method for adduct detection,<sup>17,38</sup> which enabled us to estimate DNA adduct formation based upon <sup>14</sup>C isotope bound to DNA, since the structures of the MeIgQx adducts are unknown.

MeIQx and other structurally related HAAs studied induce tumors at multiple sites in rodents during long-term feeding studies. The target organs include the oral cavity, liver, stomach, colon, pancreas, and the prostate gland in males and the mammary gland in females.<sup>1,42</sup> The total dose required to induce tumor formation ( $TD_{50}$ ) varies for each HAA and is host species-dependent. The  $TD_{50}$  values of the individual HAAs have been reported to range from 0.1 to 64.6 mg/kg/day in rodents.<sup>1</sup> MeIQx is a liver carcinogen in CDF<sub>1</sub> mice:<sup>1</sup> the TD<sub>50</sub> value of MeIQx in this mouse strain is 24 mg/kg (http://potency.berkeley.edu/chempages/MeIQx.html).

The CBI approach has been used in rodent models as a means to assess the carcinogenic potential of genotoxic chemicals.<sup>43</sup> A CBI value of 1–10 is characteristic of weak carcinogens, while moderate carcinogens display CBI values of about 100.<sup>39</sup> The CBI for both MeIQx and MeIgQx in the liver were comparable at 4 and 24 h (CBI = 7–28). These findings suggest that MeIgQx would be a weak to moderate liver carcinogen in this animal model. The [<sup>14</sup>C]-MeIgQx isotope is also associated with DNA in pancreas and colorectal tissue, suggesting that tumors could form in these organs as well. However, the presence of DNA adducts alone is insufficient to predict the development of cancer as carcinogenesis involves numerous biological events.<sup>44</sup> Moreover, dietary, environmental, and genetic factors can greatly impact the biological potency of HAAs and other genotoxicants.<sup>1,45,46</sup>

Thus far, we have not successfully identified MeIgQx-DNA adducts by LC-ESI/MS<sup>n</sup>. We surmise that the linear tricyclic ring structure of MeIgQx inhibits the efficacy of enzymatic digestion of the MeIgQx-adducted to DNA, and the MeIgQx adducts may be present as incompletely digested oligomers, which escaped detection by LC-ESI/MS<sup>n</sup>. Alternatively, the instrument tuning parameters of the mass spectrometer may not have been optimal for detection of MeIgQx-DNA adducts, or the sensitivity in response of MeIgQx-DNA adducts is at least 10-fold weaker than the signal of response for dG-C8-MeIQx. We have not been able to synthesize HONH-MeIgQx by methods previously employed for the production of other HONH-HAAs.<sup>12</sup> Studies that examine the reactivity of synthetic HONH-MeIgQx with deoxynucleosides and DNA are required to identify and elucidate the structures of MeIgQx-DNA adducts.

HAAs have been classified as probable human carcinogens by IARC and by the National Toxicology Program, sponsored by NIEHS.<sup>6,7</sup> MeIgQx is the most mass abundant HAA formed in cooked beef.<sup>4,11</sup> MeIgQx is a bacterial mutagen; however, its potency in the Ames reversion assay is about 4,000-fold lower than the potency of MeIQx, in S. typhimurium tester strain TA98. The genotoxic potency of MeIgQx is 16-fold greater in the S. typhimurium strain YG1024 than in the S. typhimurium strain TA98. The YG1024 strain is derived from strain TA98 and contains high O-acetyltransferase activity that enhances the mutagenicity of some HAAs, presumably through the formation of reactive N-acetoxy intermediates, which covalently bind to DNA.<sup>47</sup> The higher potency of MeIgQx in the YG1024 bacterial tester strain compared to TA98, indicates that the HONH-MeIgQx undergoes O-acetylation to form the N-acetoxy intermediate, as does MeIQx.<sup>11</sup> MeIgQx displays about 10-fold weaker mutagenic potency than the structurally related carcinogen PhIP (3,015 rev/ $\mu$ g) in S. typhimurium strain YG1024. It is noteworthy that the mutagenic potency of MeIgQx is comparable to the potency of 4-aminobiphenyl, a known human carcinogen, in TA98 and YG1024 (frameshift-specific) and YG1029 (primarily point mutation-specific) tester strains.<sup>48-50</sup> The strong mutagenic potencies of MeIQx and several other HAAs of angular tricyclic ring structure in the S. typhimurium TA98 strain have been attributed to the ability of these HAAs to frequently induce reversions about 9 base pairs upstream of the original CG deletion in the *hisD*<sup>+</sup> gene in a run of GC repeats.<sup>51</sup> This sequence context may not be a hotspot for MeIgQx and PhIP adduct formation, or the capacities of MeIgQx and PhIP adducts to induce mutations are weaker

