FINAL

Report on Carcinogens Background Document for

Selected Heterocyclic Amines: PhIP, MeIQ, and MeIQx

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FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of all substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS) has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP) who prepares the Report with assistance from other Federal health and regulatory agencies and non-government institutions.

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of Selected Heterocyclic Amines: PhIp, MeIQ, and MeIQx. The scientific information in this document comes from publicly available, peer reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp-server.niehs.nih.gov. The most recent RoC, the 9th Edition, was published in May 2000, and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at http://ehis.niehs.nih.gov (800-315-3010).

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Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services
National Toxicology Program

Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

Reasonably Anticipated to be Human Carcinogens:

There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen, or reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

Executive Summary

Selected heterocyclic amines: 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx).

Introduction

PhIP, MeIQ, and MeIQx are heterocyclic amines (HCAs) that are formed by condensation of creatinine with amino acids during the cooking of meat. All of these HCAs share a common imidazole-ring structure with an exocyclic amino group and, therefore, are known chemically as amino-imidazoazaarenes. Most HCAs, including MeIQx and IQ, are fully planar aromatic structures with no bulky out-of-plane functionalities; however, PhIP possesses a phenyl moiety that is not necessarily co-planar with the main bicyclic imidazopyridine. PhIP was nominated by Dr. Takashi Sugimura, President Emeritus, National Cancer Center of Japan, and MeIQ, and MeIQx were nominated by the National Institute of Environmental Health Sciences for listing in the Report on Carcinogens based on the 1993 IARC evaluation indicating there was sufficient evidence of carcinogenicity in experimental animals for each of these chemicals and that PhIP, MeIQ, and MeIQx are possibly carcinogenic to humans (Group 2B). Another HCA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was reviewed by the National Toxicology Program in 1999 and was recommended for listing in the 10th Report on Carcinogens (scheduled for publication in 2002) as *reasonably anticipated to be a human carcinogen* based on sufficient evidence of benign and malignant tumor formation at multiple tissue sites in multiple species of experimental animals.

Human Exposure

PhIP, MeIQ, and MeIQx are formed from free amino acids, creatine/creatinine, and hexoses and are produced during the cooking of meat and fish as by-products of the Maillard or browning reaction. Temperature, processing, cooking time, pH, precursor concentrations, and types of amino acid present have been reported to affect the formation of these compounds in food. In general, cooking at higher temperatures and for longer periods of time increase the amount of HCAs produced. Additionally, HCA formation increases with cooking methods that use direct or efficient transfer of heat from the source to the food; frying or grilling of muscle meats produce more HCAs than do indirect heat methods such as stewing, steaming, and poaching. The major exposure to PhIP, MeIQ, and MeIQx is through the consumption of cooked meats, although HCAs also have been detected in other products such as processed food flavorings, beverages (beer and wine), and cigarette smoke. Occupational exposure to HCAs may occur when employees make or work with broiled, grilled, or fried foods. Dietary exposures for HCAs have been estimated to range from < 1 to 17 ng/kg body weight per day, whereas diets fed to mice in carcinogenesis bioassays may deliver doses exceeding 50 mg HCAs per kg body weight per day. Formation of DNA adducts, however, has been observed following dietary-relevant doses of HCAs.

PhIP. PhIP, the most abundant HCA detected in foods, has been detected in meats (beef, pork, chicken, and fish) commonly consumed in the United States. PhIP occurs at the highest concentrations in grilled chicken and at very low concentrations in pork. Mean

intake values of PhIP (ng/day) ranged from 285.5 to 457 in three large cohort studies from the United States (two Nurses' Health Studies and the Health Professionals Follow-up Study).

MeIQ. MeIQ is found at much lower levels in food than MeIQx and PhIP. The highest concentrations apparently occur in cooked fish, with MeIQ concentrations ranging from 0.03 to 72 ng/g. Its overall occurrence in food (meat, fish, and gravy) has been reported to be less than 1 ng/g in a Swedish study.

MeIQx. MeIQx has been detected in meats (beef, pork, chicken, and fish) commonly consumed in the United States. Highest levels appear to be in well-done grilled chicken and beef (hamburger or steak). Intake values of MeIQx (ng/day) ranged from 33 to 36 in three large cohort studies from the United States (two Nurses' Health Studies and the Health Professionals Follow-up Study).

Human Cancer Studies

HCAs and cooked meats. IARC (1993) reviewed studies on cooking methods that produced HCAs as part of their evaluation of the carcinogenicity of several HCAs, including MeIQ, MeIQx, and PhIP, and concluded that they could not separate the effects of cooking methods resulting in the formation of HCAs from effects of the food item itself. Since the IARC review, the literature has expanded rapidly. Many studies have evaluated the cooking methods that produce HCAs, and several studies have reported risk estimates for specific HCAs; a few studies have attempted to separate effects of cooking methods or the formation of HCAs from the food items per se.

Some evidence suggests that cooking methods producing HCAs may be associated with cancer risk. Cooking methods producing high amounts of HCAs are possibly associated with an increased risk for lung, stomach, bladder, colon, and breast cancer, although the evidence for colon and breast cancer is less consistent. Some site concordance is apparent with studies in experimental animals; in animals, both MeIQ and PhIP induce colon cancer, PhIP induces mammary cancer, and MeIQx induces lung cancer. Moreover, HCAs are highly mutagenic, providing biological plausibility for the association. However, the studies on cooking methods cannot identify the specific agents involved, since, in addition to HCAs, other potential carcinogens, such as polycyclic aromatic hydrocarbons, are formed during cooking.

PhIP. Eleven case-control studies, reported in eight publications (the Swedish study reported results for four cancer sites), evaluated cancer effects for PhIP. Four of these studies reported positive, statistically significant associations between intake of PhIP and the risk of various cancers (the Uruguayan breast cancer study, the Iowa Women's Health breast cancer study, the Maryland colorectal adenoma study, and the Uruguayan gastric cancer study). All these studies observed a dose-response relationship. However, the association between PhIP and colon adenomas in the Maryland study was no longer statistically significant after controlling for MeIQx and DiMeIQx. One study reported a small, nonsignificant elevation in risk for bladder cancer (Swedish bladder cancer study), and three studies reported no association with kidney (Swedish kidney cancer study), lung (Missouri lung cancer study), or prostate cancer (New Zealand prostate cancer

study). Inverse associations were observed in three studies, the California breast cancer study, the Swedish colon cancer study, and the Swedish rectal cancer study, although the protective effect for breast cancer among California women was shown to be confounded by chicken consumption. In the Swedish study, four colon cases but no controls had very high intake of HCAs. In general, the studies showing inverse associations were in populations reported to have a low consumption of HCAs and/or red and grilled meats (i.e., the California breast cancer study and the Swedish study of colon and rectum cancer). PhIP induces mammary, colon, and prostate tumors and lymphomas in experimental animals. For the three of these tumor sites that have been studied in humans, there is conflicting evidence for breast and colon cancer (or adenomas) and inadequate evidence for prostate cancer.

MeIQ. MeIQ is found at low concentrations in meat; only the Swedish studies reported risk estimates and only for one level of exposure. The odds ratios (ORs) for MeIQ intake were modestly elevated for rectal and colon cancers but were null or close to null for bladder and kidney cancers. MeIQ induces colon, liver, forestomach, skin, mammary, Zymbal gland, and oral cavity tumors in experimental animals.

MeIQx. Risk estimates for intake of MeIQx were reported for nine studies. Positive associations were reported in four studies (the Uruguayan breast cancer study, the Iowa Women's Health breast cancer study, the Maryland colorectal adenoma study, and the Missouri lung cancer study), three of which were statistically significant (ORs in the Iowa breast cancer study were not significant, and the test for trend approached one after controlling for PhIP intake) and two of which showed a dose-response relationship (Uruguayan breast cancer study and Missouri lung cancer study). MeIQx intake was not associated with cancer risk in two other studies (Swedish bladder cancer and Swedish kidney cancer studies) and was inversely associated, but not significantly so, in three studies (the California breast cancer study, the Swedish colon cancer and the Swedish rectal cancer studies). The studies that reported inverse associations (Swedish and California women) were based on study populations that have low HCAs or red meat intake and reported an inverse association for PhIP. MeIQx induces lymphomas, leukemias, and tumors of the liver, lung, gastrointestinal system, Zymbal gland, and skin in experimental animals. Of the tumor sites studied to date in humans, there is concordance with the lung.

Conclusion. Some evidence indicates that PhIP increases breast and gastric cancer risk and that MeIQx increases the risk of colon adenoma, lung cancer, and possibly breast cancer. Studies reporting positive associations were based on populations with higher intake of HCAs. Moreover, limited evidence suggests that risk of colon cancer may be increased in genetically susceptible individuals, particularly in the presence of higher exposure. However, the evidence is based on only one study for a particular tumor site or is inconsistent across studies for tumor sites for which more than one study is available. Individual HCAs are highly correlated with each other and with the many constituents in cooked meats, including protein, animal fat, nitrosamines, and agents other than HCAs formed during cooking, such as polycyclic aromatic hydrocarbons, which makes it difficult to separate the effects of HCAs from the effects of cooked meats. Also, overcontrolling by including several highly correlated variables in one model, as was

done in several studies, may make it difficult to observe an association. It is therefore difficult to establish associations of cancer risk with specific HCAs. Although evidence exists to suggest that consumption of well-done or grilled meats may be associated with increased cancer risk, the available data do not provide sufficient evidence to conclude that this risk is due specifically to PhIP, MeIQx, or MeIQ present within these foods.

Studies in Experimental Animals

PhIP. PhIP was carcinogenic at multiple tissue sites and in multiple species in experimental animal studies. In most studies, PhIP was administered in the diet; however, a few studies used gavage or intraperitoneal (i.p.) injection. In long-term dietary exposure bioassays, PhIP was a potent lymphomagen in male and female mice and male rats. No other tumors were observed in mice in these assays; however, in rats, PhIP induced significantly increased incidences of tumors of the small intestine (males), colon (males), mammary gland (females) and prostate (males). Dietary administration of PhIP also was carcinogenic in short-term studies. PhIP induced aberrant crypt foci (generally considered to be a preneoplastic lesion of colon cancer) in male and female mice and male rats. In short term carcinogenicity testing using transgenic or knockout mice, increased incidences of small intestine tumors in males (dietary), liver tumors in males (i.p.) and lymphomas in males and females (dietary) were observed. Increased incidences also were observed in neonatal mice exposed to PhIP by i.p. injection. Oral exposure of PhIP to male Nagase analbuminemic rats, which are known to be sensitive to various carcinogens, induced gastrointestinal tumors.

MeIQ. MeIQ was carcinogenic at multiple sites and in multiple species in experimental animal studies. Ingestion of MeIQ by CDF_1 mice for 91 weeks (long-term) resulted in statistically significant increased incidences of forestomach tumors in males and females and liver tumors in females. In contrast, forestomach tumors were not increased in female Big Blue® or C57BL/6N mice fed MeIQ for a similar time period; however, incidences of liver, cecum, and colon tumors were significantly increased. In short-term studies (286 days), oral administration of MeIQ to rats resulted in a significantly increased incidence of tumors of the oral cavity (males and females), Zymbal gland (males and females), skin (males), mammary gland (females), and colon (females).

MeIQx. MeIQx was carcinogenic at multiple tissue sites and in multiple species in experimental animal studies. In long-term carcinogenicity studies, dietary administration of MeIQx induced liver tumors, lymphomas and leukemias (males), and lung tumors (females) in mice and liver, Zymbal gland, skin (males) and clitoral gland tumors (females) in rats. MeIQx was not carcinogenic in cynomolgus monkeys given MeIQx by nasogastric intubation for 84 months. This finding was attributed to a low level of metabolic activation via *N*-hydroxylation in this species, but a longer study period also may be needed to detect tumors. MeIQx also was carcinogenic in short-term assays. MeIQx induced liver tumors in feeding studies of transgenic mice and in neonatal male mice exposed by i.p. injection. Increased incidences of aberrant crypt foci also were observed in dietary studies in mice. Short-term studies in male F344 rats exposed to MeIQx in the diet showed dose-related increases in glutathione S-transferase placental form (GST-P)-positive foci and aberrant crypt foci. Long-Evans rats with cinnamon-like

coat color (mutant strain) fed MeIQx had increased incidences of hepatocellular adenoma and higher tumor multiplicity.

Modulators of HCA-induced carcinogenicity. HCAs were not effective promoters in most studies reviewed; however, colon tumor multiplicity was increased in rats initiated with 1,2-dimethylhydrazine dihydrochloride (DMH) and fed PhIP. Both the number and area of GST-P-positive foci were increased in rats initiated with diethylnitrosamine (DEN) and fed MeIQx. Colon tumors were increased in rats initiated with DMH and fed a low-fat diet containing high concentrations of several HCAs, primarily PhIP and MeIQx; whereas, stomach tumors were increased in all groups fed diets with high HCA content. Several studies reported that HCA-induced carcinogenesis was promoted or enhanced with exposure to other chemicals (carbon tetrachloride, phenobarbital) and dietary factors (high fat, low calcium, caffeine).

HCA mixtures. HCAs were given alone or in various combinations in the diet in a short-term assay. When given to male F344 rats (initiated with DEN followed by a partial hepatectomy) alone at the reported full carcinogenic dose level, MeIQx and MeIQ increased the number of GST-P-positive foci, but PhIP did not. MeIQ was shown to induce an increased incidence of GST-P-positive foci in the liver at the 1/5 dose level. Overall, results from mixtures of HCAs indicated that the interactions were fundamentally additive; however, some evidence of synergistic and antagonistic effects were observed.

HCA metabolites. N-hydroxy-PhIP, when given by i.p. injection, caused increased incidences of intestinal polyps in *APC* gene knockout mice and increased, but not significant, incidences of colon and rare bladder tumors in ACI/seg rats. Rats injected i.p. with *N*-hydroxy-MeIQx had significantly increased incidences of soft tissue tumors at the injection site. Furthermore, male rats treated with either compound had significantly increased incidences of atypical hyperplasia of the prostate and seminal vesicles.

Conclusion. PhIP, MeIQ, and MeIQx were carcinogenic at multiple sites and in multiple species in experimental animals, demonstrating sufficient evidence of carcinogenicity. Although chemically similar, the three HCAs produced, in general, different tumor profiles. The carcinogenic effects of these HCAs may be inhibited or enhanced by many factors including interactions of HCA mixtures. In addition, mice and rats injected with metabolites of PhIP or MeIQ developed tumors.

Genotoxicity

Adducts. HCA-induced DNA adducts have been characterized and detected both *in vitro* and *in vivo*, and the major adduct for each HCA is similar in all species examined. The predominant adduct forms at the C8 position of guanine; however, MeIQx also forms a minor adduct at the N^2 guanine position, which is a base-pairing site. In humans, DNA adducts occur at dietary-relevant doses and usually occur at higher levels than those in rodents administered an equivalent dose. Adducts have been identified in human colon, breast tissue, and prostate cancer following exposure to HCA. While HCA-DNA adducts are necessary for the mutagenesis and carcinogenesis of these compounds, their role in human cancer is unknown at this time.

Mutagenicity. In comparison to other well known mutagens, PhIP, MeIQ, and MeIQx show a high degree of potency. For example, in *S. typhimurium*, all three compounds are more potent than benzo[a]pyrene, and MeIQ and MeIQx are more potent than aflatoxin B1. The relative mutagenic potencies of these three HCAs vary with the test system. In *S. typhimurium* strain TA98, the relative potencies were MeIQ > MeIQx > PhIP; however, they were PhIP ≥ MeIQ > MeIQx in a Chinese hamster ovary system.

PhIP. PhIP was mutagenic in prokaryotes *(S. typhimurium, E.coli),* insects, rodents (*in vitro* and *in vivo* systems) and humans (*in vitro*) but was not mutagenic in yeast (based on a single test). In nonmammalian cells, PhIP induced DNA damage (insects) but did not induce mitotic recombination (insects) or gene conversion (yeast). PhIP also was genotoxic in both human and rodent *in vitro* systems and in rodent *in vivo* systems, inducing chromosomal aberrations, DNA damage, micronuclei, and sister chromatid exchange in all three systems. PhIP also induced unscheduled DNA synthesis in humans and rodents (*in vitro)* and mitotic recombination in rodents (*in vitro*).

MeIQ. MeIQ was mutagenic in prokaryotes *(S. typhimurium, E.coli),* rodents (*in vitro* and *in vivo*) and humans (*in vitro).* MeIQ did not induce mutations, chromosomal aberrations, or gene conversion in yeast (*S. cerevisiae*) albeit only a single study was performed. MeIQ also was genotoxic in both human and rodent *in vitro* systems and rodent *in vivo* systems. In rodents, MeIQ induced DNA damage *(in vitro* and *in vivo*), micronuclei (*in vitro*), sister chromatid exchange (mixed results *in vitro* and positive *in vivo*), unscheduled DNA synthesis (*in vitro*), and chromosomal aberrations (*in vivo*). In human *in vitro* systems, MeIQ induced micronuclei and unscheduled DNA synthesis.

MeIQx. MeIQx was mutagenic in prokaryotes *(S. typhimurium, E.coli)* and in rodent *in vitro* and *in vivo* systems; no mutagenic tests were available in humans. MeIQx also induced other types of genetic damage in yeast, rodents, and humans. In yeast cells, MeIQx induced gene conversion. In rodents, MeIQx induced DNA damage (*in vivo* and *in vitro*) and sister chromatid exchange (positive *in vitro* and mixed results *in vivo*) but did not induce chromosomal aberrations (*in vitro* and mixed results *in vivo*), unscheduled DNA synthesis (*in vitro*), or micronuclei (*in vivo*). In human *in vitro* systems, MeIQx induced DNA damage, micronuclei, sister chromatid exchange, and unscheduled DNA synthesis but did not induce chromosomal aberrations.

Other Relevant Data

Absorption, excretion and metabolism in animals and humans. HCAs are readily absorbed and rapidly distributed in humans and animals following oral administration. Excretion occurs by a combination of urinary and fecal routes. HCAs are distributed to most tissues; especially to the liver, GI tract, and kidneys. Tissue concentrations generally peak within 12 to 24 hours and decline rapidly thereafter. The relative order of concentration of PhIP in tissues may depend on the route of administration and the length of the exposure period. Once HCAs are absorbed from the GI tract they may be subject to recirculation by the enterohepatic system in the bile. HCAs and their metabolites also have been detected in fetuses and in the milk of lactating rodents.

HCAs are metabolized by both phase I and phase II enzymes. *N*-hydroxylation, by liver CYP1A2, is the major phase I activation pathway. However, there is some evidence that CYP1A1, 1B1, and a few other isozymes may have a role in bioactivation. Further activation by phase II enzymes is necessary for the formation of the arylnitrenium ion that ultimately binds to DNA. Four phase II cytosolic enzymes (*N*-acetyltransferase, sulfotransferase, prolyl tRNA synthetase, and phosphorylase) can catalyze the ultimate activation step. The phase II activation reactions may take place in the liver or in other tissues. HCA metabolites have been identified in human urine, thus demonstrating that the activation path occurs *in vivo* in humans. The DNA adduct data indicate that the activation steps are more efficient in human tissues than in experimental animals and that rapid acetylators may be at higher risk of cancer.

Potential mechanisms of carcinogenicity. DNA adducts have been identified in human colon and mammary tissue following exposure to dietary-relevant concentrations of HCAs. In animal studies, adducts are formed in a dose-dependent manner and are associated with carcinogenesis. In fact, all HCAs that are carcinogenic in animals also induce DNA adducts. However, the relationship is not a simple matter of cause and effect. High adduct levels do not necessarily mean that tumors will occur. Therefore, tissues from HCA-induced tumors have been examined for specific mutations in genes that are associated with human cancers. These include protooncogenes and tumor suppressor genes such as Ki-*ras*, Ha-*ras*, *Apc*, *p53*, and *β-catenin*, and others. Guanine-based mutations occurred with the greatest frequency in these genes, particularly G:C→T:A transversions, and suggest that the HCA-induced adducts are involved. The observed mutation patterns provide evidence for a mutational profile or "fingerprint" for PhIP-induced colon tumors and MeIQ-induced forestomach and Zymbal gland tumors.

Studies investigating chemical modulators have identified several inhibitory or promotional mechanisms that influence HCA-induced mutagenesis and carcinogenesis. Many potential chemical modulators have been studied, including blocking agents, suppressing agents, bioantimutagens, and tumor promoters. Many of the modulators act by more than one mechanism, which underscores the complexity of the interactions among HCAs and other components of the diet. Other potential factors in HCA-induced carcinogenesis include oxidative damage from 8-oxodG or superoxide radicals and effects on cell proliferation, apoptosis, and cell-cycle control.

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1 Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) have been nominated for listing in the Report on Carcinogens. PhIP was nominated by Dr. Takashi Sugimura, President Emeritus, National Cancer Center of Japan. MeIQ and MeIQx were nominated by NIEHS based on the International Agency for Research on Cancer (IARC) evaluation of sufficient evidence of carcinogenicity of PhIP, MeIQ, and MeIQx in experimental animals. These three compounds are part of a class of heterocyclic amines (HCAs); HCAs are naturally formed during the cooking of meat by condensation of creatinine with amino acids (Jagerstad *et al.* 1984). While only present at very low levels (see Section 2), they are very potent mutagens (Lynch *et al.* 1995).

The first published data related to carcinogenic substances present in the extracts of roasted foods were reported in 1939 by E.M.P. Widmark, who reported that female mice treated dermally with an extract made of horse muscle that was roasted at high temperature developed mammary tumors. In the 1993 evaluations by IARC, PhIP, MeIQ, and MeIQx were found to be possibly carcinogenic to humans (Group 2B) based on sufficient evidence of carcinogenicity in experimental animals. No human carcinogenicity data were available; however, laboratory studies provided sufficient evidence of benign and malignant tumor formation at multiple tissue sites in multiple species of experimental animals (IARC 1993a). PhIP induced lymphomas in male and female mice (oral exposure), liver tumors in male newborn mice (intraperitoneal injection), tumors of the small and large intestine in male rats (oral exposure), and mammary tumors in female rats (oral exposure). Oral administration of MeIQ induced tumors in the forestomach in male and female mice, tumors of the Zymbal gland, oral cavity, and colon in male and female rats, skin tumors in male rat, and tumors of the mammary gland in female rats. Oral administration of MeIQx induced liver tumors in male and female mice, lymphomas and leukemias in male mice, lung tumors in female mice, tumors of the Zymbal gland in male and female rats, liver and skin tumors in male rats, and tumors of the clitoral gland in female rats. Intraperitoneal injection of MeIQx induced liver tumors in newborn male mice.

In 1999, the NTP reviewed another HCA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), for possible inclusion in the 10th Report on Carcinogens, which is scheduled for publication in 2002. In this report, it was recommended that IQ be listed as *reasonably anticipated to be a human carcinogen* based on sufficient evidence of benign and malignant tumor formation at multiple tissue sites in multiple species of experimental animals. Oral exposure to IQ induced tumors of the lung, liver, and forestomach in male and female mice; tumors of the mammary gland, liver, small intestine, clitoral gland, oral cavity, and Zymbal gland in female rats; tumors of the liver, skin, colon, small intestine, oral cavity, and Zymbal gland in male rats; and liver tumors in cynomolgus monkeys. Intraperitoneal injection of IQ induced liver tumors in mice.

1.1 Chemical identification

PhIP, MeIQ, and MeIQx share a common imidazole-ring with an exocyclic amino group, and therefore, are known chemically as amino-imidazoazaarenes (Lynch *et al.* 1995). PhIP ($C_{13}H_{12}N_4$, mol wt 224.11, CASRN 105650-23-5) is a gray-white crystalline solid and is also known as: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine or 1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridin-2-amine. Its RTECS number is NJ5199900. The structure of PhIP is illustrated in Figure 1-1.

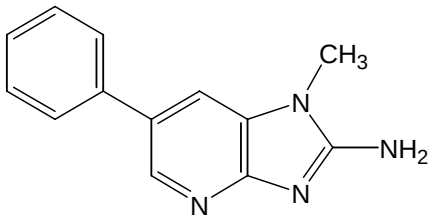


Figure 1-1. Structure of PhIP

MeIQ ($C_{12}H_{12}N_4$, mol wt 212.25, CASRN 77094-11-2) is a pale orange to brown crystalline solid and is also known as: 3,4-dimethylimidazo[4,5-*f*]quinolin-2-amine; methyl-IQ; and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline. Its RTECS number is NJ5907000. The structure of MeIQ is illustrated in Figure 1-2.

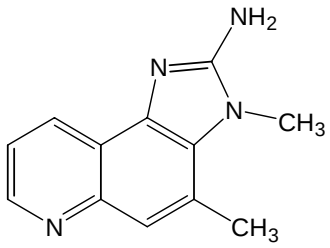


Figure 1-2. Structure of MeIQ

MeIQx ($C_{11}H_{11}N_5$, mol wt 213.24, CASRN 77500-04-0) is also known as: 3,8-dimethylimidazo[4,5-*f*]quinoxalin-2-amine; methyl-IQx; and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline. Its RTECS number is NJ5925500. Its physical state and color could not be found in the literature. The structure of MeIQx is illustrated in Figure 1-3.

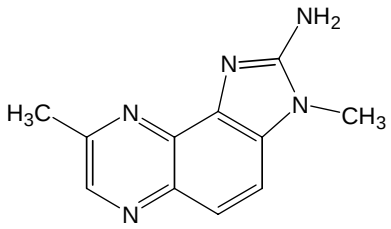


Figure 1-3. Structure of MeIQx

1.2 Physical-chemical properties

The physical and chemical properties of PhIP, MeIQ, and MeIQx are summarized in Table 1-1.

PhIP is stable under moderately acidic and alkaline conditions (IARC 1993a). MeIQ is sensitive to light, air, and prolonged exposure to heat (NTP 2001). It is stable under moderately acidic and alkaline conditions and in cold, dilute aqueous solutions protected from light (IARC 1993b). It is rapidly degraded by dilute hypochlorite. MeIQx is stable under moderately acidic and alkaline conditions and in cold, dilute aqueous solutions protected from light. It is rapidly degraded by dilute hypochlorite (IARC 1993c).

Table 1-1. Physical and chemical properties of PhIP, MeIQ, and MeIQx

Property	PhIP	MeIQ	MeIQx
Molecular weight	224.11	212.25	213.24
Color	gray-white	pale orange to brown	yellow-green
Physical state	crystalline solid	crystalline solid	crystalline solid
Melting point (°C)	327–328	296–298	295–300
Solubility:			
dimethyl sulfoxide	soluble	soluble	soluble
methanol	soluble	soluble	soluble
ethanol	na	soluble	na
Extinction coefficient	19,400 at 316 nm	48,000 at 265 nm	41,000 at 273 nm

Source: IARC 1993a, 1993b, 1993c, Knize *et al.* 1995.

na = not available

PhIP, MeIQ, and MeIQx have no commercial uses; however, all are synthesized for research purposes, and the majority of the studies reviewed in this document were performed using chemically synthesized compounds.

Most HCAs, including MeIQx and IQ, are fully planar aromatic structures (Figures 1-3, 1-4), with no bulky out-of-plane functionalities. However, PhIP possesses a phenyl moiety that is not necessarily coplanar with the main bicyclic imidazopyridine moiety (Figure 1-1). MeIQ, MeIQx, and IQ intercalate into DNA, whereas PhIP does not intercalate into DNA, but noncovalently binds in a DNA groove with some specificity for binding to A-T base pairs (Watanabe *et al.* 1982, Marsch *et al.* 1994).

The formation of HCAs in cooked meats is discussed in Section 2.2. The thermal stability of pure HCAs was assessed using fifteen purified HCA samples that were dissolved in deionized water and heated for varying lengths of time. Sealed ampoules were heated in a thermostat-controlled oil bath at 100, 150, or 200° for 1, 2, 3, 6, 12, 18, or 24 hours; after which each ampoule was inserted into an ice bath to terminate the reaction and end concentrations were analyzed by HCAs. Degradation losses for all HCAs increased with increasing temperatures and increasing cooking time (pH was not provided). PhIP was the most unstable of the HCAs studied. The degradation rates of PhIP, MeIQ, and MeIQx during heating fit a first-order model (Chiu and Chen 2000). Table 1-2 summarizes the degradation percentage of these HCAs.

Table 1-2. Degradation percentage of HCAs during heating

	Heating time (h)						
Compound	**1**	**2**	**3**	**6**	**12**	**18**	**24**
PhIP							
100°C	21.5	24.6	29.2	34.6	43.2	49.6	57.5
150°C	27.3	30.3	38.0	43.2	51.4	60.2	67.2
200°C	27.4	34.2	46.6	56.5	67.6	76.5	85.5
MeIQ							
100°C	13.5	19.2	23.5	26.5	29.9	33.3	38.8
150°C	17.1	24.2	32.0	37.9	43.7	47.6	52.8
200°C	17.3	29.8	40.2	49.5	56.4	62.9	68.7
MeIQx							
100°C	4.4	11.5	16.8	20.2	25.4	30.5	34.2
150°C	10.9	17.4	22.2	26.8	32.2	36.5	40.4
200°C	18.0	25.9	28.7	32.0	38.0	41.9	46.8

Source: Chiu and Chen 2000.

1.3 Identification of structural analogs, metabolites, and DNA adducts

HCAs are generally divided into two categories: (1) the imidazole type, which have an amino group attached to the 2 position of an imidazole ring, or (2) the nonimidazole type, which have an amino group attached to a pyridine ring (Adamson *et al.* 1996). The three compounds considered in this evaluation, PhIP, MeIQ, and MeIQx, are classified into the

imidazole category. Figure 1-4 shows several structural analogs included in the imidazole category; these analogs were not included in this current evaluation.

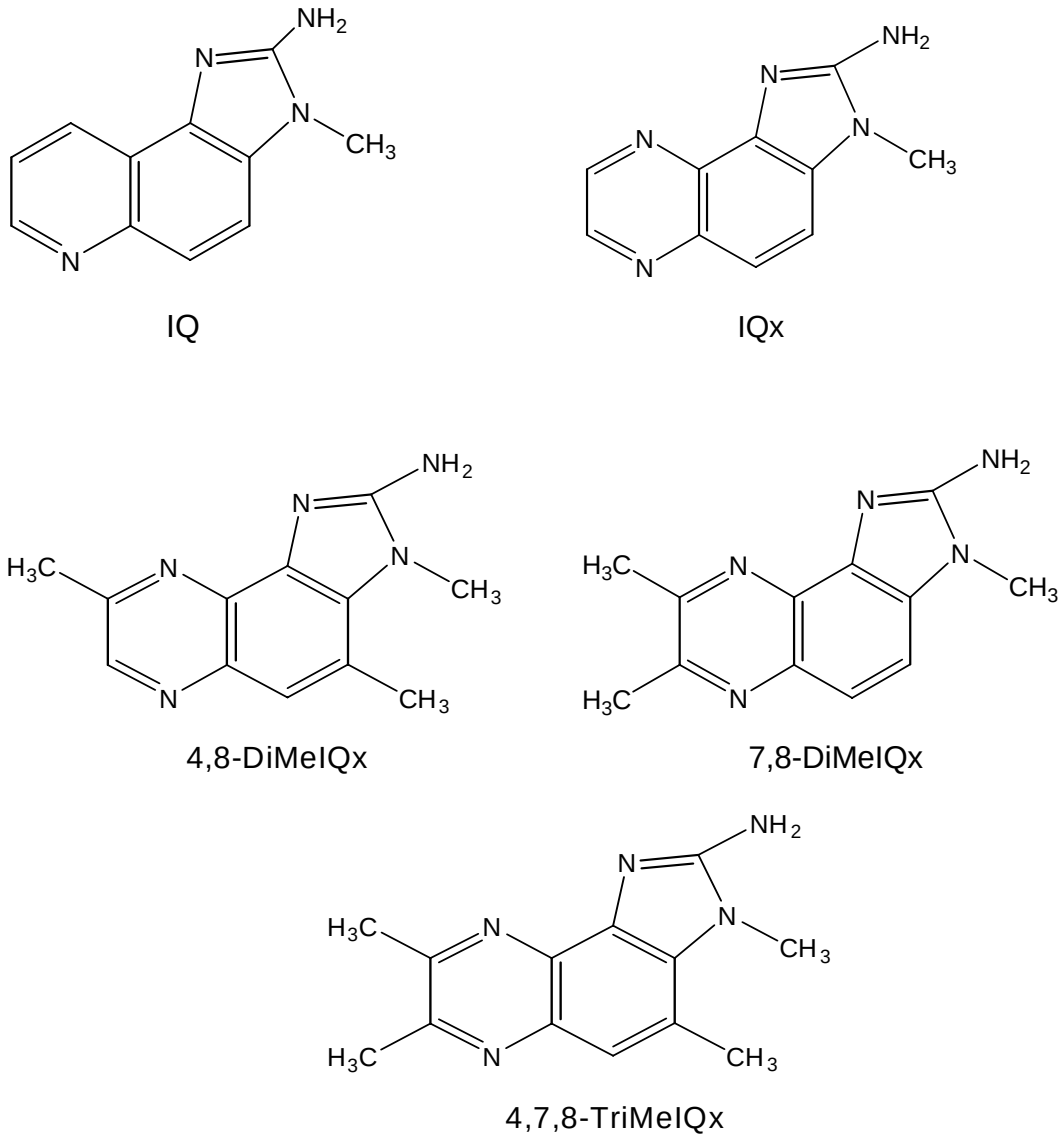


Figure 1-4. Structural analogs of selected HCAs

PhIP, MeIQ, and MeIQx are thought to be metabolically activated by *N*-hydroxylation, and catalyzed principally by cytochrome P-450 1A2; this activation is followed by further esterification, for example *N*- or *O*-acetylation catalyzed by *N*-acetyltransferases (Stone *et al.* 1998) or sulfotransferases for PhIP (Wu *et al.* 2000), and other phase II reactions. The metabolism of PhIP, MeIQ, and MeIQx is discussed further in Section 6, and a schematic diagram detailing metabolic pathways is shown in Figure 6-1.

Postmetabolic activation resulting in the formation of DNA adducts is critical for the mutagenicity and carcinogenicity of HCAs (Schut and Snyderwine 1999). For PhIP, MeIQ, and MeIQx, the predominant DNA adduct formed *in vitro* has been identified as

the *N*-(deoxyguanosin-8-yl) derivative (i.e., dG-C8-PhIP, dG-C8-MeIQ, and dG-C8-MeIQx, respectively) (Figure 5-1). For more detailed information pertaining to adduct formation of HCAs, please refer to Section 5.1.

2 Human Exposure

2.1 Use

PhIP, MeIQ, and MeIQx have no known commercial uses (IARC 1993a, 1993b, 1993c).

2.2 Production

PhIP, MeIQ, and MeIQx are produced commercially in small quantities for research purposes. Chemical synthesis, isolation, and identification of PhIP were first reported by Knize and Felton (1986). Chemical isolation and identification of MeIQ were first reported by Kasai *et al.* (1980a) who tentatively described it as a 3,5-dimethylimidazo compound. The correct structure of MeIQ as a 3,4-dimethylimidazo was later established by Kasai *et al.* (1980b) using chemical synthesis. Chemical isolation and identification of MeIQx were first reported by Kasai *et al.* (1981a). The structure of MeIQx was later confirmed by Kasai *et al.* (1981b) using chemical synthesis.

