Short Communication

Covalent Binding of 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline to Albumin and Hemoglobin at Environmentally Relevant Doses

Comparison of Human Subjects and F344 Rats

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ABSTRACT:

Covalent binding of the food-borne heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) to albumin and hemoglobin (Hb), 3.5–6.0 hr after oral administration of a single dose of either 21.3 or 228.0 μg of [14C]MeIQx (304 and 3257 ng/kg of body weight, respectively, based on a 70-kg subject weight), was studied in human volunteers using accelerator mass spectrometry. Human protein adduct levels were compared with data obtained for male F344 rats 4.5 hr after oral administration of 0.94–11,420 ng/kg of body weight [14C]MeIQx. Dose-dependent levels of MeIQx-albumin and MeIQx-Hb adducts were detected in both humans and rats. In each case, the regression coefficient (slope) of the dose-response curve was approximately 1. The highest levels of adduct formation per unit dose of MeIQx occurred with human albumin, followed by rat albumin, human Hb, and rat Hb (in that order). Although the human subjects were elderly and underwent colon resection surgery during the study period, the results indicate that formation of albumin and Hb adducts is dose dependent and that a trend exists for higher adduct levels per unit dose in humans, compared with F344 rats. Furthermore, MeIQx-albumin adducts are likely to provide a more sensitive marker of exposure to MeIQx than are MeIQx-Hb adducts.

Epidemiological studies have indicated that the ingestion of chemical carcinogens in the diet may be a major cause of human cancers (Cohen, 1987). One source of exposure is the ingestion of HCAs formed in foods by pyrolytic reactions between amino acids, glucose, and creatine/creatinine (Wakabayashi et al., 1992). Although 19 HCAs have been structurally identified, MeIQx (fig. 1) is one of the three most mass-abundant HCAs detected in cooked meat, with human exposure being estimated to be up to 2.6 μg of MeIQx/person/day (Layton et al., 1995; Ushiyama et al., 1991).

MeIQx is a mutagen in bacteria and mammalian cells (Felton et al., 1995) and a multiorgan carcinogen in rodents, primarily causing liver tumors (Ohgaki et al., 1987; Kato et al., 1988). Although the carcinogenicity of MeIQx in humans has yet to be established, epidemiological studies have linked exposure to HCAs, including MeIQx, to an increased incidence of colon, breast, and stomach cancers (Gerhaards et al., 1995; De Stefani et al., 1997; Ward et al., 1997). This evidence is supported by data suggesting that some human tissues have the capacity to activate MeIQx to a form that binds to DNA adducts with blood proteins such as albumin and Hb are frequently suitable biomarkers, because collection of blood is relatively noninvasive and these proteins are sufficiently plentiful to allow easy analysis. In addition, albumin and Hb have comparatively long biological half-lives, resulting in the dose-dependent accumulation of adducts to steady-state levels (Skipper and Tannenbaum, 1990). For some carcinogenic compounds, albumin and Hb adduct levels have been shown to correlate with DNA adduct levels in the target organ (Wild et al., 1986; Umemoto et al., 1992). Therefore, although these protein adducts are unlikely to be directly involved in the carcinogenic process, they may act as markers of formation of the corresponding DNA adducts that could result in mutations leading to tumor forma.

Fig. 1. Structure of MeIQx.
tion. Albumin and Hb adduct levels could be used as indicators of the bioactive dose and the potential cancer risk.

The aim of this study was to apply AMS to investigate the dose dependence of MeIQx adduct formation with albumin and Hb in humans, at well-characterized doses relevant to human intake. This would more accurately determine whether these protein adducts can be used as dosimeters of exposure in molecular epidemiological studies. In addition, direct comparisons between the levels of adducts in humans and male F344 rats were made, to investigate the suitability of using rodent models to predict the usefulness of these biomarkers in humans. This study was possible because of the sensitivity of AMS for the detection of carbon-14 in biological samples; this sensitivity has enabled metabolism and distribution studies with very low doses of 14C-labeled compounds to be conducted in laboratory animals (Freeman and Vogel, 1995; Turteltaub et al., 1990). Moreover, because of the requirement for the administration of only very small amounts of radioactively labeled compound, it is possible to undertake these studies in humans.

**Materials and Methods**

Materials. [2-14C]MeIQx (>95% chemical purity and radiopurity, as measured by HPLC and liquid scintillation counting, respectively) was obtained from Toronto Research Chemicals (Ontario, Canada). Male F344 rats (200–250 g) were obtained from Simonsen Laboratories (Gilroy, CA). Affi-Gel Blue Gel Columns, protein assay reagents, and bovine serum albumin standard were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemical reagents were of analytical grade.