than the potencies of adducts formed by MeIQx and other angular tricyclic HAAs. The mutagenicity of MeIgQx has not been assessed in mammalian cell systems. Such studies would provide additional data about the biological effects of MeIgQx in comparison to carcinogenic HAAs, such as MeIQx and PhIP, and aid in assessing the health risk of this newly discovered HAA.

Both MeIgQx and MeIQx undergo *N*-oxidation by mouse liver microsomes and by recombinant human P450 1A2 at similar rates. Moreover, recombinant human P450s 1A1 and 1B1, which are expressed in extrahepatic tissues,<sup>25,26</sup> are far more efficient at catalyzing the *N*-oxidation of MeIgQx than the *N*-oxidation of MeIgQx (Table 1). The differences in chemical structures of MeIgQx and MeIQx appear to affect the regioselectivities of P450-mediated *N*-oxidation of the exocyclic amine groups, as well as the quinoxaline ring structures and methyl groups of both HAAs. The higher CBI values of putative MeIgQx adducts formed in the pancreas in comparison to MeIQx may be attributed to more efficient bioactivation of MeIgQx by extrahepatic P450s.

Historically, short-term bacterial mutagenesis assays have been an effective screening tool for the identification of some mutagenic HAAs in complex food matrices, but they do not reliably predict carcinogenic potency in mammals.<sup>52</sup> MeIgQx forms apparent DNA adducts at levels that are comparable to the levels of adducts formed by its carcinogenic isomer, MeIQx, when adjusted for dose. Given the elevated concentrations of MeIgQx formed in cooked meats, further studies on the genetic toxicology and health risk of this novel HAA are clearly warranted.

## ASSOCIATED CONTENT

#### **S** Supporting Information

ESI/MS/MS product ion spectra of human liver microsomal metabolites 8-CH<sub>2</sub>OH-HONH-MeIQx and HO-HONH-MeIQX derivatized with nitrosobenzene to form the respective azoxy conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ABBREVIATIONS

AMS, accelerator mass spectrometry; CBI, carcinogen binding index; CNL/MS<sup>3</sup>, constant neutral loss-triple stage mass spectrometry scanning mode; HAA, heterocyclic aromatic amine; LC-ESI/MS, Liquid chromatography-electrospray ionization/ mass spectrometry; MeIgQx, 2-amino-1,7-dimethylimidazo-[4,5-g]quinoxaline; HONH-MeIgQx, 2-hydroxyamino-1,7dimethylimidazo[4,5-g]quinoxaline; MeIQx, 2-amino-3, 8-dimethylimidazo[4,5-f]quinoxaline; HONH-MeIQx, 2-hydroxyamino-3,8-dimethylimidazo[4,5-f]quinoxaline; 8-CH<sub>2</sub>OH-IQx, 2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5-f]quinoxaline; HONH-8-CH<sub>2</sub>OH-IQx, 2-(hydroxyamino)-8-hydroxymethyl-3methylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6phenylimidazo[4,5-*b*]pyridine; dG-C8-MeIQx, *N*-(deoxyguanosin-8-yl)-MeIQx; dG- $N^2$ -MeIQx, 5-(deoxyguanosin- $N^2$ -yl)-MeIQx; dA- $N^6$ -MeIQx, 5-(deoxyadenosin- $N^6$ -yl)-MeIQx; ppb, part per billion

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