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**Title and Subtitle:**
Breast Tissue Dosimetry of PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine) at Human-Relevant Exposures

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**Abstract:**
The aim of this research project is to define the molecular events leading to the development of PhIP-induced breast tumors and to assess if PhIP exposure at human dietary levels present a human breast cancer risk. Within the last year, we have completed pharmacokinetic studies in both male and female rats following acute oral administrations of PhIP and have determined dose-response relationships for PhIP-DNA adduct formation in the liver, colon and breast tissue. In order to identify the specific adducts formed in these tissues, we are in the process of characterizing adducts formed in vitro using the techniques of mass spectrometry and NMR. In collaboration with Miriam Poirier at NIH, we have now produced polyclonal antibodies against PhIP-DNA, which have been used in fluoroimmunoassay to quantify PhIP-DNA adducts. This assay currently has a detection limit of 33 adducts/10^6 nucleotides using 20 μg DNA per analysis. Furthermore, tritium AMS methodology has now been used in conjunction with ^14C AMS to conduct the first low-level double-labeling experiment utilizing 2 different compounds. This work was performed under the auspices of the U. S. Department of Energy under contract W-7405-ENG-48.

**Subject Terms:**
Breast Cancer
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INTRODUCTION

SUBJECT

A great deal of concern has been expressed recently that cooking meat produces genotoxic substances which may contribute to the incidence of human cancers. Of all the substances known to be produced during cooking, the most important may be a class of heterocyclic amines called the imidazo[4,5-b]pyridines (IAs). These heterocyclic amines are considered to be significant because they are produced at relatively low cooking temperatures such as occur through the grilling, frying, and broiling of red meats, poultry, fish, and grains (1-3). Several of these compounds have also been found in beer and wine and in cigarette smoke condensates (4-6). The IAs currently identified from cooked foods consist of 19 compounds classified generally as quinolines, quinoxalines, phenylpyridines, and carbolines. All quinoline, quinoxaline and carboline IAs characterized to date are very potent Salmonella mutagens (>100,000 rev/μg). 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a phenylpyridine, is a relatively weak Salmonella IA heterocyclic amine mutagen (2,000 rev/μg), but is the most potent in Chinese hamster ovary cell (CHO) genotoxicity assays (7-9). Other important food-borne carcinogens, such as aflatoxin B1 or benz[a]pyrene, are orders of magnitude less potent in genotoxicity assays than the IAs (10). Importantly, of the 19 known food-borne IAs, 10 have been tested for carcinogenicity and all ten have been found to induce tumors in both rats and mice; and in multiple organs (2,11,12). Of the IAs identified, we considered PhIP to be most important since it is present in the highest concentration in well-done beef (2), has been found in cooked grains, beer, wine, and in cigarette smoke; and, unlike most heterocyclic amines, causes breast tumors in the rat (13). Of equal importance, the human exposure of PhIP has been documented as PhIP has been detected in human urine after consumption of normal diets (14,15). Given the recent findings that mutations in the p53 gene of breast cancer patients are more similar to mutations caused by chemical mutagens than to spontaneous mutations, the role of compounds like PhIP in the etiology of human breast cancer should be critically evaluated (16).

Non-human genotoxicity & metabolism

The mechanism of PhIP's genotoxicity has been most adequately characterized in rodents, but several studies have been carried out in non-human primates and human tissue fractions. Understanding these mechanisms is critical to determining if PhIP can cause breast cancer in humans and how to predict an individual's susceptibility. Further, understanding these mechanisms is important since species and tissue specificity in metabolism can ultimately affect the extrapolation of the animal data to humans. PhIP is excreted via the urine and feces, and several stable and unstable DNA- and protein-reactive metabolites have been measured and identified (17-22), although pathways may be dose dependent (23). Pharmacokinetics, metabolism, clastogenicity, and DNA adduct formation have also been measured for PhIP, albeit at exposure levels orders of magnitude greater than found naturally and for tissues other than breast (24-33). Some data have been reported in non-human primates (34-37). The sum of the bioassay data shows conclusively that PhIP is a potent genotoxin and carcinogen. The mutagenicity, and presumably the
carcinogenicity of PhIP results from metabolic activation of the parent heterocyclic amine. This principally results from oxidation of the exocyclic amino group to its corresponding N-hydroxylated derivative (2-N-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine) by the cytochromes P450 (23,38,39). The initial oxidation of the PhIP molecule by the cytochromes P450 is followed by one of several conjugations of the exocyclic N-hydroxyl group with acetate, sulfate or other constituents (40-43). Interspecies differences in metabolism have been suggested since rabbit P450IA1 is more active with PhIP than the corresponding P450IA2, whereas human, rat, and mouse P450IA2 is more active than the corresponding P450IA1 (25,43,44). Additionally, N-hydroxy-PhIP is preferentially sulfated in mice (41) and preferentially acetylated in human tissue fractions (Turtlelab et al., unpublished). Such interspecies differences in metabolism may be significant for risk assessment and needs to be understood prior to assessing PhIP's role in human breast cancer. Likewise, the role of the breast in generating bioactive intermediates needs to be understood to develop markers for susceptibility and to understand what makes the breast a target for chemical agents like PhIP.

