Distribution and metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in female rats and their pups at dietary doses

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Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP*) is a mammary carcinogen in female rats and is present in a wide variety of cooked meats. We address here the excretion of PhIP and its metabolites into the breast-milk of lactating rats and the ability of chlorophyllin, a food product derivative with chemopreventive properties, to affect these levels at low PhIP doses. Lactating female F344 rats with suckling pups were orally administered 50, 500 and 1000 ng [14C]PhIP/kg body weight. The excretion of [14C]PhIP into milk and its distribution among the mammary tissue, liver and blood of the dam, as well as among stomach contents and liver of their suckling pups was measured using accelerator mass spectrometry (AMS). PhIP, PhIP-4'-sulfate, 4'-hydroxy-PhIP, and N2-hydroxy-PhIP-N3-glucuronide were found in the milk at all doses. The chlorophyllin (500 µg/kg) co-administration with PhIP (500 ng/kg) caused increased levels of [14C]PhIP in the milk (32%) and stomach contents (35%) of the pups relative to the animals not receiving chlorophyllin at these low PhIP doses. In contrast, lower [14C]PhIP levels in the chlorophyllin treated animals were observed in the blood (47%) and mammary tissue (68%) of the dam, as well as the pup's liver tissue (37%) compared to the animals receiving only PhIP. Chlorophyllin co-administration resulted in an increased amount of N3-hydroxy-PhIP-N3-glucuronide (42%), increased PhIP (79%) and decreased levels of PhIP-4'-sulphate (77%) relative to the animals not receiving chlorophyllin. These results suggest that PhIP and PhIP metabolites are present in the breast-milk of lactating rats at human dietary PhIP exposures and that PhIP is absorbed by the newborn. Furthermore, these results suggest that other dietary components can affect the dosimetry of PhIP in breast-feeding offspring.

Typical daily exposure for the general American public has been estimated at 16–200 ng/kg/day (11,12) and unmetabolized PhIP has been detected in the urine of human volunteers eating a normal diet (12–14).

PhIP is mutagenic in bacterial (3) and eukaryotic systems (15,16). Chronic administration of PhIP also induces lymphomas in CDF1 mice (17), and breast, prostate and colon carcinomas in the F344 rat (18,19). When exposed neonatally, Sprague–Dawley rats and B6C3F1 mice form increased levels of mammary and liver tumors respectively (20,21). Finally, epidemiological studies have linked PhIP, along with other heterocyclic amines, with human cancers of the colon (22–24) supporting the hypothesis that PhIP is a potential health concern for the human population.

Although breast-milk is not typically thought of as a major route of carcinogen elimination, previous studies have demonstrated the exposure of suckling pups to PhIP through the breast-milk in rats (25–27) and mice (28) given doses of PhIP 10 000 times greater than typical for human exposure (11,12,29). When administered at these high doses (10 mg/kg) PhIP and PhIP metabolites have also been found in the milk of lactating female rats as have DNA adducts in the tissues of breast-feeding pups (25). While these studies have shown that PhIP and its metabolites can be present in breast-milk, the distribution and metabolism of PhIP at exposure levels found in the human diet are unknown. Knowledge of breast-milk excretion at dietary relevant concentrations may help to evaluate the extent of exposure of the breast-feeding newborn to carcinogenic heterocyclic amines which may impact lifetime risk.

Additionally, exposure to dietary carcinogens occurs as part of a complex mixture along with other carcinogens, promoters and anticarcinogens. It is likely that certain dietary factors may modify the disposition of dietary heterocyclic amine carcinogens. Chlorophyllin, a water soluble derivative of chlorophyll, has been shown to have chemopreventive properties such as non-covalent complex formation with several mutagenic/carcinogenic compounds thereby either minimizing absorption or facilitating excretion (30,31). Notably, chlorophyllin has been shown to have chemopreventive effects including reduction of PhIP-induced aberrant crypts and DNA adducts in F344 rats (32) and reduction of mammary tumors in PhIP-treated female F344 rats (33).

In order to assess how a mother’s diet can affect carcinogen exposure for breast-feeding infants, we determined the amount of PhIP present in the breast-milk of lactating female rats exposed to human dietary doses of PhIP. We also examined the ability of chlorophyllin to modify levels of PhIP in breast-milk and the subsequent exposure of breast-feeding pups to PhIP and PhIP metabolites. Measurement of PhIP and its metabolites following low-dose exposure in tissue and milk samples was carried out using the technique of accelerator mass spectrometry (AMS) (34).