**Laboratory Animal Dosing and Sample Collection and Storage.** Rats were housed three/cage, maintained on a 12-hr light/dark cycle and at 24°C, 55 ± 10% humidity, and fed ad libitum. Rats (three/dose level) were fasted for 12 hr before dosing and were then dosed by gavage with 0.94, 7.4, 94, 980, or 11,420 ng/kg of body weight [2-14C]MeIQx. The rats were housed three/cage, maintained on a 12-hr light/dark cycle and at 24°C, and given water and food ad libitum. Rats (three/dose level) were fasted for 12 hr before dosing and were then dosed by gavage with 0.94, 7.4, 94, 980, or 11,420 ng/kg of body weight [2-14C]MeIQx. (45.6 mCi/mmol) in acidified water (pH 3.0 with hydrochloric acid). In addition, three animals were treated with acidified water alone, as controls.

Four and one half hours after dosing (the mean time for the collection of the human samples), rats were euthanized with carbon dioxide asphyxiation and blood was collected, by cardiac puncture, into tubes containing 50 μl of 15% (w/v) EDTA. Plasma was isolated from red blood cells by centrifugation at 3000g for 10 min. The red blood cells were washed twice with an equal volume of 0.9% (w/v) sodium chloride and centrifuged at 3000g for 10 min. Plasma and red blood cells were stored at − 20°C until albumin and Hb extraction.

**Albumin Extraction.** Plasma (1–5 ml) was dialyzed for 2 days against two changes of 250 ml of 0.1 M potassium chloride/0.05 M Tris, pH 7.0 (plasma volume/buffer volume ratio of at least 1:2500), to remove unbound MeIQx. Albumin was then purified from dialyzed plasma by affinity chromatography on Affi-Gel Blue Gel columns (Lynch et al., 1993). Albumin concentrations were determined using Bio-Rad protein assay reagents and bovine serum albumin standards, as described by the manufacturers. The albumin samples were analyzed by AMS, as described below.

**Hb Extraction.** Red blood cells were thawed, lysed with an equal volume of 0.1 M EDTA, pH 7.5, and centrifuged at 3000g for 20 min at room temperature, to remove cell debris. The Hb was precipitated by the dropwise addition of 5 ml aliquots of the supernatant to tubes containing 20 ml of ethanol, with rapid vortex-mixing to avoid clumping of precipitated protein. The precipitate was then washed sequentially with equal volumes of 80:20 (v/v) ethanol/ether, 25:75 (v/v) ethanol/ether, and ether and was air dried. The Hb samples were then analyzed by AMS, as described below.

**Human Studies.** Protocols for the human studies were approved by the National Radiological Protection Board (Didcot, UK) and the Human Subjects Review Boards at York District Hospital (York, UK) and Lawrence Livermore National Laboratory (Livermore, CA). All volunteers were undergoing surgery to remove colorectal carcinomas. [14C]MeIQx (9.3 and 43 mCi/mmol for volunteers 1 and 2 and 3–7, respectively) was packaged in gelatin capsules containing lactose filler. Volunteers 1 and 2 received a 14C-MeIQx dose of 228.0 μg/person (3257 ng/kg of body weight, based on a 70-kg subject weight), and volunteers 3–7 received a lower dose of 21.3 μg/person (304 ng/kg of body weight, based on a 70-kg subject weight). For reference, 21.3 μg corresponds approximately to the amount of MeIQx consumed in the diet in a 1-week period (Ushiyama et al., 1991), although administered in a single dose. Human volunteers participating in the study gave informed consent and completed simple questionnaires. For each subject, a predose blood sample was collected into a tube containing EDTA, as a control. The 14C-MeIQx capsule was taken with a glass of water, and 3.5–6.0 hr after an additional blood sample was collected. Red blood cells were separated from whole blood by layering 0.6 ml of 6% (w/v) dextran 500 in 0.9% (w/v) sodium chloride over 3 ml aliquots of blood. Red blood cells were allowed to sediment for 1 hr and then washed twice with an equal volume of 0.9% (w/v) sodium chloride, followed by centrifugation at 3000g for 10 min. Red blood cells were stored at −70°C until Hb extraction. Lymphocytes were removed from plasma samples derived from the dextran sedimentation by further sedimentation with Lymphoprep (Nycomed, Oslo, Norway), as described by the manufacturers. Plasma was freeze-dried and stored at room temperature until albumin extraction.

**AMS Analysis of Samples.** The carbon-14/carbon-13 ratios for the protein samples were determined by AMS, as previously described (Freeman and Vogel, 1995; Turteltaub et al., 1990). Before analysis, 2 mg of tributyrin was added to each albumin sample (0.1–1.0 mg of protein), to provide the carbon content necessary for efficient graphitization. Aliquots of 5–10 mg of Hb were graphitized without addition of tributyrin. The carbon-14/carbon-13 ratios were then converted to femtomoles of MeIQx per gram of protein by subtracting the carbon-14 contributions from tributyrin and control protein samples.