The principal detoxification pathway for PhIP in rodents and non human primates involves hydroxylation at the 4'-position of the phenyl ring by the cytochromes P450IA (23,35,43,45). The 4'-hydroxy moiety is subsequently sulfated or glucuronidated to produce several stable excreted metabolites with 4'-PhIP-sulfate [4'-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine]-sulfate being the predominant metabolite detected in plasma, bile and urine (34,35,45,46). Also detected and identified in urine, plasma and bile are the 4'-PhIP-O-glucuronide [2-amino-4'-b-glucosiduronolxy)-1-methyl-6-phenylimidazo[4,5-b]pyridine], and 4'-hydroxy-PhIP (35,45). Glucuronidation of the N2- and N3-positions of the imidazole ring system of the N-hydroxylated PhIP molecule [2-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-b]pyridine and 3-(N-b-1-glucosiduronyl)-2-hydroxyamino-1-methyl-6-phenylimidazo[4,5'-b]pyridine, respectively have also been reported (35,42). Analysis of feces has shown primarily 4'-hydroxy-PhIP and PhIP to be present (35,45). These metabolites may be useful in comparing metabolism among species and in predicting susceptibility since they can be easily measured in urine, blood, and breast fluids. The utility of using this approach, however, remains to be determined and will be addressed through this proposal.

The N2-PhIP-O-glucuronide and the N3-PhIP-O-glucuronide, like the N-O-acetylated PhIP, may be meta-stable transportable PhIP metabolites. Meta-stable metabolites may serve to cause damage in tissues where PhIP metabolism does not occur. Indeed, such meta-stable metabolites have been suggested as transportable forms of other N-hydroxylamines which are liberated following hydrolysis in extrahepatic tissues (47,48). These metabolites may be causal factors for the DNA damage seen in the blood cells of primates and rodents given PhIP and for DNA and protein damage in tissues where PhIP metabolism does not occur (35). Importantly, PhIP's metabolism has primarily been established using liver tissue fractions and male animals. Few data are available on the metabolism of PhIP in breast tissue or on metabolite levels in breast fluids. These data are needed to understand PhIP's mechanism of action in inducing breast tumors and for understanding if breast fluids can be used in molecular epidemiology studies. The data gathered through this project will specifically fill in these data voids such that the role
of compounds like PhIP in breast cancer can be better understood and used to predict, on an individual basis, who may be at risk. If such an approach proves feasible, it will help be useful in cancer prevention efforts.

DNA and protein damage

Exposure to PhIP results in DNA, and likely protein, adduct formation. However, little is known about the identity and sequence specificities of nucleic acid and protein adducts, and in which tissues these most easily form. In addition, tissue specificity in DNA repair is poorly understood. Macromolecular adduction is important since it indicates the active dose of a chemical reaching its target, and is thought to be the initiating event in chemical carcinogenesis. DNA adduct formation with MeIQx has been shown to be quantitatively, but not qualitatively, affected by metabolic capacity (49). PhIP adduct formation may be similarly affected but has not been investigated. N-(deoxyguanosin-8-yl)-3'-monophosphate adducts of IQ, MeIQx and PhIP have been identified and found in vivo (36,49-53). Other PhIP adducts also exist and are likewise due to binding at guanines (54). A deoxyguanosin-N²-yl-PhIP adduct may exist since deoxyguanosin-N²-yl-MeIQx and MeIQ adducts have been reported.

While most data on the adducts have been derived from studies in the liver, IQ, PhIP and MeIQx have been shown to form adducts in extrahepatic tissues of the rat (12,28,53). High levels of PhIP adducts have been found in the large intestine, white blood cells, pancreas, and heart, followed by stomach, small intestine, kidney, and liver (12,53,55). Some mutational sequence specificity has been demonstrated for Salmonella DNA with IQ and PhIP and both inducing GC deletions in the standard frameshift sensitive and uvrB-deficient strains TA98 and TA1538 (56). Protein binding has also been suggested for PhIP (35,57) but, to date, has only been unambiguously demonstrated for IQ (58). A major limitation of the data described above is that all have been derived from high-dose studies and no studies have been reported in the breast even though PhIP causes breast tumors. Thus, little can be determined about the toxicity, biochemistry, and macromolecular targets of PhIP in the breast at human dietary doses.

Human tumorigenesis, genotoxicity, and metabolism

Inadequate data exist on the metabolism and pathologies of all the AIA's, including PhIP, in humans. Several studies have been conducted which show that increased mutagenic activity and some heterocyclic amines can be detected in the urine of fried-meat eaters and men on normal diets, although metabolite recoveries tend to be poor (1, 59-61). Purified human cytochromes P450 and human tissue fractions have been shown to oxidize the AIA's to mutagenic intermediates in vitro (62-66). Specifically, liver fractions are known to form the N-hydroxy-PhIP metabolite (67). Further, purified acetyltransferases from human tissues have been used to show that N-hydroxy-PhIP is probably acetylated by the polymorphic arylamine acetyltransferase (68). The paucity of human data can be partially attributed to technical difficulties in measuring metabolism at the low heterocyclic amine concentrations that people are naturally exposed to, and to the difficulty in obtaining material from human subjects. Such difficulty is often
methodological in nature. A major goal of the work proposed here will be the development and validation of methods which will allow detection of molecular effects in easily accessible human tissues, such as breast fluids and blood. Development and validation of such methods are important for comparing animal and human metabolism, assessing inter-individual differences in metabolism and for eventual use in identifying high risk individuals, since individual differences in metabolism represent a potentially important determinant in risk associated with carcinogen exposure (69).