PhIP, MeIQ, and MeIQx are formed naturally during the cooking of muscle-derived foods (meat, fish) as by-products of the Maillard, or browning, reaction. Temperature, processing time, pH, precursor concentrations, and types of amino acid present have been reported to affect the formation of these compounds in food. In general, higher temperatures and longer cooking times increase the amount of HCAs produced (Knize *et al.* 1994, Skog *et al.* 1995). HCA formation also increases with cooking methods that use direct or efficient transfer of heat from the source to the food (Layton *et al.* 1995). Based on relative mutagenic activity and HCA concentrations, frying, baking, and barbecuing (term not defined by authors) of muscle meats produce more HCAs than do stewing, steaming, and poaching.

PhIP, MeIQ, and MeIQx are formed from free amino acids, creatine/creatinine, and hexoses in meat and fish (Felton *et al.* 2000, Robbana-Barnat *et al.* 1996). PhIP may be produced in model systems *in vitro* by heating mixtures of creatine and amino acids such as phenylalanine, leucine, isoleucine, and tyrosine. Heating mixtures of creatinine, phenylalanine and either sugars, aldehydes, or nucleic acids also can form PhIP (Skog *et al.* 1998). It is postulated that the amino-amidazo part of the IQ-like compounds (MeIQ and MeIQx) is formed from creatine. The remaining parts of the IQ-like compounds are likely formed from Strecker degradation products, such as pyridines or pyrazines, which are formed in the Maillard reaction between hexoses and amino acids (Jagerstad *et al.* 1984, Skog *et al.* 1998). Arvidsson *et al*. (1999) found that the rate-limiting steps in the formation of PhIP, MeIQx, and other IQx derivatives were bimolecular and followed pseudo-first-order kinetics in a model system of bovine meat juice. Neither IQ nor MeIQ was detected in their system.

In a model system studied by Jackson and Hargraves (1995), MeIQx and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) were formed in a reaction mixture of threonine, glucose, and creatine at processing temperatures of 150°C and greater; no heterocyclic amines were detected at lower reaction temperatures. At temperatures of 150°C, 175°C, and 200°C, MeIQx and DiMeIQx concentrations increased with processing time up to two hours. At higher temperatures of 225°C or 250°C, however,

MeIQx increased during the initial 15 to 30 minutes of processing time, followed by a decrease in concentration, which the authors suggested was due to decomposition.

2.3 Analysis

PhIP, MeIQ, and MeIQx are detected in foods using solid-phase extraction (SPE) and high performance liquid chromatography (HPLC) (Gross and Grüter 1992). This method uses replicate samples and spiking to allow for accurate determination of extraction losses; diode-array and fluorescence detectors are used to confirm chromatographic peak identities. The SPE-HPLC method allows for detection limits of 1 ng/g PhIP from 10 g of food sample. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is used for the quantitative analysis of PhIP released after hydrolysis of PhIP-adducted proteins from human blood and in urine; this method of detection is extremely specific and sensitive with a limit of detection of 0.2 fmol PhIP/mg protein (equivalent to 0.045 ng PhIP/g protein) (Magagnotti *et al.* 2000).

MeIQ was isolated from foods using silica-gel column chromatography and then purifying the sample using reverse-phase HPLC. Later studies used HPLC-thermospray-MS to isolate and extract MeIQ from food samples (IARC 1993b). MeIQx was identified in food using column chromatography and reverse phase HPLC with analysis by MS and ultraviolet spectrophotometry. The "blue cotton" adsorption technique, in which trisulfo-copper phthalocyanine residues are covalently bound to cellulose or cotton, also has been used extensively in several procedures to detect MeIQx in aqueous solutions. One such procedure quantifies MeIQx using LC thermospray MS. For MeIQx analysis in food extracts, monoclonal antibodies, immobilized on a support for selective immunoaffinity chromotography, were used as a clean-up procedure. MeIQx is also detected in foods using an SPE and medium-pressure LC method. Replicate samples and spiking allow for accurate determination of extraction losses, and a diode array-ultraviolet detector can confirm chromatographic peak identities. Determination of MeIQx levels using these improvements allows for detection limits of 1 ng/g MeIQx from 3 g of meat or 10 g of fish (IARC 1993c). Gas chromography/MS also has been used to identify MeIQx in fried beef (Murray *et al.* 1988).

More recently, a liquid chromatography-electrospray-mass spectrometric (LC/ES/MS) method for the determination of PhIP, MeIQ, and MeIQx has been developed. This method is more sensitive and more stable than the normally used HPLC/ultraviolet (UV) method. LC/ES/MS also allows for simultaneous determination of various HCAs in highly complex matrices such as beef extracts and produces chromatographs that are almost free of interferences. In a complex matrix like beef extracts, the detection limit for PhIP was 0.3 ng/g; for MeIQ, 0.3 ng/g; and for MeIQx, 1.1 ng/g (Pais *et al.* 1997). A similar method of high-performance liquid chromatography-electrospray tandem mass spectrometry (HPLC/ESI/MS/MS) also has been used by Richling *et al.* (1997) for analysis of heterocyclic amines in wines.

PhIP, MeIQ, and MeIQx form adducts with guanine bases in DNA, with the predominant adduct formed at the C8 position. MeIQx also forms minor adducts at the N^2 guanine position. These DNA adducts have been characterized and detected both *in vitro* and *in vivo* using methodologies such as HPLC, MS, proton nuclear magnetic reasonance

(NMR) spectroscopy, accelerator mass spectrometry (AMS), ^{32}P-postlabeling, and thin layer chromatography. AMS is an extremely sensitive analytical technique that has been used to quantify low-dose HCA-DNA adduct levels. Experiments in animals and humans can be conducted at levels more representative of human exposure levels using this method (Turteltaub and Dingley 1998).

2.4 Environmental occurrence

2.4.1 Cooking methods

Many studies have investigated the occurrence of the HCAs in food, particularly in meat. Some of these studies are reviewed below. Each study used different cooking methods, and the original reference must be consulted to determine the details of the cooking method used. However, the following definitions should apply to most of the U.S. studies discussed below:

- Bake or roast: To cook in a closed environment (namely, an oven) using indirect heat. Roasting is generally done at temperatures above 450°F (232°C); baking refers to cooking at lower temperatures (Digsmagazine 2002).

- Broil: To cook by direct exposure to radiant heat, as beneath a gas flame or electric coil (*Websters Third New International Dictionary, Unabridged 1993*).

- Grill: To broil on an open grill or griddle (*Websters Third New International Dictionary, Unabridged 1993*).

- Barbeque: To cook food directly over or under hot coals, a fire, or some other heat source (Digsmagazine 2002).

- Fry/Deep fry: To cook in a large amount of hot oil (enough to cover the food completely). The ideal frying temperature is between 350°F (177°C) and 375°F (191°C) (Digsmagazine 2002).

Barbecue is a particularly variable method of cooking meat that is defined in many ways. It has been described as a method characterized by slowly cooking meat at low temperatures. However, it should be remembered that, in the United States, cooking methods differ greatly depending on regional area, ethnic background, and other factors. In addition, cooking methods in other countries are quite different from those in the united States. In Sweden, for example, frying is the most common way of preparing meat, and food is often fried before it is added to dishes such as casseroles and stews (Augustsson *et al.* 1999a).

2.4.2 Occurrence in food

The HCAs reviewed in this document, i.e., PhIP, MeIQ, and MeIQx, are found in a variety of cooked meats and fish. PhIP is one of many HCAs formed when various meats and fish are cooked, with concentrations ranging from 0.56 to 48.5 ng/g in beef, and

nondetectable to 69.2 ng/g in various fish. Originally, PhIP was isolated from fried ground beef cooked at 300°C and has been detected in cooked beef, chicken, fish, and pork. PhIP is generally found to be the most abundant HCA detected in cooked meats and has been isolated from a complete human diet prepared in a manner to simulate domestic cooking conditions (IARC 1993a).

MeIQx has been detected in some samples of cooked beef, fish, chicken, and mutton; the concentrations ranged from nondetectable to 30 ng/g (IARC 1993c). Upon heating, MeIQx was formed in concentrated meat juice, and the concentration increased with increasing cooking time and temperature (Arvidsson *et al.* 1999).

MeIQ is present in most foods at much lower concentrations than MeIQx and PhIP. Augustsson *et al*. (1999b) reported that about 25% of the population were exposed to measurable amounts of MeIQ in a case-control study from Sweden. In an earlier publication (Augustsson *et al.* 1997), the same research group reported that the intake of MeIQ ranged from zero to two ng/day per person from meat, fish, and gravy. The primary source of MeIQ was from gravy as the intake from meat and fish was listed as zero ng/day per person. MeIQ is one of many HCAs formed in various cooked fish at concentrations ranging from 0.03 to 72 ng/g. MeIQ also was detected in fried ground beef cooked at 250°C, but only at concentrations of less than 0.1 ng/g; this compound also was present, but not quantified, in ground pork (IARC 1993b).

Layton *et al*. (1995) reviewed the literature to research concentrations of the principle HCAs identified in cooked food; the resultant database contains 261 records categorized by food item, cooking method and conditions, and the HCAs detected. Cooking temperatures ranged from 190°C to 260°C, and cooking times were generally between 6 and 10 minutes per side when reported.

Table 2-1 summarizes selected HCAs detected in cooked meats and fish. Data for DiMeIQx are included in this table and in some later tables in which published reports included analysis of this HCA. However, DiMeIQx is not reviewed in this document.

Table 2-1. Concentrations (ng/g) of selected HCAs in cooked meats/fish

Food type	Cooking method	Concentration (ng/g cooked weight)		
		PhIP	MeIQx	DiMeIQx
Beefsteak	broiled/fried	39	5.9	1.8
Ground beef/hamburgers	fried	7.5	1.8	0.4
Lamb	broiled	42	1.0	0.67
Bacon	fried	1.5	11	2
Pork	broiled/barbequed[a]	6.6	0.63	0.16
Pork, ground	fried	4.4	1.3	0.59
Chicken	broiled/barbequed[a]	38	2.3	0.81
Fish	broiled/barbequed[a]	69	1.7	5.4
Fish	fried	35	5.2	0.1
Fish	baked	12	3.8	nd

Source: Layton *et al.* 1995.

nd = not detected

[a]Cooking method not defined.

2.4.2.1 Factors affecting HCA formation in meat

Cooking temperatures and time are among the most important factors that affect HCA formation. As cooking times and temperatures increase, both PhIP and MeIQx content in fried beef patties also increase. Knize *et al.* (1994, 1995) studied HCA formation from ground beef (labelled as containing 15% fat) cooked on a commercial steel griddle. MeIQ was not analyzed in this study, which is summarized in Table 2-2.

Table 2-2. MeIQx and PhIP content (ng/g) in fried beef patties

Cooking temperature (°C/°F)	Cooking time (minutes)	MeIQx	PhIP
150/302	2	nd	nd
	4	nd	nd
	6	0.2 ± 0.2	0.25 ± 0.1
	10	0.6 ± 0.5	1.8 ± 0.7
190/374	2	0.1 ± 0.1	nd
	4	0.25 ± 0.1	0.15 ± 0.1
	6	1.3 ± 0.3	1.9 ± 0.2
	10	1.3 ± 1.1	9.8 ± 2.2
230/446	2	0.7 ± 0.4	1.3 ± 0.7
	4	0.4 ± 0.1	1.3 ± 0.1
	6	5.6 ± 3.2	7.8 ± 4.7
	10	7.3 ± 2.7	32 ± 10

Source: Knize *et al.* 1994.

nd = not detected

Skog *et al*. (1995) and Augustsson *et al.* (1997) investigated the intake of HCAs in the Swedish diet, concluding that the relative formation of HCAs depends on cooking method, cooking temperature, type of meat dish, and shape of meat. They found that the formation of HCAs increases with frying temperature, and with the degree of surface browning correlated with the frying temperature for each dish. In addition, the extent to which pan residue and gravy were consumed affected the amount of HCAs ingested.

Johansson *et al*. (1995a) reported that the use of different frying fats influenced the production of HCAs in hamburger patties and pan residues, with a greater effect noted in the pan residues. A significantly ($P < 0.05$) higher concentration of MeIQx (1.6 ng/g) was found in hamburgers fried at 200°C in rapeseed oil than in any other oil (1.0 to 1.2 ng/g) tested, which included margarine, margarine fat phase, liquid margarine, butter, and sunflower seed oil. Pan residues also contained increased concentrations of MeIQx, with the lowest concentration found in sunflower seed oil (2.1 ng/g) and the highest detected in liquid margarine (4.3 ng/g). PhIP levels ranged from 0.5 to 1.5 ng/g in the meat and from 2.0 to 13.3 ng/g in the residues. At a frying temperature of 165°C, the amount of HCAs in beefburgers and pan residues were lower than those at 200°C. No MeIQ was recovered from the fried burger or the pan residues.

Recent studies have shown that marinating meats may reduce the formation of PhIP and affect the concentration of MeIQx. Marinating in a mixture of brown sugar, olive oil, cider vinegar, and other spices reduced PhIP concentration by 92% to 99% in grilled chicken breasts cooked for 10, 20, 30, or 40 minutes (Salmon *et al.* 1997). In contrast, MeIQx increased over 10-fold with marinating, but this increase was only observed at the

30- and 40-minute cooking intervals by which time the chicken was overcooked. When compared to unmarinated meat, beefsteaks marinated with teriyaki sauce had 45% and 67% reductions in PhIP levels at 10 and 15 minutes of cooking, respectively, and 44% and 60% reductions in MeIQx levels at 10 and 15 minutes of cooking time, respectively (Nerurkar *et al.* 1999). In contrast, marinating in a commercial honey barbecue sauce resulted in a 2.9- and 1.9-fold increase in PhIP and a 4- and 2.9-fold increase in MeIQx concentrations at cooking times of 10 and 15 minutes, respectively. The mechanisms of the marinade effect are not known; however, several hypotheses, including the pH or the water component in the marinade, have been suggested (Salmon *et al.* 1997, Nerurkar *et al.* 1999). Nerurkar *et al.* (1999) speculated that the increase in HCA formation with the commercial honey barbecue sauce may be attributed to the increase in fructose (present in honey and corn syrup), which is a more efficient precursor for HCA formation than sucrose (found in sugar).

2.4.2.2 HCAs in beef cooked by different methods

Beef is the most frequently consumed meat in the United States and is likely to be an important contributor to HCA exposure. Table 2-3 shows PhIP and MeIQx formation measurements in cooked beef using cooking methods that were representative of U.S. cooking practices (Sinha *et al.* 1998a). The beef was cooked to four levels of doneness: rare, medium, well-done, and very well-done. The doneness of the meat was defined by internal temperature: 60°C as rare, 70°C as medium, 80°C as well-done, and 90°C as very well-done. No detectable formation of PhIP was noted in roast beef, but gravy made from its drippings did contain PhIP (4.1 ng/g gravy) (Sinha *et al.* 1998a). Using HPLC-UV detection, Gross *et al.* (1993) found that PhIP levels in cooked beef increased from 0.7 to 3.1 ng/g with three and five minutes/side of cooking, respectively, while MeIQx levels increased from 0.8 to 2.0 ng/g, respectively. When cooking time was increased to seven minutes/side neither PhIP nor MeIQx was detectable (< 0.1 ng/g). The combined pan scrapings from meat and fish cooked for seven minutes contained the most HCAs, 144 ± 38 ng/g PhIP and 29 ± 3 ng/g MeIQx. HCA content in grilled meat pan scrapings ranged from < 0.5 to 45 ± 3.3 ng/g PhIP and < 0.5 to 32 ± 4.8 ng/g for MeIQx. Because fat drippings and residue scrapings are often used as a base for gravies and sauces, HCA exposure is impacted strongly by cooking practices and dietary habits (Gross *et al.* 1993).

Table 2-3. PhIP and MeIQx content in beefsteak and hamburger patties (ng/g of cooked meat) cooked by various methods and degrees of doneness

Type of meat, cooking method, and degree of doneness	Selected HCAs content (ng/g meat)	
	MeIQx	PhIP
Steak—pan-fried		
rare	1.3	1.9
medium	1.9	nd
well-done	4.1	6.5
very well-done	8.2	23.2
Steak—oven-broiled		
rare	nd	6.1
medium	nd	2.1
well-done	1.7	4.6
very well-done	1.5	7.1
Steak—grilled/barbequed[a]		
rare	0.2	2.5
medium	0.6	4.7
well-done	0.8	7.3
very well-done	2.7	30.0
Hamburger—pan-fried		
rare	0.34	nd
medium	1.0	nd
well-done	2.4	nd
very well-done	4.3	2.3
Hamburger—oven-broiled		
rare	nd	nd
medium	nd	nd
well-done	nd	nd
very well-done	1.6	nd
Hamburger—grilled/barbequed[a]		
rare	nd	nd
medium	nd	nd
well-done	1.3	nd
very well-done	4.6	16.8

Source: Sinha *et al.* 1998a.

nd = not detected; limit of detection - < 0.2 ng/g of cooked meat

[a]Gas barbecue unit with ceramic briquettes and thermometers on the grill surface.

2.4.2.3 HCAs in pork cooked by different methods

Table 2-4 lists PhIP and MeIQx concentrations assayed in pork cooked using methods that were representative of U.S. cooking practices (Sinha *et al.* 1998b). The pork was cooked to three levels of doneness: just until done, well-done, and very well-done/crisp. Internal temperatures defined the degrees of doneness: 70°C as just until done, 80°C as well-done, and 90°C as very well-done. MeIQx was detected in well-done and very well-done pan-fried pork chops (1.3 and 3.8 ng/g, respectively) and in well-done and very well-done pan-fried sausage patties (1.6 and 5.4 ng/g, respectively). MeIQ has not been detected in pork, except in some laboratory tests, where small amounts (approximately 0.6 ng/g) were found (Pais *et al.* 1999). Lee *et al.* (1994) reported that MeIQ was present in an extract of boiled pork juice at a concentration of 1.2 ng/g; this HCA accounted for 38.1% of the total mutagenic activity present in the extract. In bacon, PhIP was detected at < 1 to 53 ng/g, while MeIQx was detected at 0.9 to 18 ng/g. Bacon fat contained elevated levels of HCAs ranging from < 0.1 to 17 ng/g PhIP and 1.4 to 27 ng/g MeIQx (Gross *et al.* 1993).

Skog *et al*. (1997) found the highest concentration of PhIP (32.0 ng/g) in the pan residue from a pork fillet cooked at 225°C in a study of 84 samples of cooked fish, meat, and pan residues. A dramatic increase in the formation of HCAs was noted when the cooking temperature increased from 175°C to 200°C; the total amount of HCAs present at 200°C was typically at least twice the concentration detected at 175°C. HCA formation increased substantially at 225°C, with a notable increase in the pan residues.

Table 2-4. PhIP and MeIQx content in pork and pork products (ng/g of cooked meat) cooked by various methods and degrees of doneness

Type of meat, cooking method, and degree of doneness	Selected HCAs content (ng/g meat)	
	MeIQx	PhIP
Sausage links—pan-fried		
just until done	nd	nd
well-done	0.4	nd
very well-done	1.3	0.1
Sausage patties—pan-fried		
just until done	nd	nd
well-done	1.6	nd
very well-done	5.4	nd
Ham slice—pan-fried		
just until done	nd	nd
well-done	0.6	0.3
very well-done	1.8	nd
Pork chop—pan-fried		
just until done	nd	nd
well-done	1.3	nd
very well-done	3.8	nd
Bacon fat—pan-fried		
just until done	nd	nd
well-done	nd	nd
very well-done	0.6	2.3
Hot dog—pan-fried		
just until done	nd	nd
well-done	nd	nd
very well-done	nd	nd
Hot dog—oven-broiled		
just until done	nd	nd
well-done	nd	nd
very well-done	nd	nd
Hot dog—grilled/barbequed[a]		
just until done	nd	nd
well-done	nd	nd
very well-done	nd	nd
Hot dog—boiled		
well-done	nd	nd

Source: Sinha *et al.* 1998b.

nd = not detected; limit of detection - < 0.2 ng/g of cooked meat

[a]Gas barbecue unit with ceramic briquettes and thermometers on the grill surface.

2.4.2.4 HCAs in chicken cooked by different methods

In laboratory tests, PhIP, MeIQ, MeIQx, and DiMeIQx were detected in chicken cooked by different methods. In pan-fried, very well-done boneless, skinless chicken breasts, the levels of PhIP (70 ng/g) were 2-fold greater than those previously found in very well-done pan-fried hamburgers (32.8 ng/g) (Sinha *et al.* 1994, 1995). MeIQ was found in small amounts (0.3 ng/g) in chicken thighs (Pais *et al.* 1999). Using improved HPLC methods, HCA concentrations were determined in chicken legs that were fried at 200°C for 10 minutes; PhIP (0.21 ng/g), MeIQ (0.11 ng/g), and MeIQx (0.13 ng/g) were all found in these chicken legs (Chen and Yang 1998). Table 2-5 provides a brief overview of the HCA concentrations detected in chicken breasts (Sinha *et al.* 1995).

Other studies have reported much lower levels of PhIP in chicken. Solyakov and Skog (2002) determined the amount of PhIP and other HCAs in chicken samples that were broiled, deep-fried, pan-fried, oven-roasted, cooked in an unglazed clay pot or in a roasting bag in the oven, and oven-broiled. . Of the 30 chicken samples analyzed, 15 had no detectable levels of PhIP, while the remaining samples had PhIP levels ranging from trace to 38.2 ng/g. Literature reviews on HCAs in cooked food have reported typical PhIP levels in chicken of 0 to 40 ng/g (Solyakov and Skog 2002).

Marinating of chicken also may affect the concentration of HCAs formed upon cooking. In an unmarinated, flame-grilled chicken breast, PhIP was detected at 315 ng/g and MeIQx at 0.9 ng/g after 26 minutes of cooking time. When the chicken was marinated for 4 hours in olive oil, lemon juice, cider vinegar, and other spices, the PhIP levels decreased to 4 ng/g, while the MeIQx concentration rose to 6.1 ng/g at 26 minutes of cooking time. While these results seem similar to laboratory results, cooking styles and degree of doneness do seem to affect the amount of PhIP produced (Knize *et al.* 1997a, 1997b).

Table 2-5. Concentration (ng/g of cooked meat) of selected HCAs detected in skinless, boneless chicken breasts

Cooking method	Degree of doneness	Concentration (ng/g cooked meat		
		PhIP	MeIQx	DiMeIQx
Pan-fried	just until done	12	1	1
	well-done	37	2	2
	very well-done	70	3	4
Oven-broiled	just until done	6	nd	nd
	well-done	64	nd	nd
	very well-done	150	3	nd
Grilled/barbequed[a]	just until done	27	nd	nd
	well-done	140	2	1
	very well-done	480	9	2

Source: Sinha *et al.* 1995.

nd = not detected

[a]Gas barbecue unit with ceramic briquettes and thermometers on the grill surface.

2.4.2.5 HCAs in fish cooked by different methods

Recent studies have attempted to characterize HCA concentrations in fish, factoring in cooking preparation. For fish, cooking temperature seems to dictate HCA content as it does in other meats, with higher cooking temperatures producing higher HCA content (Johansson and Jagerstad 1994, Skog *et al.* 1997). Gross and Grüter (1992) used HPLC with UV fluorescence detection to determine HCA content in salmon that was prepared and cooked at different temperatures. Salmon was prepared by pan-frying over a gas flame, cooking in a hot-air oven, or barbecuing (cooking method not defined) (Gross and Grüter 1992). Table 2-6 summarizes the HCA concentrations in selected fish.

Table 2-6. HCA concentrations in selected fish

Type of fish and preparation	MeIQ (ng/g)	MeIQx (ng/g)	PhIP (ng/g)
Salmon, pan-broiled at 200°C[a]			
2 x 3 min	nt	1.4 ± 0.01[b]	1.7 ± 0.7
2 x 6 min	nt	5 ± 0.01[b]	23 ± 7
2 x 9 min	nt	4.7 ± 0.3[b]	14 ± 2.8
2 x 12 min	nt	3.7 ± 0.2[b]	17 ± 0.6
Salmon, oven-cooked at 200°C			
20 min	nt	< 1[b]	nd
30 min	nt	4.6 ± 0.3[b]	18 ± 0.1
40 min	nt	3.1 ± 0.02[b]	5.9 ± 1.4

Type of fish and preparation	MeIQ (ng/g)	MeIQx (ng/g)	PhIP (ng/g)
Salmon, barbecued at 200°C[c]			
2 x 4 min	nt	< 1[b]	2 ± 0.3
2 x 6 min	nt	< 1[b]	6.2 ± 0.6
2 x 9 min	nt	< 1[b]	69 ± 23
2 x 12 min	nt	< 1[b]	73 ± 0.45
Cod fillet, breaded			
150°C	nt	nd	0.02
175°C	nt	nd	0.05
200°C	nt	nd	0.4
225°C	nt	0.9	2.2
Cod fillet, pan residue			
150°C	nt	< 0.01	< 0.01
175°C	nt	0.1	0.01
200°C	nt	0.1	0.05
225°C	nt	0.2	0.01
Baltic herring, breaded			
150°C	nt	0.2	0.07
175°C	nt	nd	0.06
200°C	nt	nd	0.3
225°C	nt	0.2	0.1
Baltic herring, pan residue			
150°C	nt	nd	< 0.01
175°C	nt	nd	< 0.01
200°C	nt	< 0.01	< 0.01
225°C	nt	0.01	< 0.01
Baltic herring	0.1	0.6	nd
Salmon[d]	1.3 ± 0.3	0.6	3.0 ± 3
Salmon - pan residue	nd	0.5	nd
Smoked salmon	nd	1.3 ± 0.1	nd
Smoked flounder	0.3	1.2 ± 1.7	nd

Source: Gross and Grüter 1992, Johansson and Jagerstad 1994, Skog *et al.* 1997.

nt = not tested, nd = not detected

[a]Two sides of the fish, times number of minutes cooked

[b]Values were corrected for incomplete recovery

[c]Barbecued indicates cooking over an open flame on a grill.

[d]Salmon was cooked on a stove with frying fat at a temperature of 155 – 175°C for 0.5 – 4 minutes per side to create an edible dish.

2.4.2.6 HCAs in restaurant-prepared foods

Analysis of restaurant foods shows that HCA content in these samples is similar to that obtained in laboratory cooking. This finding suggests that these studies are a good indicator for U.S. consumption of HCAs in meat. Analysis of HCA content in fast-food products showed undetectable levels of HCAs in 10 of 17 samples. Studies found low levels of PhIP (0.1 to 0.6 ng/g in fast-food hamburgers not detectable [< 0.1 ng/g] levels in fast-food sausage, chicken, and fish)and MeIQx (< 0.1 to 0.3 ng/g in fast-food hamburgers and not detectable to 0.3 in fast-food sausage, and not detectable levels in fish and chicken samples) in fast-food samples (Knize *et al.* 1995).

Commercially cooked meats from conventional restaurants have shown PhIP levels from undetectable to 13 ng/g; MeIQx was detected at lower concentrations (0.42 to 0.89). Table 2-7 summarizes PhIP and MeIQx concentrations in commercially cooked foods.

Table 2-7. Selected HCA concentrations (ng/g) in commercially cooked foods

Type of sample	PhIP (ng/g)	MeIQx (ng/g)
Blackened pork	3.0	0.53
Chicken fajita	6.4	0.54
Hamburger	11	0.89
Blackened beef	1.0	0.48
Top sirloin steak	13	0.87
Pork rinds	nd	0.42

Source: Knize *et al.* 1997a.

nd = not detected; limit of detection - < 0.1 ng/g

In a more recent restaurant study, very well-done charbroiled hamburgers (pooled samples) had the highest levels of PhIP (18.4 ng/g) while very well-done griddle-fried beefsteak had the highest levels of MeIQx (2.4 ng/g). Table 2-8 summarizes HCA content in restaurant food. No correlation between doneness of meat and HCA content was found in this study; however, rare samples were not tested and this may have skewed the results. Chicken was found to have HCAs at levels much lower than hamburgers, steaks, or the composite pork rib samples. It appears that the cooking method and type of preparation may affect the HCA content in chicken, as these concentrations differ greatly with previous studies (Sinha *et al.* 1995). These results also differ from fast-food restaurants, most likely caused by the different preparation styles between fast-food and food that is cooked-to-order. The short cooking times of fast-food restaurants may explain the lower HCA levels (Knize *et al.* 1998, Pais *et al.* 2000).

Table 2-8. HCA concentrations in restaurant foods

Sample	HCA content (ng/g)	
	PhIP	MeIQx
Hamburger - griddle fried		
medium	1.9 ± 0.30	1.5 ± 0.17
well-done	4.4 ± 0.47	1.8 ± 0.33
very well-done	2.6 ± 0.07	1.3 ± 0.04
Hamburger - charbroiled		
medium	5.2 ± 0.20	0.2 ± 0.13
well-done	1.8 ± 0.12	0.4 ± 0.02
very well-done	18.4 ± 7.1	1.8 ± 0.30
Beefsteak - griddle fried		
medium	10 ± 1.01	1.7 ± 0.40
well-done	6.8 ± 0.39	1.8 ± 0.01
very well-done	9.0 ± 1.08	2.4 ± 0.08
Beefsteak - charbroiled		
medium	12 ± 0.81	1.1 ± 0.05
well-done	15 ± 2.11	1.6 ± 0.15
very well-done	5.7 ± 0.16	1.2 ± 0.09
Pork rib - smoked		
regular	7.4 ± 1.77	nd
well-done	0.7 ± 0.50	nd
Pork ribs - baked		
regular	0.5 ± 0.01	nd
well-done	2.3 ± 0.32	nd
Chicken - grilled, sandwich		
Vendor A	0.81 ± 0.11	0.72 ± 0.37
Vendor B	nd	0.54 ± 0.01
Vendor C	0.38 ± 0.04	0.38 ± 0.07
Vendor D	1.44 ± 0.19	0.27 ± 0.02
Chicken - rotisserie grilled		
white meat	0.75 ± 0.19	0.45 ± 0.08
dark meat	0.59 ± 0.06	0.40 ± 0.06
Top sirloin – well-done	1.8	1.2
Top sirloin steak – well-done	7.8	2.0
New York steak – well-done	0.86	0.12
New York steak – well-done	7.7	1.3
Tenderloin steak – well-done	16	1.9

Sample	HCA content (ng/g)	
	PhIP	MeIQx
Tenderloin steak – well-done	49	0.67
London broil steak – well-done	182	3.0
Prime rib – well-done	nd	nd
Beef (French dip sandwich)	nd	nd
Beef (fajitas)	1.7	0.93
Au jus gravy	nd	nd
Pork chop	2.4	0.4

Source: Knize *et al.* 1998, Pais *et al.* 2000.

nd = not detected; limit of detection - < 0.1 ng/g.

2.4.2.7 Summary of HCA occurrence in foods

The dietary intake of PhIP, MeIQ, and MeIQx is a function of cooking method, doneness preference, and consumption frequency (Keating *et al.* 1999). PhIP, MeIQ, and MeIQx have been detected in all of the meats commonly consumed by the U.S. population, i.e., beef, pork, chicken, and fish. While very well-done grilled/barbequed (gas barbecue unit with ceramic briquettes and thermometers on the grill surface) chicken produced the greatest levels of PhIP (480 ng/g) (Sinha *et al.* 1995), beefsteaks, hamburger patties, and bacon account for more than 60% of the red meat consumed in the United States (Sinha *et al.* 2000a). Pork, which is the second most frequently consumed meat in the United States, contains very little PhIP, with the exception of bacon. MeIQ is rarely detected in cooked beef, pork, or chicken, but this HCA has been found in cooked fish. As discussed in Section 2.6, below, the variation in HCA content of various foods based on cooking temperature, time, and other factors makes the estimation of individual exposure very difficult.

2.4.3 Occurrence in other environmental media

Manabe *et al*. (1993) reported that they had measured PhIP in airborne particles, diesel-exhaust particles and incineration ash from garbage-burning plants. They propose that PhIP is likely to be a widespread environmental pollutant. While no studies identified MeIQ, or MeIQx in the environment, food processing facilities and restaurants may release these HCAs into the environment from exhaust emissions and solid or liquid waste disposal.PhIP and MeIQx were found in organic extracts by the blue rayon hanging method from the Yodo River (Japan) (Ohe 1997). The geometric mean in extracts collected from 11 different locations was 11.9 ng/g (PhIP) and 4.8 ng/g (MeIQx) blue rayon equivalent (i.e., the amount of HCA recovered from one gram of blue rayon). This study indicates the possible occurrence of these compounds in the environment.

2.4.4 Airborne occurrence

PhIP and MeIQx are found in aerosol droplets that form during the cooking process, thereby leading to possible inhalation exposure. While increased cooking temperatures have been shown to increase HCA production, a concomitant increase in the formation of airborne by-products also was found (Thiébaud *et al.* 1995). Table 2-9 summarizes PhIP, MeIQx, and DiMeIQx formation in fried food samples and trapped smoke condensate obtained while cooking. PhIP was the most abundant HCA detected in all fried meat samples, with a relative amount representing 47% to 65% of the total HCAs detected, followed by MeIQx (15% to 41%) and DiMeIQx (7% to 12%). While the temperature ranges used in this study were higher than those normally found in actual food preparation, relative amounts of PhIP, MeIQx, and DiMeIQx did increase in both fried meats and trapped smoke condensates as the cooking temperatures rose. Further, the formation of PhIP is disproportionate to the other HCAs at higher temperatures. Fried bacon contained the highest amount (163 ng/g) of these selected HCAs.

Table 2-9. Selected HCA concentrations (mean ± SE) in fried food samples and trapped smoke condensate

	Fried food sample			Trapped smoke condensate		
Food sample[a]	PhIP (ng/g[b])	MeIQx (ng/g[b])	DiMeIQx (ng/g[b])	PhIP (ng/g[b])	MeIQx (ng/g[b])	DiMeIQx (ng/g[b])
Beef patties (198°C)	4.9 ± 1.4	4.3 ± 0.3	1.3 ± 0.3	0.14 ± 0.002	0.14 ± 0.005	0.006 ± 0.001
Beef patties (277°C)[c]	68 ± 14	16 ± 0.8	4.5 ± 0.8	1.8 ± 0.09	1.1 ± 0.1	0.25 ± 0.006
Bacon strips (208°C)	106 ± 20	45 ± 3.5	12 ± 2.5	1.0 ± 0.2	nd	nd
Soy-based patties (226°C)	nd	nd	nd	0.007 ± 0.006	nd	nd

Source: Thiébaud *et al.* 1995.

nd = not detectable; limit of detection - 0.1 ng/g

[a]Average frying temperatures in parenthesis

[b]ng/g of cooked meat

[c]From Thiébaud *et al.* 1994.

Significant amounts of MeIQx were detected in cooking aerosol from stir-fried fish (Yang *et al.* 1998). This elevated concentration of MeIQx (268.1 ng/dish of fish) in cooking aerosols coupled with the time spent cooking (approximately 1 hour) may lead to the exposure of cooks to MeIQx from cooking aerosols.

2.4.5 Occurrence in processed food flavors

Zimmerli *et al*. (2001) evaluated 16 Swiss commercial samples including bouillon (cubes), gravy concentrates, and oxtail soup powder, for HCA content. Nine samples (one soup powder, three gravy concentrates, and five bouillon) did not contain detectable quantities of HCAs (< 1 ng/g). In the remaining seven samples, total HCA concentrations ranged from 1 to 4 ng/g. MeIQx was detected in 31% of the commercial samples, whereas PhIP was detected in only 6%; MeIQ was not detected in the commercial

samples. MeIQ was detected in 4% of samples from homes and restaurants, and the intake of MeIQ was estimated at 0.6 ng/kg body mass per day in the Swiss population.

MeIQ was not found in seven process flavors, five process flavor ingredients, and four bouillon concentrates tested for HCA content (Solyakov *et al.* 1999). A trace amount of PhIP was found in two samples, while MeIQx was found in four samples (range 1.0 to 13.8 ng/g), 4,8-DiMeIQx in three samples (1.3 to 2.9 ng/g), and 7,8-DiMeIQx in one sample (0.3 ng.g).