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References

Materials and methods

Chemicals

[14C]PhIP (10 mCi/mmol) was purchased from Toronto Research Chemicals (Downsview, Canada) and repurified by HPLC. Radiopurity was determined to be >99%. Chlorophyllin (sodium-copper salt, C-6003, >99% pure) was obtained from Sigma Chemicals (St Louis, MO). All other chemicals and solvents were analytical grade.

Animals

F344 rats (225–245 g, 60–90 days of age) with newborn litters were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and were provided NIH soy-based lab chow and water ad libitum. Animals were maintained in an AAALAC-accredited facility on a 12-h light/dark cycle.

Tissue distribution and excretion of PhIP and PhIP metabolites into breast-milk and influence of chlorophyllin

[14C]PhIP was dissolved in a small amount of ethanol (1 µCi/30 µl; 0.75 mg/ ml) prior to preparation of the dosing solution in phosphate-buffered saline (PBS). Lactating female F344 rats with 5-day old pups were administered a single oral dose of 50, 500, or 1000 ng [14C]PhIP/kg body weight in PBS (<0.1% ethanol; 1 ml dosing solution/kg body weight). In studies with chlorophyllin, 500 ng [14C]PhIP/kg was co-administered per os with 500 µg chlorophyllin/kg body weight also in PBS. After dosing, the dams were returned to their pups for a 3-h nursing period. The dams and pups were then killed by decapitation and hexamethylenetetramine. Liver, inguinal mammary gland and heparinized blood (from the descending aorta) was collected from the dams. Stomach and liver were collected from the pups. All samples were frozen at −20°C until analysis.

In a parallel study to examine the excretion of PhIP and PhIP metabolites into breast-milk, lactating female F344 rats were separated from their pups and dosed with [14C]PhIP or [14C]PhIP plus chlorophyllin as described above. Three h later, breast-milk was manually expressed into microcentrifuge tubes as previously described (25). To obtain sufficient amounts of PhIP metabolites for mass spectral analysis, breast-milk was also collected 3 h after administration of 10 mg PhIP/kg to a single lactating female rat. Samples were stored at −20°C prior to analysis.

Analysis of PhIP metabolites

Milk samples (100–500 µl) were mixed with 2 vols methanol, vortex mixed for 5 min at room temperature and then centrifuged at 14 000 g for 5 min to remove proteins. The supernatant was then removed, evaporated to dryness in a vacuum centrifuge (Jouan RC110 Concentrator, Winchester, VA) and the resulting solids resuspended in 50 µl 0.1% trifluoroacetic acid. The samples were immediately analysed by reverse phase HPLC on a rainin (Rainbow, MA) binary system equipped with a Rainin variable wavelength UV detector, a rhodochromix injection valve with a 50-µl injection loop, a microsorb C18 precolumn (3-µl packing material, 4.6 mm×2.5 cm; Rainin) and a ecomerse c18 analytical column (3-µl packing material, 4.6 mm×10 cm; Rainin) using Rainin data acquisition software. The samples were eluted at 1 ml/min with 0.1% trifluoroacetic acid (buffer A) and acetonitrile at room temperature. The column was equilibrated at 95% buffer A:5% acetonitrile for 20 min before the start of the injection. The column was held at 95% buffer A:5% acetonitrile for 5 min followed by a linear gradient to 70% buffer A:30% acetonitrile over 30 min. The system was held at 70% buffer A:30% acetonitrile for 5 min before terminating the analysis. Throughout the analysis, 1-min fractions were collected in 1.5-ml microcentrifuge tubes and were evaporated to dryness immediately following the HPLC analysis. The dried samples were resuspended in 200 µl 50% water:50% methanol (v/v) and prepared for AMS analysis as described below. Recovery of PhIP metabolites from the breast-milk was found to be >95% based on the radiocarbon levels measured before and after extraction and analysis by AMS (data not shown).

AMS sample preparation and measurement

Tissue (5–10 mg), blood (20 µl), milk (20 µl) or HPLC fractions (200 µl) were placed in 6×50 mm ID quartz tubes and dried by vacuum centrifugation. In the case of HPLC fractions, 2 mg tributyrin was added as an additional source of carbon for AMS analysis. The dried samples were then converted to graphite and measured by AMS as previously described (35).