PURPOSE AND SCOPE OF THE RESEARCH

The scope of this proposal is to determine if the dietary breast carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) causes macromolecular damage in the breast, and the mechanism by which this damage occurs at human levels of exposure. The proposed work will be carried out in female animals for which, as we point out, few data are available. Our purpose is to define the molecular events leading to the development of PhIP-induced breast tumors, and to assess the likelihood that PhIP exposure at human dietary levels present a human breast cancer risk. A crucial step in risk determination is the estimation of the dose of a reactive carcinogen reaching the critical molecular target. DNA adducts are particularly relevant for this purpose since the adduct, if not repaired, can be considered the initial step in the multistage process of cancer. Protein adducts may likewise be useful since they are indicators of the active carcinogen dose in the tissues. Our goals are to understand the effects of chemical dose (exposure) on adduct formation and metabolism, the types of adducts formed, how adducts are repaired, and the ability of the breast to metabolize PhIP at exposure levels expected to occur via the human diet. This low-dose work will be possible by use of AMS, a highly sensitive and novel technique for tracing $^{14}$C-labeled xenobiotics with sensitivity in the zeptomole ($10^{-21}$ moles) range. The data collected through this project will help determine if exogenous factors present in the diet can be linked to breast cancer and how best to extrapolate breast cancer risk from standard high-dose tumor assays. Further, this work will lead to a better understanding of the utility of using adducts or metabolites for identifying women at risk for cancer, either because of exposure to high levels of exogenous compounds or due to metabolism genotype. Finally, the data gathered through this work will be used to develop a sensitive assay for assessing PhIP metabolism, exposure and, potentially, risk in humans. If successful, this work will lead in out years to directly studying the molecular epidemiology of PhIP in human breast samples and to defining the role of compounds like PhIP in the etiology of breast cancer.

BACKGROUND OF PREVIOUS WORK

In years 1-2 of this research project we have studied the pharmacokinetics of PhIP in both chronic feeding experiments and acute oral administrations of PhIP in accordance with specific aim 1. We have included both male and female F344 rats to provide useful comparisons between gender and to credibly assess our results versus published data performed with higher doses. In these studies we have been able to demonstrate dose-response trends for tissue concentrations of PhIP and DNA adduct levels. Initial difficulties in working with mammary tissue were resolved.
We conducted studies to determine if PhIP is present in the breast tissue of lactating rats and if PhIP is passed from the milk to suckling pups in accordance with specific aim 2. Additionally, we investigated the effect of chlorophyllin treatment on the distribution of $^{14}C$-PhIP. These studies have revealed that even at low human dietary equivalent doses, PhIP and PhIP metabolites are passed to suckling pups and may pose a carcinogenic risk to the pups. Further, while chlorophyllin appears to be a reasonable detoxifying agent for the dams, it actually increases the exposure of the pups to PhIP.

The chemistry to optimize PhIP-DNA adduct formation has been performed and adduct synthesis can be directed towards either mainly C-8 adduct or towards the uncharacterized polar adducts. These adducts have been compared to in vivo adducts using $^{32}$P-Postlabeling. In addition, a portion of these polar adducts have been characterized by triple-quadrupole mass spectrometry, UV absorbance and fluorescence spectroscopy.

We started to determine the effect of dose of PhIP on PhIP-DNA adduct formation in female rodents. Acute oral exposures to female F344 rats have been performed and linear dose-response relationships observed. Therefore, even at low dietary relevant doses, DNA adducts are formed and therefore may be involved in the carcinogenic effects of PhIP. This data supports the role of PhIP in breast cancer as indicated from epidemiological studies linking the consumption of food likely high in heterocyclic amine content with breast cancer.

We synthesized 15mg of highly adducted PhIP-DNA and sent it to Miriam Poirier at NIH for antibody production. It was hoped that high affinity polyclonal antibodies would be produced, which could be employed in an immunoassay to quantify PhIP-DNA adducts in laboratory animal and human samples.

Finally, tritium AMS methodology was developed for the sensitive measurement of $^3$H-labeled compounds in small biological samples. This now enables us to conduct unique low-level double-labeling experiments, in conjunction with $^{14}$C AMS, utilizing 2 different compounds.