**Statistical Analysis.** The data for rats represent one AMS measurement for each rat sample, with an accompanying AMS measurement error. The AMS measurement error represents the larger of the AMS counting uncertainty or the spread of repeated isotope ratio determinations. Three rats were treated at each dose level. The data for human subjects represent the weighted mean of three AMS measurements for each human sample, where the weights used were inversely proportional to the square of the AMS measurement error. The AMS measurement errors associated with the weighted means were calculated using the usual Pythagorean formula (i.e. the square root of the sum of the squares of the errors associated with the individual measurements).

Dose-response data were analyzed by ordinary linear regression, regressing logarithmic response versus logarithmic dose. SE values for functions of regression coefficients were calculated using the δ method (Agresti, 1990). All CIs are asymptotic normal approximations. CIs for ratios were calculated by determining a CI for the logarithm of the ratio and then taking exponents. All analyses were performed using S-PLUS software (Mathsoft, 1997).

**Results and Discussion**

We have quantified the amounts of 14C-MeIQx bound to blood proteins in humans at doses of MeIQx relevant to dietary intake, using the technique of AMS. The human subjects were in the age range of 58–87 years and were undergoing colorectal surgery during the study period; hence, they may not be a good representation of the general population. Nevertheless, this small pilot study provides preliminary information on the dose dependence of protein adduct formation in humans, which is useful for comparison with data for other species and for assessment of the value of protein adducts as markers of exposure.

AMS provides a measure of the total amount of carbon-14 associated with the sample of interest. To determine the amount of a compound covalently bound to macromolecules, it is necessary to remove all unbound carbon-14 before analysis. In the case of protein samples analyzed in this study, plasma was dialyzed extensively and the albumin was purified by affinity chromatography. In addition, red blood cells were washed and Hb was extracted with solvent, to remove unbound 14C-MeIQx. Consequently, carbon-14 detected in the protein samples by AMS is considered to be the result of covalent MeIQx adducts.

Covariant 14C-MeIQx binding to albumin and Hb, as measured by AMS, was detectable in all human subjects 3.5–6.0 hr after oral administration of a single 21.3- or 228.0-μg dose of 14C-MeIQx (fig.
shown (y = 5(95% CI, 4.2–99) in humans, compared with 10 (95% CI, 1.4–69) in rodents. Although no attempt was made here to use interspecies scaling factors to adjust the data, our results suggest that F344 rats may not be a suitable model for judging the usefulness of protein adducts as biomarkers of exposure in humans.

AMS provides only a measure of carbon-14 present in the sample; hence, these analyses did not provide structural information concerning the protein adducts formed. Previous studies have indicated that a minor portion of MeIQx adducts with albumin and Hb are released as MeIQx upon acid hydrolysis, indicating the presence of adducts with sulphydryl-containing amino acids, such as cysteine (Turesky et al., 1987; Lynch et al., 1991, 1993). Lynch et al. (1991, 1993) used acid hydrolysis of albumin and Hb and GC/MS quantification of liberated MeIQx to measure protein adduct levels in blood samples from MeIQx-dosed rodents and human subjects eating normal diets. However, the data indicated that MeIQx-albumin and MeIQx-Hb adducts are not formed in sufficient quantities to be suitable biomarkers in humans. Our study examined levels after single treatments but, assuming that steady-state adduct levels resulting from chronic exposure are 30 times greater than adduct levels after a single dose (Skipper and Tannenbaum, 1990), the MeIQx-adduct levels present in humans eating an average amount of cooked meat are likely to be below the detection limit of the GC/MS assay.

**Acknowledgments.** We thank Kurt Haack (Lawrence Livermore National Laboratory) for AMS sample preparation and Dr. Belinda Cupid (University of York) for extraction of Hb from the red blood cells from volunteers 3–7.

**References**


**FIG. 2.** Dose-response curves for covalent binding of MeIQx to human and rat albumin and Hb after oral administration of a single dose of [14C]MeIQx.

**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Regression Coefficient</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Albumin</td>
<td>0.94</td>
<td>0.11</td>
</tr>
<tr>
<td>Human</td>
<td>Hb</td>
<td>1.27</td>
<td>0.16</td>
</tr>
<tr>
<td>Rat</td>
<td>Albumin</td>
<td>1.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Rat</td>
<td>Hb</td>
<td>1.03</td>
<td>0.11</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Mean Adduct Level per Unit Dose (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Albumin</td>
<td>3.70 (1.95–7.01)</td>
</tr>
<tr>
<td>Human</td>
<td>Hb</td>
<td>0.18 (0.04–0.77)</td>
</tr>
<tr>
<td>Rat</td>
<td>Albumin</td>
<td>0.46 (0.24–0.92)</td>
</tr>
<tr>
<td>Rat</td>
<td>Hb</td>
<td>0.05 (0.01–0.29)</td>
</tr>
</tbody>
</table>

**Human and rat MeIQx-albumin and MeIQx-Hb adduct levels per unit dose after oral administration of a single dose of [14C]MeIQx**


Hamilton WF and Dow P eds (1963) *Handbook of Physiology, Section 2, Circulation*, vol 2, American Physiological Society, Washington DC.