AMS measures the concentration of rare isotope relative to a stable isotope. For this work, the [14C]/[12C] ratio is measured and normalized to the [14C]/[12C] ratio of 1950 carbon using the Australian National University standard as reference (34,36–38). The concentration of radiocarbon in the graphite sample is converted to the concentration of PhIP by normalizing to the carbon content of the original sample and by accounting for the specific activity and fraction of the carbon resulting from the radioassay of the samples for animals and sample handling processes. The carbon content of the tissue samples were determined on a Carlo-erba Nitrogen NA 1500 Series 2 analysers (Milan, Italy) using protocols supplied from the manufacturer. As AMS measures the total concentration of radiocarbon in a sample and does not differentiate between PhIP, PhIP metabolites or PhIP adducts, the tissue and milk data are collectively referred to as PhIP equivalents.

Characterization of PhIP metabolites by mass spectrometry.

PhIP metabolites found in the breast-milk of an animal given a single 10 mg PhIP/kg dose were analysed by mass spectrometry to verify metabolite identity in milk since metabolite analysis at the low dose were based on retention time only. Milk samples were either analysed as described above with collection of fractions for mass spectral analysis or directly injected onto a Michrom μLC system connected to a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer through a Finnigan electrospray interface. Either neat milk samples or dried fractions resuspended in 0.1% acetic acid were injected onto a Zorbax C18 SB 1×150 mm (5-μm packing material, Michrom Bioresources, Inc.) base-deactivated column and eluted with 0.1% acetic acid (buffer A) and acetonitrile. PhIP metabolites were eluted with a linear gradient of 100% buffer A to 30% buffer A:70% acetonitrile over 30 min. PhIP metabolites were detected using multi-selected-reaction monitoring (SRM) (39).

Statistical analysis

All experiments were performed using three animals per treatment group. For analysis of pups, the litters were combined from the three dams per group and sampled at random. Each tissue sample was analysed at least in duplicate and each resulting sample was measured for radiocarbon content at least three times. Values were averaged. In general, intra-sample coefficients of variation (AMS measurement errors) were ±5%. When appropriate, statistical analysis was carried out using the statistics package available with Excel 5.0 (Microsoft Corp., Redmond, WA). Data were tested for equality using one-tailed homoscedastic Student t-tests. Means were considered to be significantly different for P values <0.01.

Results

Distribution of dietary doses of PhIP in female F-344 rats and their suckling pups

PhIP equivalents were detected in the blood, liver and mammary gland of lactating rats given 50, 500 or 1000 ng PhIP/kg by AMS. At all doses examined, the highest concentration of PhIP was found in the expressed breast-milk, followed by the liver, mammary gland, and then blood (Figure 1). With the exception of the blood, dose-dependent increases in PhIP equivalents were observed.

At all three doses examined, PhIP equivalents were found in the stomach contents and liver of nursing pups. The concentration of PhIP equivalents in the pups’ stomach contents (which largely consisted of milk curds) were dose-dependent as well. At the 1000 ng/kg dose, 1177 ± 36 pg PhIP equivalents/ml milk were found. Notably the concentration of PhIP in pup liver did not correlate with the dose administered to the mothers (P > 0.1).

PhIP metabolites in the milk of lactating F-344 rats

In addition to the total levels of PhIP equivalents in the milk, aliquots of milk were also measured for individual PhIP metabolites by HPLC-AMS. Metabolite identification was based on retention time and comparison to well characterized standards. At all three doses used, 3 metabolite peaks and 3-((4'-hydroxy-PhIP) and 3-((4'-hydroxy-PhIP) and 3-((4'-hydroxy-PhIP) were detected using multi-selected-reaction monitoring (SRM) (39).
ions (M + H⁺) and first-order fragmentation patterns (39). Fragmentation of the PhIP metabolites are described in the legend to Figure 3. In addition to the PhIP, N²-hydroxy-PhIP-N³-glucuronide, 4' hydroxy-PhIP and PhIP-4'-sulfate observed at the lower doses, two additional peaks were observed. The parent ion (M + H⁺) and fragmentation pattern of these two additional peaks is consistent with N²-PhIP-glucuronide and N³-PhIP-glucuronide which have been previously reported (40). However, no detectable ¹⁴C peak consistent with the presence of N²-PhIP-glucuronide or N³-PhIP-glucuronide was observed during the analysis of the low dose milk samples.

**Effect of chlorophyllin in tissue distribution of PhIP at dietary exposure levels**

The effects of chlorophyllin co-administration on PhIP levels in milk at low PhIP doses in lactating female rats with 5-day-old pups are shown in Figure 4. The chlorophyllin co-treatment significantly decreased (P < 0.005) the concentration of PhIP in the blood and mammary gland of the dams and liver of the pups. The PhIP in the dam’s livers also appeared to be lower, but the difference was not statistically significant (P > 0.1). In contrast, the concentration of PhIP equivalents in the milk and stomach contents of the pups were significantly increased (P < 0.005). Notably, hepatic levels of PhIP were lower (P < 0.005) in pups from dams given chlorophyllin than in pups from dams not receiving chlorophyllin.