**BODY (PROGRESS TO DATE)**

The progress made towards each of the specific aims of the research project completed in the period October 1, 1996 to September 30, 1997 are described as follows:

1. **Ultra-low level pharmacokinetics by accelerator mass spectrometry**

Towards meeting the goals of specific aim 1, in years 1-2 we administered $^{14}$C-PhIP to female F344 rats, in order to examine the amount of PhIP reaching the mammary tissue in female rodents as a function of dose. In order to understand if PhIP bioavailability in female rat tissues relates to PhIP-induced tumor sensitivity, within the last year we have repeated this acute dosing study
experiment in male F344 rats. Rats were acutely dosed by gavage with $^{14}$C-PhIP in the dose range 5 ng/kg to 100 mg/kg [36 animals total, 3 animals/dose group], a dose range that incorporates environmentally relevant and rodent bioassay levels. The $^{14}$C-PhIP utilized had a specific activity of 46.7 mCi/mmol, with doses above 10 μg PhIP/kg serially diluted in unlabeled PhIP. The specific activity of $^{14}$C-PhIP used in this study was higher than used for the female dosing study (46.7 mCi/mmol compared to 10 mCi/mmol), which increases the sensitivity of detection of the $^{14}$C-PhIP in tissue and bound to DNA by AMS, which was a problem in the female rat dosing study. Animals were sacrificed 6 hours after dosing, a time point chosen to reflect the initial peak of tissue uptake. Liver, kidney, mammary gland, colon, prostate and spleen, which included target and non-target organs for PhIP-induced carcinogenicity, were removed and frozen at -20°C until either AMS analysis or DNA extraction were performed. The results of AMS analysis of liver, colon and mammary tissue of male rats are presented in figure 1.

![Graph showing dose-response for $^{14}$C-PhIP in male F344 rats.](image)

**Figure 1. Dose-response for $^{14}$C-PhIP in male F344 rats. Data from liver, colon and mammary tissue following a single acute dose are shown. Lines illustrate the power fit and the data points are the means ± SD of three replicate animals.**

The concentration of PhIP in the liver, colon and mammary tissue samples increased linearly with administered dose. The highest levels of PhIP in the dose range 5 ng/kg to 1μg/kg were observed in the liver and colon tissue. Doses above this were not measured in the liver and colon tissue due to the high levels of $^{14}$C, which were too high to measure by AMS. These samples are currently being analyzed by liquid scintillation counting. Fundamentally, the mammary tissue of male rats contained measurable levels of PhIP. The mean mammary tissue levels were 1.23x10⁶ pg PhIP/g tissue at 100 mg/kg dose and 48.20 pg PhIP/g tissue at 10 μg/kg dose. Below 500 ng/kg $^{14}$C-PhIP was not
detectable in the mammary tissue, which may partially be due to the very high levels of carbon in this tissue [67.7% carbon] compared to the liver [29.7% carbon] and colon [11.8% carbon], which would effectively dilute any $^{14}$C signal. However, due to the higher specific activity of $^{14}$C-PhIP used in this study, we could detect $^{14}$C-PhIP at much lower levels in the mammary tissue than in the previous female rat study.

The dose-response curves for PhIP levels in colon tissue in females and males were very similar (plot not shown). Both male and female rats develop colon tumors following chronic exposure to PhIP, although males have been shown to have a higher incidence than females (13). Therefore, the results indicate that tissue bioavailability alone does not account for these sex differences in PhIP induced carcinogenicity in the colon. In the liver, it appears that female rats have consistently lower levels of PhIP (plot not shown), although the liver is not a target organ for PhIP carcinogenicity in either sex.

Exposure to PhIP in the diet causes mammary tumors in female rats, but not in males (13). Therefore, in order to investigate if differences in PhIP bioavailability in the mammary tissue may account for these sex differences in carcinogenicity, male and female mammary tissue dose-response data were compared (figure 2).

![Graph showing dose-response curves for PhIP in mammary tissue of male and female rats.](image)

**Figure 2. Summary of dose-response for $^{14}$C-PhIP in the mammary tissue of male (m) and female (f) F344 rats following a single, acute dose. Data points are the means ± SD of three replicate animals. Lines and equations represent the power fits of the data.**

It can be seen that for both male and female rats there is a clear dose-response for levels of $^{14}$C-PhIP in the mammary tissue following an acute exposure to PhIP. Interestingly, the slope of the dose-response curve is greater for the
female rats than the male rats. For example, at the higher PhIP doses it appears that bioavailability of PhIP to female mammary tissue is greater than to male mammary tissue. Therefore, although at high doses (mg/kg body-weight) there appear to be significant sex differences in bioavailability, at the lower doses the tissue bioavailability alone dose not appear to clearly indicate that female rats would be more likely to develop mammary tumors.

In conclusion, the acute PhIP dosing studies have demonstrated that PhIP is distributed to mammary tissue in both male and female rats. Furthermore, the levels of PhIP in the mammary tissues increase as a linear function of dose. Although the slopes of the dose-response curves are slightly different between the sexes, there appear to be no clear differences in the data that would suggest why female rats are at greater risk of breast cancer than male rats. Furthermore, there appear to be no differences in the amounts of PhIP distributed to the colon between the male and female rats, demonstrating that levels of PhIP bioavailability also do not correlate with carcinogenicity in this organ.

2. Breast metabolism of PhIP

Towards specific aim 2 we have previously conducted a study to determine if PhIP is present in the mammary tissue of lactating rats and if PhIP is passed from the milk to suckling pups. Additionally, we have developed HPLC/AMS separation protocols for determination of metabolite levels in the milk from these animals. Lactating female F344 rats with suckling pups were gavaged with doses ranging from 50-1000 ng/kg 14C-PhIP. The excretion of the 14C-PhIP in the milk and distribution of 14C-PhIP into the mammary tissue, liver and blood of the dam as well as in the stomach contents, liver and urine of their suckling pups were measured using AMS. Within the last year a manuscript describing these studies has been submitted for publication.