The amount of MeIQ in a commercial beef extract (source not identified) was reported by two methods. Galceran *et al.* (1993) measured 5.8 ng of MeIQ per g of the extract using HPLC with electrochemical detection. Later, Puignou *et al.* (1997), from the same research group, used capillary zone electrophoresis to separate HCAs and reported 9.3 ng of MeIQ per g of what was described as a food-grade beef extract.

2.4.6 Occurrence in beverages

MeIQ concentration in beverages was low compared to food levels, but findings with beverages have been inconsistent. MeIQ was detected in coffee beans in one study (0.016 to 0.15 ng/g) (Kikugawa *et al.* 1989), but was not detected in a follow-up study (Gross and Wolleb 1991). PhIP was found in 11 brands of beer and 10 brands of wine at mean concentrations (± SD) of 14.1 ± 6.18 ng/L and 30.4 ± 16.4 ng/L, respectively (Manabe *et al.* 1993). Using HPLC/ESI/MS/MS, Richling *et al.* (1997) found HCAs in 23 of 24 wines, including both red and white wines from Germany, Spain, Italy, and France. MeIQ was found in two German white wines, though below the level of quantification. PhIP was found in a French white wine (not quantifiable) and a German white wine (83 ng/L). MeIQx was the most widely distributed HCA in this study, found in three French white, three Spanish white, three Spanish red, one Italian red, one German red, and one French red wine; all levels were below the limit of quantification.

2.4.7 Occurrence in cigarette smoke

The formation of mutagenic and carcinogenic compounds in cigarette smoke is well established. PhIP has been detected in mainstream cigarette smoke condensate at 11 to 23 ng/cigarette, with an average of 16.4 ng/cigarette in six samples (Manabe *et al.* 1991). In mainstream smoke, 0.28 to 0.75 ng MeIQ/cigarette was found; in sidestream smoke, 0.25 to 0.45 ng MeIQ/cigarette was found (reviewed by Felton *et al.* 2000).

2.4.8 Methods to reduce HCA content

When compared to concurrent controls, the addition of phenolic antioxidants (i.e., vitamin E and oleoresin rosemary each at 1% and 10%) directly to the ground beef or on the surface of the beef before frying greatly inhibited the formation of PhIP, MeIQ, MeIQx, and DiMeIQx. The greatest inhibition of HCA formation was observed following the addition of 1% vitamin E to the ground beef (reduced by 48.0% to 79.2% compared to controls, $P < 0.05$). Application of 1% vitamin E to the ground beef also caused inhibition of HCA formation (reduced by 45.1% to 75.6% compared to controls, $P < 0.006$) (Balogh *et al.* 2000).

2.4.9 HCA content and pathogens in meats

While lower cooking temperatures and times may minimize HCA content in meats, food safety concern exists as pathogens must be thermally inactivated. Salmon *et al.* (2000) showed that HCA formation could be minimized and bacteria in meat inactivated by cooking the meat in a preheated pan at 160°C, turning the meat once every minute, and cooking the meat to an internal temperature of 70°C. The study shows that the frequency of turning the meat patty affects the HCA content of the meat. This result can be explained by heat transfer. When the meat patties are turned more frequently, less heat is lost from the exposed surface, resulting in quicker cooking times. Longer cooking times have been associated with higher levels of HCAs (Salmon *et al.* 2000).

2.4.10 Other compounds

Polyaromatic hydrocarbons (PAHs) are another group of chemicals that includes a number of potentially carcinogenic compounds formed as a result of cooking meat. Similar to the HCAs, PAHs have been detected in many foods, including smoked meat and fish products. In addition, method of cooking, time of cooking, distance from the heat source, and drainage of fat during cooking all influence the presence of PAHs in foods. Charcoal broiling has been shown to increase the levels of PAHs in meat (ATSDR 1995). The ATSDR (1995) reported that PAHs formed after cooking of meat, poultry, and seafood included benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, and indeno[1,2,3,-c,d]pyrene. These five PAHs and ten other related compounds are listed in the Ninth Report on Carcinogens (2000) as *Reasonably Anticipated to Be Human Carcinogens*.

The concentrations of PhIP, MeIQ, and MeIQx reported above are mostly within the range of 1 to 100 ng/g of cooked meats, and similar concentrations have been found for PAHs. In one U.S. study, total PAH concentrations ranged from 2.6 to 29.8 ng/g, 2.8 to 22.4 ng/g, and 9.3 to 86.6 ng/g, in smoked red meat products, smoked poultry products, and smoked fish products, respectively. Total PAH concentrations in liquid smoke flavorings and seasoning ranged from 6.3 to 43.7 ng/g. Benzo[a]pyrene was detected in pork sausage (1.8 to 2.3 ng/g), grilled pork chops (2.5 ng/g), whole ham (1.1 ng/g), beef sausage (1.1 ng/g), salmon (3.9 ng/g), and oysters (3.0 ng/g) (ATSDR 1995).

2.5 Environmental fate

No data on environmental fate were found. The persistence of PhIP, MeIQ, and MeIQx in the environment after any potential release is unknown.

2.6 Environmental exposure

The occurrence of PhIP, MeIQx, and DiMeIQx in a variety of commonly consumed cooked meats is summarized in Section 2.4, above. While these three compounds are among the most abundant of the HCAs present in cooked meats, MeIQ is present only in small amounts. MeIQ was not found in fried or broiled beef, but was found in pork (0.02 ng/g) and fish (0.03 to 20 ng/g) cooked by these methods (Lynch *et al.* 1995).

Dietary exposure to PhIP, MeIQ, and MeIQx has been difficult to quantify because many studies fail to include descriptions of the cooking methods. These studies, which are

concerned primarily with method development, typically report results for samples that are cooked to maximize HCA production; they are not representative of general domestic cooking methods. Published data, however, indicate that humans are exposed daily to HCAs in the normal diet. Based on the Continuing Survey of Food Intakes of Individuals (CSFIIs), Layton *et al.* (1995) published estimates of the average daily intake of HCAs in the U.S. diet. The group used 3,563 individual 3-day diet records obtained in the 1989 study. Using a compiled database of HCA concentrations measured from various cooked food sources, the group estimated average intake of PhIP, MeIQx, and DiMeIQx to be 16.64 ng/kg b.w. per day, 2.61 ng/kg b.w. per day, and 0.81 ng/kg b.w. per day, respectively.

Data also are available that indicate that these compounds can be absorbed into the systemic circulation and excreted renally. In a review by Lynch *et al*. (1995), data were provided for urinary PhIP and MeIQx measurements in five male and five female volunteers (Ushiyama *et al.* 1991) eating a mixed Japanese-Western style diet; PhIP was excreted at 0.53 ± 0.59 ng/24 h (mean ± SD) urine collection, while MeIQx was excreted at 29 ± 14 ng/24 h urine collection. However, in patients receiving parenteral nutrition, both compounds were below the limit of detection (< 0.01 ng/24 h urine collection).

2.6.1 Estimation of HCA intakes with food-frequency questionnaire and HCA database

A common method of estimating the intake of dietary components, such as HCAs, is to administer a food-frequency questionnaire that includes commonly consumed foods and the amount consumed on a daily (or other regular) basis. The consumption of the specific dietary component is then assessed by multiplying the quantity of food consumed by the concentration of the component of interest. Sinha and coworkers developed a 100-item food-frequency questionnaire that included an evaluation of meat cooking practices (e.g., pan frying, broiling, grilling) (Swanson *et al.* 1997). This food-frequency questionnaire has been coupled with a database for three HCA components, MeIQx, DiMeIQx, and PhIP, developed by the same research group for meats prepared by different cooking techniques and to different doneness levels. The food-frequency questionnaire and HCA database were used in the assessment of HCA consumption summarized in Table 2-10 from Byrne *et al.* (1998). The database contains information derived from publications by Sinha and coworkers on beef (Sinha *et al.* 1998a, see Table 2-3), pork (Sinha *et al.* 1998b, see Table 2-4), chicken (Sinha *et al.* 1995, see Table 2-5), and fast-food meat products (Knize *et al.* 1995, see Section 2.4.2.6). In those publications, the authors reported the HCA concentrations in different types of meats cooked by different methods and to varying degrees of doneness. Photographs were made of the cooked meats to show the internal coloring and external browning; the - photographs were used in conjunction with the food-frequency questionnaire to standardize the responses of individuals assessed.

The HCA database was used to assess HCA intakes in four U.S. population-based case-control studies published by Delfino *et al.* (2000) and Sinha *et al.* (2000a, 2000b, 2001) and discussed in Section 3.3.2, below. Sinha and Potter (1997) described their intended approach to validation of their dietary instrument (food-frequency questionnaire), especially the portion related to cooking practices, by comparison of questionnaire-derived data with 24-hour and 12-day dietary records and the use of biomarkers such as

urinary HCA parent compounds and metabolites; however, this validation has not yet been published at the time of this review.

2.6.2 Data on environmental exposure

Byrne *et al.* (1998) estimated overall daily dietary PhIP, MeIQx, and DiMeIQx intake based on 673 survey responders (a total of 750 questionnaires were mailed) from three large prospective cohorts (250 questionnaires per cohort): the two Nurses' Health Studies (NHS and NHS II) and the Health Professionals Follow-up Study (HPFS). Table 2-10 shows the exposure estimates in each cohort. The eight food items and portion sizes were predetermined for the survey. The survey requested information pertaining to specific cooking methods, e.g., pan-fried, broiled, grilled/barbequed (gas barbecue unit with ceramic briquettes and thermometers on the grill surface), fried, microwaved; frequency of consumption; degree of doneness; and for chicken and fish, whether the skin was cooked or consumed. A wide range of exposures to HCAs was observed among the participating health care professionals. The ratios of the 90th to the 10th percentile values for each HCA were as follows: 20-fold for PhIP intake, ~30-fold for MeIQx intake, and > 110-fold for DiMeIQx intake. The study authors reported that a greater number of food and cooking combinations was necessary in all cohorts to explain 90% of the variance in MeIQx and DiMeIQx when compared to PhIP.

In a Swedish study (Augustsson *et al.* 1999a), information on food consumption was gathered by an extensive semiquantitative questionnaire containing 188 food items and photographs of fried meat. The number of yearly servings for all fried meat dishes was 301, corresponding to 0.8 consumption events per day. For fish, 142 yearly servings were reported, corresponding to 0.4 daily consumption events. In an earlier study from the same research group, Augustsson *et al.* (1997) summarized the mean daily intake of heterocyclic amines for 544 men and women from a group of 692 subjects randomly selected from a population register for Stockholm. The total intake of five HCAs, IQ, MeIQ, MeIQx, DiMeIQx, and PhIP, ranged from 0 to 1,816 ng per day, and the mean daily intake was 160 ng. When the daily intakes were divided into quintiles, the cut-off points between the quintiles for the HCAs were as follows: IQ (0.02, 0.09, 0.20, 0.55 ng/day), MeIQ (0, 0, 0, 0.01 ng/day), MeIQx (12.12, 25.37, 46.35, 98.89 ng/day), DiMeIQx (3.27, 6.84, 12.63, and 23.54 ng/day), PhIP (8.31, 19.66, 43.36, 105.09 ng/day), and total HCAs (24.77, 52.85, 104.15, and 241.28 ng/day). The HCA intakes in this Swedish population represent a skewed distribution, with only 20% of the population accounting for the highest intakes of HCAs, between 240 and 1800 ng/day. [Comparable data are not available for the U.S. population; thus, we do not know to what extent HCA intake in different sections of the United States has a skewed distribution.]

The Western diet, due to its abundance of meat cooked at temperatures above 200°C, is considered to contain the highest levels of HCA compounds (Felton *et al.* 1986, Robbana-Barnat *et al.* 1996). Several studies have described an increased risk for colorectal adenoma with the consumption of well-done/very well-done red meats (see Section 3).

Table 2-10. Mean (± SD) PhIP and MeIQx intake values (ng/day) in each cohort overall and by food type (g per day consumed)

NHS				
Food type	**Daily food consumption[a]**	**PhIP**	**MeIQx**	**DiMeIQx**
Chicken	21.2 ± 21.4	204.0 ± 370.0	6.0 ± 8.1	1.5 ± 3.7
Fish	8.1 ± 9.7	40.8 ± 56.8	nr	nr
Hamburger	10.8 ± 10.5	1.2 ± 12.9	9.9 ± 16.4	0.5 ± 0.8
Steak	7.6 ± 7.7	31.8 ± 39.3	7.9 ± 12.6	0.5 ± 1.4
Beef gravy	1.7 ± 2.7	2.7 ± 7.1	5.7 ± 12.1	0.7 ± 1.9
Bacon	0.6 ± 1.2	2.9 ± 7.3	1.7 ± 4.5	0.2 ± 0.5
Sausage	0.4 ± 0.6	0.02 ± 0.05	0.9 ± 1.6	nr
Overall	–	285.5 ± 426.5	33.0 ± 35.6	3.5 ± 5.1
NHS II				
Food type	**Daily food consumption**	**PhIP**	**MeIQx**	**DiMeIQx**
Chicken	22.8 ± 20.7	288.8 ± 514.9	7.7 ± 10.1	2.6 ± 4.9
Fish	6.3 ± 9.6	31.0 ± 57.1	nr	nr
Hamburger	13.8 ± 14.0	1.1 ± 7.8	15.4 ± 24.6	0.7 ± 0.9
Steak	7.9 ± 8.8	37.3 ± 46.5	7.9 ± 11.4	0.4 ± 0.9
Beef gravy	1.1 ± 1.9	1.5 ± 4.7	3.2 ± 8.0	0.4 ± 1.3
Bacon	0.5 ± 0.8	1.6 ± 3.1	1.0 ± 2.0	0.09 ± 0.2
Sausage	0.4 ± 0.8	0.02 ± 0.08	1.0 ± 2.6	nr
Overall	–	346.2 ± 524.9	36.0 ± 36.7	4.1 ± 5.2
HPFS				
Food type	**Daily food consumption.**	**PhIP**	**MeIQx**	**DiMeIQx**
Chicken	26.8 ± 25.8	313.8 ± 653.7	10.9 ± 17.2	1.8 ± 3.9
Fish	14.6 ± 14.4	69.8 ± 89.1	nr	nr
Hamburger	15.6 ± 15.5	5.8 ± 52.2	15.0 ± 25.6	0.9 ± 1.5
Steak	11.6 ± 13.0	62.7 ± 145.2	12.7 ± 21.9	0.7 ± 1.6
Beef gravy	1.3 ± 2.8	1.7 ± 9.4	3.9 ± 16.3	0.5 ± 2.5
Bacon	0.9 ± 1.6	2.7 ± 6.3	1.7 ± 2.9	0.1 ± 0.3
Sausage	0.8 ± 2.5	0.05 ± 0.2	1.9 ± 7.6	nr
Overall	–	457.9 ± 735.2	44.8 ± 53.7	4.0 ± 4.9

Source: Byrne *et al.* 1998.

nr = not reported

[a]The following amounts equal one serving of each food type: 141.75 g of chicken, steak, or roast; 113.4 g of fish or hamburger; 75 g of beef gravy; 26 g of sausage; 13 g of bacon.

2.7 Occupational exposure

Occupational exposure to PhIP and MeIQ may occur when workers prepare broiled or fried foods, such as chicken, beef, fish, or pork. Although the studies by Thiébaud *et al.* (1995) and Yang *et al*. (1998) did seem to suggest that cooks may be potentially exposed to higher levels of airborne HCAs than are individuals in other occupations (see section 2.4.4), no studies were found that researched PhIP, MeIQ, or MeIQx intake among workers who prepared or served broiled or fried foods.

2.8 Biological indices of exposure

Urinary PhIP metabolites appear to be the most widely used biological markers. Methods employing LC/MS/MS detection are a requirement because UV or fluorescence detection cannot be used due to the complexity of the urine extracts and the low levels of metabolites present. The four major urinary PhIP metabolites in humans are N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4'-PhIP-sulfate, and N^2-OH-PhIP-*N*3-glucuronide. Kulp *et al.* (2000) performed a study in which female volunteers consumed samples of cooked chicken. Urine analysis was performed to quantify the amount of PhIP metabolites excreted. PhIP dose in the urine as PhIP metabolites accounted for 4% to 53% of the ingested dose. The rate of metabolism varied among the volunteers, with the majority (62% to 85%) of the metabolites excreted in the first 12 hours (Kulp *et al.* 2000).

Ji *et al*. (1994) studied the urinary excretion of MeIQx among 47 African-American, 41 Asian-American (Chinese or Japanese), and 43 non-Hispanic white male residents of Los Angeles County. Significant interracial differences were observed. Mean levels of MeIQx excretion were 1.3- and 3.0-fold higher in African-Americans than in Asian-Americans and whites, respectively. Urinary levels of MeIQx were positively associated with intake frequencies of bacon, pork/ham, and sausage/luncheon meats among study subjects.

In a subsequent study, Kidd *et al*. (1999) utilized a similar study design and population to examine the urinary excretion of PhIP. Geometric mean levels of PhIP excretion were 2.8-fold higher in African- and Asian-Americans than in whites. However, unlike the previous study (Ji *et al.* 1994), the levels of urinary excretion were not associated with intake frequencies of any cooked meat.

While urinary PhIP metabolites are a good indicator of recent exposure, endogenous macromolecule adducts could provide more information about long-term exposure. Serum albumin and globin appear to be suitable biomarkers to assess dietary exposure and internal PhIP doses. LC/MS/MS analysis can provide very sensitive results (limit of detection is 0.2 fmol PhIP/mg protein) (Magagnotti *et al.* 2000).

The study by Magagnotti *et al.* (2000) of 35 volunteers with different dietary habits demonstrated that diet was a major factor in the formation of both PhIP-serum albumin and PhIP-globin adducts. The PhIP-blood protein adduct data summarized in Table 2-11 represent the integral value of repeated exposure over the lifespan of the blood proteins. These adduct levels were much higher (by a factor of 3 to 50) than those seen in volunteers administered a single dose of 70 to 84 μg radiolabeled PhIP (Dingley *et al.* 1999). Diet was a major factor in formation, with vegetarians having significantly lower

PhIP-serum albumin and globin adduct levels than meat consumers (serum albumin, P = 0.04; globin, P = 0.02) (see Table 2-11 for adduct values).

Table 2-11. PhIP-blood protein adduct levels (mean ± SD)

	fmol/mg protein (ng/g protein)	
Group	**PhIP-albumin**	**PhIP-globin**
Entire group	5.2 ± 1.3 (1.2 ± 0.3)	2.3 ± 0.6 (0.5 ± 0.1)
Smokers	5.3 ± 1.4 (1.2 ± 0.3)	2.9 ± 0.9 (0.7 ± 0.2)
Nonsmokers	5.0 ± 2.2 (1.1 ± 0.5)	1.6 ± 0.6 (0.4 ± 0.1)
Meat eaters	6.7 ± 1.6 (1.5 ± 0.4)	3.0 ± 0.8 (0.7 ± 0.2)
Vegetarians	0.7 ± 0.3 (0.2 ± 0.1)	0.3 ± 0.1 (0.1 ± 0.02)

Source: Magagnotti *et al.* 2000.

While the metabolism of PhIP is well characterized in animals (see Section 6), little is known about the metabolism of PhIP in humans. It has been postulated that N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N3-glucuronide represent activation pathways, whereas PhIP-N^2-glucuronide and 4'-PhIP-sulfate represent detoxification pathways (Kulp *et al.* 2000). Lynch *et al*. (1992) reported that 10 human volunteers who ingested PhIP, MeIQx, and DiMeIQx in fried beef patties excreted 1.1 ± 0.5% of PhIP and 2.1 ± 1.1% of MeIQx as unchanged parent compounds in the urine. Levels of DiMeIQx in urine, if present, were below the limit of detection of the assay (20 pg/mL).

For PhIP and MeIQx, the major DNA adduct formed *in vitro* is the *N*-(deoxyguanosin-8-yl) derivative. This C8-guanine adduct also has been identified *in vivo* in various tissues from rodents administered PhIP and from cynomolgus monkeys exposed to MeIQx and PhIP (Lynch *et al.* 1995).

For MeIQ, one single adduct, dG-C8-MeIQ, has been characterized *in vitro* by Tada *et al.* (1994). This adduct also was detected as a major adduct formed *in vivo* in mice treated with MeIQ.

Stillwell *et al*. (1999a, 1999b) evaluated the contribution of *N*-oxidation to the *in vivo* metabolism of MeIQx by analyzing the N^2-glucuronide conjugate of 2-hydroxy-MeIQx (*N*-OH-MeIQx-N^2-glucuronide) in human urine. Following consumption of a diet containing known amounts of MeIQx, *N*-OH-MeIQx-N^2-glucuronide was readily detectable in human urine (2.2% to 17.1% of dose) at levels as low as 10 pg/mL of urine when using 8 mL volumes of urine; further, the levels of excretion correlated with the ingested dose.

In one study, PhIP was found in 12 of 14 human hair samples, with concentrations ranging from 50 to 5,000 pg/g hair, while two other samples were below the limit of detection (50 pg/g hair) (Reistad *et al.* 1999). White/gray hair had an approximately 50% lower PhIP concentration than mixed pigmented, gray, and white hair samples, indicating that melanin participates in PhIP binding. While no dose-response relationship was found and an exposure assessment was not performed, PhIP could be reliably quantified,

leading to the possibility that human hair could be used as a biomarker for dietary exposure.

HCAs also were found in breast milk of healthy Canadian women (DeBruin *et al.* 2001). PhIP was detected in 9 of 11 milk samples using an SPE and LC/MS analysis. PhIP levels were as high as 0.059 ng/mL (59 ppt). No PhIP was detected in the breast milk of a vegetarian woman, with a limit of detection estimated to be 0.003 ng/mL (3 ppt).

2.9 Regulations

No PhIP-, MeIQ-, or MeIQx-specific regulations were found.

2.10 Summary

PhIP, MeIQ, and MeIQx are formed from free amino acids, creatine/creatinine, and hexoses during the cooking of meat and fish; these compounds are by-products of the Maillard reaction. Temperature, processing time, pH, precursor concentrations, and types of amino acid present have been reported to affect the formation of these compounds in food. In general, higher temperatures and longer cooking times increase the amount of HCAs produced. In addition, HCA formation increases with cooking methods that use direct or efficient transfer of heat from the source to the food; frying, baking, and barbecuing of muscle meats produce more HCAs than do stewing, steaming, and poaching. The major exposure to PhIP, MeIQ, and MeIQx is through the consumption of cooked meats, although HCAs also have been detected in other products such as processed food flavors, beverages (beer and wine), and cigarette smoke. Occupational exposure to HCAs may occur when employees work with broiled, grilled, or fried foods. Dietary exposures for HCAs have been estimated to range from < 1 to 17 ng/kg b.w. per day, wheras diets fed to mice in carcinogenesis bioassays may deliver doses exceeding 50 mg of HCAs per g body weight per day. DNA adducts have been observed, however, following dietary relevant doses of HCAs.

PhIP, the most abundant HCA detected in foods, has been detected in all the meats (beef, pork, chicken, and fish) commonly consumed in the United States. PhIP occurs at the highest concentration in grilled chicken and at very low concentrations in pork. Intake values of PhIP (ng/day) ranged from 285.5 to 457 in three large cohort studies from the United States (two Nurse's Health Studies and the Health Professionals Follow-up Study).

MeIQ is found at much lower levels in food than MeIQx and PhIP. The highest concentrations apparently occur in cooked fish with MeIQ concentrations ranging from 0.03 to 72 ng/g). Its overall occurrence in food (meat, fish, and gravy) was reported to be less than 1 ng/g in a Swedish population.

MeIQx has been detected in all the meats (beef, pork, chicken, and fish) commonly consumed in the United States. The highest levels appear to be in well-done grilled chicken and beef (hamburger or steak). Intake values of MeIQx (ng/day) ranged from 33 to 36 in three large cohort studies from the United States (two Nurse's Health Studies and the Health Professionals Follow-up Study).

3 Human Cancer Studies

HCAs are formed during cooking of meats and fish (see Section 2). The formation of HCAs is primarily dependent on high cooking temperatures and long cooking times, although the amount of HCA formed also varies with cooking method; HCAs are formed more readily by cooking methods that involve direct contact with the heating source, such as frying, grilling, and broiling. Cooking methods such as stewing, boiling, and baking are not typically associated with the formation of HCAs (Knize *et al.* 1999). Gravy and pan residues also contain high amounts of HCAs (Skog *et al.* 1995). The amount of HCAs formed during cooking can be reduced by certain methods of cooking preparations, such as marinating the meat before grilling (Knize *et al.* 1999). As discussed in Section 2, PhIP is the most abundant HCA found in cooked meat; MeIQ is found at much lower levels than MeIQx and PhIP. The amount of a specific HCA found in cooked meats and fish depends on the cooking conditions and type of meat (see Section 2).

The cancer literature used to evaluate the role of the three specific HCAs, PhIP, MeIQ, and MeIQx, consists of three categories of studies: (1) studies measuring consumption of meat; (2) studies measuring consumption of meat cooked under conditions associated with forming HCAs, such as high temperature, degree of browning, degree of doneness, and cooking method (e.g., grilling, barbecuing, and frying); and (3) studies measuring intake of the specific HCAs (PhIP, MeIQx, and MeIQ). This section briefly discusses recent reviews or evaluations of the first two categories of studies and focuses on a study-by-study evaluation of the third category because these studies provide the most specific information for the evaluation of potential cancer effects of PhIP, MeIQ, and MeIQx. Most of these studies have created databases in which individual HCAs have been measured in specific foods cooked by different methods and doneness. The consumption (quantitative) of those foods was measured by food frequency questionnaires. The amount of specific HCA was then calculated from the food frequency questionnaire and the database listing those HCAs' content in the food items. A food frequency questionnaire and database developed by Sinha *et al*. (1999) were used in four of the studies (see Section 2.6.1).

3.1 Meat, fish, and cancer

The World Cancer Research Fund (WCRF) in association with the American Institute for Cancer Research (AICR) published a report on diet and cancer (WCRF/AICR 1997). The report was based on a three-year review of more than 4,500 studies on diet and cancer The relationship between various food items or components and cancer at multiple sites was evaluated and discussed in detail. The panel then reached a consensus about the strength of the evidence (convincing, probable, possible, or insufficient) and the direction of the risk relationship (increasing, decreasing, or none) between cancer and various nutritional constituents or food items. Meat in this report was defined as red meat and essentially limited to beef, lamb and pork from farmed, domesticated cattle, sheep, and pigs in both fresh and preserved states. The panel judgments concerning meat, poultry, and fish were the following: (1) there is probable evidence that consumption of meat increases the risk of colon and rectal cancer; (2) there is possible evidence that

consumption of meat increases the risk of cancers of the pancreas, breast, prostate, and kidney; (3) there is possible evidence that there is no relationship between poultry consumption and breast cancer; and (4) there is possible evidence that there is no relationship between fish consumption and colon and rectal cancer (WCRF/AICR 1997). For many tumor sites, the panel did not make a judgment because of limited evidence or inconsistent results. The report reviewed studies published through the mid-1990s.

Other panels or review groups (the World Health Organization's Consensus statement on the role of nutrition in colon cancer and the Working Group on Diet and Cancer of the Committee on Medical Aspects of Food and Nutrition Policy) have supported the conclusions reached by the WCRF/AICR panel for colon cancer risk and meat (red or total meat) consumption. However, recent meta-analyses and reviews have reported somewhat conflicting or weaker evidence for the association between meat consumption and colon cancer and breast cancer. Sandhu *et al.* (2001) conducted a meta-analysis of prospective studies on meat consumption and colorectal cancer risk published through June 1999. They reported that an increase of 100 g in the daily intake of all meats or red meats was associated with a significant 12% to 17% increased risk of colorectal cancer. In their analysis, a broad definition of meat was used that included red meat, lamb, pork, and processed meats but excluded white meat such as poultry when possible. Norat *et al.* (2002) reported a weakly elevated relative risk of borderline significance for meat (including red and white meat) intake and colon cancer (RR = 1.14, 95% CI = 0.99 to 1.31). Higher and statistically significant elevated risks were observed for red meat consumption (RR = 1.35, 95% CI = 1.21 to 1.51). In contrast to the WCRF/AICR conclusion, Missmer *et al.* (2002) found no statistically significant association between breast cancer risk and total meat, red meat or white meat consumption in a pooled analysis of eight prospective cohorts from the United States, Canada, and Europe. The potential association between meat and cancer may be due to HCAs or PAHs that are formed when meats are cooked at high temperatures; or it may be attributed to other components of meat such as the protein itself, fat content, or production of *N*-nitroso compounds.

3.2 Cooking conditions (temperature, doneness, and methods) and cancer risk

The methods by which cooking conditions associated with HCAs were evaluated varied among studies. Some studies assessed consumption of meats cooked to different degrees of doneness or at high cooking temperature; other studies assessed consumption of meats cooked by different methods, such as grilling or pan frying; and still others evaluated consumption of specific foods associated with high HCAs, such as gravy or bacon. As discussed in Section 2.4.1, barbecuing is sometimes used interchangeably with grilling (cooking at high temperatures over direct heat), but barbecuing also can mean cooking at lower temperatures over indirect heat. The latter definition of barbecuing is probably not associated with the formation of high levels of HCAs since HCAs are produced at high temperatures. Most epidemiological studies did not specify the definition of barbecuing used in their study. The WCRF/AICR (1997) panel also reviewed the effects of cooking conditions and cancer risk and concluded the following: (1) there is possible evidence that grilling and barbecuing meats and fish increases the risk for stomach cancer; (2) there is possible evidence that cooking meat at high temperatures increases the risk of colon and rectal cancer; and (3) there is insufficient evidence that frying various foods

increases the risk of bladder cancer. This section on cooking conditions will briefly review the literature for cancer sites that have at least four studies available; these studies include esophageal, stomach, lung, bladder, colon, and breast. The approach will be to review the WCRF/AICR report and update the information using literature published since that report.

The WCRF/AICR's conclusion that cooking meat at high temperatures possibly increases the risk of colon cancer was based on three case-control studies reporting significantly elevated risks for colon cancer or adenomas and eating fried or well-done meats (Gerhardsson de Verdier and Longnecker 1992, Schiffman and Felton 1990, Probst-Hensch *et al.* 1997, as reviewed by WCRF/AICR report). However, a more recent review of nine case-control studies (including one reported by the WCRF/AICR)and three cohort studies on cooking methods concluded that the results were not consistent across studies. More consistent results were observed for preparation by smoking and barbecuing than for degree of meat doneness (Norat and Riboli 2001). Two of these studies or study populations reported in the review are described in Section 3.3.1 (Augustsson *et al.* 1999b, Sinha *et al.* 2001).

For breast cancer, the WCRF/AICR suggested that not enough data existed to make a judgment concerning cooking methods (frying, grilling/barbecuing, or degree of doneness) and cancer; and studies published since the WCRF/AICR review also are either conflicting or provide little evidence to reach a conclusion. The WCRF/AICR cited three studies (Phillips 1975, Knekt *et al.* 1994, Ronco *et al.* 1996, as cited in WCRF/AICR), which reported an increased risk of breast cancer and fried foods; however, one study did not adjust for fat intake (Phillips 1975, as cited in WCRF/AICR). Three studies of U.S. populations, the Iowa Women's Health Study (Zheng *et al.* 1998), the Nurse's Health Study (a prospective study) (Gertig *et al.* 1999), and a case-control study of New York women (Ambrosone *et al.* 1998) have been published since the WCRF/AICR report on cooking methods and breast cancer. Two studies that evaluated fried/broiled/grilled or barbecued foods did not find an association with these cooking methods (Zheng *et al.* 1998, Gertig *et al.* 1999). The Iowa Women's Health Study also evaluated doneness and reported an increased risk for both well-done foods as a category and some individual well-done foods (Zheng *et al.* 1998) (a later publication of this study population is described in Section 3.3.1. Consumption of food types associated with high HCAs, such as bacon and gravy, also was evaluated in two studies. The study of New York women reported elevated but non-significant risks for the highest intake of foods fried in bacon fat and for gravy consumption (increased risk for foods fried in bacon fat was only observed in postmenopausal women), but no dose-response pattern was observed; the authors did not consider consumption of either food to be associated with breast cancer. An elevated risk was observed for consumption of well-done bacon in the Iowa Women's Health study (Zheng *et al.* 1998).

For esophageal cancer, the WCRF/AICR (1997) also suggested that insufficient data existed to reach a conclusion concerning an association with cancer, citing three case-control studies that evaluated barbecuing of meat (Victora *et al.* 1987, De Stefani *et al.* 1990, Brown *et al.* 1988, as cited in WCRF/AICR). Two of these three studies reported an increased risk, and one case-control study that evaluated doneness reported a

nonsignificant increased risk (Ward *et al.* 1997, as cited in WCRF/AICR 1997). Since that report, an additional case-control study conducted in Uruguay has been published that reported a nonsignificant increased risk of esophageal cancer for certain cooking methods (fried, boiled, and broiled) and for consumption of bacon (De Stefani *et al.* 1999).

Studies on lung, bladder, and stomach cancer provide some evidence for an association between cooking methods that produce high HCA levels and cancer. The WCRF/AICR (1997) did not discuss any studies on cooking method and lung cancer. Current studies suggest that consumption of foods cooked by methods that generate high amounts of HCAs increases the risk of lung cancer. Three studies, the National Health Interview Survey Cohort study (Breslow *et al.* 2000), a hospital-based case-control study in Uruguay (De Stefani *et al.* 1997a, Deneo-Pellegrini *et al.* 1996), and a population-based case-control study of Missouri women (Sinha *et al.* 1998c) reported an increased risk for fried foods (fried chicken in the cohort study) and lung cancer. A dose-response relationship was observed in two of the studies that adjusted for energy and fat (Breslow *et al.* 2000, De Stefani *et al.* 1997a). Cooking methods also were evaluated in the two case-control studies, and both studies reported nonsignificantly elevated risks associated with broiled foods; however, elevated risks also were associated with boiled foods in the Uruguayan study. Consumption of grilled foods was associated with lung cancer risk in women from Missouri but was not measured in the Uruguayan study. Cooking temperature was evaluated in a case-control study conducted among Iowa women (Alavanja *et al.* 2001). Dose-response relationships were observed for red meat consumption cooked at both high and low temperatures, thus, they did not provide support for a link between HCAs and lung cancer. With respect to specific foods associated with high HCA intake, the National Health Interview Survey Cohort observed an increased risk associated with bacon consumption.

The WCRF/AICR (1997) panel reviewed one prospective (Chyou *et al.* 1993, as cited in WCRF/AICR) and three case-control studies on cooking methods and bladder cancer, all of which reported an increased risk from consumption of either fried foods or gravy (Steineck *et al.* 1990, Bruemmer *et al.* 1996, Vena *et al.* 1992, as cited in WCRF/AICR 1997). The panel concluded that fried foods may increase urinary bladder cancer risk but felt the evidence at that time was insufficient. A hospital-based case-control study from Uruguay, published after the panel report, did not observe an increased risk for consumption of fried foods but did observe a dose-response relationship for consumption of barbecued foods, with a three-fold risk observed for the highest intake category (Balbi *et al.* 2001).