**Effect of chlorophyllin on PhIP metabolites in expressed milk**

Individual levels of PhIP metabolites found with chlorophyllin co-treatment were analysed by HPLC-AMS. Although the same three metabolites and PhIP were observed in the milk samples from the rats co-administered chlorophyllin, the amount of the individual metabolites differed. When compared to rats receiving only a 500 ng/kg dose of [¹⁴C]PhIP, the chlorophyllin co-treated animals had a 1.77-fold reduction of PhIP-4'-sulfate and a 1.42-fold increase in N²-hydroxy-PhIP-23-glucuronide and a 1.79-fold increase in PhIP (Figure 5).

**Discussion**

In this study, lactating female rats with suckling pups were administered human dietary doses of PhIP. The distribution of the PhIP and PhIP metabolites in both the dam and the suckling pups was measured using accelerator mass spectrometry (AMS). At doses comparable to an average human daily dose of PhIP, radiocarbon in excess of the natural abundance was detected in milk demonstrating that PhIP and PhIP metabolites are present in the breast-milk, as well as the other tissues examined. The results also demonstrate that the suckling pups are exposed to the carcinogen PhIP even at these low doses. In the case of the dams administered 500 ng [¹⁴C]PhIP/kg, PhIP metabolites in milk account for ~0.25% of the administered dose per ml milk. Further, this low dose to the pups is well absorbed, with concentrations of PhIP in the pup livers of 36 pg PhIP/g pup liver.

Previous studies have shown the excretion of heterocyclic amines, including PhIP, into the breast-milk of rodents at much higher doses than used here (25–28). Although the three doses used are not sufficient for detailed line fitting analysis, the best fit through the three doses for the milk data is an exponential fit (y = 83.76 e⁻⁰.⁰⁰⁰²⁷x; R² = 0.9887) suggesting that less PhIP is excreted into the milk as the exposure decreases. Consistent with this observation, linear extrapolation of high dose data (26) to the low doses used in this study...
Fig. 3. SRM-MS analysis of metabolites observed in the 3-h milk collection from a female F344 rat administered a 10 mg/kg PhIP dose. Following protein precipitation, milk aliquots (50 µl) were injected onto a µLC system and PhIP metabolites were eluted with a linear gradient of 0.1% acetic acid and acetonitrile. Optimal fragmentation conditions and characteristic fragments were determined for each metabolite (data not shown). The specific transitions were measured sequentially for 5 s with a total loop time of 30 s. The transitions measured are: PhIP (m/z 225) to m/z 210 (loss of methyl group); PhIP-hydroxy-glucuronide (m/z 417) to m/z 241 (loss of glucuronide) or to m/z 225 (loss of glucuronide-OH); PhIP-glucuronide (m/z 401) to m/z 225 (loss of glucuronide); 4’-hydroxy-PhIP (m/z 241) to m/z 226 or m/z 224 (loss of hydroxyl group); and PhIP-4’-sulfate (m/z 321) to m/z 241 (loss of SO₃). The final panel displays the total ion current obtained from summing the above scans.

Fig. 4. Effect of co-treatment of chlorophyllin and [14C]PhIP on tissue distributions. Lactating female rats with 5-day-old suckling pups were treated with 500 ng [14C]PhIP/kg or 500 ng [14C]PhIP/kg and 500 µg chlorophyllin/kg. Data represent the average of three dams or three litters of pups per group ± standard error. *Significantly different (Student’s t-test, P < 0.005). **Significantly different (Student’s t-test, P < 0.0005).

Fig. 5. Effect of co-treatment of chlorophyllin and [14C]PhIP on PhIP metabolites in milk 3 h post-exposure. Lactating female F-344 rats were removed from their pups and were treated with 500 ng/kg [14C]PhIP and 500 µg/kg chlorophyllin. The percentage increase or decrease relative to the animals given PhIP alone ± standard error in metabolite concentration is shown.

nearly predicts the data at the 1000 ng/kg dose, but overestimates PhIP concentrations for the lower doses of 50 ng/kg and 500 ng/kg (not shown).