Within the next few months we will conduct metabolism studies using mammary tissue homogenates from male and female rats dosed with 14C-PhIP. We will use the developed HPLC/AMS separation protocols to determine the metabolites formed in the mammary tissue and plasma. The levels of the various metabolites will then be correlated to dose and to DNA adduct levels in the mammary tissue.

3. Determination of DNA and protein adducts of PhIP in the breast

The third objective of these studies is to characterize the macromolecular adducts formed in the mammary tissue of female rodents dosed with PhIP. Characterization of the PhIP-DNA adducts formed in vivo is being undertaken by comparison to synthetic PhIP-DNA adducts. These synthetic adducts are being characterized by mass spectrometry and NMR technology.

During the last year, much progress has been made in the production and characterization of synthetic PhIP-DNA adducts to be used in the identification of the DNA adducts formed in vivo in rats dosed with PhIP. Synthetic DNA adducts have been produced by the reaction of N-acetoxy-PhIP with synthetic oligonucleotides in preparation for NMR (Dr. Monique Cosman of LLNL) and mass spectrometry studies (Dr. Robert Hettich of Oakridge National Laboratory and Dr. Hugh Gregg of LLNL).
Initial adduction experiments were conducted utilizing single-stranded 11 base oligonucleotide containing a 5'-GGA-3' 'hot-spot' for PhIP modification. Based upon preliminary studies we expected modification levels on the order of 15 - 20%, which has been sufficient for solution structure determinations of other adducts. However, large-scale adduction reactions using this sequence yielded less than 5% adduction. Therefore, experiments were conducted in order to determine how to increase adduct yields.

Initially, the effect of pH on adduction efficiencies were investigated. It was found that pH5 yielded more adducts than pH7 or pH9, possibly because the formation of the positively charged putative nitrenium ion intermediate would be favored by acidic reaction conditions. Further experiments demonstrated that yields could be greatly increased by adduction of duplex DNA, rather than single-stranded DNA and by slower addition of N-acetoxy PhIP to the reaction over longer periods of time. As a result of these studies, adduct yields were increased to 40%. However, use of duplex DNA in NMR studies is more complex to interpret than a single-stranded sequence, hence the self-complementary 8 base-pair oligonucleotide 5'GATCGATC3' was designed. In a provisional adduction experiment using 100 ODs of oligonucleotide, many 'adduct' reaction products were seen upon HPLC analysis. All the products had characteristic PhIP-adduct absorbance maxima at approximately 350nm. Some of these oligonucleotide adduct products were sent to Bob Hettich for analysis by mass spectrometry, although unfortunately a buffer contaminant prevented analysis.

In order to investigate which nucleosides in the oligonucleotide were modified by the N-acetoxy-PhIP, the individual adduct peaks were HPLC purified, enzymatically digested to nucleosides and the digestion products analyzed by HPLC. In addition, some of the digested nucleoside peaks were sent for analysis by mass spectrometry by Bob Hettich and Hugh Gregg.

HPLC analysis of the digested adducted oligonucleotide peaks suggested that mainly deoxyguanosine was modified by N-acetoxy-PhIP and that in many cases the adduct was the dG-C8 PhIP adduct. Other adduct peaks appeared to be very unstable and yielded inconclusive digestion results.

Within next few months, we will repeat the synthesis to produce sufficient amounts of adducted oligonucleotide for NMR studies. In addition, we will attempt to produce sufficient amounts of the minor oligonucleotide adducts in a suitable buffer to be characterized by Bob Hettich.

4. Dose-response relationships

Objective 4 is concerned with the understanding of the effects of the dose of PhIP on DNA adduct formation in mammary and non-mammary tissues, hence establishing data to be employed for the extrapolation to breast cancer risk in humans at low-dose exposures.

As part of these studies, the dosimetry of PhIP on PhIP binding to the DNA in the liver, colon and mammary tissue is being determined in acute PhIP female and male dosing studies [for details of the dosing regimens refer to objective 1]. DNA from liver, colon and mammary tissue samples in these studies were
extracted and the covalent $^{14}$C-PhIP binding measured by AMS. In the second year report, results of the analyses of DNA from colon and liver of female rats were shown. Within the last year, the mammary DNA from the female rats has been extracted and measured. The results of the complete set of female colon, liver and mammary DNA analyses are shown in figure 3.

Figure 3. Dose-response curves for DNA adduct formation by $^{14}$C-PhIP in female F344 rat liver, colon and mammary following a single acute dose. The power fit lines are shown. Data are means ± SD of three replicate animals.

Analogous to the tissue binding data, the dose-response curves for DNA adduct formation in the liver, colon and mammary were linear over the measurable range with a mean peak adduct level at 10 µg/kg dose of 15.2, 41.5 and 139.6 adducts/10^{12} nucleotides in the liver, colon and mammary, respectively. DNA adducts were not detectable in either the liver or colon at doses below 10 µg/kg, or the mammary below 1 µg/kg using this acute dosing regimen. Importantly, mammary PhIP-DNA adduct levels were higher than adduct levels in colon and liver DNA, indicating that DNA-adduct levels may correlate with organ susceptibility.