The WCRF/AICR (1997) concluded that consumption of grilled (broiled) or barbecued meats possibly increases the risk of stomach cancer based on positive results in four of five studies reviewed (Kono *et al.* 1988, Lee *et al.* 1995, Kato *et al.* 1992, Wu-Williams *et al.* 1990, Ward *et al.* 1997, as cited in WCRF/AICR). The panel did not make a judgment concerning consumption of fried foods and stomach cancer, due to conflicting results observed in a review of 11 studies (for review, see WCRF/AICR). Since the WCRF/AICR study, De Stefani *et al.* (1998) reported a two-fold risk for consumption of barbecued meat (see Table 3.1 and Section 3.3.1). Besides containing HCAs, cooked

meats also can contain PAHs, which are products of pyrolysis and combustion of organic compounds and are formed at high temperatures (see Section 2). The amount of PAHs formed is strongly dependent on method of cooking, distance from the heat source, and fat content of the food (Knize *et al.* 1999). Kazerouni *et al*. (2001) found that the concentration of PAHs in foods was not as dependent on temperature as it was on cooking method, with the highest production occurring in foods cooked over an open flame; high concentrations of PAHs are not typically observed in fried foods.

3.3 Specific HCAs and cancer risk

Eight publications describing 11 case-control studies (one publication reported on four cancer sites) have calculated odds ratios (ORs) for individual HCAs (such as IQ, MeIQ, MeIQx, DiMeIQx, and PhIP) and cancer risk. These studies, including three studies on breast cancer, one study evaluating multiple cancers (colon, rectum, bladder, and kidney), and one study each on colorectal adenomas, lung cancer, and prostate cancer, are described below and in Table 3.1. The studies are organized by geographical location because dietary patterns and cooking practices vary geographically.

3.3.1 South America - Uruguay

Two hospital-based case-control studies on breast and gastric cancer were conducted in Uruguay. Dietary habits appear to be homogenous in Uruguay and are characterized by a high consumption of barbecued red meat (definition of barbecued not described by authors) and salted meats (De Stefani *et al.* 1998). The consumption of beef is approximately 14% of total dietary energy supply (WCRF/AICR 1997).

De Stefani *et al*. (1997b) conducted a hospital-based case-control study of 352 patients with breast cancer and 382 matched (age, gender, and residence) hospitalized controls from Uruguay. Patients and controls were interviewed and completed a food frequency questionnaire that queried the food intake and cooking methods (meats cooked by frying, broiling, and boiling) of 64 food items consumed two years prior to the development of symptoms. The food frequency questionnaire did not include portion size, which was determined by local practices, but did include a sufficient number of food items to calculate total energy intake. Total intake (ng/g) of specific HCAs (IQ, MeIQx, and PhIP) was calculated by multiplying the frequency of consumption of fried and broiled meats by the product of the portion size and the HCA content per 100 g for each fried and broiled meat according to published literature. That is, the content is not based on Uruguayan data.

Each food item (e.g., meat or red meat), cooking method (e.g., fried meat, boiled meat) and individual HCA (e.g., IQ, MeIQx, and PhIP) was categorized according to the quartile distribution of the population. The reference group for each category was the lowest quartile of exposure. The ORs were adjusted for age, residence, dietary factors (total energy intake, vegetable intake, and total fat intake), and risk factors for breast cancer (family history of breast cancer, age at menarche, parity, previous history of benign breast disease). A statistically significant positive dose-response relationship was observed for intake of total meat, red meat, fried meat, IQ, MeIQ, and PhIP, and a statistically significant negative dose-response relationship was observed for the intake of

white meat and broiled meats. ORs for the highest quartile of HCA intake were 3.3 (95% CI = 1.9 to 6.0) for IQ, 2.1 (95% CI = 1.3 to 3.6) for MeIQx and 2.6 (95% CI = 1.4 to 4.7) for PhIP. The authors also calculated risks stratified by menopausal status. Statistically significant dose-response relationships were observed for all three HCAs (analyzed separately) in post-menopausal women but not for premenopausal women. In premenopausal women, two-fold, nonsignificant risks were observed in all quartiles for IQ and MeIQx, and risks were close to null for PhIP.

Strengths of this study include an adequate number of cases and controls and multivariate analyses adjusting for other dietary factors, including energy and fat intake. A limitation of the study is the use of hospital controls. Other limitations were related to exposure assessment and potential of recall bias due to the case-control design. The study did not obtain any information on portion size, and it used published values of HCA content in prepared foods such as meats and fish rather than creating a database from locally prepared foods. It is unclear if this study disentangles the effects of HCAs from the effect of other constituents in meat.

De Stefani *et al.* (1998) conducted a population case-control study of gastric cancer in Uruguay that included 340 cases and 698 controls who were hospitalized patients without conditions related to digestive tract, nutritional disorders, or tobacco or alcohol consumption. Exposure to food items, such as meat, and to PhIP, was assessed similarly to that described for the Uruguayan breast cancer study described above, except the food frequency questionnaire was more limited and did not allow for the calculation of total energy intake. Risk estimates were calculated using both continuous and categorical models and were adjusted for matched variables (age, sex, and residence) and relevant confounders (urban/rural status, tobacco use, alcohol use, and maté drinking). PhIP was the only HCA for which intake was assessed.

Red meat, barbecued meat (definition not described by authors), salted meat, PhIP, and nitrosodimethylamine (NDMA) were statistically significantly associated with gastric cancer in the continuous analyses; the OR for PhIP was 1.9 (95% CI = 1.6 to 2.2). Intake of all these substances remained statistically significant in the multivariate analyses in which all foods, micronutrients, and related substances were adjusted for each other; the OR for PhIP was 1.5 (95% CI = 1.2 to 1.9). In the categorical analyses, a statistically significant dose-response relationship was observed for PhIP intake, with a three-fold risk observed for the highest quartile of exposure. The combined effects of NDMA and PhIP were also evaluated. Individuals who had high intake of both compounds (OR = 12.7) had a three- or four-fold higher risk than individuals with high exposure to only one compound (OR = 3.1, high NDMA; and OR = 4.4, high PhIP); this finding is suggestive of an interaction.

High consumption of salted, fried, or broiled red meat is common in Uruguay, a country that has a high incidence of gastric cancer. The authors stated that limitations of their study were related to the use of hospital controls, who may have altered their diet, and to the limitations of the food frequency questionnaire to assess exposure. The questionnaire had not been validated, and the limited number of items precluded calculation of total energy intake. The former may lead to misclassification, which generally would limit the

ability to detect an effect, while the latter may result in uncontrolled confounding. A strength of the study was sufficient power to detect an effect due to adequate population size and high exposure.

3.3.2 United States

Overall, meat consumption in the United States is one of the highest worldwide, making up approximately 14% to 15% of the total dietary energy supply (WCRF/AICR 1997). However, dietary habits in the United States are probably much more heterogeneous than those in Uruguay or Sweden (see below). U.S. diets vary geographically, and even in the same geographical locations a greater diversity of foods may be consumed than in Sweden or Uruguay. Moreover, the term barbecued can have different meanings in different geographical locations; in some regions it is used interchangeably with grilling, while in other regions it refers to cooking for long periods of time using indirect heat from charcoals or coals. The four studies in the United States were all population-based, case-control studies. Theyincluded two breast cancer studies, one from California and the other from Iowa; a colorectal adenoma study from Maryland; and a lung cancer study from Missouri.

Delfino *et al*. (2000) evaluated the relationship between HCAs and breast cancer in a case-control study from California. The study population consisted of 394 women > 39 years old who were scheduled for a biopsy because of a suspicious breast mass and who had completed a self-administered questionnaire, the Health Habits and History Questionnaire, which contained questions on 112 food items that account for > 90% of the intake of major dietary nutrients. After biopsy, 114 women were found to have malignant tumors (cases) and 280 had benign tumors (controls). Information about meat and gravy consumption was obtained from an interview using a questionnaire and color photographs (meat types and doneness levels) developed by Sinha *et al.* (1999) (see Section 2.6.1 for a description of that database). For this particular study, red meats included hamburger patty/cheeseburger, beefsteak, pork chops, bacon, and breakfast meat sausage and white meats included chicken, turkey, and fish. For each type of meat, the frequency of intake (10 frequency groups ranging from never to ≥ 2/day) of meats cooked by different cooking methods (e.g., pan-fried, grilled/barbecued, baked, etc.) was assessed. Intake of specific HCAs was estimated by the product of the intake of meat type estimated by the questionnaire and the corresponding concentration of the HCA in the database.

Risk estimates for intake (g/day) of meat, specific types of meat, specific types of meat cooked by various methods, and intake (ng/day) of specific HCAs were analyzed as continuous or categorical variables based on distribution in the controls; the lowest category was used as the reference group. Models were tested for potential risk factors that could confound or modify the effect, including dietary factors such as total energy intake, total fat, and total protein, fruits, and vegetable intake; a confounding effect was assumed if the estimate changed by at least 10%. Decreased ORs (ranging from 0.4 to 0.8) were observed for the highest category of intake of white meat, chicken (cooked by various methods), red meat, red meat cooked to different degrees of doneness, PhIP, MeIQx, and DiMeIQx with the ORs being statistically significant for variables related to

white meat and PhIP. Statistically significant negative dose-response relationships were observed for intake of white-meat related categories. The ORs observed for PhIP approached one after adjusting for chicken intake.

The patients in the California study were interviewed before diagnosis, thus giving the potential for minimization of recall, participation, and interviewer biases. Several study factors limited the power to detect an effect. The study size was small, and the population had a low level of red meat consumption. Moreover, meat intake was assessed during the previous year, which may not reflect diets in earlier life; the authors commented that meat consumption in California had changed in the last decade. The major concern of the study was the use of patients with benign disease as controls; the controls may have shared some of the same risk factors as breast cancer cases, thereby limiting the ability to detect an effect. Nevertheless, protective effects were observed for white meat after restricting the analyses to a subgroup of controls having a lower risk of benign breast disease based on histological criteria. “Low-risk” controls had nonproliferative disease, whereas “high-risk” controls had proliferative disease.

Sinha *et al.* (2000a) conducted a case-control study nested within the Iowa Women’s Health Study cohort of over 41,000 members. Cases (273) were women who developed breast cancer between 1992 and 1994, and controls (657) were randomly selected from among women who were cancer free and participated in the 1992 follow-up study. Exposure to HCAs was assessed after diagnosis of cancer (1995 to 1996) using the HCA database and the responses to the food frequency questionnaire using color photographs (see above). In this study, cooking methods were only queried for three types of meat--steak, hamburger, and bacon--and intake of HCAs was based only on those types of meats.

The ORs for HCAs (DiMeIQx, MeIQx, and PhIP) were calculated using both continuous models (increments of 10 ng/day) and categorical models (quintiles of exposure based on distribution in control population); both models were adjusted for age, dietary factors (total energy intake), and risk factors for breast cancer (family history of breast cancer, use of hormone replacement therapy, and waist-to-hip ratio). A statistically significant dose-response relationship was observed for PhIP intake and breast cancer risk with an almost two-fold risk in the highest quintile of intake (OR = 1.7, 95% CI = 1.1 to 2.8). Risks for exposure to MeIQx also increased with increasing intake (test for trend, P = 0.05), but the magnitudes of the ORs were lower (1.2 for the highest quintile) and not statistically significant. After adjusting for the intake of other HCAs, the test for trend approached unity for MeIQx, but it remained statistically significant for PhIP. Intake of DiMeIQx was not associated with breast cancer risk. In the continuous model that controlled for the presence of the other HCAs, a statistically significant elevated OR was observed for each 10 ng increment of PhIP. Risks for 10 ng increments of MeIQx and DiMeIQx were no longer elevated after adjusting for the other two HCAs. Consumption of well-done red meat had been previously reported to be associated with breast cancer in this study population (see Section 3.2) (Zheng *et al.* 1998). This association may be explained by HCA intake; the risk estimate for well-done meats decreased from 1.27 per 10 g of very well-done meat to 1.04 after adjustment for PhIP, whereas the risk estimate for 10 ng of PhIP did not decrease after adjusting for well-done red meat. Intake of

MeIQx was highly correlated to DiMeIQx (0.76) and PhIP (0.6), whereas the correlation between PhIP and DiMeIQx was weaker (0.4).

Advantages of this study (Sinha *et al.* 2000a) include a larger study population than the one reported by Delfino *et al*. (2000) and the use of statistical models in an attempt to separate effects of HCAs from red meat and individual HCAs from each other. Limitations include the use of only three meats to estimate HCA intake and possible recall bias due to retrospective assessment of exposure. However, the authors stated that similar results on total red meat intake were obtained in both the prospective (cohort) and case-control studies.

Sinha *et al.* (2000b) also conducted a population-based case-control study of lung cancer among Missouri women, using the same database on HCAs and the same food frequency questionnaire used for the breast cancer study. The study population consisted of 593 cases and 623 smoking and nonsmoking controls, who were randomly selected from driver's license files (ages 30 to 64) and from health care lists (ages 65 to 84). The ORs were calculated separately for individual HCAs using both categorical and continuous data and were adjusted for age, smoking, body mass index (BMI), calories, fat, fruit/fruit juices, vegetables, and education level. Additional analyses were performed to control for cooking practices and doneness levels.

High intake (90th percentile) of MeIQx was associated with a modest risk of lung cancer. The risk estimate remained statistically significant after adjusting for other HCAs and after adjusting for characteristics related to meat and was elevated but not statistically significant after adjusting for well-done red meat or fried red meat. Furthermore, a statistically significant dose-response relationship (test for trend, $P = 0.006$) was observed between intake level and cancer risk. A slightly elevated risk was observed for high intake of DiMeIQx, but no increase in risk was observed for high intake of PhIP. In the continuous analyses, a four-percent excess of risk was observed for each 10 ng/day increment of MeIQx. Stratified analysis according to smoking and carcinoma cell type also was executed. The elevated risk of MeIQx was not observed in heavy smokers (OR = 1.0, 95% CI = 0.7 to 1.5) and was higher in nonsmokers (OR = 3.6, 95% CI =1.3 to 10.4) than light smokers (OR = 2.1, 95% CI = 1.3 to 3.3). The test for interaction was statistically significant between nonsmokers and heavy smokers ($P = 0.04$) and was of borderline significance between light and the heavy smokers ($P = 0.09$). Higher risks were observed for squamous cell carcinoma and other histological types than for adenocarcinomas and small cell carcinomas.

Intake of HCAs was comparable to, but somewhat higher than, that observed in the colorectal adenoma study in Maryland. The mean intake (ng) per day in the study by Sinha *et al.* (2000b) was 4.1 for DiMeIQx, 64.0 for MeIQx, and 137.5 for PhIP. An earlier study of this population reported that consumption of well-done meat and consumption of fried red meat were associated with elevated risks for lung cancer. The authors proposed that MeIQx intake may partially explain the risk observed with fried or well-done meats but they also speculated that the excess risk may be due to an unidentified meat pyrolysis byproduct that is correlated with MeIQx intake.

Sinha *et al.* (2001) evaluated the relationship between HCAs and colorectal adenomas in a clinic case-control study consisting of 146 cases and 228 controls, who did not have colorectal adenomas at sigmoidoscopy. HCA intake was assessed using a food frequency questionnaire and the database described above. Mutagenic activity of extracts of meats cooked according to different degrees of doneness (same database as HCA content) was measured using *Salmonella typhimurium* assay. Intake of mutagenic activity was calculated from the food frequency questionnaire and the database containing the mutagenic activity measurements. The ORs and 95% confidence intervals were calculated for quintiles of exposure and for each 10-ng increment using a continuous model and were adjusted for age, gender, total caloric intake, fiber intake, reason for screening, physical activity level, smoking, and use of nonsteroidal anti-inflammatory drugs (NSAIDs). Additional analyses included adjustment for the other HCAs, mutagenic activity, and different types of meat groups.

High intake (i.e., the fifth quintile) of each of the HCAs (MeIQx, DiMeIQx, and PhIP) was associated with two to two and a half-fold increased risks of colorectal adenomas. Mutagenic activity increased the risk of adenomas by three-fold. Statistically significant dose-response relationships were observed for intake of MeIQx, DiMeIQx, and PhIP; however, after adjusting for the other two HCAs, the test for trend was only significant for MeIQx. Intake of each of the HCAs was correlated with each other, with higher correlations observed between DiMeIQx and MeIQx (Spearman correlation (r) = 0.74) than between PhIP and either MeIQx (r = 0.44) or DiMeIQx (r = 0.48). The risks for MeIQx remained significantly associated with risk after adjusting for red meat but not vice versa.

The mean intake of HCAs (ng/day) of the specific HCAs were 2.2 for DiMeIQx, 32.7 for MeIQx, and 109.7 for PhIP. The authors noted that limitations of the study included the exposure assessment, the lack of validation of the database using biomarkers for HCAs, and possible differential recall of exposure between cases and controls. However, the authors stated that the cases had adenomas rather than cancer, which may have potentially minimized this type of bias.

3.3.3 Europe - Sweden

Dietary surveys have estimated that the number of daily servings of meat is 1.4 in the Swedish population, and meat makes up approximately 10% of the total dietary energy supply (WCRF/AICR 1997, Augustsson *et al.* 1999a) The most common method of cooking is frying (61%); grilling of meats is relatively rare (4%). Meats may be fried at different temperatures for different lengths of time, resulting in varying degrees of browning or doneness. The top seven Swedish dishes are sausage, steak casserole, meatball, pork chops, pork belly, and ground beef patty. Gravy is often consumed with the meat dishes (Skog *et al.* 1995).

Augustsson *et al.* (1999b) conducted a population-based case-control study in Sweden that consisted of 352 cases of colon cancer, 249 cases of rectal cancer, 273 cases of bladder cancer, and 138 cases of kidney cancer as identified through the cancer registry. Age- and gender-matched controls were randomly selected from the population registry. Subjects completed a food frequency questionnaire (188 food items) and were shown

color photographs of six dishes, each fried at four different temperatures, resulting in different degrees of browning; these photographs corresponded to a database of HCA content in each of the dishes. Intake of HCAs was assessed by linking the questionnaire to the HCA database and to the Swedish National Food Administration's food-composition database. The HCA database contained information on the HCA content in 22 dishes with corresponding pan residues (15 fried meat dishes, 3 roasted meat dishes, 2 fried fish dishes, fried eggs, and black pudding). Meat, fish, and HCA intake were categorized into quintiles based on the distribution in the controls; the lowest quintile was used as the reference group for calculating risk. The ORs were adjusted for age, sex, and smoking (bladder and kidney cancer only). Adjusting for other potential confounding factors (fat, protein, dietary fiber, vegetables, fruits, and physical activity) had little or no effect on the risk estimates. Additional analysis included a multivariate model in which nutrient density was calculated by dividing the exposure of interest (meat, fish, and HCAs) by the total energy intake, but this adjustment also had little effect on risk estimates.

Total meat and fish intake was associated with increased risks (ORs = 1.4 to 1.9) of colon, rectal, and bladder cancers, albeit the risks were generally observed for quintiles 2 to 4 rather than the highest exposure category (fifth quintile). Intake of total HCAs was associated with decreased risks of colon and rectal cancer and an increased risk for bladder cancer; intake had little effect on the risk for kidney cancer. Seven cases (four colon, two bladder, and one kidney cancer), but no controls, had a very high intake of total HCAs (> 1,900 ng). Risks also were calculated for individual HCAs. Because of the lower occurrence of MeIQ in food, risk estimates were only calculated for one category of exposure. The ORs for MeIQ intake were slightly elevated for colon and rectal cancer but were null or close to null for bladder and kidney cancer. Further, intake of the other specific HCAs was not associated with colon, rectal, bladder, or kidney cancer; in general, ORs were either close to one or were inconsistently increased or decreased across quintiles. However, for colon cancer, nonsignificant decreases in ORs were observed for the intake of MeIQx, DiMeIQx, and PhIP, which is consistent with the findings for total HCAs.

A strength of the study was the assessment of HCAs using dishes cooked according to local practices. The failure to detect an association with HCAs may be due to limited power because of the low intake of HCAs in this study population (median = 1 ng/kg of body weight, 77 ng/day in controls, 63 to 96 ng/day in cases). Moreover, all individuals, although there were only seven, with the highest estimated intake of HCAs had cancer.

3.3.4 New Zealand

Norrish *et al*. (1999) studied the effects of HCA intake on prostate cancer risk in a population-based case-control study of 317 patients and 480 age-matched controls in New Zealand. Participants completed a 107 food-item food frequency questionnaire, which queried food frequency, portion size, usual cooking methods (cooked in liquid, microwaved, or baked, fried, grilled, or barbecued) and doneness for seven commonly consumed types of meats, including beefsteaks, lamb/mutton chops, pork, minced beef, chicken, bacon, and sausage. HCAs were measured in each meat type, which were

obtained from local sources, and fried at 200°C until different target temperatures were reached. Subjects were assigned to one of three categories of doneness for each of the seven meat types: low (no consumption, cooked rare or baked/cooked in liquid/microwaved), medium (fried, grilled, or barbecued to medium brown) and high (fried, grilled, or barbecued to well-done). Combined effect from all meat types was assessed by assigning a score to the individual meats based on doneness and then summing the scores for each meat type. HCA (individual and total) concentrations (ng/g of meat) were assigned to the doneness categories based on the laboratory analyses of the cooked meats, as discussed above. HCA intake (ng/day) was calculated by multiplying the frequency of consumption (obtained from the food frequency questionnaire) by the estimated HCA content for the usual meat cooking method.

Risk estimates were calculated for quartiles of exposure based on the distribution of intake in the control groups and using the lowest exposure category as the reference group. Risk estimates were adjusted for age, potentially confounding nutrients, socioeconomic status, and energy consumption. For the analyses of risks for individual foods, meat-doneness was associated for some, but not all, of the seven meat types. Elevated risks were observed for medium and well-done beefsteaks, with higher and statistically significant risks observed for the latter, and for well-done chicken. However, when all meats were considered together, doneness was not associated with prostate cancer risk. High intake (fourth quartile) of total HCAs, MeIQx, and PhIP was not associated with prostate cancer risk. Elevated but nonsignificant risks were observed for high intake of DiMeIQx and another HCA, 2-amino-1,6 dimethylfuro[3,2-*e*]imidazo[4,5-*b*] pyridine (IFP).

In the New Zealand study, the median intake (ng/day) of total HCAs was 146 ng/day, which is higher than that of the Swedish study (discussed above) but lower than other estimates. The authors stated several limitations of the study, including moderate response rates (71% for controls 77% for cases), misclassification of self-reported dietary intake, imprecision of the database, and unrecognized confounding. Many of these limitations are inherent in most dietary case-control studies. An advantage of this study was the assessment of dietary patterns before disease diagnoses, which would have attenuated any recall bias.

3.4 Genetic susceptibility

HCAs are thought to be activated by *N*-oxidation by the liver CYP1A2 enzyme and to undergo further *O*-acetylation either in the liver or the target tissue by *N*-acetyltransferase (NAT2). This activation results in the formation of the arylamine-DNA adduct and, thus may contribute to the mutagenicity and carcinogenicity of HCAs (see Sections 5.1 and 6.2.2). Activity of these metabolic enzymes varies in the general population and is thought to be determined by polymorphisms in these genes (Sinha and Potter 1997); therefore, individuals with genes resulting in increased metabolic activation may be at a greater risk for any cancer effects of HCAs.

The impact of genetic variation in these metabolic enzymes on modification of the cancer effects caused by HCAs has been evaluated in several studies on colon and breast cancer. Studies on breast cancer have assessed the role of polymorphisms in the *NAT2* and

s*ulfotransferase (SULT) 1A1* genes. Polymorphisms in the *NAT2* gene locus have been identified that result in decreased *N*-acetyltransferase activity. Rapid *O*-acetylation in the target tissue of HCA metabolites is hypothesized to lead to an increased risk of breast cancer. However, most studies evaluating the *NAT2* polymorphism did not find an increased risk for HCAs (PhIP, MeIQx, or DiMeIQx) (Delfino *et al.* 2000) or surrogates for HCAs such as gravy or bacon (Ambrosone *et al.* 1998) or charred meat (Gertig *et al.* 1999) among individuals who were rapid acetylators. These studies also reported no association with HCAs or surrogates of HCAs, whereas studies from the Iowa Women's Heath cohort, which observed a positive association (Sinha *et al.* 2000a, Zheng *et al.* 1998), reported that the risk from the consumption of well-done meat was higher in women with higher metabolic enzyme activity. Postmenopausal women who were *NAT2* rapid acetylators or had the *SULT 1A1 Arg/Arg* genotypes were at a greater risk than those with lower enzyme activities (Deitz *et al.* 2000, Zheng *et al.* 2001). *SULT 1A1* is involved in both detoxification (sulfonate conjugation) and bioactivation reactions; the *Arg/Arg* genotype is thought to be associated with higher bioactivation of procarcinogens than the *His/His* genotype.

The evidence for genetic susceptibility appears to be stronger for colon cancer. Several studies have reported some evidence to suggest that the risks from high intake of meat or fried or well-done meat are higher in fast or presumed fast acetylators (NAT1 and or NAT2) (reviewed by Kampman *et al.* 1999, Roberts-Thomson *et al.* 1999) or in rapid NAT2 (genotypes) and CYP1A2 phenotypes (Lang *et al.* 1986, as cited by Roberts-Thomson 1999, Le Marchand *et al.* 2001). Ishibe *et al*. (2002) reported that intake of MeIQx was associated with a higher risk for colorectal adenoma in individuals who were rapid NAT1 acetylators than in those who were slow acetylators (same study population as Sinha *et al*. [2001] discussed above). Identification of individuals who are more susceptible to the carcinogenic effects of HCAs would help to increase the power to detect effects; negative studies may not be able to detect effects because risks are only elevated for a subset of the population.

3.5 Summary of the findings for individual HCAs

3.5.1 PhIP

Section 3.3 describes eight publications that evaluated cancer effects for PhIP for 11 case-control studies (the Swedish study reported results for four cancer sites). Four of these studies reported positive, statistically significant risk associations between intake of PhIP and various cancers (two studies on breast and one study each on colon adenoma and gastric), all of which showed a dose-response relationship. The association between PhIP and colon adenomas was no longer statistically significant after controlling for MeIQx and DiMeIQx. One study reported a weakly elevated but nonsignificant risk for bladder cancer, and three studies reported no association with kidney, lung, and prostate cancer (one study for each site). Protective effects were observed in three studies, for breast, colon, and rectal cancer (one study for each site), although the protective effect for breast cancer among California women was shown to be confounded by chicken consumption. In general, the studies that showed protection were in populations reported to have a low consumption of HCAs and red and grilled meats (i.e., the breast cancer study in California women and the Swedish study of colon and rectal cancer). In the

Swedish study, four colon cases but no controls had very high intake of HCAs. As will be discussed in Section 4, PhIP induces mammary, colon, and prostate tumors and lymphomas in experimental animals. Three of these tumor sites have been studied in humans; the results show conflicting evidence for breast and colon cancer (or adenomas) and inadequate evidence for prostate cancer.

3.5.2 MeIQ and MeIQx

MeIQ is found at low concentrations in meats (see Section 2); only the Swedish study reported risk estimates and only for one level of exposure. The ORs for MeIQ intake were modestly elevated for rectal and colon cancers but were null or close to null for bladder and kidney cancers. MeIQ induces colon, liver, forestomach, skin, mammary, Zymbal gland, and oral cavity tumors in experimental animals (see Section 4). Risk estimates for intake of MeIQx were reported for nine studies. Positive associations were reported in four studies (two studies on breast cancer, colon adenomas, and lung cancer), three of which were statistically significant (ORs in the Iowa breast cancer were not statistically significant, and the test for trend approached unity after controlling for PhIP intake) and two of which showed a dose-response relationship (Uruguayan breast cancer and Missouri lung cancer studies). MeIQx intake was not associated with cancer risk in two studies (one each on bladder and kidney cancer) and was protective but not significantly so in three studies (one each on breast, colon, and rectal cancers). The studies that reported protective effects (Swedish and California women) were based on study populations that have low HCAs or red meat intake and reported a protective effect for PhIP. MeIQx induces lymphomas, leukemias, and tumors of the liver, lung, gastrointestinal system, Zymbal gland, and skin in experimental animals. Of the tumor sites studied to date in humans, there is concordance with the lung.

3.6 Discussion: Issues related to the study designs

The inconsistency observed among the studies evaluating cancer risks related to exposure to MeIQ, MeIQx, and PhIP may be explained in part by differences in the tumor sites studied, study population, and study designs. Some of the problems inherent to dietary studies, such as imprecision in measuring exposure and limited power due to low exposure, would more likely bias toward the null hypothesis; however, other problems such as recall bias and uncontrolled confounding may lead to a false positive.

3.6.1 Power considerations

The role of specific HCA intake in cancer risk was evaluated in eight study populations, which varied in size, selection of subjects, geographical location, and level of exposure. These characteristics can affect the power of a study to detect an effect. Most of the studies had a moderate sample size (greater than 200 cases) except for the Maryland colorectal adenoma study (146 cases), the California breast cancer study (114 cases) and the Swedish kidney study (138 cases). Small sample size could limit the power to detect an effect and limit further analyses, such as multivariate analyses that attempt to separate the effects of individual HCAs from each other or from well-done or grilled meat. Moreover, further stratification by categories of HCA intake could result in small sample size in each stratum and unstable risk estimates. Nevertheless, small sample size probably did not greatly affect the conclusions of the previously mentioned studies. Statistically

significant, positive associations and statistically significant, positive dose-response relations were observed for PhIP intake and colorectal adenomas (Maryland study). The ORs for PhIP and MeIQx intake appeared to be stable across exposure strata (decreasing risk with increasing exposure) in the breast cancer study. Small numbers may have explained the inconsistent risk estimates observed across quintiles of MeIQx intake and kidney cancer.

Another characteristic that may limit detection of an association is low levels of exposure (HCA intake). High consumption of fried or broiled red meats was reported for the two Uruguayan studies (breast and gastric cancer); both studies reported positive associations with PhIP intake, and one study (breast cancer) reported a positive association with MeIQx intake (MeIQx intake was not reported in the gastric study). In contrast, two studies (Swedish and Californian breast cancer) reported that HCA intake or red meat consumption was low in their study population. The California population had a low intake of red meats and of grilled or barbecued red or white meats.

3.6.2 Selection of cases and control

In general, a study base comprises the person-time the investigators attempt to describe. In a case-control study, one typically tries to obtain information from all cases with a newly detected disease and a representative sample of the study base control (controls). In a population-based study, such a representative sample can often be attained; however, in a hospital-based study, investigators are less certain of the study base, which compromises the usefulness of the controls. Seven case-control studies were population based (Missouri lung cancer, the four Swedish studies, Iowa Women's Health Study of breast cancer, and New Zealand prostate cancer), two were hospital based (both Uruguayan studies), and two were clinic based (Maryland colon adenoma and the California breast cancer). In the clinic-based studies, controls were subjects who did not have disease, based on the diagnostic procedure (sigmoidoscopy or biopsy). Hospital patients may have changed their diets as a result of hospitalization. One concern about the California study is that the controls had benign breast disease; thus, if HCAs were a risk factor for benign breast disease, the risk would probably not be detected in this study.

3.6.3 Measurement errors: misclassification

Measuring errors, that is, incorrect measurement of HCA intake, may result in misclassification. Misclassification of self-reported dietary intake is a concern with food frequency questionnaires. Important components of the methodology used to measure HCA intake include the measurement of consumption of foods containing HCAs and potential confounders using the food frequency questionnaire, the use of color photographs to assess typical cooking methods, the database that contains HCA concentration of foods cooked by various methods, whether the diet was assessed retrospectively, and the dietary period that was assessed. Measuring errors in these components could contribute to misclassification. Misclassification also can result from latency considerations, that is, the time needed for cancer to develop after exposure. Most of these studies have evaluated only recent exposure (last five years), assuming that it is representative of past exposure, whereas the critical exposure for cancer induction may have occurred prior to the period of measurement.

3.6.3.1 Nondifferential misclassification

Nondifferential misclassification occurs when the study subjects are assigned to the wrong exposure category (HCA intake) due to measuring errors that affect cases and controls equally. This type of error is probably related to the accuracy of the food frequency questionnaire and HCA database. All of the studies discussed in Section 3.3 used an food frequency questionnaire that queried food intake, including cooking methods, and calculated the intake of specific HCA by multiplying the HCA content for specific meats cooked to a certain degree of doneness by the frequency of consumption of that meat. Most studies used color photographs to assess cooking methods and doneness. Four of the studies, all of U.S. populations, used the same database of HCA content and food frequency questionnaire that were developed by Sinha *et al*. (1999), although the number of food items in the database varied across studies. The Swedish and New Zealand studies (prostate cancer) developed their own databases of HCA content in meats cooked according to local practices. The two Uruguayan studies did not develop their own databases of HCA content and used reported values from the literature. They also did not query portion size but used portion sizes typical of local dietary practices. Both of these features--the use of reported values and standardization of portion size--would lead to less precise measurements and probably greater misclassification of exposure, which is more likely to bias toward the null hypothesis. Nevertheless, both studies reported a positive association between an individual HCA and cancer risk.

The databases that were prepared varied in the number of meat items used to calculate HCA exposure. Most studies used both white and red meats to assess HCA content. The Iowa Women's Health breast cancer study, which reported a positive association between breast cancer and PhIP intake, only used three meats, all of them red (beefsteak, hamburger, and bacon), to estimate PhIP intake. High concentrations of PhIP have been found in well-done chicken (grilled or fried) (see Section 2); therefore, it is unclear how the inclusion of white meats would have affected the risk estimates for PhIP.

The use of color photographs of meats cooked by various methods and to varying degrees of doneness has facilitated the evaluation of individual HCAs; these photographs correlate with the specific HCA content that has been measured in those foods. However, the database and food frequency questionnaire still have limitations for exposure assessment. Besides having a limited number of food items, the questionnaires did not query about other cooking techniques, such as marinating of meats, that can greatly modify MeIQx and PhIP levels in foods (Nerurkar *et al.* 1999, Salmon *et al.* 1997). Also, none of the databases have been validated using biomarkers. Most of these limitations are related to imprecise exposure assessment, resulting in possible misclassification and generally biasing toward the null hypothesis.

The other concern of most dietary studies is that they are surveying recent dietary patterns (1 to 5 years), whereas past dietary habits may be more important due to the long latency of cancer. Dietary patterns do change over time, especially as the authors cited in the Californian study, where changes in meat consumption had occurred.

3.6.3.2 Differential misclassification

Differential misclassification can occur when there is a different quality of exposure information for controls than for cases; thus, errors in classification would differ for cases and controls. A type of differential misclassification is recall bias, which can occur when exposure is assessed retrospectively, as was done in most of the studies discussed in this section. Individuals who have been diagnosed with cancer may remember their dietary habits differently than those without cancer. The two studies that assessed exposure before cancer diagnoses were the California breast cancer study and the New Zealand prostate cancer study, in which 60% of the men were interviewed before completion of diagnosis. The California study showed a protective effect for PhIP and MeIQx intake, whereas the New Zealand study was null for both HCAs.

3.6.4 Confounding

Most studies used multivariate analyses that controlled or evaluated any effects from age, gender, potential confounders for that particular cancer, and dietary factors, including energy intake. The Uruguayan gastric case-control study did not control for energy intake due to the limitations of the number of food items in the food frequency questionnaire. Due to the high consumption of salted meats, the Uruguayan studies also were complicated by the presence of nitrosamines. A few studies attempted to separate the effects of individual HCAs from each other or from the consumption of meat or well-done meats. The Swedish studies reported positive associations between total meat and fish intake and colon and bladder cancer; however, negative associations between total HCAs, PhIP, and MeIQx and colon cancer suggested that other components of meat may be responsible for the carcinogenic effects. In contrast, the Maryland study on colorectal adenomas suggested that the risks observed from red meats were due to MeIQx intake; the risks for MeIQx intake remained statistically significant after adjusting for red meat or for the other HCAs, whereas risk for red meat was no longer statistically significant after adjusting for MeIQx. Similar relationships were observed between MeIQx and lung cancer among Missouri women and between PhIP intake and breast cancer among Iowa women. For the breast cancer study, the risk for PhIP was still statistically significant after adjusting for well-done red meats, whereas the association between MeIQx and breast cancer was no longer statistically significant. A few studies--the Iowa breast cancer study, the Missouri lung cancer study, and the Maryland colon adenoma study--also have tried to separate the effects of individual HCAs. The association between MeIQx and lung and colon adenomas and the association between PhIP and breast cancer were still statistically significant after adjusting for other HCAs, whereas the association between PhIP and colon adenomas was no longer statistically significant. However, these types of analyses are difficult to evaluate because intakes of individual HCAs are correlated with each other.