Analysis of the PhIP metabolites found in the milk by HPLC-AMS shows the presence of 3 PhIP metabolites, plus the excretion of unmetabolized PhIP. The presence of PhIP metabolites hydroxylated at the 4’ position represent a measure of detoxification pathways while the presence of N²-hydroxy-PhIP-N³-glucuronide and PhIP are a measure of potential bioactive PhIP dose to the pups and breast tissue. Although direct characterization and positive identification of the metabolites at these low levels is not possible due to the low amounts of metabolite present in the milk samples, retention times are consistent with authentic PhIP metabolites formed in the milk of animals administered a 10 mg/kg PhIP dose (Figure 3) or in the urine of rodents fed high levels of PhIP. Although the putative PhIP-N²-glucuronide and PhIP-N³-glucuronide conjugates appeared present in the high dose (10 mg/kg) milk sample, no evidence for these metabolites is seen at low dose. Previous analysis of milk metabolites from high dose (10 mg PhIP/kg) studies (25) show the presence of only three peaks: an unidentified early eluting peak, 4’-hydroxy-PhIP and PhIP. Additional processing of the PhIP or N²-hydroxy-PhIP-N³-glucuronide in the pups may be responsible for the macromolecular damage that has been reported previously (25).

Chlorophyllin has been associated with reduced cancer risk in exposed adult animals for a number of potential carcinogens given its ability to non-covalently bind procarcinogens and prevent their absorption (31,41). Although chlorophyllin was administered at a 1000 fold higher dose than PhIP, the 500 µg/kg dose is at least 10-fold lower than typically used in chemoprevention studies and is well within the range of
individuals eating even small amounts of green vegetables (42). When a 500 µg/kg chlorophyllin dose was co-administered with a 500 ng/kg PhIP dose, significant decreases in tissue levels of PhIP in chlorophyllin co-treated animals were observed in this study at the 3 h post administration time point (Figure 4). This effect is consistent with previous reports of chlorophyllin response in adult animals (32,33,43,44). However, increased levels of PhIP in the breast-milk of chlorophyllin co-treated animals suggests a second mechanism for chlorophyllin involving absorption of chlorophyllin and subsequent complexation of PhIP in the blood, and increased distribution into the lipophilic milk. The significance of the increased levels of PhIP in the breast-milk are unknown and warrant further investigation to determine if an alternation in DNA adduct formation can be observed as well.

Analysis of PhIP metabolites by HPLC-AMS demonstrates that co-treatment with chlorophyllin does not result in different milk metabolites, but does cause an alternation in the amount of individual metabolites at the 3-h time point examined. Consistent with the ability of chlorophyllin to complex hydrophobic chemicals (31), chlorophyllin co-treatment results in increased levels of non-polar PhIP and N2′-hydroxy-PhIP-N3-glucuronide to be excreted into the milk. At the same time, the chlorophyllin co-treatment has little effect on the level of the more polar 4′-hydroxy-PhIP and the chlorophyllin co-treatment decreased the level of the even more polar PhIP-4′-sulfate excreted into the milk. Whether this affect is due to differing affinities for metabolite complexation by the absorbed chlorophyllin, interaction with metabolic enzymes or alteration of other metabolic modifiers is unknown. The modulation of xenobiotic metabolizing enzymes by chlorophyllin has been observed previously in vivo (45) and in vitro (46,47). Regardless of mechanism, our metabolite analysis demonstrates that the chlorophyllin co-treatment not only results in increasing the overall level of PhIP and its metabolites into the milk, but also increases the ratio of unmetabolized PhIP and the glucuronide conjugate of N-hydroxy-PhIP to the deactivated 4-hydroxy-PhIP and PhIP-4′-sulfate.

In conclusion, we find that PhIP and its metabolites are excreted into milk and absorbed by breast-feeding offspring at dietary doses. We also find that excretion and pup exposure is dose-dependent within the human exposure range. Co-administration of chlorophyllin alters the distribution of PhIP by decreasing the concentration of PhIP found in the dam tissues, but increases the exposure of the pups by increasing the excretion of PhIP and N2′-hydroxy-PhIP-N3-glucuronide into the milk. Although it is believed that chlorophyllin complexes PhIP in the gut thus preventing its absorption, these metabolism and distribution data suggest that some of the chlorophyllin is also absorbed and modifies the distribution and metabolism in a presently unknown fashion. The 3-h time point used in this study provides a limited assessment of the overall distribution and metabolism of PhIP, and studies with additional time points are needed for a complete understanding of these factors. Regardless of the mechanism, these data suggest that PhIP is excreted into the milk of lactating animals and that the consequences of chemoprevention strategies to breast-feeding infants must be considered. The impact of such results for human females and breast-feeding offspring consuming well cooked meat remains to be determined.

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