In order to look at sex differences in PhIP distribution and macromolecular binding, the acute PhIP dosing study was repeated in male F344 rats (see specific aim 1 for experimental details) and the DNA extracted from the liver, colon and mammary tissue. The PhIP-DNA adduct levels in these tissues in male rats is shown in figure 4:
Figure 4. Dose-response curves for DNA adduct formation by $^{14}$C-PhIP in male F344 rat liver, colon and mammary following a single acute dose. The power fit lines are shown. Data are means ± SD of three replicate animals.

In the male rats, the dose-response for PhIP-DNA adduct formation up to a dose of 10 mg/kg in the colon, liver and mammary were very similar. At a dose of 10 μg/kg, the mean adduct levels in the liver, colon and mammary tissue were 67.2, 145.1 and 143.1 adducts/10$^{12}$ nucleotides respectively. This would imply that DNA adduct levels do not correlate with PhIP induced carcinogenicity in the male rat using this dosing regimen. At doses of 10-100 mg/kg, it appeared that DNA adduct formation in the mammary tissue reached a plateau in which adduct levels did not increase despite increases in dose.

In order to investigate if there are sex differences in PhIP-DNA adduct levels in the mammary tissue between the male and female rats that may account for the differences in PhIP's carcinogenicity, the dose-responses for PhIP-DNA adduct formation in this organ were compared (figure 5). At doses of 10-100 mg/kg in the male rats the dose-response plateaued. Therefore, in order to compare the slopes of the curves, results from doses of 10 mg/kg and below are plotted.
Figure 5. Dose-response curves for DNA adduct formation by $^{14}$C-PhIP in male (m) and female (f) F344 rat mammary tissue following a single acute dose. The power fit lines and equations are shown. Data are means± SD of three replicate animals.

From the comparison of the DNA adduct formation in the male and female rat mammary tissue, it can be seen that DNA adduct formation is greater in the female mammary tissue and the slope of the curve is greater. At doses of 10-100 mg/kg, there was a plateau in DNA adduct formation in the male mammary tissue, but the female tissue dose-response continued to increase.

In conclusion, PhIP formed DNA adducts in the liver, colon and mammary tissue in both male and female rats following acute oral exposure. In the female rats, the mammary tissue had the highest levels of DNA binding, followed by the colon and then liver. Levels of DNA binding in these tissues were all dose-dependent in the dose range measured. In the male rats adducts were also detected in the liver, colon and mammary tissue. Unlike the female rats, adduct levels detected in these organs were similar in males. Comparing the male and female mammary adduct levels, the female mammary tissue had higher levels of binding than the males and adduct levels continued to increase up to 100 mg/kg. However, in the males adduct levels plateaued at 10-100 mg/kg dose, indicating possible saturation of enzymes involved in metabolic activation of PhIP. This suggests that at high dose the female rats may be at greater risk from PhIP-induced mammary tumors, although at lower doses the difference in adduct levels between the sexes was much smaller.
5. Development of an AMS isotope-labeled immunoassay

The purpose of specific aim 5 is to produce antibodies against PhIP modified DNA. The antibodies will then be utilized in a selective and sensitive immunoassay to detect and quantify PhIP-DNA adducts in various biological samples from laboratory animal and human studies. The immunoassay will potentially have applications in a wide range of molecular epidemiology studies to investigate the link between breast cancer and PhIP exposure. For example, in the validation of PhIP-DNA adducts as biomarkers in assessing exposure to PhIP and in determining susceptibility to breast cancer. In addition, it may also be useful for assessing the effectiveness of cancer chemopreventive agents. During years 1-2 of the grant, methods established by Marsch et al. (70) for the modification of DNA by PhIP have been utilized in order to obtain 15 mg of DNA with a modification level of 1 adduct/115 nucleotides. The adducts were characterized by \(^{32}\)P-Postlabeling and sent to Dr. Miriam Poirier [NIH] for polyclonal antibody production.

Within the last year, polyclonal anti-PhIP-DNA antibodies have been successfully produced in Miriam Poirier’s laboratory by immunization of rabbits with the PhIP-DNA. These antibodies have been used in a fluoroimmunoassay to detect and quantify the in vitro highly modified PhIP-DNA used in antibody production and to assess antibody cross-reactivity.

In this assay, 96 well micro titer plates are coated with highly modified PhIP-DNA. Plates are then coated with a blocking agent to reduce non-specific binding of the antibodies to the plates. Serial dilutions of PhIP-DNA in carrier calf thymus DNA are made to achieve standards containing a range of amounts of PhIP-DNA. Antibody is added to these standards (or unknowns) and allowed to equilibrate for 30 minutes. During this time, the antibodies bind to the PhIP-DNA adducts. The mixture is then added in triplicate to the coated titer plate wells and incubated for a further 1.5 hours, in which time any free antibody will bind to the wells in the plate. The wells are then washed to remove unbound material and a secondary biotinylated anti-rabbit antibody added, incubated and washed. A streptavidin labeled enzyme is then added, which binds to the biotin. Finally, a substrate is added, which is converted to a fluorescent product by the enzyme. The fluorescent product is detected using a plate reader. A standard curve is produced using the fluorescence data of the serially diluted standards, which can be used to quantify adduct levels in unknown samples.