3.7 Summary

IARC reviewed the carcinogenicity of several HCAs, including MeIQ, MeIQx, and PhIP, in 1993 (IARC 1993d). At that time no studies on the evaluation of individual HCAs existed, but there were some studies on cooking methods that produced HCAs. IARC concluded that “the available evidence was insufficient to establish whether cooking methods that result in the formation of HCAs are a risk factor for cancer independent of

the food item itself." Since the IARC review, the literature has expanded rapidly. Several studies have reported risk estimates for specific HCAs, and a few studies have attempted to separate effects of HCAs from the food items; however, the literature is still difficult to evaluate.

Some evidence suggests that cooking methods that produce HCAs may be associated with cancer risk. As discussed in Section 3.2, cooking methods that produce high amounts of HCAs are possibly associated with an increased risk for lung, stomach, bladder, colon, and breast cancer, although the evidence for colon and breast cancer is less consistent. Some site concordance is apparent with studies in experimental animals; both MeIQ and PhIP induce colon cancer, PhIP induces mammary cancer, and MeIQx induces lung cancer. Moreover, HCAs are highly mutagenic (see Section 5), providing evidence of biological plausibility. Although the studies on cooking methods provide support for positive findings observed in some studies on individual HCAs and well-done and grilled meat, they are confounded by coexposure to other potential carcinogens, such as PAHs.

With respect to the evaluation of MeIQx, MeIQ, and PhIP, the current evidence is difficult to evaluate. Some evidence indicates that PhIP increases breast and gastric cancer risk and that MeIQx increases the risk of colon adenoma, lung cancer, and possibly breast cancer. Studies reporting positive associations were based in populations with higher intake of HCAs. Moreover, limited evidence suggests that risk of colon cancer may be increased in genetically susceptible individuals, particular in the presence of higher exposure. Nevertheless, the evidence is based either on only one study for a particular tumor site or is inconsistent across studies for tumor sites for which more than one study is available. The inconsistencies observed in the breast and colon cancer studies may be explained by different levels of exposure in the study populations, but at the present time that is still difficult to conclude. The literature on MeIQ is very sparse due to the low occurrence of this HCA in meats, thus precluding any conclusions on its carcinogenicity in humans. Individual HCAs are highly correlated with each other and with the many constituents in cooked meats, including protein, animal fat, nitrosamines, and agents other than HCAs formed during cooking, such as polycyclic aromatic hydrocarbons, which makes it difficult to separate the effects of HCAs from the effects of cooked meats. Also, overcontrolling by including several highly correlated variables in one model, as was done in several studies, may make it difficult to observe an association. .

In conclusion, although evidence exists to suggest that consumption of well-done or grilled meats may be associated with increased cancer risk, the available data do not provide sufficient evidence to conclude that this risk is due specifically to PhIP, MeIQx, or MeIQ present within these foods.

Table 3-1. Recent case-control studies

Reference	Cancer site	Population	Exposure	Effects	Comments
De Stefani *et al.* 1997b Uruguay Uruguayan breast cancer study	breast cancer	hospital-based case-control study cases: 352 patients diagnosed with breast cancer controls: 382 patients hospitalized for non-neoplastic diseases and matched for age, gender, and residence	Patients were interviewed and completed a food frequency questionnaire, which queried food intake and cooking methods over 2 years prior to interview or symptoms. Portion sizes were determined according to local practices. HCAs were calculated as [frequency of consumption of fried and broiled meats] x [portion size] x [HCA content for each fried and broiled meat according to literature data]/100 g. HCA intakes were categorized according to quartile distribution in cases and controls.	Adjusted ORs for breast cancer – for the 4th quartile vs. 1st quartile <u>Meat variable and cooking methods for all subjects</u> Total meat 2.3 (1.2–4.1)* Red meat 2.6 (1.4–4.9)* White meat 0.6 (0.4–1.0)* Fried meat 2.7 (1.6–4.6)* Broiled meat 1.6 (1.0–2.6) Boiled meat 0.4 (0.2–0.8)* <u>HCA - all subjects</u> IQ 3.3 (1.9–6.0)* MeIQx 2.1 (1.3–3.6)* PhIP 2.6 (1.4–4.7)* Premenopausal IQ 2.2 (0.6–8.1) MeIQx 2.0 (0.6–6.4) PhIP 1.2 (0.3–4.0) Postmenopausal IQ 3.8 (1.9–7.6)* MeIQx 2.1 (1.2–3.9)* PhIP 3.3 (1.6–6.9)* * test for trend significant ($P < 0.05$)	ORs were adjusted for age, residence, family history of breast cancer, age at menarche, parity, previous history of benign breast disease, total energy intake, vegetable intake, and total fat intake. <u>Limitations</u> failure to obtain information on portions estimates of HCAs not very complete recall bias due to retrospective design use of hospital controls

Reference	Cancer site	Population	Exposure	Effects	Comments
De Stefani *et al.* 1998 Uruguay Uruguayan gastric cancer study	gastric cancer	hospital-based case-control study (1993–1996) cases: 340 newly diagnosed and confirmed cases at four major hospitals controls: 698 hospital patients without conditions related to digestive tract, nutritional disorders, tobacco or alcohol consumption	Patients completed an interview and a limited food frequency questionnaire that included meat types and cooking methods but precluded calculation of total energy intake. PhIP intake was computed by multiplying [the frequency of consumption of fried and broiled meat] x [standard portion size] x [the PhIP content for each fried food according to the literature data]/100 g.	<u>Continuous analyses-</u> Risk for changes per unit of the variable Red meat 1.4 (1.2–1.7) Barbecue 2.0 (1.7–2.5) PhIP 1.9 (1.6–2.2) <u>Categorical analyses/PhIP</u> Dose/response, $P < 0.001$ highest quintile 3.9 (2.3–6.4) <u>Interaction with NDMA</u> NDMA PhIP low low 1.0 (ref) high low 3.1 (1.9–5.0) low high 4.4 (2.7–7.1) high high 12.7 (7.7–21.2)	ORs were adjusted for matched variables (age, sex, residence) and relevant confounders (urban/rural status, tobacco, alcohol consumption, and maté drinking). Effect modification was tested. High consumption of fried or broiled red meats. <u>Limitations</u> possibility that hospital controls had changed diet patterns not adjusted for total energy intake food frequency questionnaire not validated HCA content obtained from literature <u>Strengths</u> high participation rate (94% cases, 92% controls) hospital controls may have reduced recall bias.

Reference	Cancer site	Population	Exposure	Effects	Comments
Delfino *et al.* 2000 California, USA California breast cancer study	breast cancer	case-control study population: women >39 years with a suspicious breast mass scheduled for a biopsy and who completed a questionnaire prior to the biopsy date cases: 114 who had malignant tumors controls: 280 with benign tumors	Subjects completed a self-administered questionnaire and were interviewed about meat intake using a questionnaire and photographs developed by Sinha *et al.* Red meats included hamburger patty/cheeseburger, beefsteak, pork chops, bacon, and sausage. White meats included chicken, turkey and fish. A database of PhIP and other HCA concentrations for meats cooked to different degrees of doneness/technique was used. HCAs were estimated from the food frequency questionnaire and data-base. Gram consumption was measured for each meat type by cooking technique and doneness. HCA intake was assessed by multiplying grams of meat by HCA measured for each cooking technique/doneness level for that meat and summed across all meats	Adjusted ORs for breast cancer 4th vs. 1st quartile White meat > 67 g/day 0.5 (0.2–0.9)* Red meat > 15 g/day 0.6 (0.3–1.0) Red meat, very well-done > 6.7 g/day 0.6 (0.3–1.1)** PhIP > 240 ng/day 0.4 (0.2–0.9)+ MeIQx > 22.5 ng/day 0.7 (0.3–1.3) DiMeIQx > 2.5 ng/day 0.5 (0.3–1.1) * test for trend 0.02 ** test for trend 0.065 + results for PhIP confounded by chicken No interaction was observed between N-acetyltranferase 2 (NAT2) genetic polymorphism and meat or HCAs.	Recall, participation, and interviewer biases were reduced due to the interviewing of patients before diagnosis. Small sample size limited the power to detect an effect and especially to detect gene-environment interactions Controls had benign breast disease, which may share some of the same risk factors as breast cancer. Population had a low intake of red meat, and meat intake for the last year was assessed, which may not reflect diets earlier in life. For cases, median g/day of red meat was 6.6 and white meat was 31.5, with 0 for grilled red meat or grilled chicken.

Reference	Cancer site	Population	Exposure	Effects	Comments
Sinha *et al.* 2000a Iowa, USA Iowa Women's Health breast cancer study	breast cancer	case-control study Population was a subset of women participating in the Iowa Women's Health study cohort (N = 41,836) but exposure was assessed retrospectively cases: 273 women with breast cancer diagnosed from 1992 to 1994 controls: 657 free of cancer, who participated in the 1992 follow-up survey	Subjects completed a self-administered food frequency questionnaire and interviewer-administered meat cooking module, which consisted of color photographs of hamburger patty, beefsteak and bacon cooked at increasing levels of doneness. Subjects were interviewed about cooking methods after diagnosis of cancer (1995 to 1996) HCAs were calculated as described above (Delfino *et al.* 2000) using the database developed by Sinha *et al.*	Adjusted ORs for breast cancer, Categorical analyses: fifth quintile (ng/d) DiMeIQx (2.42–30.84) 1.0 (0.7–1.7) MeIQx (35.9–204.5) 1.2 (0.8–2.0) * PhIP (55.8–523.1) 1.7 (1.1–2.8) ** * test for trend, $P = 0.05$ ** test for trend, $P < 0.001$, still significant after adjusting for other HCAs Continuous analyses per 10-ng increments DiMeIQx 1.32 (0.93–1.86) MeIQx 1.03 (1.00–1.07) PhIP 1.03 (1.01–1.05)* *OR remained significant after adjusting for other HCAs or adjusting for well-done red meat; however, OR for very well-done red meat decreased after adjusting for PhIP	ORs were adjusted for age, total energy intake, family history of breast cancer, use of hormone replacement therapy, and waist-to-hip ratio. Spearman correlation coefficients DiMeIQx/MeIQx 0.76 PhIP/DiMeIQx 0.43 PhIP/MeIQx 0.60 Intake of HCA based only on three meats (beefsteak, hamburger patty, and bacon) that account for more than 60% of red meat consumed in this population.

Reference	Cancer site	Population	Exposure	Effects	Comments
Sinha *et al.* 2000b Missouri, USA Missouri lung cancer study	lung cancer	population-based case-control study in women (1993–1994) cases: 593 cases reported to the Missouri cancer registry controls: 623 women randomly selected from driver's license files (30–64 years old) and from Health Care Finance Administration list (65–84 years old), including both nonsmoking and smoking women and matched for age	Subjects completed a food frequency questionnaire, the Health Habits and History Questionnaire, which queried consumption frequency and portion size for 22 meat and fish items and other foods along with methods of cooking. HCAs were estimated from the food frequency questionnaire and database as described above (Delfino *et al.* 2000)	Adjusted ORs for lung cancer <u>10th vs. 90th percentile</u> DiMeIQx 1.2 (0.9–1.6) MeIQx 1.5 (1.1–2.0)* PhIP 0.9 (0.8–1.1) *Still significant after adjusting for other HCAs (OR=1.8, 1.2–2.7) and after adjusting for total meat, red meat, barbecued red meat, and smoked meat; elevated but not significant after adjusting for well-done red meat or fried red meat. <u>OR for 10 ng per increment difference</u> DiMeIQx 1.27 (0.90–1.80) MeIQx 1.04 (1.01–1.07)* PhIP 1.00 (0.99–1.00) *Still significant after adjusting for other HCAs.	ORs were adjusted for age, pack-years of smoking, smoking status, years-quit-smoking, BMI, calories, fat, fruit/fruit juices, vegetables, and education.level HCA database limited to a fraction of compounds associated with high-temperature cooking of meat; association with MeIQx may be explained by a meat byproduct correlated with MeIQx.

Reference	Cancer site	Population	Exposure	Effects	Comments
Sinha *et al.* 2001 Maryland, USA Maryland colorectal adenoma study	colorectal adenomas	clinic-based case-control study cases: 146 cases with colorectal adenomas at sigmoidoscopy or colonoscopy controls: 228 without colorectal adenomas at sigmoidoscopy	Database -developed by Sinha *et al*. and described above. Mutagenic activity also was measured. HCAs were estimated from the food frequency questionnaire and database as described above Delfino *et al.* 2000.	Adjusted ORs for colorectal adenomas 5th vs. 1st quintile (ng/day) DiMeIQx 2.2 (1.2–4.1)* MeIQx (27.00–179.65) 2.1 (1.0–4.3) + PhIP (140.24–728.50) 2.5 (1.1–5.5)+* mutagenic activity 3.1 (1.4–6.8) * no longer significant after adjusting for other HCAs + test for trend significant ng/day not reported for DiMeIQx Continuous analyses (10 ng/day increments) total meat MeIQx 1.15 (1.05–1.25) PhIP 1.02 (1.00–1.04) red meat only MeIQx 1.13 (1.03–1.23) PhIP 1.05 (1.01–1.10) white meat only MeIQx 1.95 (0.98–3.85) PhIP 1.01 (0.99–1.34)	ORs were adjusted for age, gender, total caloric intake, fiber intake, reason for screening, physical activity, smoking, and use of NSAIDs. Spearman correlation between HCAs estimated using verbal doneness response and photographs was 0.92 for MeIQx and 0.93 for PhIP. HCA modestly or highly correlated with each other and with mutagenic activity. Possible recall bias because cases were interviewed before diagnosis; however, endpoint was precancerous not cancerous lesions. Cases also less likely to have changed dietary patterns after diagnosis.

Reference	Cancer site	Population	Exposure	Effects	Comments
Augustsson *et al.* 1999b Sweden Swedish studies	colon, rectal, bladder, and kidney cancer	population-based case-control study population: people born in Sweden between 1918 and 1942 and who had a permanent address in Stockholm for at least one month between 1992 and 1994 cases: 352 colon cancer, 249 rectal cancer, 273 bladder cancer, and 138 kidney cancer identified by cancer registry controls: 553 randomly chosen from population register and matched for age and gender for colon cancer cases	HCA database: HCA content was measured from 22 dishes fried or roasted according to Swedish recipes. Diet was assessed by a food frequency questionnaire on eating habits for the prior 5 years. Color photographs showing 6 dishes cooked to different degrees of doneness were used. HCA concentrations were calculated by linking the database with food frequency questionnaire.	Adjusted ORs for colon cancer 5th vs. 1st quintile Total meat and fish 0.9 (0.5–1.4) Total HCAs 0.6 (0.4–1.0) IQ 1.1 (0.7–1.6) MeIQ 1.3 (0.9–1.8)* MeIQx 0.6 (0.4–1.0) PhIP 0.6 (0.4–0.9) Adjusted ORs for rectum cancer 5th vs. 1st quintile Total meat and fish 1.0 (0.6–1.6) Total HCAs 0.7 (0.4–1.1) MeIQ 1.5 (1.0–2.1)* MeIQx 0.7 (0.4–1.2) PhIP 0.6 (0.4–1.1) Adjusted ORs for bladder cancer 5th vs. 1st quintile Total eat and fish 0.8 (0.5–1.5) Total HCAs 1.2 (0.7–2.1) MeIQ 1.0 (0.7–1.5)* MeIQx 1.1 (0.6–1.9) PhIP 1.2 (0.7–2.1) Adjusted ORs for kidney cancer 5th vs. 1st quintile Total eat and fish 1.0 (0.5–1.9) Total HCAs 1.0 (0.5–1.9) MeIQ 1.1 (0.7–1.8) * MeIQx 0.9 (0.5–1.9) PhIP 0.9 (0.5–1.7) *reference category quintiles 1-4	ORs were adjusted for age and gender and for smoking (bladder and kidney only). Adjusting for other potential factors had little or no effect (fat, protein, dietary fiber, vegetables, fruits, and physical activity and energy). Limitations misclassification due to estimated intake of HCA, relationship between cooking condition browning, and HCA concentrations judged by photos is limited low power because of low intake of HCAs

Reference	Cancer site	Population	Exposure	Effects	Comments
Norrish *et al.* 1999 Auckland, New Zealand New Zealand prostate cancer study	prostate cancer	population-based case-control study of men aged 40–80 cases: 317 men, 60% diagnosed from clinic attendees with prostate-related symptoms who were recruited before completion of diagnosis; the remainder were identified retrospectively within 3 weeks of diagnosis controls: 480 men with no history of prostate cancer and randomly selected from electoral rolls and matched for age	Subjects completed self-administered questionnaires and a validated food frequency questionnaire on frequency, portion size, and usual cooking methods for seven types of meats (white and red). HCAs were measured for each type of meat cooked to different degrees of doneness (by frying at 200ºC). Participants were assigned to one of three categories of doneness for each of the seven meat types: low, (no consumption, cooked rare, or baked/cooked in liquid/microwave) medium (fried/grilled/ barbecued to medium brown state), and high (fried/ grilled/barbecued to well-done). HCA concentrations were assigned to the meat-cooking data based on laboratory analyses and categories were defined by quartiles.	Elevated ORs for different types of meat beefsteak medium 1.4 (0.8–2.2) well-done 1.7 (1.0–2.8) chicken well-done 1.3 (0.9–1.9) ORs for HCAs – 4th quartile (ng/day) Total HCA (> 420.9) 1.1 (0.7–1.7) MeIQx (> 95.2) 1.0 (0.6–1.5) DiMeIQx (> 22.0) 1.2 (0.8–1.9) IFP† (> 84.5) 1.3 (0.9–2.0) PhIP (> 223.8) 1.1 (0.7–1.6) † 2-amino-1,6 dimethylfuro[3,2-*e*]imidazo[4,5-*b*] pyridine	ORs were adjusted for age, NSAIDs, socioeconomic status, energy consumption. Population had a low intake of HCAs, median daily intake was 146 ng. Median intake (g/day) of red meat was 104 for cases and 98 for controls. Strengths diet assessed for some cases before diagnosis

4 Studies of Cancer in Experimental Animals

At the time of the IARC monographs, (1993a, 1993b, 1993c) relatively few studies had investigated the carcinogenicity of PhIP, MeIQ, or MeIQx in experimental animals. However, in recent years, the carcinogenicity of these compounds has been investigated in many studies. These included long-term (> 1 year) and short-term (≤ 1 year) studies, initiation and promotion studies, effects of other chemicals on HCA carcinogenesis, and carcinogenic effects of HCA mixtures. Compared to concentrations in the human diet of < 1 ng/g to about 500 ng/g (< 0.001 to 0.5 ppm) (see Sections 2.4 and 2.6), most of the animal studies administered HCAs at much higher concentrations (100 to 600 ppm). In terms of daily intakes, Layton *et al.* (1995) estimated that HCA intakes in humans ranged from < 1 to about 17 ng/kg b.w. per day. For comparison, mice fed a diet of 300 ppm PhIP ingested about 40 to 50 mg/kg b.w. per day (Sørensen *et al.* 1996). However, a few studies have used concentrations that are similar to those found in the human diet. The results of short-term and long-term studies for each of the HCAs are presented in Sections 4.1 to 4.3. Modulators of HCA-induced carcinogenicity and the carcinogenicity of HCA metabolites are discussed in Sections 4.4 and 4.5, respectively.

4.1 PhIP

PhIP is one of the most extensively studied of the HCAs. IARC (1993a) reviewed three studies that tested PhIP by oral administration and two studies that used intraperitoneal (i.p.) injection and concluded that there was *sufficient evidence in experimental animals for the carcinogenicity of PhIP*. In recent years, many more carcinogenicity studies of PhIP became available and are reviewed in Sections 4.1.1 and 4.1.2 for mice and rats, respectively.

4.1.1 Mice

PhIP's carcinogenicity was investigated in mice using short-term and long-term studies and both oral and i.p. administration. Short-term studies generally used transgenic or mutant mice or examined incidences of preneoplastic lesions. Long-term studies included dietary exposure to 400 ppm for 579 days, dietary exposure to 300 ppm for 31 weeks and followed for an additional 48 weeks, and i.p. administration of 6.5 or 26.2 mg/kg given by day 15 and followed for 15 months.

4.1.1.1 Short-term studies

In recent years, transgenic animal models have been used for short-term carcinogenicity testing. Sørensen *et al.* (1996) used Eμ-*pim*-1 transgenic mice and nontransgenic littermates in a seven-month carcinogenicity study of PhIP. Eμ-*pim*-1 mice over express the *pim*-1 oncogene and are predisposed to T-cell lymphomas. Mice were divided into four groups of 15 male and 15 female mice each; however, group III contained 14 male mice and 15 female mice. Nontransgenic, wild-type mice were used in groups I and II. Group I mice were the controls, and group II mice received 0.03% (300 ppm) PhIP in the diet (average intake of 46.8 mg/kg per day for males and 47.7 mg/kg per day for females). Eμ-*pim*-1 transgenic mice were used in groups III and IV. Group III mice were not given PhIP, and group IV mice received 0.03% (300 ppm) PhIP in the diet (average

intake of 44.0 mg/kg per day for males and 49.6 mg/kg per day for females). Body weights of mice exposed to PhIP were reduced compared to their respective control groups. Spontaneous lymphomas did not occur in the nontransgenic controls (group I) but were observed in one male (7%) and three female (20%) mice in the transgenic controls (group III). However, the incidence of spontaneous lymphomas in the transgenic mice was not statistically different from the incidence in nontransgenic controls. PhIP exposure resulted in significantly higher incidences of lymphomas (80%) and a shorter latency period in female transgenic mice compared to transgenic controls (20%) or PhIP-fed, nontransgenic littermates (27%). Incidences of lymphoma were higher in male transgenic mice but were not statistically significant compared to transgenic controls or PhIP-fed, nontransgenic littermates (Table 4-1).

The group A xeroderma pigmentosum gene-knockout mouse ($XPA^{-/-}$) is deficient in nucleotide excision repair (NER) but has a relatively low incidence of spontaneous cancer (15% incidence of hepatocellular adenomas after 1.5 years, 6% to 10% incidences of lymphomas after 8 to 14 months). PhIP was given at 10 to 200 ppm in the diet to $XPA^{-/-}$ mice (van Steeg *et al.* 1998). Mice given the highest dose died within two weeks. At lower doses (10 to 25 ppm for 26 weeks), some $XPA^{-/-}$ mice (incidence not specified) developed lymphomas and adenomas of the small intestine. In another study, $XPA^{-/-}$ mice were highly susceptible to PhIP-induced toxicity, as evidenced by body weight retardation and poor survival (Imaida *et al.* 2000). Although $XPA^{-/-}$ mice developed twice as many PhIP-DNA adducts in the liver, colon, and lung as wild type mice, tumor incidence was not significantly different between the groups (Table 4-1). Lymphomas and leukemias were the only tumors observed.

Table 4-1. Lymphomas in mice exposed to PhIP in the diet

		Exposure		Tumor incidence[a] (%)		
Test strain	Sex	Concentration (ppm)	Duration (wk)	Test	Controls	Reference
C57BL/6ByA	male	300	31	0/15 (0)	0 /15 (0)	Sørensen *et al.* 1996
	female	300	31	4/15 (27)	0/15 (0)	
Eμ-pim-1	male	300	31	4/15 (27)	1/14 (7)	
	female	300	31	12/15 (80)**	3/15 (20)	
B57BL/ACI/CBA						Imaida *et al.* 2000
$XPA^{+/+}$	male	40[b]	40	1/7 (14.3)	0/2 (0)	
	female	40[b]	40	3/5 (60)	1/2 (50)	
$XPA^{-/-}$	male	40[b]	40	0/20 (0)	5/18 (27.8)	
	female	40[b]	40	5/18 (27.8)	8/15 (53.3)	

***P* < 0.01 vs. controls

[a]The number of animals bearing tumors/number of animals examined.

[b]Some animals were treated with 80 ppm (0.008%) PhIP for 4 weeks then reduced to 40 ppm (0.004%) after a 2-week recovery period.

Aberrant crypt foci (ACF) were identified as early preneoplastic lesions following treatment with colon carcinogens (Bird 1987). Kristiansen *et al.* (1997) reported that C57BL/6J female mice fed a diet containing 0.03% (300 ppm) PhIP developed ACF in the colon after four weeks. Other groups of animals that received two weekly i.p. injections of the known colon carcinogens 1,2-dimethylhydrazine dihydrochloride (DMH) or azoxymethane (AOM) developed ACF after 10 weeks. ACF were not observed in the controls. The ACF were grouped according to small (1 to 3 crypts), medium (4 to 6 crypts), and large (7 to 9 crypts). Female C57BL/6J mice on the PhIP diet developed an average of 0.5 and 3.7 ACF/animal after 4 and 10 weeks, respectively (Table 4-2). All ACF induced by PhIP were small; whereas, those induced by DMH or AOM had a higher percentage of medium and large ACF (Kristiansen *et al.* 1997). Longer-term studies suggested that the PhIP-induced ACF (high number with low crypt multiplicity) were not likely precursors of colon cancer (Kristiansen 1996). Colons from mice in three PhIP feeding studies were examined for ACF. Although the number of ACF increased over time, no colonic tumors were found and 95% to 100% of the ACF were classified as small. The authors concluded that these results support the hypothesis that tumor formation is predicted by a high number of ACF with high crypt multiplicity.

Oshima *et al.* (1996) developed an adenomatous polyposis coli (*Apc*) gene knockout mouse to use in short-term carcinogenicity testing. These mice have a truncation mutation at codon 716 ($Apc^{\Delta 716}$) and develop intestinal polyps shortly after birth. A diet containing PhIP at 0.04% (400 ppm) was fed to 10 mutant mice (five males and five females) for eight weeks. Five mutant mice (two males and three females) maintained on normal diets served as controls. Other groups of mice were fed diets containing IQ or MeIQx. Results with MeIQx are reported in Section 4.2.1.1. The mice were five weeks old at the beginning of the study. Although the mutant mice showed normal activity and food intake throughout the experiment, they stopped gaining weight or lost weight after seven weeks. This was not observed in wild-type littermates receiving the PhIP diet or in mutant mice fed a normal diet. Mice were sacrificed at 13 weeks of age and examined for intestinal polyps along the entire length of the gut. Although the total number of polyps (167 ± 62) in the test mice was not statistically different from controls (147 ± 42) (Table 4-2), there was a shift toward larger polyps in the PhIP-fed mice. Polyp morphology was similar between both groups. The authors suggested that PhIP affected the growth of microadenomas in intestinal polyps without affecting the first step of microadenoma formation.

Sørensen *et al.* (1997) conducted a short-term carcinogenicity test with PhIP in transgenic C57BL6/J-Apc^{+}/*Apc*1638N mice. These mice have a frameshift mutation at codon 1638 of the *Apc* gene and develop intestinal adenomas and adenocarcinomas as well as mammary tumors. Thirty male and 28 female mice (four weeks old) were randomized based on body weight and assigned to the test group or control group. Each group contained 15 male and 14 female mice. Control mice received a standard diet for 33 weeks. Test mice were fed a diet with 0.03% (300 ppm) PhIP for 32 weeks and the standard diet during the last week of the study. Animals were sacrificed on day 233. No difference was reported in clinical appearance or survival of the test mice compared to the controls; however, food intake and body weight gain was lower in the test mice. At sacrifice, almost all animals had macroscopically detectable tumors in the small intestine,

and most animals had a swollen spleen. Male mice fed PhIP had a statistically significant increase in the number of tumors per mouse (10 ± 13) compared to controls (2.9 ± 2.4). Treated male mice also had a nonsignificant increase in ACF (21.3 ± 5.1) compared to controls (14.0 ± 3.4) (Table 4-2). No difference was reported between the female mice in the number of intestinal tumors or ACF. In addition, colonic adenomas were found in four dosed and one control male, adenocarcinomas of the mammary gland were found in two dosed and one control female, and a squamous cell carcinoma of the skin was found in one dosed female.

Table 4-2. Intestinal tumors and preneoplastic lesions in mice exposed to PhIP in the diet

Test strain	Sex	Exposure		Lesions per mouse[a] (N)		Reference
		Conc. (ppm)	Duration (wk)	Test	Controls	
C57BL/6J	female	300	4	0.5 ± 0.3[b, c] (4)	0 (4)	Kristiansen *et al.* 1997
	female	300	10	3.7 ± 1.0[b, c] (10)	0 (10)	
Apc$^{\Delta 716}$ knockout	male and female	400	8	167 ± 62[d] (10)	147 ± 42[d] (5)	Oshima *et al.* 1996
C57BL6/J-*Apc*$^{+}$/*Apc*1638N	male	300	32	10 ± 13*[e] (15)	2.9 ± 2.4[e] (15)	Sørensen *et al.* 1997
	male	300	32	21.3 ± 5.1[b] (14)	14 ± 3.4[b] (13)	
	female	300	32	3.1 ± 3.5[e] (14)	2.7 ± 1.8[e] (14)	
	female	300	32	9.4 ± 1.9[b] (14)	9.0 ± 2.5[b] (14)	

*$P < 0.05$ vs. control

[a]Mean number of lesions per animal ± SE unless otherwise specified.

[b]Number of ACF per colon.

[c]The significance level was not reported.

[d]Intestinal polyps of the small and large intestine (male and female data combined).

[e]Total number of macroscopically detectable adenomas and adenocarcinomas in the small intestines.

N = Number of animals.

Mice having the multiple intestinal neoplasia (*Min*) mutation in the *Apc* gene develop spontaneous intestinal adenomas. Steffensen *et al.* (1997) gave four weekly i.p. injections of 50 mg/kg PhIP to C57BL/6J-*Min*/+ and wild-type mice (4 to 7 mice per group) to investigate the effect on early intestinal neoplasia. PhIP treatment did not affect body weight in *Min*/+ or wild-type mice. After 10 weeks, PhIP exposure produced a significant increase in the numbers of small tumors (adenomas) and cystic crypts in the proximal section of the small intestine of male *Min*/+ mice and the numbers of ACF in the large intestines of both sexes. Effects were more pronounced in males than in females. Tumor size was not affected by PhIP treatment. No tumors or cystic crypts were found in the intestines of wild-type mice exposed to PhIP.

Several studies investigated liver and lung tumorigenesis in neonatal mice following i.p. injections of PhIP dissolved in dimethyl sulfoxide (DMSO). Dooley *et al.* (1992) investigated the carcinogenicity of four HCAs, including PhIP and MeIQx (see Section 4.2.1.2) administered at the maximum tolerated dose (MTD) and one-half the MTD. A total dose of 0.625 µmol (~140 µg) or 1.25 µmol (~280 µg) PhIP was administered by i.p injection to male $B6C3F_1$/nctr mice. The mice received 1/7, 2/7, and 4/7 of the total dose at 1, 8, and 15 days of age. Mortality at the high dose (~25%) was comparable to that for the group treated only with DMSO; however, mortality at the lower dose was higher (~50%) than for the DMSO only group. The researchers noted that the higher mortality was associated with rejection of the pups by the foster mothers rather than to treatment-associated toxicity. Hepatocellular adenomas were significantly increased in three of the treatment groups compared to vehicle controls (Table 4-3). One adenoma (2%) was observed after 8 months, and five (11%) were observed after 12 months in the control group. In addition, one hepatocellular carcinoma occurred in the high-dose group after 12 months. No other treatment-related lesions were observed.

Von Tungeln *et al.* (1996) gave i.p. injections to two groups of male CD1 mice with 0.625 µmol PhIP using the same protocol as Dooley *et al.* (1992). Both groups were fed *ad libitum* for the first 14 weeks after which one group was maintained on the *ad libitum* diet while the other was placed on a calorie-restricted diet. The calorie-restricted group received 90% of the calories of the *ad libitum* group for one week, 75% for the next week, and 60% from week 16 to sacrifice at 12 months. Liver and lung tumors developed in the *ad libitum* group but not in the calorie-restricted group (Table 4-3). These results are consistent with the hypothesis that excess calories enhance tumorigenicity.

Male C57BL/6 ($p53^{+/+}$) neonates and transgenic *p53*-deficient heterozygous mice ($p53^{+/-}$) received i.p. injections of PhIP dissolved in DMSO at 8 and 15 days of age (Dass *et al.* 1999). The total doses were 5.9 mg/kg and 23.7 mg/kg with one-third given on day 8 and the remainder on day 15. Other groups received either 2.4 or 9.5 mg/kg dimethylnitrosamine (DMN) or 7.9 or 15.7 mg/kg of 6-nitrochrysene (6-NC). Transgenic mice were examined seven months after treatment, but wild-type mice were examined at one year of age. No tumors occurred in the controls for either strain. Liver tumors (predominantly adenomas) occurred in two of the $p53^{+/-}$ mice exposed to the high-dose level. One liver tumor occurred in each of the other three groups of PhIP-treated mice (Table 4-3). These results were not significant and indicated that PhIP was not tumorigenic under the conditions of this study. Furthermore, *p53* deficiency did not accelerate liver tumorigenesis in these mice. Wild-type mice treated with either dose of DMN or 6-NC had significantly increased incidences of liver tumors at 12 months.

Table 4-3. Lung and liver tumors produced in male neonatal mice after i.p. injections of PhIP

Test strain	Total dose	Age at sacrifice (months)	No. of mice	Tumor incidence[a] (%)			Reference
				Liver adenoma	Liver carcinoma	Lung adenoma	
$B6C3F_1$	0	8	44	1 (2)	0	0	Dooley *et al.* 1992
	0.625 (µmol)	8	19	2 (11)	0	0	
	1.25 (µmol)	8	24	7 (29)**	0	0	
	0	12	44	5 (11)	0	0	
	0.625 (µmol)	12	16	8 (50)**	0	0	
	1.25 (µmol)	12	21	14 (67)***	1 (5)	0	
CD1	0.625 (µmol)	12	24	8 (33)**[c]	2 (8)	4 (17)	Von Tungeln *et al.* 1996
	0.625 (µmol)[b]	12	23	0	0	1 (4)	
C57BL/6							Dass *et al.* 1999
p53$^{+/+}$	0	12	17	0 (0)	nr	nr	
	5.9 mg/kg	12	18	1 (5.6)	nr	nr	
	23.7 mg/kg	12	14	1 (7.1)	nr	nr	
p53$^{+/-}$	0	7	15	0	nr	nr	
	5.9 mg/kg	7	17	1 (5.9)	nr	nr	
	23.7 mg/kg	7	15	2 (13.3)	nr	nr	

$^{**}P < 0.01$, $^{***}P < 0.001$ vs. controls unless otherwise noted

nr = not reported

[a]Number of animals bearing tumors/number of animals examined.

[b] Calorie restricted diet.

[c] Compared to the PhIP-exposed group on a calorie restricted diet.

4.1.1.2 Long-term studies

Esumi *et al.* (1989) fed CDF_1 mice a diet containing 0.04% (400 ppm) PhIP for 579 days. Both the control and experimental groups initially contained 40 mice of each sex. Mean body weights of the mice given PhIP were 15% to 20% less than in the control group. Lymphomas occurred in 11 male (31%) and 26 female (68%) mice exposed to PhIP compared to 2 (5.6%) and 6 (15%) in male and female controls, respectively; however, no statistical analyses were presented (Table 4-4).