The sensitivity of this assay using the highly adducted PhIP-DNA is 33 adducts/\(10^9\) nucleotides, using 20 \(\mu\)g of DNA per sample. Using this assay, the cross-reactivity of the antibodies has been determined with unmodified calf thymus DNA and PhIP instead of PhIP-DNA in the initial incubations. In both cases, no appreciable binding of the antibodies to these compounds was seen, indicating that the polyclonal antibodies produced are specific to PhIP-DNA adducts.

Within the next few months, the assay will be repeated using a PhIP-DNA standard with a lower adduct level, as it has previously been shown that highly modified standards contain clusters of adducts which are recognized more efficiently by the antibodies (71). This may lead to erroneous data when using these standards to quantify the low levels of modification likely to be
present in vivo. In order to achieve a standard with a lower level of modification, calf thymus DNA has been adducted with smaller amounts of N-acetoxy PhIP than used previously to achieve a high level of modification. However, the resulting adduct levels are too low to quantify by UV spectrophotometry. Therefore, it is now necessary to quantify the adduct levels by 32P-Postlabeling prior to sending the DNA to Miriam Poirier for use in the immunoassay.

Once the assay has been repeated using the new standard, the assay will be used to quantify PhIP-DNA adducts in DNA samples from rats dosed with known amounts of 14C-PhIP. The adduct levels calculated by AMS will then be compared to the adduct levels calculated by immunoassay. In this way it will be possible to see if the assay may be useful in quantifying PhIP-DNA adducts in human samples. Furthermore, more highly modified PhIP-DNA has been synthesized in order to boost the rabbit immune response and hopefully obtain antibodies with a higher affinity for the adducts. In this way, an assay with a greater sensitivity than 33 adducts/10⁹ nucleotides may be achieved. In addition, instead of using a fluorescence assay to quantify adduct levels, coupling the assay to AMS will be investigated.

Methods of Procedure used in these studies: Development of tritium AMS

Tritium-Accelerator Mass Spectrometry (3H AMS) has been developed in order to measure the 3H content of mg-sized biological research samples (72). The ability to perform 3H AMS measurements at sensitivities equivalent to those obtained for 14C enables us to perform experiments using compounds that are not readily available in 14C-labeled form. In addition, unique double-labeling experiments could be performed in which the fate, distribution, and metabolism of separate biological compounds can be studied.

In years 1-2 of the grant we demonstrated that 3H AMS can precisely and accurately measure 3H in small biological samples. Within the last year, we have used 3H AMS to study two independent compounds, one 3H-labeled and the other 14C-labeled in the first low-level double-labeling experiment. The aim of this experiment was to demonstrate that two compounds could be studied separately within the a single animal, enabling us to investigate possible interactive effects of the compounds at doses relevant to human exposures. Such studies have important applications in this research project.

The two compounds selected for this first study were 14C-MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) and 3H-PhIP. MeIQx is a heterocyclic amine also formed during the cooking of meat, hence human exposure to both MeIQx and PhIP is likely to occur. Therefore, it is important to investigate if co-administration alters the pharmacokinetics or pharmacodynamics of the compounds by altering such things as absorption and/or distribution. Additionally, MeIQx and PhIP have different target organs for carcinogenicity in rodents (the target organ for MeIQx is liver while the target organs for PhIP are the colon and mammary tissue), hence it is of interest to understand if differences in pharmacokinetics of these compounds may explain the differences in target organs.
In this experiment, three groups of 12 rats were used (4 doses, 3 animals/dose), plus three controls. The first group was dosed by gavage with $^{14}$C-MelIQx (specific activity of 45.57 mCi/mmol) in the dose range 1 ng/kg-1 μg/kg, which are exposures relevant to humans. The second group was administered $^3$H-PhIP (specific activity of 9.05 Ci/mmol) in the same dose range. The third group received both 1 ng/kg-1 μg/kg $^{14}$C-MelIQx and 1 ng/kg-1 μg/kg $^3$H-PhIP. Four and a half hours later, rats were euthanized and the liver collected and frozen until analysis. Aliquots of liver tissue were then analyzed by either $^{14}$C or $^3$H AMS for $^{14}$C-MelIQx and $^3$H-PhIP, respectively. Results obtained from animals co-administered both compounds are displayed in Figure 6.

![Graph showing liver MelIQx and PhIP levels](image)

**Figure 6.** $^{14}$C-MelIQx and $^3$H-PhIP levels in the liver tissue of rats co-administered $^{14}$C-MelIQx and $^3$H-PhIP in the dose range 1 ng-1 μg/kg body-weight by gavage. $^{14}$C-MelIQx levels were measured by $^{14}$C AMS and $^3$H-PhIP levels measured by $^3$H AMS. Data points are the mean ± the standard deviation of 3 animals/dose point.