Kristiansen *et al.* (1998) extended the study by Sørensen *et al.* (1996) (see Section 4.1.1.1). Animals that survived 31 weeks in the Sørensen *et al.* (1996) study were placed on a standard diet and followed for another 48 weeks. This study confirmed that PhIP was a potent mouse lymphomagen but demonstrated very weak or no carcinogenicity in other tissues. Seventy percent of the treated nontransgenic mice (males and females)

developed lymphomas compared to 10% to 20% in the controls (Table 4-4). Lymphomas developed in 90% of the transgenic controls compared to 80% (males) to 100% (females) of the treated transgenic mice. Other tumors in PhIP-treated mice included one intestinal papilloma (transgenic male), one adenocarcinoma (transgenic male), and one hepatocellular adenoma (nontransgenic male). Tumors reported in control mice included an intestinal papilloma in one male and a pituitary adenoma in one female.

Table 4-4. Lymphomas in mice exposed to PhIP in the diet

		Exposure		Tumor incidence[a] (%)		
Test strain	Sex	Conc. (ppm)	Duration	Test	Controls	Reference
CDF_1	male	400	579 days	11/35 (31)[b]	2/36 (5.6)	Esumi *et al.* 1989
	female	400	579 days	26/38 (68)[b]	6/40 (15)	
C57BL/6ByA	male	300	79 wk[c]	7/10 (70)[b]	2/10 (20)	Kristiansen *et al.* 1998
	female	300	79 wk[c]	7/10 (70)[b]	1/10 (10)	
Eμ-pim-1	male	300	79 wk[c]	8/10 (80)[b]	9/10 (90)	
	female	300	79 wk[c]	3/3 (100)[b]	9/10 (90)	

[a]The number of animals bearing tumors/number of animals examined.

[b]The significance level not reported.

[c]Animals were treated for 31 weeks then maintained on standard diet for 48 weeks.

Dass *et al.* (1998) treated male C57BL/6 neonates by i.p. injection with 6.5 or 26.2 mg/kg PhIP. The high dose was identified as the MTD in preliminary experiments. One-third of the dose was given on day 8, and the remaining two-thirds were given on day 15. No tumors occurred after 15 months. Only 15 mice (9 at the high dose and 6 at the low dose) were treated. Lung and liver tumors did develop in mice treated with DMN in this study.

4.1.2 Rats

Since the IARC (1993a) monograph was published, the carcinogenicity of PhIP in rats has been investigated in many short-term studies and a few long-term studies. In most studies, PhIP was administered in the diet to F344 rats.

4.1.2.1 Short-term studies

Preneoplastic lesions

ACF are induced by colon carcinogens in a dose-dependent manner and are generally considered to be preneoplastic lesions (Bird 1987, Tsukamoto *et al.* 1999, Paulsen *et al.* 2000). Therefore, ACF are frequently used as an intermediate end point to study colon carcinogens. PhIP at dietary concentrations of 50 to 500 ppm induces ACF in the rat colon within a few weeks to a few months (Takahashi *et al.* 1991, Tsuda *et al.* 1999).

Takahashi *et al.* (1991) conducted two experiments in male F344 rats; however, only three or four rats were used per group. Rats were exposed to 500 ppm PhIP in the diet for

2 to 16 weeks. Each group had a matched control group. Only 2 of 20 rats in the control group developed ACF compared to 16 of 20 in the various treatment groups (Table 4-5). The difference was highly significant after 12 weeks of PhIP exposure. The researchers concluded that the data show that PhIP is possibly carcinogenic to rat colon.

Ochiai *et al.* (1996) investigated the different responses to PhIP exposure between male and female F344 rats. Seven rats of each sex were fed 400 ppm PhIP in the diet for 14 weeks. The colons were examined for the number, size, and location of ACF. Although there was no difference in the distribution of ACF, the mean number of ACF was significantly higher in male versus female rats (Table 4-5). No ACF were observed in untreated controls of either sex. DNA adduct levels in male and female rats were similar. PhIP-enhanced cell proliferation was not observed in female rats or in male rats after 4 or 12 weeks but was observed in male rats after 8 weeks (42% increase compared to controls). Ishiguro *et al.* (1999), Nakagama *et al.* (1999), and Purewall *et al.* (2000a) investigated strain differences in susceptibility to PhIP-induced ACF. BUF, Wistar, F344, Norway, and ACI rats were fed a basal diet containing 400 ppm PhIP for two weeks. then placed on a high-fat diet for four weeks (Ishiguro *et al.* 1999). Non-treatment groups for each strain were given the basal diet for two weeks followed by the high-fat diet for four weeks. The BUF strain was the most susceptible, the ACI strain was the most resistant, and F344 and Wistar strains showed intermediate susceptibility. The number of ACF per rat in the BUF, Wistar, and F344 strains were significantly higher than in the ACI strain (Table 4-5). Very few rats in the non-treatment groups developed ACF.

Nakagama *et al.* (1999) examined F1 hybrids of F344 and ACI rats. The treatment protocol was identical to that used by Ishiguro *et al.* (1999) described above. The hybrids had a similar number of ACF as observed in the F344 strain (Table 4-5). Candidate susceptibility genes were identified in a genome-wide linkage analysis of 170 (F344 × ACI)F1 × ACI backcross progeny. A significant linkage was reported for markers on chromosome 16.

Purewall *et al.* (2000a) fed a fast-acetylator strain (F344) and a slow-acetylator strain (Wistar Kyoto) diets containing 0.01% (100 ppm) or 0.04% (400 ppm) PhIP for eight weeks. No ACF were observed in control groups of either strain. At 0.01% PhIP, there were no strain differences in the number of ACF; however, at 0.04% PhIP, the number of ACF in F344 rats was twice that in Wistar rats. Both strains had a significant increase in ACF from the low to high dose (Table 4-5). The authors concluded that these results support human data showing increased risk of colorectal cancer in rapid acetylators who frequently consume well-done meat.

Tsukamoto *et al.* (1999) developed a crypt isolation technique and studied the sequential morphology and location of ACF in the rat colon. PhIP was dissolved in water, and 100 mg/kg was administered by gavage three times per week for seven weeks. The control group received water only. Ten rats from the treated group and five rats from the control group were examined at weeks 12, 25, 50, and 75. ACF were isolated and classified as “single” or “bifurcating” based on morphology. Their data indicated that crypt proliferation occurred predominantly during the early stages and that the location of ACF shifted from the distal to the proximal part of the colon between weeks 25 and 50. A total

of 12 adenocarcinomas and seven adenomas were detected at weeks 50 and 75 combined, with the majority found in the proximal region. These results suggested that ACF forming in different segments of the colon may progress in different manners. Many early-stage ACF may disappear, whereas those developing later in the proximal region may be more likely to progress to neoplasia.

Paulsen *et al.* (2000) investigated age-dependent induction of ACF in F344 rats exposed to PhIP. Thirteen male pups and eight adult F344 rats were exposed three times per week to PhIP (50 mg/kg) by oral gavage. Treatments were stopped after the seventh exposure and ACF were scored eight weeks after the first exposure. In an additional experiment, 25 pups were exposed via lactating dams that received 50 mg/kg PhIP by gavage, three times per week for three weeks. Body weight gain was not affected in adults but was significantly reduced in pups exposed to PhIP by gavage; however, 9 of the13 pups were removed from the experiment because of toxicity (sudden weight reduction, loss of hair, or bruises). ACF were not observed in controls. Pups were more susceptible to ACF induction by PhIP than were adult rats. Incidences of ACF and crypt multiplicity were 2.2- and 3.5-times higher in pups than in adult rats. Pups exposed via breast milk did not show a significant increase in ACF (Table 4-5).

Table 4-5. Aberrant crypt foci in rats orally exposed to PhIP

Test strain (group)	Sex	Exposure		Aberrant crypt foci		Reference
		Conc. (ppm)	Duration (wk)	Incidence[a] (%)	No. of Foci/rat[b]	
F344						Takahashi *et al.* 1991
Experiment 1	male	0	2–4	0/4 (0)	0	
	male	500	2	1/4 (25)	0.3 ± 0.4	
	male	500	4	4/4 (100)	1.3 ± 0.8	
Experiment 2	male	0	4 or 12	0/3 (0)	0	
	male	0	8 or 16	1/3 (33.3)	0.3 ± 0.6	
	male	500	4	3/3 (100)	1.3 ± 0.6	
	male	500	8	2/3 (67)	0.7 ± 0.6	
	male	500	12	3/3 (100)	3.0 ± 0***	
	male	500	16	3/3 (100)	11.3 ± 7.0	
F344	M/5	0	14	nr	0	Ochiai *et al.* 1996
	M/7	400	14	nr	6.6 ± 1.5***[c]	
	F/5	0	14	nr	0	
	F/7	400	14	nr	1.9 ± 0.5	

Test strain (group)	Sex	Exposure		Aberrant crypt foci		Reference
		Conc. (ppm)	Duration (wk)	Incidence[a] (%)	No. of Foci/rat[b]	
BUF	male	0	6	2/5 (40)	0.4 ± 0.5	Ishiguro *et al.* 1999
	male	400	6[d]	5/5 (100)	12.2 ± 1.7*[e]	
Wistar/Crj	male	0	6	0/5 (0)	0	
		400	6[d]	5/5 (100)	5.6 ± 1.7*[e]	
	male					
F344	male	0	6	0/5 (0)	0	
		400	6[d]	21/21 (100)	3.5 ± 1.8*[e]	
	male					
Brown Norway	ale	0	6	0/5 (0)	0	
		400	6[d]	5/5 (100)	2.8 ± 1.6[e]	
	male					
ACI	male	0	6	1/5 (20)	0.2 ± 0.4	
		400	6[d]	14/20 (70)	0.9 ± 0.7	
F344	nr	400	6[d]	nr/21	3.5 ± 1.8*[e]	Nakagama *et al.* 1999
ACI	nr	400	6[d]	nr/20	0.9 ± 0.7	
(F344 × ACI)F1	nr	400	6[d]	nr/5	3.4 ± 1.4[f]	
(ACI × F344)F1	nr	400	6[d]	nr/5	3.4 ± 1.0[f]	
(F344 × ACI)F1 × ACI	nr	400	6[d]	nr/170	2.2 ± 1.4[f]	
F344	male	100	8	nr/10	0.7 ± 0.4	Purewal *et al.* 2000a
	male	400	8	nr/10	4.9 ± 1.0*[g,h]	
Wistar Kyoto	male	100	8	nr/10	0.5 ± 0.2	
	male	400	8	nr/10	2.4 ± 0.4*[g]	
F344						Paulsen *et al.* 2000
pups (gavage)	male	50[i]	8	4/4 (100)	2.0 ± 0.0*[k]	
adults (gavage)	male	50[i]	8	5/8 (62.5)	0.9 ± 0.8	
pups (breast milk)	male	50[i,j]	8	3/25 (12)	0.1 ± 0.3	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls unless otherwise noted

nr = not reported

[a]Number of animals bearing tumors/number of animals examined (significance level not reported).

[b]Number of foci ± SD (Takahashi *et al.* 1991, Paulsen *et al.* 2000), ± SE (Purewall *et al.* 2000a), SD or SE not specified in the remaining studies.

[c]Significantly elevated compared to the corresponding female group.

[d]Mice were treated with PhIP for two weeks then maintained for four weeks on a high-fat diet before sacrifice.

[e]Data were compared to the ACI rats receiving PhIP and not to the respective controls.

[f]Statistical comparisons not provided.

[g]Significantly elevated compared to the low-dose (intrastrain) group.

[h]Significantly elevated compared to the high-dose (interstrain) group.

[i]Dose in mg/kg administered in seven to nine doses (three per week).

[j]Dams were exposed by gavage.

[k]Significantly elevated compared to the corresponding adult group.

Tumors

Several other studies show that male rats orally exposed to PhIP also develop adenomas and adenocarcinomas in the small or large intestine. Ito *et al.* (1991a) fed male and female F344 rats a diet containing 400 ppm PhIP for 52 weeks. PhIP exposure resulted in reduced body weight gain in both males (36%) and females (26%) compared to controls. No tumors occurred in controls, but 16 of 29 male rats and 2 of 30 female rats developed colon adenocarcinomas (Table 4-6). The increase was statistically significant for male rats.

Ochiai *et al.* (1991) used Nagase analbuminemic rats (NARs), which are known to be sensitive to various carcinogens, in a PhIP feeding study. Thirteen male rats were initially fed a diet containing 0.04% (400 ppm) PhIP; however, this dose was toxic and was subsequently reduced to 0.03% (300 ppm) after 108 days and finally to 0.01% (100 ppm) on day 144. The experiment was terminated on day 311. Animals were sacrificed when they became moribund. Only two animals survived until the end of the experiment. The first tumors were observed on day 136. Thirty-six intestinal tumors (2 adenomas and 34 adenocarcinomas) were identified in 10 of 13 rats resulting in an average of 2.8 tumors per rat. Twenty-four tumors were found in the small intestine compared to 12 in the large intestine (including the cecum). Tumors of the small intestine, large intestine, and cecum were observed in nine, six, and three animals, respectively. In addition, four animals had Zymbal gland tumors, two had pancreatic tumors, and one had a skin tumor. None of these tumor types were reported in historical controls; however, no statistical comparisons were reported.

Colon tumors developed in 9 of 20 male F344 rats fed 400 ppm PhIP in the diet for at least 43 weeks (Table 4-6) (Kakiuchi *et al.* 1995). Eighteen tumors were identified. Eight of the larger tumors (> 3 mm in diameter) were examined for mutations of the *Apc* gene. PhIP-induced tumors were found to have a specific and unique frameshift mutation at sites having three contiguous guanines (5'-GGGA-3' → 5'-GGA-3') (see Section 6.3.2.2).

Table 4-6. Gastrointestinal tumors produced in rats orally exposed to PhIP

Test strain	Sex	Conc. (ppm)	Duration (wk)	Tumor incidence[a] (%)			Reference
				Small Intestine	Large Intestine	Total GI	
F344	male	0	52	nr	0/40 (0)	0/40 (0)	Ito *et al.* 1991a
	male	400	52	nr	16/29 (55)***	16/29 (55)***	
	female	0	52	nr	0/40 (0)	0/40 (0)	
	female	400	52	nr	2/30 (7)	2/30 (7)	
NAR	male	400 ↓ 100	44.4	9/13 (69)[b]	9/13 (69)[b]	10/13 (77)[b]	Ochiai *et al.* 1991
F344	male	400	> 43	nr	9/20 (45)[b]	nr	Kakiuchi *et al.* 1995

***$P < 0.001$ vs. controls

nr = not reported

[a]Number of animals bearing tumors/number of animals examined.

[b]Significance level was not reported.

PhIP also is an established mammary gland carcinogen in female rats (Nagao *et al.* 1994, Ito *et al.* 1997, Venugopal *et al.* 1999a, Yu *et al.* 2000). PhIP induced mammary gland tumors when administered in the diet or by gavage. Some studies investigated the modifying effects of dietary fat and other chemicals on PhIP-induced mammary gland carcinogenesis (see Section 4.4.2). Overall, the carcinogenic potency of PhIP was similar to that of benzo[a]pyrene, producing both malignant and benign mammary tumors, but it was more potent than 1-nitropyrene (El-Bayoumy *et al.* 1995).

Administration of PhIP in the diet to female F344 or Sprague-Dawley rats resulted in a statistically significant increased incidence of mammary tumors in several studies. Forty-seven percent of female F344 rats exposed to 400 ppm PhIP in the diet for 52 weeks developed mammary adenocarcinoma compared to none in the controls (Ito *et al.* 1991a). Imaida *et al.* (1996) demonstrated a dose-dependent response in female Sprague-Dawley rats fed diets containing PhIP at 0, 25, 100, or 200 ppm (experiment 1) and 0, 12.5, or 50 ppm (experiment 2) for 48 weeks. Mammary adenocarcinoma occurred in 4.8% to 72% of rats fed PhIP, but none were observed in the control groups (Table 4-7).

In a pilot study, Ghoshal *et al.* (1994) dosed groups of female Sprague-Dawley rats by gavage with 100 mg/kg PhIP dissolved in sesame oil containing 1% DMSO. One group was treated on five consecutive days and the other for eight consecutive days. Rats in the control groups received five or eight consecutive doses of the vehicle (sesame oil). More than half of the rats in the 800 ppm group developed mammary tumors within 27 weeks, while only 10% of those in the 500 ppm group developed tumors by 43 weeks (Table 4-7).

Table 4-7. Mammary tumors produced in rats orally exposed to PhIP

Test strain	Sex	Conc. (ppm)	Duration (wk)	Incidence of adenocarcinoma[a] (%)	Reference
F344	male	0	52	0/40 (0)	Ito *et al.* 1991a
	male	400	52	0/29 (0)	
	female	0	52	0/40 (0)	
	female	400	52	14/30 (47)***	
Sprague-Dawley					Imaida *et al.* 1996
Experiment 1	female	0	48	0/20 (0)	
	female	25	48	1/21 (5)	
	female	100	48	5/20 (25)*	
	female	200	48	13/18 (72)***	
Experiment 2	female	0	48	0/20 (0)	
	female	12.5	48	2/20 (10)	
	female	50	48	7/20 (35)**	
Sprague-Dawley					Ghoshal *et al.* 1994
Pilot study	female	0	43	0/10 (0)	
	female	500[b]	43	1/10 (10)[c]	
	female	800[b]	27	5/9 (56)[c]	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls

[a]Number of animals bearing tumors/number of animals examined.

[b]Cumulative dose given in 5 to 8 daily doses of 100 mg/kg by gavage.

[c]Statistical significance level was not reported.

Transplacental and trans-breast milk exposure to PhIP increased the risk of developing mammary adenocarcinoma in Sprague-Dawley rats (Hasegawa *et al.* 1995). Female Sprague-Dawley rats were fed a diet containing 100 ppm PhIP beginning four weeks before mating and continuing through gestation and lactation (Group A). A second group of dams was not exposed to PhIP (Group B). Male and female offspring of both groups were nursed by their own dams for three weeks and were divided at weaning into three groups receiving diets containing 0, 25, or 100 ppm PhIP for 44 weeks. Body weights were lower in offspring from the PhIP-treated dams. Both the incidence and multiplicity of adenocarcinomas were higher in female offspring from the PhIP-fed dams than in offspring from the control dams (Table 4-8), but the difference was statistically significant only in the offspring receiving the 25 ppm diet. No significant effects were observed in male offspring.

Table 4-8. Effects of transplacental and transbreast milk exposure on PhIP-induced mammary carcinogenesis in Sprague-Dawley rats

Sex of pups	Group	Treatment (ppm)		Duration[a] (wk)	Mammary adenocarcinoma	
		Dams	Offspring		Incidence[b] (%)	Multiplicity (no./rat)[c]
Female	A1	100	0	44	6/36 (17)	0.22 ± 0.54
	A2	100	25	44	9/21 (43)**[d]	0.62 ± 0.86**[d]
	A3	100	100	44	14/20 (70)***[e]	1.55 ± 1.85**[e]
	B1	0	0	44	1/30 (3)	0.07 ± 0.25
	B2	0	25	44	1/21 (5)	0.05 ± 0.22
	B3	0	100	44	13/21 (62)***[e]	0.90 ± 1.14**[e]
Male	A1	100	0	44	1/35 (3)	0.03 ± 0.17
	A2	100	25	44	0/20 (0)	0
	A3	100	100	44	3/20 (15)	0.15 ± 0.37
	B1	0	0	44	0/30 (0)	0
	B2	0	25	44	0/21 (0)	0
	B3	0	100	44	3/21 (14)	0.14 ± 0.36

Source: Hasegawa *et al.* 1995.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[a]Dams in group A given PhIP in the diet beginning 4 weeks before mating and continuing through gestastion and lactation. Pups in both groups nursed for 3 weeks then placed on treatment diets for 44 weeks.

[b]Number of animals bearing tumors/number of animals examined.

[c]The authors did not state whether variation was expressed as SD or SE.

[d]Compared to the respective group B.

[e]Compared to the respective 0 ppm group.

Kimura *et al.* (1996) suggested that pregnancy and lactation might provide some protection against PhIP-induced mammary carcinogenesis. However, when the tumorigenicity of PhIP was assessed in pregnant rats the only statistically significant difference was in the latency period for tumor development. Female Sprague-Dawley rats were divided into four groups. Group 1 received PhIP in the diet at 100 ppm for 15 weeks beginning four weeks before mating with untreated males. Group 2 rats received the same PhIP treatment but were not mated, group 3 rats were not treated with PhIP but were mated, and group 4 rats were not treated with PhIP and were not mated. Animals were sacrificed at 48 weeks. The relatively low dose of PhIP and short treatment duration failed to induce many tumors (Table 4-9). Tumor latency was slightly increased in mated rats (group 1) compared to nulliparous rats (group 2), and the total tumor incidence and tumor multiplicity in group 2 was slightly higher than in group 1. Although, these differences were not statistically significant, the authors concluded that these results were partly supported by epidemiological evidence that breast cancer incidence is higher in nulliparous than in parous women.

Table 4-9. Effects of parity on PhIP-induced mammary carcinogenesis in female Sprague-Dawley rats

Treatment[a]	No. rats	Adenocarcinoma		Gross mammary tumors	
		Incidence (%)	Multiplicity (no./rat)[b]	Incidence (%)	Multiplicity (no./rat)
PhIP (mated)	29	4 (13.8)	0.14 ± 0.35	4 (13.8)	0.14 ± 0.35
PhIP (not mated)	20	4 (20.0)	0.30 ± 0.66	5 (25.0)	0.45 ± 0.89*
Controls (mated)	30	1 (3.3)	0.03 ± 0.18	1 (3.3)	0.03 ± 0.18
Controls (not mated)	21	1 (4.8)	0.05 ± 0.22	1 (4.8)	0.05 ± 0.22

Source: Kimura *et al.* 1996.

*$P < 0.05$ compared to controls (not mated).

[a]100 ppm PhIP in the diet for 15 weeks.

[b]The authors did not state whether variation was expressed as SD or SE.

Male F344 rats fed a diet containing 400 ppm PhIP for 52 weeks had statistically significant ($P < 0.001$) increased incidences of atypical hyperplasia of the ventral prostate and seminal vesicles and carcinoma of the ventral prostate compared to untreated controls (Shirai *et al.* 1997). Atypical hyperplasia of the ventral prostate or seminal vesicles occurred in 22 of 27 (81.5%) and 24 of 27 (88.9%) of treated rats, respectively, compared to 1 of 37 (2.7%) and 2 of 37 (5.4%) of controls, respectively. Carcinoma of the ventral prostate was not observed in controls but occurred in 18 of 27 (66.7%) treated rats.

4.1.2.2 Long-term studies

Thirty F344 rats of each sex were fed diets containing 0, 25, or 100 ppm PhIP (Hasegawa *et al.* 1993). Average PhIP intakes ranged from 0.26 (females) to 0.39 (males) mg/kg day for the 25 ppm groups and 1.05 (females) to 1.47 (males) mg/kg per day for the 100 ppm groups. The study was terminated at the end of week 104 when all surviving animals were sacrificed and autopsied. Survival rates were 83%, 70%, and 30% in males and 87%, 77%, and 23% in females in the controls, 25 ppm group, and 100 ppm group, respectively. Body weight gain was slightly reduced in males in the high-dose group throughout the experiment and after 1.5 years in the high-dose female group. Colon adenocarcinomas were induced in 13 males and 4 females in the high-dose group (Table 4-10). The increase was statistically significant for males. No tumors occurred in the controls or low-dose groups of either sex. Female rats exposed to 100 ppm had a statistically significant ($P < 0.001$) increased incidence of mammary adenocarcinomas compared to controls. Mammary tumors did not occur in male rats exposed to either 25 or 100 ppm or in male or female controls. Incidences of mammary tumors in female rats were 2/30 (7%) and 14/30 (47%) in the 25 and 100 ppm groups, respectively.

Table 4-10. Colon tumors produced in rats orally exposed to PhIP

Test strain	Sex	Conc. (ppm)	Duration (wk)	Tumor incidence[a] (%) Adenocarcinomas	Reference
F344	male	0	104	0/30 (0)	Hasegawa *et al.* 1993
	male	25	104	0/30 (0)	
	male	100	104	13 (43)**	
	female	0	104	0/30 (0)	
	female	25	104	0/30 (0)	
	female	100	104	4 (13)	

***P* < 0.01 vs. controls

[a]Number of animals bearing tumors/number of animals examined.

Although Rao *et al.* (1996) investigated the chemopreventive effects of dietary oltipraz on PhIP-induced lymphoma in male F344 rats (see Section 4.4.1), they also reported that approximately 28% of these rats exposed to variable amounts of PhIP in the diet (400 ppm decreasing to 100 ppm) from 5 to 58 weeks of age developed intestinal tumors. The incidence of tumors in the small intestine and colon were 20% and 8%, respectively. Statistical analyses were not provided.

Shirai *et al.* (1999) demonstrated that dietary exposure to 400 ppm PhIP for the first 20 weeks of a 60-week study was sufficient to induce prostate carcinomas (Table 4-11). Incidences of prostate carcinoma in mice exposed to 25 ppm or 100 ppm for 104 weeks were 52% and 50%, respectively. However, these incidences were not significant because prostate carcinoma occurred in 40% of the control group. The authors offered no explanation for the high incidence of prostate carcinomas or other lesions in the control group even though the historical background incidence for prostate carcinoma in F344 rats is 0% (0 of 899) in NTP studies (NIEHS 1998).

Table 4-11. Prostate tumors produced in F344 rats orally exposed to PhIP

Conc. (ppm)	Duration (wk)	No. of rats	Carcinoma (%)	Atypical hyperplasia (%)		
			Ventral prostate	Ventral prostate	Anterior prostate	Seminal vesicles
Experiment 1						
0	104[a]	25	10 (40)	11 (44)	na	2 (8)
25	104	25	13 (52)	16 (64)	na	5 (20)
100	104	22	11 (50)	13 (59)	na	10 (45)[b]
0	52	37	0 (0)	1 (3)	na	2 (3)
400	52	27	18 (67)***	22 (81)***	na	24 (89)***
Experiment 2						
0	60[c]	15	0 (0)	2 (13)	0 (0)	0 (0)
400	60[c]	20	11 (55)**	18 (90)***	5 (25)	18 (90)***

Source: Shirai *et al.* 1999.

$^{**}P < 0.01$, $^{***}P < 0.001$

na = not available.

[a]Typographical error in the paper was corrected per Shirai (personal communication).

[b]*P* value not provided.

[c]Animals were treated for 20 weeks.

4.2 MeIQx

The IARC (1993c) monograph reviewed two carcinogenicity studies (one oral and one i.p. administration) of MeIQx in mice, one feeding study in rats, and three short-term studies in which MeIQx was administered with other carcinogens and concluded that *there is sufficient evidence in experimental animals for the carcinogenicity of MeIQx*. In recent years, several more carcinogenicity studies of MeIQx in experimental animals have been published. This section summarizes the results of available carcinogenicity studies conducted in experimental animals, including those reviewed by the IARC (1993c).

4.2.1 Mice

Four dietary studies and one i.p study are summarized in the following sections. Only one long-term study was reviewed.

4.2.1.1 Short-term studies

Dooley *et al.* (1992) administered a total dose of 0.625 µmol (~133 µg) or 1.25 µmol (~266 µg) MeIQx by i.p injection to male B6C3F_1/nctr mice. These doses were selected to represent the MTD and half the MTD. The mice received 1/7, 2/7, and 4/7 of the total dose at 1, 8, and 15 days of age, respectively, and were sacrificed at either 8 or 12 months of age. The control groups were injected with DMSO, the solvent vehicle. There were no significant differences in body weights. Mortality at the high dose (35%) was comparable to the group treated only with DMSO (30%); however, mortality at the lower dose was lower (~10%) than in the comparable control group (25%). Liver tumors were the only neoplasms observed (Table 4-12). Hepatocellular adenomas were significantly increased

in both dose groups after 12 months but not after 8 months. One adenoma (2%) was observed after eight months, and five (11%) were observed after 12 months in the control groups. In addition, one hepatocellular carcinoma occurred in the high-dose group after 12 months. Tumor multiplicity was slightly higher in the treated groups at 12 months.

Table 4-12. Liver tumors produced in male neonatal B6C3F$_1$ mice after i.p. injections of MeIQx

Total dose (µmol)	Age at sacrifice (months)	No. of mice	Tumor incidence (%)		Tumor multiplicity[a]
			Hepatocellular adenoma	Hepatocellular carcinoma	
Control	8	44	1 (2)	0 (0)	1.0
0.625	8	24	2 (8)	0 (0)	1.0
1.25	8	22	1 (5)	0 (0)	1.0
Control	12	44	5 (11)	0 (0)	1.0
0.625	12	24	8 (33)*	0 (0)	1.3
1.25	12	20	7 (35)*	1 (5)	1.4

Source: Dooley *et al.* 1992.

*$P < 0.05$ (two-tailed Fisher's Exact test) vs. controls

[a]Number of confirmed adenomas and carcinomas per tumor-bearing mouse (confidence intervals not provided).

Oshima *et al.* (1996) fed a group of nine (five males and four females) *Apc*$^{\Delta 716}$ gene knockout mice a diet containing 400 ppm MeIQx for 11 weeks. Four mutant mice (two males and two females) maintained on normal diets served as controls. The mice were five weeks old at the beginning of the study and were sacrificed at 16 weeks of age. The total number of intestinal polyps (213 ± 100) in the test mice was not statistically different from controls (254 ± 38); however, there was a slight increase in average polyp size. Polyp morphology was similar between both groups.

Groups of seven-week-old C57BL/6N mice (five or six per group) were fed a diet containing 0, 400 ppm, or 600 ppm MeIQx for seven weeks followed by a basal diet for another seven weeks (Okonogi *et al.* 1997a). ACF formation was measured as a biomarker for cancer of the large intestines. ACF formation was dose dependent. No ACF occurred in the control groups. Incidences of ACF were 2/5 (40%) and 3/5 (60%) in low-dose males and females, respectively, but the number of ACF/mouse was not significantly different from controls. All mice in the high-dose groups developed ACF and the number of ACF/mouse was significantly increased compared to controls. The response in the treated male and female mice was not significantly different.

Ryu *et al.* (1999) investigated the hepatocarcinogenicity of MeIQx in mice carrying the *lacZ* mutation reporter gene (C57Bl/*λlacZ*) and in bitransgenic mice carrying the *lacZ* gene and overexpressing the *c-myc* oncogene *(c-myc*/*λlacZ*). Liver tumor incidence was recorded after feeding the mice a diet containing 0.06% (600 ppm) MeIQx or a control diet for 30 weeks (3 to 4 mice per group) or 40 weeks (10 to 13 mice per group). After 30

weeks, hepatocellular carcinomas were detected in all male *c-myc*/*λlacZ* mice treated with MeIQx but not in the other groups. At 40 weeks, hepatocellular carcinomas were identified in all groups of mice treated with MeIQx; incidences were 100%, 80%, 44%, and 17% in male *c-myc*/*λlacZ* mice, female *c-myc*/*λlacZ* mice, male C57Bl/*λlacZ* mice, and female C57Bl/*λlacZ* mice, respectively. Further, approximately 17% of male *c-myc*/*λlacZ* mice on the control diet developed hepatocellular carcinomas after 40 weeks. These findings, along with mutant frequency (see Section A.2.3.2) suggest that *c-myc* overexpression enhances MeIQx hepatocarcinogenicity by increasing MeIQx-induced mutations.

Groups of male and female *p*53 gene knockout mice ($p53^{+/-}$) and wild-type littermates ($p53^{+/+}$) were fed diets containing 0, 0.1, 1, 10, or 100 ppm MeIQx for one year (Park *et al.* 1999). There were 10 male and 10 female $p53^{+/-}$ mice in each treatment group, 12 male and 11 female $p53^{+/-}$ mice in the heterozygous control group, and five male and five female mice in each of the wild-type groups, including controls. There were no differences in food consumption or body weight changes between treated and control groups. Tumors of the lung, liver, and hematopoietic system developed in some of the treated heterozygotes; however, incidences were not statistically different from the $p53^{+/-}$ control group or the wild-type mice except for hepatocellular adenomas in male $p53^{+/-}$ treated with 10 ppm MeIQx. Thus, $p53^{+/-}$ heterozygotes were not more susceptible to MeIQx carcinogenesis than were wild-type mice.

4.2.1.2 Long-term studies

CDF_1 mice were fed 0.06% (600 ppm) MeIQx in the diet for up to 84 weeks (Ohgaki *et al.* 1987). Both the control and exposed groups included 40 male and 40 female mice. The mice were six weeks old at the beginning of the experiment. Body weights were recorded once each week, and food consumption was measured each month. The body weights in treated mice were approximately 5% lower than in the controls throughout the experiment. Survival was not affected in male mice but was significantly lower ($P < 0.01$) in female mice. The average survival times were 76 and 77 weeks (males) and 75 and 82 weeks (females) in treated and control mice, respectively. Tumor incidences were compared using both the χ^2 and age-adjusted statistical tests. Treated male and female mice developed statistically significant increased incidences of liver tumors. In addition, female mice had significantly higher incidences of lung tumors, and male mice had significantly higher incidences of leukemia and lymphoma (Table 4-13). Other tumors (gastrointestinal, harderian gland, kidney, uterus, thyroid, mammary gland, and salivary gland) were observed but were not significantly increased compared to controls.

Table 4-13. Tumor incidences in mice exposed to MeIQx administered in the diet

Test strain and sex	Conc. (ppm)	Duration (wk)	Tumor incidence[a] (%)			
			Liver[b]	Lung[c]	Lymphoma and leukemia	Intestinal[c]
CDF_1						
Male	0	84	6/36 (17)	10/36 (28)	2/36 (5)	9/36 (25)
	600	84	16/37 (43)**	16/37 (43)	11/37 (29)**	13/37 (35)
Female	0	84	0/39 (0)	4/39 (10)	11/39 (28)	6/39 (15)
	600	84	32/35 (91)***	15/35 (43)**	10/35 (28)	6/35 (17)

Source: Ohgaki *et al.* 1987.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. controls (age-adjusted statistical test).

[a]Number of animals bearing tumors/number of animals examined.

[b]Includes all tumors, mostly hepatocellular adenomas and hepatocellular carcinomas.

[c]Includes adenomas and adenocarcinomas.

4.2.2 Rats

Three short-term studies and two long-term studies were reviewed. MeIQx was administered in the diet in all five studies and all but one used F344 rats.

4.2.2.1 Short-term studies

Kato *et al.* (1996) fed groups (four to six animals per group) of 8-week-old, male F344 rats powdered diets containing 0, 0.05, 0.2, 0.8, 3.2, 12.5, 50, or 200 ppm MeIQx. All rats were subjected to a two-thirds partial hepatectomy after the first week and were sacrificed at the end of week six. The average body weights at the end of the experiment were similar in all groups except the highest dose group, which was significantly lower than in controls. A few rats died during the experiment, possibly due to surgery. Glutathione S-transferase placental form (GST-P)-positive foci were categorized into five groups: single cell, 2 to 4 cells, 5 to 10 cells, 11 to 20 cells, and > 20 cells and expressed as the number of foci per cm^2 of liver section. No foci were observed with > 20 cells. A dose-related increase was observed in the number of foci, with larger foci appearing in the higher dose groups. Single-cell foci were significantly greater at 0.8 ppm and 12.5 ppm and higher. Incidences of foci with 2 to 4 cells or 5 to10 cells were significantly increased at 50 and 200 ppm, and foci with 11 to 20 cells were significantly increased at 200 ppm.