The data illustrated in Figure 6 shows that the levels of MelIQx and PhIP absorbed from the digestive system and distributed to the liver are equal (despite the fact that MelIQx is a rodent liver carcinogen and PhIP is not). Additional data collected (but not shown), show that animals dosed with either $^{14}$C-MelIQx or $^3$H-PhIP alone displayed very similar dose-response curves. This indicates that at low levels, co-administration of MelIQx and PhIP does not alter either their absorption or distribution to the liver and that total levels in the tissue are additive (no interaction).
There are numerous mechanisms by which compounds can interact. For example, one compound may alter the metabolism or excretion of another compound, or it may interact with an enzyme or receptor needed to exert the other compound's toxic response. Therefore, we wanted to analyze protein and DNA from the liver tissue to look for interactions between the compounds at the macromolecular level. In addition, we wanted to determine if binding to protein and DNA correlated with a compound's carcinogenicity in this organ.

Using the animals described above, protein was extracted from liver tissue and 0.5 mg aliquots analyzed by $^3$H AMS. Due to the requirement of larger amounts of carbon necessary for graphitization, 2 mg of protein was required for $^{14}$C AMS. Results of the analyses of protein are shown in Figure 7.

![Graph](image)

**Figure 7.** Dose-response for $^{14}$C-MeIQx and $^3$H-PhIP binding to liver protein in animals dosed by gavage with either $^{14}$C-MeIQx, $^3$H-PhIP or $^{14}$C-PhIP plus $^3$H-PhIP. Data are the mean ± standard deviation of 3 animals/dose point. Binding was not detectable above the controls at the 1 ng/kg dose for $^{14}$C-MeIQx, or below the 100 ng/kg dose for $^3$H-PhIP.

As illustrated in Figure 7, levels of MeIQx-protein binding are approximately 30 times greater than binding of PhIP to liver protein. This may indicate that metabolic activation of MeIQx to the reactive form is greater for MeIQx, which could account for the carcinogenicity of MeIQx in this organ. Furthermore, co-administration of PhIP and MeIQx does not significantly effect their binding to protein.
Similar to the protein binding studies, DNA was extracted from the liver tissue and analyzed using AMS. For $^3$H AMS analysis, 0.5 mg of DNA was used. For $^{14}$C AMS analysis, carrier carbon was added to the DNA due to the requirement for 1 mg carbon for graphitization. Although the data is not shown, DNA binding was only detectable at the dose of 1 $\mu$g/kg for both compounds. Binding of MeIQx to liver DNA at this dose was approximately 15 times greater than PhIP binding to DNA. Therefore, DNA binding also correlates with the carcinogenicity of these compounds in the liver.

Our preliminary work has demonstrated the viability and usefulness of $^3$H AMS for detecting and quantifying extremely low levels of $^3$H tracer. We have shown that $^3$H AMS has a sensitivity comparable to $^{14}$C AMS for samples as small as 0.5 mg. We have also demonstrated the usefulness of $^3$H AMS as a tool to be used in conjunction with $^{14}$C AMS for investigating the distribution and metabolism of very low levels of two independent compounds in biological systems. In this way, it is possible to examine interactions between compounds at doses relevant to human exposures. We now plan to use this methodology to look at the effects of 2 component mixtures in mammary tissue.

A manuscript containing this data is currently being prepared for publication.

CONCLUSIONS

To date, a significant amount of progress has been made in our specific aims.

We have studied the pharmacokinetics of PhIP following acute oral administrations in accordance with specific aim 1. We have included both male and female F344 rats to provide useful comparisons between gender and to credibly assess our results versus published data performed with higher doses. In these studies we have been able to demonstrate dose-response trends for tissue concentrations of PhIP and DNA adduct levels in liver, colon and mammary tissue in both male and female F344 rats.

A manuscript has been written on our studies of PhIP metabolism in the breast tissue of lactating rats and our investigations of the effect of chlorophyllin treatment on the distribution of $^{14}$C-PhIP in accordance with specific aim 2. Work using mammary tissue homogenates is underway.

We are in the process of characterizing the DNA adducts formed by PhIP in accordance with specific aim 3. Despite initial difficulties in achieving high adduction efficiencies, we have now established methodology for production and separation of oligonucleotides adducted with N-acetoxy PhIP. We have established that the majority of adducts formed are with deoxyguanosine and the major adduct is dG-C8 PhIP. Based upon HPLC separation, many other adducts were formed and we are in the process of analyzing these by mass spectrometry.

We have determined the effect of dose of PhIP on PhIP-DNA adduct formation in female and male rodents in accordance with specific aim 4. Acute oral exposures to female and male F344 rats have been performed and linear dose-response relationships observed. Therefore, even at low dietary relevant doses, DNA adducts are formed and therefore may be involved in the
carcinogenic effects of PhIP. Although this data supports the role of PhIP in breast cancer, it is not yet clear how these relationships relate to PhIP-induced tumor sensitivity.

We have now produced antibodies against PhIP-DNA in accordance with specific aim 5. This DNA has been used in a fluoroimmunoassay to detect and quantify PhIP-DNA adducts. In the following few months the assay will be validated using DNA from PhIP-dosed rats.

Furthermore, tritium AMS has been successfully used to do the first low-level double-labeling experiment. This will enable us to investigate the effects of 2 compound mixtures of chemicals which may be involved in the development of breast cancer.

REFERENCES


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