Fukushima (1999) fed MeIQx to 2,140 three-week-old male F344 rats in the diet at 0, 0.001, 0.01, 0.1, 1, 10, or 100 ppm for 16 or 32 weeks. Incidences of GST-P-positive foci were not increased in rats exposed to 0.001 to 1 ppm but were significantly increased at 10 and 100 ppm ($P < 0.001$). Fukushima (1999) also reported that transplacental or trans-breast milk exposure to low doses of MeIQx did not induce GST-P foci in F344 rats. The author concluded that no observed effect level exists for MeIQx-induced preneoplastic lesions in rats.

Sone *et al.* (1996) investigated the carcinogenicity of low doses of MeIQx in Long-Evans rats with cinnamon-like coat color (LEC) and Long-Evans rats with agouti coat color (LEA). LEC rats are a mutant strain characterized by high incidences of spontaneous hepatocellular carcinomas. Groups of eight male LEC and eight male LEA rats were fed a diet containing 40 ppm MeIQx beginning at 23 weeks of age to 63 weeks of age. A control group included eight male LEC rats that were maintained on a basal diet. The average intakes of MeIQx were 780 µg/day and 840 µg/day for LEC and LEA rats, respectively. Body weights were lower in the treated LEC rats compared to the controls; however, the relative liver weights were 46% higher. Liver tumors occurred in control and treated LEC rats but not in LEA rats fed MeIQx. In the control group, five rats developed hepatocellular adenoma and two developed hepatocellular carcinoma compared to four with hepatocellular adenoma and eight with hepatocellular carcinoma in the treated LEC group. There was a significant increase ($P < 0.05$) in tumor multiplicity from 0.3 hepatocellular carcinomas per rat in the controls to 2.8 per rat in the treated group. There was no evidence of tumors in organs other than the liver.

In another short-term assay, dietary exposure to MeIQx for 16 weeks induced ACF in the colons of male F344 rats (Tanakamaru *et al.* 2001). Groups of rats were fed diets containing 0, 0.001, 0.01, 0.1, 1, 10, or 100 ppm MeIQx. Ten animals were included in the two highest dose groups, while the other groups included 30 animals. No animals died during the experiment. Criteria used to identify ACF included increased size, thicker epithelial cell lining, and increased pericryptal zone relative to normal crypts. Incidences of ACF were 57% in the control group and 50%, 60%, 50%, 57%, 80%, and 90% in the low-dose to high-dose groups, respectively. ACF multiplicity was significantly increased ($P < 0.01$) only in the high-dose group (4.1 ± 3.2) compared to the control group (1.0 ± 1.2). Histopathological examination of the spontaneous and MeIQx-promoted ACF did not reveal any morphological differences.

4.2.2.2 Long-term studies

F344 rats were fed 0.04% (400 ppm) MeIQx or a basal diet for 429 days (Kato *et al.* 1988). Each group originally included 20 male or female, seven-week-old rats. Food consumption was similar in treated (17.1 and 11.6 g/day for males and females) and control groups (17.3 and 12.5 g/day for males and females); however, average body weights were 15% and 6% lower in treated male and female rats, respectively, compared to controls. All animals that survived for at least 177 days (the day the first tumor was observed) were included in the analyses. Survival was similar in all groups for the first 40 weeks but then began to decline in treated groups because of cancer-related deaths. Mean survival times were 427, 429, 326, and 364 days in control males, control females, treated males, and treated females, respectively. Increased incidences of liver and Zymbal gland tumors occurred in both male and female rats. All but one of the liver tumors in male rats were hepatocellular carcinomas. The remaining liver tumor was a neoplastic nodule, as were all liver tumors observed in female rats. Hepatocellular carcinomas had metastasized to the lungs in six of the male rats. In addition, skin tumors (mostly squamous cell carcinomas) were increased in males, and clitoral gland squamous cell carcinomas were increased in females (Table 4-14).

Kushida *et al.* (1994) conducted a dose-response study of MeIQx carcinogenicity in male F344 rats. Groups of seven-week-old rats were exposed to 0, 100, 200, or 400 ppm MeIQx in the diet for up to 56 weeks. Body weights were not affected in the low-dose group but were decreased by 6% and 19% in the mid- and high-dose groups, respectively. Rats that survived longer than 259 days (the date of the first liver tumor) were included in the analyses. The average times of survival were 377, 379, 373, and 315 days in the control, low-dose, mid-dose, and high-dose groups, respectively. Only the high-dose group had a significantly decreased survival. Dose-related increased incidences of liver, Zymbal gland, and skin tumors were observed in the treatment groups (Table 4-14). There was also a dose-related increase in malignancy and multiplicity of tumors. All liver tumors in the low-dose group were hepatocellular adenomas. In the mid-dose group, half the tumors were hepatocellular adenomas and half were hepatocellular carcinomas. Only one hepatocellular adenoma occurred in the high-dose group compared to 15 hepatocellular carcinomas. Similarly, all the Zymbal gland tumors observed in the low-dose group were sebaceous gland adenomas or squamous cell papillomas. At higher doses, incidences of squamous cell carcinomas increased.

Table 4-14. Tumor incidences in F344 rats administered MeIQx in the diet

Sex	Conc. (ppm)	Duration	Tumor incidence[a] (%)				Reference
			Liver[b]	Zymbal gland	Skin	Clitoral gland	
Male	0	429 d	0/19 (0)	0/19 (0)	0/19 (0)	na	Kato *et al.* 1988
	400	429 d	20/20 (100)***	15/20 (75)***	7/20 (35)**	na	
Female	0	429 d	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)	
	400	429 d	10/19 (53)***	10/19 (53)***	1/19 (5)	12/19 (63)***	
Male	0	56 wk	0/15 (0)	0/15 (0)	0/15 (0)	na	Kushida *et al.* 1994
	100	56 wk	5/30 (17)	1 (3)	0 (0)	na	
	200	56 wk	26/29 (90)**	5 (17)	3 (10)	na	
	400	56 wk	16/16 (100)**	13 (81)**	6 (38)*	na	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls

[a]Number of animals bearing tumors/number of animals examined.

[b]Includes neoplastic nodules and hepatocellular carcinomas.

na = not applicable

4.2.3 Other animals

MeIQx was not carcinogenic when administered to cynomolgus monkeys. Ogawa *et al.* (1999) exposed three male and six female monkeys to 10 mg/kg and six males and four females to 20 mg/kg by nasogastric intubation five times per week for 84 months. The monkeys were one year old at the start of the experiment. Autopsies conducted at 92 months did not reveal any preneoplastic or neoplastic lesions that were related to MeIQx exposure. The lack of carcinogenicity was attributed to the lack of constitutive expression

of CYP1A2 and the inability of other P450s to catalyze *N*-hydroxylation of MeIQx in cynomolgus monkeys. Because the animals in this study were sacrificed before they were 10 years old, it is not known if tumors would have developed later in life had they been maintained for another 10 years or more.

4.3 MeIQ

The IARC (1993b) monograph reviewed one carcinogenicity study of MeIQ in mice and three studies in rats and concluded that *there is sufficient evidence in experimental animals for the carcinogenicity of MeIQ*. One of the rat studies reviewed by the IARC (1993b) was an initiation-promotion study using MeIQ and phenobarbital (Kristiansen *et al.* 1989) and another examined synergistic effects of HCA mixtures (Ito *et al.* 1991b). These are discussed in Sections 4.4.1.2 and 4.4.2.3, respectively. In recent years, a few more carcinogenicity studies of MeIQ in mice were published; however, no additional rat studies were identified. This section summarizes the results of the available carcinogenicity studies conducted in experimental animals, including those reviewed by the IARC (1993b).

Ohgaki *et al.* (1991), the IARC (1993b), Eisenbrand and Tang (1993), and Nagao and Sugimura (1993) reviewed the carcinogenicity data for MeIQ and other HCAs in experimental animals. Dietary administration of MeIQ induced hepatocellular adenomas and carcinomas in female mice, forestomach papillomas and carcinomas in both male and female mice, skin squamous-cell carcinomas in male rats, mammary adenocarcinomas in female rats, Zymbal gland and oral cavity squamous-cell carcinomas in male and female rats, and colon adenomas and adenocarcinomas in male and female rats. Studies conducted after these reviews confirmed that MeIQ, administered in the diet, was associated with these and other types of cancer in mice and rats, including adenocarcinomas of the colon and cecum in female mice.

4.3.1 Mice

All carcinogenicity studies of MeIQ in mice were long-term studies. Ohgaki *et al.* (1986a) reported interim results for an ongoing study of the carcinogenicity of MeIQ in CDF_1 mice. Mice were fed a diet containing 0.04% (400 ppm) MeIQ. By week 83, squamous cell carcinomas of the forestomach were observed in 10 of 16 male mice and in 11 of 22 female mice. Ten of these tumors had metastasized to the liver. In addition, hepatocellular adenomas or carcinomas were observed in 9 female mice. In a subsequent study, Ohgaki *et al.* (1986b) administered MeIQ to groups of 40 male and 40 female CDF_1 mice at dietary concentrations of 0, 100, or 400 ppm for 91 weeks. Throughout the experiment, body weights in the treated mice were approximately 20% to 30% lower in the high-dose group and approximately 10% lower in the low-dose group than in the controls. Survival in the high-dose females was lower than in control females. However, survival in high-dose males and low-dose males and females was similar to that of the control group. Increased incidences of forestomach tumors were observed in both sexes, and increased incidences of liver tumors were observed in females (Table 4-15). Incidences of squamous-cell carcinomas of the forestomach also were significantly increased with incidences of 0%, 7.9%, and 78.9% (males) and 0%, 30.6%, and 63.2% (females) in the control, low-dose, and high-dose groups, respectively. Approximately

40% of the forestomach squamous-cell carcinomas metastasized to the liver. Incidences of lymphoma, leukemia, intestinal tumors, and lung tumors were similar among treated and control groups.

Nagao *et al.* (1998) examined the relationship between mutation frequencies in various organs and carcinogenicity in female Big Blue® mice (C57BL/6N genetic background) fed MeIQ in the diet. A group of 20 mice received 300 ppm MeIQ in the diet for 92 weeks. A control group of 20 mice was maintained on a basal diet throughout the experiment. Body weights and food intakes were monitored weekly throughout the experiment, and animals that died or became moribund during the experiment were autopsied. Body weights in the experimental group were 19% to 37% less than in the controls throughout the experiment, and survival was lower in the experimental group after 75 weeks (the time the first tumor appeared). The incidences of liver and intestinal tumors in the experimental group were statistically higher than in controls (Table 4-15). However, the incidence of malignant lymphoma was higher in the controls (32%) than in the experimental group (11%). There was no correlation between mutant frequencies and cancer incidence (see Section 5). These results, compared to those for CDF_1 mice, indicated differences in target organs and tumor susceptibility in different strains of mice. However, the authors recognized that the *lac*I transgene in the Big Blue® mouse might have affected susceptibility. Therefore, a follow-up study (Fujita *et al.* 1999) was conducted to determine whether MeIQ could induce colon cancer in mice without an artificially modified genetic composition.

Female C57BL/6NCrj were divided into two groups of 40 animals each (Fujita *et al.* 1999). The control group was maintained on a basal diet, and the experimental group was fed a diet containing 300 ppm MeIQ for 92 weeks. Body weights in the experimental group were approximately 20% lower than in the control group throughout most of the experiment. Survival rates were lower in the experimental group after 50 weeks, and all mice in the experimental group were sacrificed by the 85th week. One or more tumors developed in 38 of the 40 mice in the experimental group. Increased incidences of tumors in the colon and cecum (adenocarcinomas) and liver (hepatocellular adenomas, hepatocellular carcinomas, and fibrosarcomas) were observed (Table 4-15). As described in the Big Blue® mouse, incidences of lymphomas were higher in controls. These results demonstrated that tumor susceptibility and target organs following exposure to MeIQ vary among different mouse strains.

Table 4-15. Tumor incidences in mice exposed to MeIQ administered in the diet

Test strain and sex	Conc. (ppm)	Duration (wk)	Tumor incidence[a] (%)				Reference
			Fore-stomach[b]	Liver[b]	Cecum[c]	Colon[c]	
CDF_1							Ohgaki *et al.* 1986b
Male	0	91	0/29 (0)	4/29 (14)	nr	9/29 (31)[d]	
	100	91	7/38 (18)*	11/38 (29)	nr	8/38 (21)[d]	
	400	91	35/38 (92)***	7/38 (18)	nr	15/38 (39)[d]	
Female	0	91	0/40 (0)	0/40 (0)	nr	7/40 (18)[d]	
	100	91	19/36 (53)***	4/36 (11)*	nr	13/36 (36)[d]	
	400	91	34/38 (89)***	27/38 (71)***	nr	6/38 (16)[d]	
Big Blue®							Nagao *et al.* 1998
Female	0	92	0/19 (0)	0/19 (0)	0/19 (0)	0/19 (0)	
	300	92	3/19 (16)	16/19 (84)***[e,f]	13/19 (68)***	8/19 (42)**	
C57BL/6N							Fujita *et al.* 1999
Female	0	92	0/39 (0)	0/39 (0)	0/39 (0)	0/39 (0)	
	300	92	1/40 (3)	10/40 (25)**[e,g]	12/40 (30)**	7/40 (18)*	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. controls

nr = not reported

[a]Number of animals bearing tumors/number of animals examined.

[b]Total tumors (includes papillomas, squamous-cell carcinomas, and sarcomas of the forestomach and hepatocellular adenomas, hepatocellular carcinomas, and fibrosarcomas of the liver).

[c]Includes only adenocarcinomas.

[d]Includes total intestinal tumors.

[e]Includes only hepatocellular carcinomas (data for total tumors was not provided).

[f]Incidences of hepatocellular adenomas (53%) also were significantly higher ($P < 0.001$) than in controls (0%).

[g]Incidences of hepatocellular adenomas (20%) and fibrosarcomas (68%) also were significantly higher ($P < 0.005$) than in controls (0%).

Ramsey *et al.* (1998) reported that female CDF_1 mice developed chromosome aberrations and tumors following exposure to MeIQ in the diet. Mice were fed a diet containing 0 or 400 ppm MeIQ beginning at seven weeks of age. Body weights and food consumption were recorded weekly. After 70 weeks, five control mice and eight exposed mice were sacrificed and examined. Body weights in exposed mice dropped by 20% in the first few weeks of the experiment but began to recover after four to six weeks. Although body weight gains in the exposed mice were similar to controls after the recovery period, they remained lower than controls throughout the experiment. Seven of the eight mice exposed to MeIQ developed tumors in one or more tissues. These included the liver, forestomach, hematopoietic system, and lung. These tumors were not observed in the control group; however, a malignant lymphoma, hemangiosarcoma in the ear, and a forestomach papilloma were observed in one control mouse each. The authors were not

able to make statistical inferences regarding any possible associations between chromosome aberrations and tumor occurrence.

4.3.2 Rats

Fischer 344 rats fed a diet containing 300 ppm MeIQ for 286 days developed tumors of the oral cavity, Zymbal gland, skin, colon, and mammary gland (Kato *et al.* 1989) (Table 4-16). None of the treated males and only four of the treated females lived until the end of the experiment. Most of the tumors observed in the Zymbal gland, oral cavity, and skin were squamous-cell carcinomas. Colon tumors were diagnosed as adenomas or adenocarcinomas and most of the mammary gland tumors were adenocarcinomas.

Table 4-16. Tumor incidences in rats exposed to MeIQ administered in the diet for 286 days

Test strain and sex	Conc. (ppm)	Tumor incidence (%)[a]				
		Zymbal gland	Oral cavity	Skin	Mammary gland	Colon
F344						
Male	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	300	19 (95)***	7 (35)**	10 (50)***	0 (0)	7 (35)**
Female	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	300	17 (85)***	7 (35)**	1 (5)	5 (25)*	5 (25)*

Source: Kato *et al.* 1989.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. controls

[a]20 rats per group.

4.4 Modulation of HCA-induced carcinogenesis

Sex (Ochiai *et al.* 1996), strain differences (Ishiguro *et al.* 1999, Nakagama *et al.* 1999, Purewal *et al.* 2000a), dietary levels of fat and calcium (Weisburger *et al.* 1994), caffeine (Tsuda *et al.* 1999), age (Paulsen *et al.* 2000), and many other factors may modulate HCA-induced carcinogenicity. More than 180 modulators of HCA-induced carcinogenesis or mutagenesis have been described (Dashwood 2002). This section provides a brief overview of some of the factors that suppress or enhance HCA carcinogenicity. In addition, these studies have provided some important insights into the mechanisms of action of HCAs and are discussed further in Section 6.2.4.1.

4.4.1 Chemoprevention of HCA carcinogenesis

Both natural and synthetic chemicals, including many phytochemicals, nutrients, and antioxidants, may provide protection against HCA-induced mutagenesis and carcinogenesis. Preneoplatic lesions (e.g., ACF, GST-P-postive foci), lymphomas, colon tumors, and mammary tumors in F344 rats, as well as DNA adducts in these tissues, have been used in many studies to identify potential chemopreventive agents. Some of the more effective inhibitors of HCA-induced carcinogenesis include constituents in green

and black tea (catechins and polyphenols), green vegetables (chlorophyllin), cruciferous vegetables (indole-3-carbinol), and dairy products (conjugated linoleic acids) (Xu and Dashwood 1999, Schut and Yao 2000, Dashwood 2002). The purpose of this section is not to review all the potential chemoprevention agents (see Dashwood 2002). Instead, some of these studies are briefly mentioned as relevant to the question of HCA carcinogenicity (i.e., HCAs administered to experimental animals induced either preneoplastic lesions or tumors; however, other chemicals were introduced to study their chemopreventive properties and the mechanisms involved).

4.4.1.1 Lymphomas

Rao *et al*. (1996) investigated the effects of dietary oltipraz on the carcinogenic effects of PhIP in male F344 rats. Oltipraz is a substituted dithiolethione drug that inhibits chemically-induced carcinogenesis in many target organs. The animals were divided into five groups and results are summarized in Table 4-17. The initial concentration of PhIP in the diet was 400 ppm; however, this level was too toxic and was subsequently reduced to 200 ppm from the 10th to the 13th week and then to 100 ppm from the 14th week to the end of the experiment at 52 weeks. Body-weight gains and survival rates were depressed in animals receiving PhIP. Seventy-five percent of the rats receiving PhIP alone developed lymphomas, most of which were of thymic origin (thymomas). Dietary oltipraz increased survival and significantly reduced the incidence of lymphomas.

Table 4-17. Lymphomas in male F344 rats exposed to PhIP alone or combined with oltipraz in the diet for one year

Experimental group	Initial no. of rats	Survival[a] (%)	Total lymphomas[b] (%)	Thymoma (%)
Control diet	12	100***	0	0
Oltipraz	12	100***	0	0
PhIP	36	38	75	64
PhIP + 200 ppm oltipraz	36	72*	6[c]	6[b]
PhIP + 400 ppm oltipraz	36	89**	0[c]	0[b]

Source: Rao *et al.* 1996.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[a]Significance level is based on a comparison to the PhIP diet group.

[b]No statistical comparisons were made with the control or oltipraz only groups.

[c]Statistically lower by life-table analysis than in the PhIP group but the *P* value was not provided.

4.4.1.2 Gastrointestinal tumors and preneoplastic foci

Chlorophyllin or indole-3-carbinol were given either before and during PhIP exposure (initiation), after PhIP exposure (post-initiation), or continuously for 16 weeks, to F344 rats (Guo et al. 1995). PhIP was given (50 mg/kg) by gavage during weeks 3 and 4 of the 16 week study. Chlorophyllin (0.1%) given in the drinking water reduced the number of ACF with ≥ 4 crypts/focus when given continuously (0.3 ± 0.5) or during post-initiation (0.7 ± 0.3) compared to controls (1.4 ± 0.9). Indole-3-carbinol (0.1%) given in the diet reduced ACF formation regardless of the treatment protocol, and ACF were completely inhibited when indole-3-carbinol was given during the initiation phase.

Hagiwara *et al.* (1999) investigated the modifying effects of caffeine and naturally occurring antioxidants on PhIP-induced colon and mammary tumors. The effects on colon carcinogenesis are reported here. A total of 120 female F344/DuCrj rats were divided into eight groups. Four groups of 20 rats each received 200 ppm PhIP in the diet alone (group 1) or in combination with 0.1% caffeine (group 2), 0.5% α-tocopherol (group 3), or 0.1% *n*-tritriacontane-16,18-dione (TTAD) (group 4). Groups 5 through 8 consisted of 10 rats each and received the basal diet alone or in combination with caffeine, α-tocopherol, or TTAD. The average PhIP intake in each group ranged from 10.2 mg/kg per day to 11.9 mg/kg per day. The final survival rates at 54 weeks in groups 1 through 4 were 85%, 95%, 90%, and 90%, respectively; the average final body weight in group 4 was significantly lower than that in group 1 (189.4 ± 6.3 g vs. 201.0 ± 7.8 g). No tumors occurred in non-PhIP-treated groups; however, colon tumors occurred in all groups exposed to PhIP (Table 4-18). Although caffeine decreased the amount of PhIP metabolites in a dose-dependent manner, PhIP in combination with caffeine in the drinking water resulted in significantly more colon tumors (75%) than exposure to PhIP alone (15%). The antioxidants α-tocopherol or TTAD did not significantly reduce colon tumor incidence. This finding is in contrast to the effects on mammary carcinogenesis (see Section 4.4.1.3).

Table 4-18. Colon tumors produced in female F344 rats orally exposed to PhIP, caffeine, and antioxidants for up to 54 weeks

Treatment group	No. of rats	Tumor incidence (%)			Multiplicity (no./rat ± SD)
		Adenoma	Adeno-carcinoma	Total	
200 ppm PhIP	20	1 (5)	2 (10)	3 (15)	0.15 ± 0.37
+ 0.1% caffeine	20	8 (40)**	10 (50)**	15 (75)**	0.90 ± 0.64**
+ 0.5% α-TP	20	0 (0)	1 (5)	1 (5)	0.05 ± 0.22
+ 0.1 % TTAD	20	0 (0)	3 (15)	3 (15)	0.15 ± 0.37

Source: Hagiwara *et al.* 1999.

***P* < 0.01 vs. PhIP only group

α-TP = α-Tocopherol

TTAD = *n*-Tritriacontane-16,18-dione

The chemopreventive effects of dietary arctiin (a plant lignan), given during or after initiation by PhIP, were investigated in female Sprague-Dawley rats (Hirose *et al.* 2000). This study was designed primarily to investigate mammary tumors (see Section 4.4.1.3); however, preneoplastic and neoplastic lesions of the colon and pancreas also were investigated. Groups of 20 rats each were initiated with 100 mg/kg of PhIP by gavage, once each week for eight weeks. Arctiin treatment began three days before PhIP administration in the initiation groups and continued for 8 weeks. Animals in the promotion group were initiated with PhIP for 8 weeks and then received arctiin for 40 weeks. Control rats were fed the basal diet and given 0.02% or 0.2% arctiin throughout both stages. Body weights were significantly lower in all PhIP-treated groups and the arctiin alone groups compared to the basal diet alone group. PhIP treatment alone did not induce tumors in the colon of female Sprague-Dawley rats. The average number of ACF per colon was significantly decreased in groups given 0.02% arctiin during the initiation or promotion period and in the group given 0.2% arctiin during the promotion period. Two colon carcinomas occurred in the low-dose-arctiin promotion group. Pancreatic lesions occurred in all groups exposed to PhIP without any clear effects of arctiin. Basophilic foci were found in all animals given intragastric administration of 100 mg/kg PhIP once a week for eight weeks, compared to one animal in the control groups. Acidophilic foci occurred in 26% of PhIP-treated animals compared to none in the control groups (Table 4-19). In addition, one pancreatic adenoma occurred in a rat treated with PhIP alone. Other lesions (liver foci, adrenal and pituitary hyperplasias, and pituitary tumors) were observed in rats, but there were no significant differences between groups. Statistical comparisons between the PhIP-exposed groups and non-PhIP-exposed groups were not reported because the purpose of the study was to evaluate the effects of arctiin. The authors concluded that PhIP was a weak pancreatic carcinogen in female Sprague-Dawley rats and that arctiin had a protective effect on PhIP-induced carcinogenesis in the promotion period.

Table 4-19. Colon and pancreatic lesions produced in female Sprague-Dawley rats orally exposed to PhIP and arctiin

Treatment group	Duration (wk)[a]	Colon		Pancreas	
		Aberrant crypt foci[b]	Carcinoma (%)[c]	Basophilic foci (%)[c,d]	Acidophilic foci (%)[c,d]
Basal diet	48	nr	nr	1/9 (11)	0/9 (0)
0.02% arctiin	48	nr	nr	0/9 (0)	0/9 (0)
0.2% arctiin	48	nr	nr	0/9 (0)	0/9 (0)
100 mg/kg PhIP	8	38.2 ± 15.6	0/20 (0)	17/17 (100)	7/17 (41)
Initiation stage					
+ 0.02% arctiin	8	18.6 ± 6.8**[e]	0/20 (0)	19/19 (100)	3/19 (16)
+ 0.2% arctiin	8	26.0 ± 13.5	0/20 (0)	18/18 (100)	3/18 (17)
Promotion stage					
+ 0.02% arctiin	40	21.0 ± 12.1*[e]	2/19 (11)	19/19 (100)	3/19 (16)
+ 0.2% arctiin	40	21.7 ± 10.8*[e]	0/20 (0)	20/20 (100)	8/20 (40)

Source: Hirose *et al.* 2000.

$^{**}P < 0.01$, $^{***}P < 0.001$

[a]See text for details

[b]Only 10 rats per group examined for ACF (mean number of ACFs/colon, variability not specified as SD or SE).

[c]Number of animals bearing lesion/number of animals examined.

[d]No statistical comparisons with controls were provided.

[e]Significantly less than the PhIP-alone group.

nr = not reported

In a second experiment (Hirose *et al.* 2000), seven -week-old male F344 rats were given a single i.p. injection of 200 mg/kg diethylnitrosamine (DEN), and two weeks later, groups of 15 rats were placed on diets containing 0.03% (300 ppm) MeIQx alone, MeIQx plus 0.5% arctiin, 0.1% arctiin alone, or basal diet alone for six weeks. Partial hepatectomies were performed on all rats at the end of week 3. All surviving animals were killed at week 8, and the livers were examined for GST-P-positive foci greater than 0.2 mm in diameter. MeIQx increased the number and area of GST-P foci. Treatment with arctiin alone did not increase liver foci, but treatment with both MeIQx and arctiin significantly increased both the area and number of foci compared to the MeIQx alone group (Table 4-20). In contrast to the interactions with PhIP, the authors concluded that arctiin may have a weak cocarcinogenic effect on MeIQx-induced liver cancer.

Table 4-20. GST-P-positive foci in male F344 rats fed MeIQx and arctiin

Treatment group[a]		Number of rats	Number of foci/cm^2 [b]	Area (mm^2/cm^2)[b]
MeIQx (ppm)	Arctiin (%)			
0	0	13	6.3 ± 1.4	0.5 ± 0.2
0	0.1	15	6.1 ± 1.8	0.4 ± 0.2
300	0	15	21.1 ± 9.6***[c]	1.7 ± 1.0***[c]
300	0.5	15	28.0 ± 5.6*[d]	2.6 ± 0.6*[d]

Source: Hirose *et al.* 2000.

***P* < 0.01, ****P* < 0.001

[a]All groups were pretreated with an i.p. injection of DEN two weeks prior to treatment with MeIQx and/or arctiin for six weeks.

[b]Variability not specified as either SE or SD.

[c]Compared to the control (basal diet only) group.

[d]Compared to the group with MeIQx and basal diet.

Collett *et al.* (2001) investigated the effects of PhIP and curcumin when given in the diet to *Apc*^{min} heterozygous C57BL/6J mice. Treatment groups of 10 male *Apc*^{min} or wild-type mice included curcumin only (2,000 ppm), PhIP only (300 ppm), or PhIP and curcumin. Control groups of 10 *Apc*^{min} or wild-type mice received pelleted AIN-76 diet. After 10 weeks, dietary PhIP significantly increased adenomas in the proximal third of the small intestine of *Apc*^{min} mice (Table 4-21). Tumors were not increased in other sections of the small intestine, and no tumors were observed in any of the wild-type mice. Curcumin treatment significantly inhibited PhIP-induced tumorigenesis in *Apc*^{min} mice.

Table 4-21. Effects of PhIP and curcumin treatment on formation of adenomas in the small intestine of male *Apc*^{min} mice

Treatment	Duration (wk)	Proximal small intestine[a]	Middle small intestine[a]	Distal small intestine[a]	Total small intestine[a]
Control	10	2.1 ± 2.5	6.0 ± 5.9	6.1 ± 5.2	14.1 ± 11.6
Curcumin	10	1.5 ± 2.2	6.5 ± 6.2	5.1 ± 6.7	13.2 ± 15.1
PhIP	10	4.6 ± 2.7*	7.8 ± 10.2	4.6 ± 10.3	17.1 ± 22.5
PhIP + curcumin	10	2.2 ± 1.5*[b]	6.7 ± 6.6	7.4 ± 5.6	16.3 ± 12.3

Source: Collett *et al.* 2001.

**P* < 0.05 (Mann-Whitney test) vs. control unless otherwise specified

[a]Average number of adenomas per mouse (N = 10), variability not specified as SD or SE.

[b]Significantly reduced compared to the PhIP-only group.

Seventeen male F344 rats were dosed twice with 200 mg/kg MeIQ by gavage to initiate colon carcinogenesis (Corpet *et al.* 2000). All rats were given tap water for seven days after initiation and then were randomly divided into treatment groups. Nine rats were maintained on a standard diet and tap water. The remaining eight rats were given 5% polyethylene glycol in the drinking water for 30 days. The mean number of ACF (± standard deviation) was significantly lower ($P = 0.003$) in the group receiving polyethylene glycol (0.5 ± 0.5) than in controls (3.0 ± 1.8).

4.4.1.3 Mammary tumors

Six studies were reviewed that examined inhibition of PhIP-induced mammary cancer by antioxidants and other chemicals (Hirose *et al.* 1995a, Suzui *et al.* 1997, Hagiwara *et al.* 1999, Hirose *et al.* 2000, Nakatsugi *et al.* 2000, Ohta *et al.* 2000). PhIP administered in the diet at 200 ppm for one year or by gavage (8 doses) at 85 to 100 mg/kg over 10 days to 8 weeks induced a high yield of mammary tumors. Treatment with the antioxidant 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ), caffeine, and nimesulide (a cyclooxygenase inhibitor) significantly reduced tumor incidence and multiplicity. Treatment with diallyl disulfide, aspirin, α-tocopherol, TTAD, arctiin, and fermented soy milk significantly reduced tumor multiplicity. Results are summarized in Table 4-22.

Table 4-22. Chemoprevention of PhIP-induced mammary tumors

Test strain (treatment)	PhIP Conc. (ppm)	Duration (wk)[a]	Adenocarcinoma		Reference
			Incidence (%)[b]	Multiplicity (no./rat)[c]	
Dietary studies					
F344					Hirose *et al.* 1995a
PhIP alone	200	52	8/20 (40)	0.40 ± 0.50	
+ 0.5% HTHQ	200	52	1/21 (5)**	0.05 ± 0.22**	
F344					Hagiwara *et al.* 1999
PhIP alone	200	54	8/20 (40)	0.50 ± 0.51	
+ 0.1% caffeine	200	54	2/20 (10)*	0.10 ± 0.31**	
+ 0.5% α-TP	200	54	4/20 (20)	0.20 ± 0.41*	
+ 0.1% TTAD	200	54	3/20 (15)	0.15 ± 0.37*	
Gavage studies					
Sprague-Dawley					Suzui *et al.* 1997
PhIP alone	85 mg/kg × 8	10 days	21/31 (68)	2.32 ± 2.47	
+ 0.02% DAD	85 mg/kg × 8	10 days	16/31 (52)	0.90 ± 1.12**	
+ 0.04% aspirin	85 mg/kg × 8	10 days	14/30 (47)	1.27 ± 1.91	

Test strain (treatment)	PhIP Conc. (ppm)	Duration (wk)[a]	Adenocarcinoma		Reference
			Incidence (%)[b]	Multiplicity (no./rat)[c]	
Sprague-Dawley					Hirose *et al.* 2000
PhIP alone	100 mg/kg × 8	8	12/20 (60)	2.05 ± 2.50	
Initiation					
+ 0.02% arctiin	100 mg/kg × 8	8	11/20 (55)	1.45 ± 2.04	
+ 0.2% arctiin	100 mg/kg × 8	8	13/20 (65)	1.50 ± 1.85	
Promotion					
+ 0.02% arctiin	100 mg/kg × 8	8	10/19 (53)	1.00 ± 1.11*	
+ 0.2% arctiin	100 mg/kg × 8	8	11/20 (55)	0.65 ± 0.67*	
Sprague-Dawley					Nakatsugi *et al.* 2000
PhIP alone	85 mg/kg × 8	2	30/42 (71)	2.6 ± 0.5	
+ 0.04% NSE	85 mg/kg × 8	2	19/37 (51)	1.2 ± 0.2*	
Sprague-Dawley					Ohta *et al.* 2000
PhIP alone	85 mg/kg × 8	2	30/42 (71)	2.6 ± 0.5	
+ 0.1% FSM	85 mg/kg × 8	2	20/39 (51)	1.2 ± 0.2*	
+ 0.04% IF	85 mg/kg × 8	2	25/41 (61)	1.5 ± 0.3*	

*$P < 0.05$, **$P < 0.01$ (significantly lower vs. the PhIP only groups).

[a]Duration for PhIP exposure only and not necessarily the study duration.

[b]Number of animals bearing tumors/number of animals examined.

[c]Variability is SD for Hirose *et al.* 1995a, Hagiwara *et al.* 1999, Suzui *et al.* 1997; SE forNakatsugi *et al.* 2000 and Ohta *et al.* 2000, and not specified for Hirose *et al.* 2000.

DAD, diallyl disulfide; α-TP, α-tocopherol; TTAD, *n*-tritriacontane-16,18-dione; NSE, nimesulide; FSM, fermented soy milk; IF, isoflavones

4.4.2 Promotion or tumor enhancement studies

HCAs have been used as both initiators and promoters in experimental animal studies. In general, HCAs were not very effective at promoting carcinogenesis. However, certain dietary factors and chemicals may promote or enhance HCA-induced carcinogenesis.

4.4.2.1 HCAs as promoters

The studies reviewed for this section indicate that PhIP and MeIQx are weak promoters at best. GST-P-positive foci were not increased in number or size in male Wistar rats initiated with 200 mg/kg DEN, given a partial hepatectomy, and given 20 to 40 mg/kg MeIQx by gavage (Kleman *et al.* 1993). PhIP given in the diet at 25 to 200 ppm did not have a clear promotional effect on 3,2'-dimethyl-4-aminobiphenyl (DMAB)-induced pancreatic and intestinal tumorigenesis in male F344 rats (Ogawa *et al.* 1998). Lung, liver, nasal cavity, and pancreatic tumors were not increased in neonatal Crj:CD-1 mice pretreated with *N*-nitrosobis(2-oxopropyl)amine (BOP) and fed PhIP (10, 50, or 200 ppm) or MeIQx (3, 30, or 300 ppm) for 23 weeks, with the exception of a significantly increased ($P < 0.05$) multiplicity of lung adenomas in mice that received 10 ppm PhIP

(Miyauchi *et al.* 1999). Syrian hamsters inititated with 30 mg/kg BOP, followed by daily i.p. injections of DL-ethionine (500 mg/kg) on days 12 to 15, an i.p injection of L-methionine (800 mg/kg) on day 16, an additional s.c. injection of BOP (20 mg/kg) on day 18, and fed diets containing 600 ppm MeIQx or 400 ppm PhIP did not show an increased incidence of hyperplasia or carcinoma of the pancreatic duct compared to the animals maintained on the basal diet (Yoshimoto *et al.* 1999). However, a few studies showed some slight promotional effects of HCAs.

Hirose *et al.* (1999a, 1999b) gave male F344 rats four s.c. injections of 40 mg/kg DMH to initiate colon cancer. One week later, groups of 19 to 21 rats were placed on diets containing 300 ppm PhIP alone or in combination with 0.125% or 0.5% HTHQ, a synthetic antioxidant, for 32 weeks. A control group was placed on a basal diet after DMH treatment. All animals were sacrificed after 36 weeks. The incidence of colon tumors was not statistically different among the four groups; however, tumor multiplicity was increased by treatment with PhIP alone compared to the basal diet and was reduced by the addition of HTHQ to PhIP treatment (Table 4-23).

Table 4-23. Incidence and multiplicity of colon tumors in male F344 rats treated with combinations of DMH, PhIP, and HTHQ

Treatment	Duration (weeks)[a]	No. of rats	Incidence (%)	Multiplicity (No./rat ± SD)
DMH + basal diet	32	20	15 (75)	1.3 ± 1.2
DMH + PhIP (300 ppm)	32	21	20 (95)	9.1 ± 6.2***[b]
DMH + PhIP + 0.125% HTHQ	32	21	21 (100)	6.2 ± 3.2
DMH + PhIP + 0.5% HTHQ	32	19	19 (100)	3.6 ± 1.8***[c]

Source: Hirose *et al.* 1999a, 1999b.

***$P < 0.001$

DMH - 1,2-dimethylhydrazine.

HTHQ - 1-*O*-hexyl-2,3,5-trimethylhydroquinone.

[a]Duration of the treatment diet after DMH initiation.

[b]Compared to the basal diet group.

[c]Compared to the PhIP group.

The modifying effects of propolis (a resinous plant material used in folk medicine in Europe and in health foods in Japan) on MeIQx promotion of hepatocarcinogenesis was studied in male F344 rats (Kawabe *et al.* 2000). Six groups of 15 or 16 rats were injected with 200 mg/kg DEN. Two weeks later, rats were fed diets containing 0.03% (300 ppm) MeIQx alone or combined with 0.5% water-extracted propolis or ethanol-extracted propolis for six weeks. Other groups were not pretreated with DEN but received basal diets containing 0.1% or 0.5% propolis extracts or the basal diet alone. At the end of week 3, all rats were given a partial hepatectomy. All surviving animals were sacrificed after eight weeks and examined for hepatic GST-P-positive foci > 0.2 mm in diameter. Body weights in the group given MeIQx alone were significantly lower than in the

control group. Relative liver weights were increased in several treatment groups compared to the control group. MeIQx treatment clearly increased the numbers and areas of GST-P-positive foci. Furthermore, treatment with MeIQx and 0.5% water-extracted propolis significantly increased the numbers and areas of foci compared to MeIQx alone. These results indicate that the water extract of propolis acted as a co-carcinogen with MeIQx (Table 4-24).

Table 4-24. Modifying effects of propolis on MeIQx promotion of DEN-initiated hepatocarcinogenesis in male F344 rats

Experimental group[a]	No. of mice	Body weight (g)[b]	Relative liver weight (g/100 g bw)[b]	GST-P-positive foci[b]	
				No./cm^2	Area (mm^2/cm^2)
Control 1	13	264.6 ± 17.3	3.06 ± 0.14	6.3 ± 1.4	0.5 ± 0.2
300 ppm MeIQx	15	248.2 ± 12.4**	3.60 ± 0.25***	21.1 ± 9.6[c]	1.7 ± 1.0[c]
+ 0.5% w-propolis	15	254.0 ± 14.3	3.52 ± 0.15***	29.4 ± 6.9*[d]	2.9 ± 0.9**[d]
+ 0.5% e-propolis	16	254.3 ± 12.5	3.68 ± 0.19***	24.2 ± 5.9[c]	2.1 ± 0.7[c]
+ 0.1% w-propolis	15	270.1 ± 10.0	3.10 ± 0.14	14.2 ± 1.8**	0.5 ± 0.1
+ 0.1% e-propolis	15	269.1 ± 10.2	3.22 ± 0.11**	9.3 ± 3.8*	0.5 ± 0.2
Control 2	14	276.7 ± 13.4	3.32 ± 0.10	7.2 ± 3.0	0.4 ± 0.2
+ 0.5% w-propolis	15	268.4 ± 16.0	3.28 ± 0.17	5.9 ± 2.6	0.3 ± 0.2
+ 0.5% e-propolis	15	273.6 ± 10.5	3.46 ± 0.11**	7.5 ± 2.0	0.4 ± 0.2

Source: Kawabe *et al.* 2000.

*P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group unless otherwise noted).

[a]All animals were pretreated with DEN two weeks prior to treatment (six weeks) with MeIQx and propolis. At three weeks, all animals were given a partial hepatectomy.

[b]± SD

[c]Comparison to basal diet group (Control 1) not reported.

[d]Compared to MeIQx alone.

w-propolis - water extract

e-propolis - ethanol extract

4.4.2.2 Enhancement of HCA-induced preneoplastic or neoplastic lesions

Kleman *et al.* (1989) reported that PhIP and MeIQx were weak initiators in the resistant hepatocyte model. Male Wistar rats were given a single i.p. injection of PhIP at 50 or 75 mg/kg, or MeIQx at 50 mg/kg, within 24 hours after undergoing a two-thirds partial hepatectomy. Two weeks later, the animals were placed on a diet containing 0.02% 2-acetylaminofluorene (2-AAF) for two weeks. Three weeks after treatment with PhIP or MeIQx, a single dose of carbon tetrachloride (2 mL/kg) was given. All animals were sacrificed six weeks after the start of the experiment. A statistically significant increase (P < 0.01) in γ-glutamyl-transferase-positive hepatic foci was observed in rats injected with 75 mg/kg PhIP (P < 0.01) or 50 mg/kg MeIQx (P < 0.05).

Male F344 rats given 0.4, 4, or 40 ppm MeIQx in the diet for 8 to 12 weeks did not develop hepatic GST-P-positive foci (Sone *et al.* 1992). However, when 40 ppm MeIQx was co-administered with 0.7 mL/kg carbon tetrachloride given twice a week by s.c. injection, the numbers ($P < 0.001$) and areas ($P < 0.01$) of foci showed significant increases after 8 weeks compared to rats exposed to carbon tetrachloride only. The numbers ($P < 0.01$) and areas ($P < 0.05$) of hyperplastic nodules also were significantly increased at 12 weeks.

Kristiansen *et al.* (1989) conducted an initiation-promotion assay in male Wistar rats using MeIQ as the initiator and phenobarbital as the promoter. The test animals were given MeIQ (10 mg/kg per day) by gavage for 14 consecutive days. Animals in the control group received water acidified with citric acid to a pH of 3.5. One week later, both the test and control groups were divided into two groups each. One group received 500 ppm sodium phenobarbital in their drinking water throughout the rest of the study, and the other group received no further treatment. Body weights and food and water consumption were recorded periodically throughout the experiment. An interim sacrifice of 10 animals from each group was conducted during week 42, and the experiment was terminated after week 58. Body weights of mice treated with MeIQ were significantly lower than the controls throughout the experiment; however, no animals died before sacrifice in any group. Zymbal gland tumors occurred in five of 40 rats treated with MeIQ alone ($P < 0.05$) and in three of 40 rats treated with MeIQ and phenobarbital. These tumors did not occur in controls or in animals receiving phenobarbital only. Squamous-cell carcinoma of the skin and lymphatic leukemia each occurred in one animal in the MeIQ group. Hepatocellular carcinomas occurred in two rats, and a liver sarcoma occurred in one rat treated with MeIQ and phenobarbital. Although the incidence of liver tumors in the MeIQ plus phenobarbital group was lower than expected, gamma-glutamyltranspeptidase (GGT) activity was significantly increased by exposure to MeIQ or phenobarbital when given alone or combined.

Tsuda *et al.* (1990) investigated the tumor initiation potential of MeIQx and the effects of cell proliferation on the initiation step. Two experiments, each with three groups of 5 to 10 male F344 rats, were conducted. In the first experiment, group 1 rats were given a single intragastric dose of 80 mg/kg MeIQx dissolved in corn oil. Two weeks later, these animals were placed on a basal diet containing 0.05% phenobarbital for six weeks. One week after the animals were first exposed to phenobarbital, a two-thirds partial hepatectomy was performed. The rats in groups 2 and 3 were treated identically except group 2 was given corn oil instead of MeIQx, and group 3 was exposed to MeIQx but was not given phenobarbital. In experiment two, the groups were the same as in the first experiment; however, the partial hepatectomy was performed 12 hours before administration of the test compound or corn oil. Furthermore, the animals in experiment two were given an i.p. injection of a toxic dose (300 mg/kg) of D-galactosamine one week after the first exposure to phenobarbital to stimulate cell proliferation during the promotion phase. All animals in both experiments were sacrificed at eight weeks. Body weights of group 1 rats were significantly higher than group 2 rats in experiment one but were significantly lower in experiment two. There were no differences in liver weights among the groups. The number and size of GST-P-positive foci were not significantly different among groups in experiment one. However, in experiment two, incidences of

GST-P-positive foci were significantly higher ($P < 0.001$) in group 1 (3.09 ± 2.14) compared to group 2 (0.45 ± 0.32). The average size of the foci also was significantly increased in group 1. There were no significant differences between group 3 and group 2 rats in either experiment in body weights, liver weights, incidences of GST-P-positive foci, or average size of the foci.

Weisburger *et al.* (1994) investigated the interaction of dietary fat and calcium levels with PhIP-induced ACF in rat colon. Eight groups of five male F344 rats were exposed to various combinations of low or high fat and calcium levels. Four of these groups were exposed to 400 ppm PhIP in the diet, and the other four groups served as controls. All groups exposed to PhIP had significantly more ACF per rat than their respective control groups (Table 4-25). The high-fat diet produced significantly more ACF than the low-fat diet, and high calcium levels reduced the number of ACF in animals on the low-fat diet.

Table 4-25. Effects of dietary fat on incidences of ACF in male rats orally exposed to PhIP

Test strain (group)	No. of rats	Exposure		Aberrant crypt foci	
		Conc. (ppm)	Duration (wk)	Incidence (%)	No. of foci/rat (± SE)
F344					
low fat + low Ca^{2+}	5	0	9	0	0
	5	400	9	5 (100)	17 ± 0.6*
high fat + low Ca^{2+}	5	0	9	1 (20)	1 ± 02
	5	400	9	4 (80)	30 ± 7.6*
low fat + high Ca^{2+}	5	0	9	1 (20)	1 ± 02
	5	400	9	4 (80)	13 ± 1.6*
high fat + high Ca^{2+}	5	0	9	0	0
	5	400	9	5 (100)	20 ± 0.8*

Source: Weisburger *et al.* 1994.

*$P < 0.05$ vs. respective controls (no statistical comparisons of incidence data)

Tsuda *et al.* (1999) examined the interactions of PhIP with other HCAs and caffeine in male F344 rats. In the first experiment, PhIP, IQ, and MeIQx were added to the diet (50 ppm) for 16 weeks and were studied alone or in combinations of two or three HCAs (see Section 4.4.2.3). In the second experiment, rats received 400 ppm PhIP in the diet alone or in combination with 10 ppm, 100 ppm or 1,000 ppm caffeine in the drinking water for 10 weeks. Control groups received only the basal diet or 1,000 ppm caffeine. All rats administered PhIP alone at 400 ppm for 10 weeks developed ACF. Administration of caffeine at 1,000 ppm did not produce ACF but did induce CYP1A2. Caffeine at 1,000 ppm co-administered with PhIP significantly increased the number of ACF and CYP1A2

protein levels compared to PhIP alone (Table 4-26). These data indicate that caffeine induces CYP1A2, which may increase the carcinogenic risk of PhIP.

Table 4-26. Aberrant crypt foci and CYP1A2 levels in rats orally exposed to PhIP alone or in combination with caffeine for 10 weeks

Test strain (group)	CYP1A2 levels (A.U.)	Aberrant crypt foci	
		Incidence (%)[a]	No. of foci/rat[b]
F344			
Basal diet	1.00	0	0
Caffeine (1,000 ppm)	3.92	0	0
PhIP (400 ppm)	3.53	100	17.0
+ 10 ppm caffeine	1.86	100	17.9
+ 100 ppm caffeine	2.36	100	14.7
+ 1,000 ppm caffeine	6.31*	100	26.0**

Source: Tsuda *et al.* 1999.

*$P < 0.05$, **$P < 0.01$ (compared to PhIP alone group)

A.U. - arbitrary units based on the basal diet group.

[a]Number of animals was not reported.

[b]Confidence interval was not reported.

Female Sprague-Dawley rats on a high-fat diet had more mammary tumors than identically treated rats on a low-fat diet (Table 4-27). Female rats were given 10 doses of 75 mg/kg PhIP dissolved in corn oil (Ghoshal *et al.* 1994). The animals were dosed on five consecutive days, followed by two days off, and then were dosed for another five days. Animals were placed on a high-fat (23.5%) or low-fat (5%) diet two days after the last dose and maintained for 25 weeks. Control groups received 10 doses of the corn oil vehicle using the same protocol as the treated groups and were placed on low-fat or high-fat diets. No tumors occurred in control groups treated with vehicle only. Tumor incidences in animals on low-fat and high-fat diets were 16% and 53%, respectively. All tumors in the low-fat group were benign. In contrast, 80% of the tumors in the high-fat group were malignant. Snyderwine *et al.* (1998a) reported similar results in an identical study using more animals. Tumor incidences in animals on the low-fat and high-fat diets were 31% and 49%, respectively. In contrast to the Ghoshal *et al.* (1994) study, 24% of the animals in the low-fat group had malignant tumors. The authors suggested that the sample size may explain this difference. Only 12 rats were used in the earlier study compared to 88 in the latter. Eighty-nine percent of the tumors in animals on the high-fat diet in the latter study were malignant (Snyderwine *et al.* 1998a).

Tumor incidence was not affected by dietary fat levels in Sprague-Dawley × F344 hybrid rats fed a diet containing 150 ppm PhIP for 11 weeks then changed to 100 ppm for 41 weeks (Weisburger *et al.* 1997). Groups of 30 rats each were exposed to PhIP and maintained on a low-fat (5%) or high-fat (23.5%) diet. The low- and high-fat control

groups included 18 animals. One mammary gland tumor occurred in the low-fat control group. Twenty-one rats in each of the PhIP-exposed groups developed mammary gland tumors (Table 4-27). The mean number (± S.D.) of tumors per rat was slightly higher in the high-fat group (1.33 ± 1.47) than in the low-fat group (1.14 ± 1.08), but the difference was not significant.

Table 4-27. Modulation of PhIP-induced mammary tumors in female rats fed high-fat diets

Test strain	Conc. (ppm)	Duration (wk)	Incidence of adenocarcinoma[a] (%)	Reference
Sprague-Dawley				Ghoshal *et al.* 1994
Main study				
Low-fat diet	750[b]	27	2/12 (16)[c]	
High-fat diet	750[b]	27	8/15 (53)[c]	
Sprague-Dawley				Snyderwine *et al.* 1998a
Low-fat diet	0	27	0/43 (0)	
	750[b]	27	27/88 (31)	
High-fat diet	0	27	0/44 (0)	
	750[b]	27	44/90 (49)*[d]	
SD × F344				Weisburger *et al.* 1997
Low-fat diet	0	52	1/18 (3)	
	100-150[e]	52	21/30 (72)[f]	
High-fat diet	0	52	0/18 (0)	
	100-150[e]	52	21/30 (70)	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. respective controls unless otherwise specified.

[a]Number of animals bearing tumors/number of animals examined.

[b]Cumulative dose given in 10 daily doses of 75 mg/kg by gavage.

[c]Statistical significance level was not reported.

[d]Compared to the low-fat group.

[e]Rats were dosed with 150 ppm for the first 11 weeks, followed by 100 ppm for 41 weeks.

[f]As listed by Weisburger *et al.* 1997; however, the percentage appears to be based on 21 of 29 animals.

4.4.2.3 HCA mixtures

Ito *et al*. (1991b, 1995) and Hasegawa *et al.* (1991, 1994, 1996) conducted a series of three experiments with 10 HCAs administered alone or in various combinations. Groups of male F344 rats were initially given i.p. injections of 200 mg/kg DEN. Two weeks later, these animals received various HCAs (alone or in combination) in the diet for six weeks. A two-thirds partial hepatectomy was performed at week 3, and animals were

sacrificed at week 8. In the first experiment (Ito *et al.* 1991b), five HCAs, including MeIQx and MeIQ, were administered alone at 1/1, 1/5, or 1/25 carcinogenic doses, or in mixtures of all five at 1/5 or 1/25 dose levels. The second experiment was identical to the first experiment but with a different group of five HCAs, including PhIP (Hasegawa *et al.* 1991). The concentrations of PhIP and MeIQx were 400, 80, or 16 ppm. Concentrations of MeIQ were 300, 60, or 12 ppm. In experiment 3, all 10 HCAs were given alone at 1/10 dose levels or in mixtures of all 10 HCAs at 1/10 or 1/100 dose levels (Hasegawa *et al.* 1994, Ito *et al.* 1995). The number of hepatic GST-P-positive foci in the full-dose groups was proportional to their liver carcinogenicities with MeIQx > MeIQ > PhIP. The full dose level of PhIP did not increase the number of GST-P-positive foci compared to controls. The number of GST-P-positive foci also was increased in some combination groups compared to the sum of the effects for the individual groups at the same dose levels. However, results for the combination groups generally were similar to the average values for the individual groups given at the next higher dose level. For example, the average number of GST-P-positive foci for all five chemicals at the full dose in experiment 1 was 54.32 compared to 56.85 when these five compounds were combined at the 1/5 dose level. In experiment 1, MeIQ at the 1/5 dose level significantly increased the number and area of foci. At the 1/25 dose level, none of the five HCAs increased foci number or area above the control value; however, the mixture of these five HCAs (each at the 1/25 level) significantly increased both the number and area of GST-P-positive foci. In experiment 2, results for the mixture of all five chemicals at the 1/5 dose level were significantly greater than the mean of the five given alone at full doses ($P < 0.05$). However, PhIP alone did not significantly increase GST-P foci at any dose level. None of the 10 HCAs significantly increased GST-P-positive foci when given alone at 1/10 the full dose. However, the mixture of all 10 HCAs at 1/10 the full dose significantly increased GST-P-positive foci, indicating a clear synergistic response. The response for the mixture of 10 HCAs was lower than that observed in controls. Overall, the data suggested that the effects of HCAs are fundamentally additive rather than synergistic. However, the authors suggested that some combinations including PhIP and MeIQ may be synergistic because they induce key metabolic enzymes.

Colon and stomach carcinogenesis were enhanced in male Sprague-Dawley rats induced with 10 weekly injections of DMH and fed diets with high concentrations (85.6 ng/g) of HCAs (Pence *et al.* 1998). PhIP and MeIQx accounted for approximately 83% and 14%, respectively, of the total HCA content in the high-HCA diet. Lean beef was cooked by different methods at different temperatures to produce various levels of HCAs. Eight groups of 25 animals each were maintained on various combinations of high- or low-fat diets and low- or high-HCA content. An additional group was maintained on a high-fat-high HCA diet without DMH treatment. The combination of a low-fat diet with high-HCA content significantly increased the incidence of colon adenocarcinomas; however, colon adenocarcinomas were reduced in rats receiving a high-fat diet with a high-HCA content. The apparent protective effect of the high-fat diet may be due to induction of CYP2E, which is responsible for DMH *N*-demethylation or other interactions. Diets with high-HCA content also increased the incidence of stomach tumors in all DMH-treated rats, which suggests a synergistic effect. This finding was unexpected because DMH does not usually induce stomach tumors.

Tsuda *et al.* (1999) examined the interactions of PhIP, IQ, and MeIQx in male F344 rats. PhIP was added to the diet (50 ppm) for 16 weeks and was studied alone or in combination with one or two other HCAs. The rats were examined for hepatic GST-P-positive foci (> 1 mm^2 diameter) and colonic ACF (> 1 crypt). PhIP did not induce GST-P-positive foci and generally reduced GST-P foci when given in combination with other HCAs. PhIP induced more ACF than either IQ or MeIQx, but when combined with MeIQx or IQ resulted in fewer or only slightly more ACF than PhIP alone. MeIQx induced about 4-fold fewer GST-P-positive foci than IQ (about 5/cm^2 compared to 19/cm^2). However, MeIQx combined with IQ induced approximately 50 GST-P foci/cm^2, which was significantly greater than an additive effect. Although MeIQx alone induced only about half as many ACF as PhIP alone, the combination of MeIQx and IQ showed an enhanced response and was even greater than the response observed when all three HCAs were combined. Therefore, interactions of HCA mixtures are variable and do not always result in an additive response.

4.5 Carcinogenicity of HCA metabolites

Gastrointestinal tumors occurred in mice exposed to *N*-hydroxy-PhIP by i.p. injection. Oshima *et al.* (1996) dosed $Apc^{\Delta 716}$ heterozygous mutant mice with 50 mg/kg *N*-hydroxy-PhIP for five consecutive days beginning at 26 days of age. Unlike the results of the feeding studies with PhIP or MeIQx (see Sections 4.1.1.1 and 4.2.1.1), the number of intestinal polyps in male mice increased three-fold over controls. The mean number of polyps in female mice increased slightly, but was not statistically significant. Most of the difference in male mice was from a marked increase in small polyps. Polyp size and distribution did not differ between female mice and controls.

Archer *et al.* (2000) injected (i.p) male ACI/seg rats twice a week for 10 weeks with *N*-hydroxy-PhIP or *N*-hydroxy-MeIQx (the reactive metabolites of PhIP and MeIQx). Animals in the control group were injected with the solvent vehicle. Animals were sacrificed at 68 weeks. Colon carcinomas and bladder transitional cell tumors were each found in 4 of 31 ($P < 0.06$) animals treated with *N*-hydroxy-PhIP. Incidences of soft tissue tumors at the injection site were significantly higher ($P < 0.05$) in animals treated with *N*-hydroxy-MeIQx (6/27) than in controls (1/31). In addition, atypical hyperplasia of the ventral prostate, anterior prostate, and seminal vesicles was significantly higher ($P < 0.05$) in rats treated with either *N*-hydroxy-PhIP or *N*-hydroxy-MeIQx than in controls. These researchers noted that bladder tumors are rare in ACI rats and concluded that *N*-hydroxy-PhIP and *N*-hydroxy-MeIQx may be potential carcinogens of the prostate and bladder.

4.6 Summary

Although there is some overlap, tumor profiles of PhIP, MeIQx, and MeIQ in rats and mice were variable (Table 4-28). Nevertheless, each of these chemicals caused tumors at multiple sites in several strains of mice and rats. Furthermore, the carcinogenic effects of HCAs may be inhibited or enhanced by many factors including interactions of HCA mixtures. In addition, mice and rats injected with the *N*-hydroxy metabolites of PhIP or MeIQx developed tumors. This section summarizes the findings of carcinogenicity

studies in experimental animals for the individual HCAs and mixtures, their modulators, and their metabolites.

4.6.1 PhIP

Many carcinogenicity studies of PhIP in experimental animals were reported after the IARC (1993a) monograph was published. PhIP was administered in the diet in most of these studies; however, a few studies used gavage or i.p. injection. PhIP is a potent lymphomagen in male and female mice but shows weak to no carcinogenicity in other tissues. Although some mice developed ACF in the colon following PhIP exposure, the data suggested that the ACF were unlikely to progress to colon cancer. One dietary study and two i.p. injection studies with transgenic mice predisposed to develop intestinal tumors, showed a statistically significant increase in the number of some intestinal lesions (ACF, adenomas, polyps, etc.) in male mice. Male neonatal mice injected (i.p.) with PhIP showed a significant increase in liver adenomas in two of three studies reviewed.

Lymphomas occurred in 75% of male F344 rats initially exposed to dietary concentrations of 400 ppm PhIP; these dietary concentrations were later reduced to 200 ppm and 100 ppm. PhIP at 400 ppm in the diet induced gastrointestinal and prostate tumors in male rats. Female rats were generally resistant to gastrointestinal tumors. Many studies demonstrated that PhIP exposure was associated with an increased incidence of mammary tumors in female rats. Furthermore, transplacental and transbreast milk exposure increased the risk of developing mammary adenocarcinomas.

4.6.2 MeIQx

Although not as extensively investigated as PhIP, several carcinogenicity studies of MeIQx in experimental animals were reported after the IARC (1993c) monograph was published. MeIQx was administered in the diet in all but one study that used i.p. injection.

Mice fed MeIQx in the diet developed ACF, liver tumors, lymphomas and leukemias (males only), and lung tumors (females only). As seen with PhIP, neonatal male mice exposed by i.p. injection also developed hepatocellular adenomas within 12 months. All mice fed diets containing 600 ppm MeIQx for 7 weeks developed ACF within 14 weeks compared to none in the controls. Another short-term study with double transgenic mice (*c-myc/λlacZ*) indicated that hepatocarcinogenicity was enhanced by *c-myc* overexpression. Incidences of liver tumors in CDF_1 mice fed 600 ppm MeIQx for 84 weeks were 43% and 91% in males and females, respectively, compared to 17% and 0% in male and female controls, respectively. Incidences of lung tumors in female mice (43%) and lymphomas and leukemias in male mice (29%) were significantly higher than in controls (10% and 5%, respectively).

Short-term studies in male F344 rats exposed to MeIQx in the diet showed dose-related increases in GST-P-positive foci and ACF. LEC rats fed 40 ppm MeIQx for 40 weeks had increased incidences of hepatocellular adenoma and higher tumor multiplicity. Male F344 rats fed diets containing 400 ppm MeIQx for 429 days developed increased

incidences of liver (100%), Zymbal gland (75%) and skin (35%) tumors. In this same study, female rats had increased incidences of liver (53%), Zymbal gland (53%), and clitoral gland (63%) tumors. None of these tumors were observed in the control groups. Another long-term feeding study in male F344 rats fed diets containing 0, 100, 200, or 400 ppm MeIQx for 56 weeks gave very similar results and showed dose-related increases in these tumor types. In addition, incidences of liver tumors were significantly higher in the 200 ppm group.

MeIQx was not carcinogenic in cynomolgus monkeys given 10 to 20 mg/kg MeIQx by nasogastric intubation for 84 months. This finding was attributed to a low level of metabolic activation via *N*-hydroxylation in this species.

4.6.3 MeIQ

The carcinogenicity of MeIQ has not been investigated as extensively as PhIP or MeIQx; however, a few long-term feeding studies in mice and short-term feeding studies in rats were available. The tumor spectrum varied with species and strain.

Ingestion of 100 or 400 ppm MeIQ for 91 weeks resulted in statistically significant increased incidences of forestomach tumors in male and female CDF_1 mice and liver tumors in female mice. Forestomach tumors occurred in approximately 90% of mice that received the high dose compared to none in the controls. Approximately 70% to 85% of the forestomach tumors were classified as squamous-cell carcinomas and approximately 40% of these had metastasized to the liver. Liver tumors included hepatocellular adenomas and carcinomas, and fibrosarcomas. The incidences were 71% in the high-dose group and 0% in the controls. In contrast, forestomach tumors were not increased in female Big Blue® or C57BL/6N mice fed 300 ppm MeIQ for 92 weeks. However, incidences of liver, cecum, and colon tumors were significantly increased. None of these tumors were observed in controls of either strain.

Male and female F344 rats fed a diet containing 300 ppm MeIQ for 286 days developed increased incidences of several tumors. These included Zymbal gland (85% to 95%), oral cavity (35%), and colon (25% to 35%) tumors in both sexes, skin tumors (50%) in male rats, and mammary tumors (25%) in female rats. None of these tumors were observed in controls. In another study, male F344 rats that were administered 200 mg/kg MeIQ twice by gavage developed ACF after 30 days.

4.6.4 Modulators of HCA-induced carcinogenicity

More than 150 modulators of HCA mutagenicity and carcinogenicity have been studied. Because of the widespread occurrence of HCAs in the human diet and the carcinogenic effects of HCAs in a variety of tissues in experimental animals, many of these studies focused on identifying potential chemopreventive agents in foods. These include caffeine, catechins and other antioxidants, polyphenols, chlorophyllin, indole-3-carbinol, conjugated linoleic acids, and others. Synthetic agents (such as oltripraz) also have been tested. Results were mixed, but this research provides inportant insights into the mechanisms involved in HCA carcinogenesis.

HCAs were not effective promoters in most studies reviewed. However, colon tumor multiplicity was increased in rats initiated with DMH and fed PhIP, and both the number and area of GST-P-positive foci were increased in rats initiated with DEN and fed MeIQx. Another study investigated the promotional effects of HCA concentration and fat content in the diet. Colon tumors were increased in rats initiated with DMH and fed a diet containing high concentrations of several HCAs, primarily PhIP and MeIQx, and a low-fat diet, whereas stomach tumors were increased in all groups fed diets with high HCA content.

Several studies reported that HCA-induced carcinogenesis was promoted or enhanced with exposure to other chemicals (carbon tetrachloride, phenobarbital) and dietary factors (high fat, low calcium, caffeine). Co-administration of carbon tetrachloride with MeIQx increased the incidence of GST-P-postive foci and hyperplastic nodules in male F344 rats. Phenobarbital did not increase the number of Zymbal gland tumors in male Wistar rats exposed to MeIQ but did increase GGT activity. GST-P-postive foci were increased in male F344 rats given a partial hepatectomy and treated with MeIQx, phenobarbital, and D-galactosamine. PhIP combined with 0.1% caffeine produced a statistically significant increase in colon tumors and total gastrointestinal tumors in female rats compared to occurrence in a group exposed only to PhIP. The incidences of ACF and mammary tumors were increased in male F344 rats and female Sprague-Dawley rats, respectively, treated with PhIP. However, high-fat diets did not affect the incidences of mammary tumors in hybrid (Sprague-Dawley × F344) female rats.

Finally, several short-term studies examined the possible synergistic effects of HCAs administered in mixtures. HCAs were given alone or in various combinations in the diet. When given alone at their full carcinogenic dose level, MeIQx and MeIQ increased the number of GST-P postive foci but PhIP did not. MeIQ was shown to induce an increased incidence of GST-P-positive foci in the liver at the 1/5 dose level. Overall, results from mixtures of HCAs indicated that the interactions were fundamentally additive; however, some evidence of synergistic and antagonistic effects was observed.

4.6.5 HCA metabolites

Male *Apc*$^{\Delta 716}$ heterozygous mutant mice injected with 50 mg/kg *N*-hydroxy-PhIP on five consecutive days developed increased incidences of intestinal polyps. ACI/seg rats injected with *N*-hydroxy-PhIP twice a week for 10 weeks had higher, but not significant ($P < 0.06$), incidences of colon and rare bladder tumors; rats injected with *N*-hydroxy-MeIQx had significantly higher incidences of soft tissue tumors at the injection site. Furthermore, male rats treated with either compound had significantly increased incidences of atypical hyperplasia of the prostate and seminal vesicles.

Table 4-28. Tumorigenic effects of PhIP, MeIQx, and MeIQ in rats and mice

	PhIP		MeIQx		MeIQ	
Tumor site	**Mice**	**Rats**	**Mice**	**Rats**	**Mice**	**Rats**
Preneoplastic lesions (short-term studies)						
ACF	4	4m	4	4m		
GST-P+ foci				4m		
Neoplastic lesions (short-term or long-term studies)						
Clitoral gland				4f		
Cecum					4f	
Colon		4m			4f	4
Forestomach					4	
Leukemia			4m			
Liver	$4m^n$		4	4	4f	
Lung			4f			
Lymphoma	4	4m	4m			
Mammary gland		4f				4f
Oral cavity						4
Prostate		4m				
Skin				4m		4m
Small intestine	4m	4m				
Zymbal gland				4		4

4= Positive results in both sexes.

4m = Positive results reported only in males.

$4m^n$ = Positive results reported only in male neonates (i.p. administration).

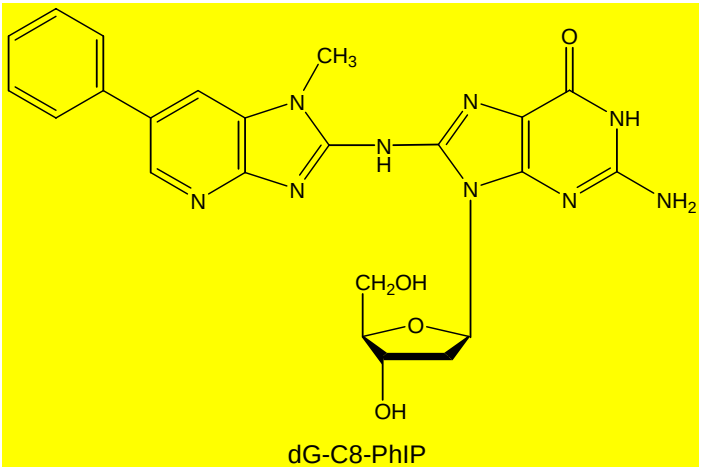
4f = Positive results reported only in females.

5 Genotoxicity

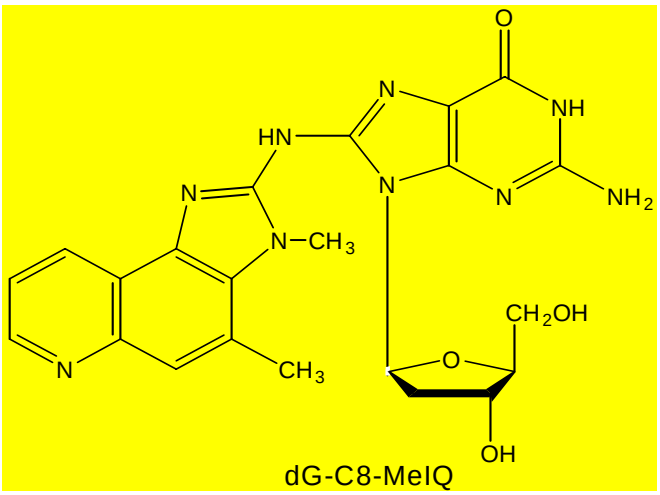
Many studies have been conducted on the metabolic activation and genotoxicity of PhIP, MeIQ, and MeIQx. These HCAs are metabolically activated by phase I hepatic CYP-mediated *N*-hydroxylation followed by esterification of the *N*-hydroxylamines by phase II pathways (see Section 6.1.2). These ester derivatives are highly reactive and form adducts with DNA. HCAs are highly mutagenic in *Salmonella typhimurium*, *Escherichia coli*, cultured human cells, cultured rodent cells, and mice. Additional genotoxicity assays have shown micronuclei (MN) formation, unscheduled DNA synthesis (UDS), DNA single-strand breaks, chromosomal aberrations (CA), and sister chromatid exchanges (SCE) as well as DNA damage. This section discusses DNA adduct formation and removal in both humans and animals, summarizes of the genotoxicity of these compounds, reviews genotoxicity studies published after the IARC review, and compares the mutagenicity of these compounds with each other and with other known mutagens. Detailed descriptions of these individual genotoxicity studies are found in Appendix A.

5.1 DNA Adducts

PhIP, MeIQ, and MeIQx adduct to the guanine base, with the predominant adduct formed at the C8 position (see Figure 5-1) (Lin *et al.* 1992, Tada *et al.* 1994, Fukutome *et al.* 1994, Ghoshal *et al.* 1995). MeIQx also forms a minor adduct at the N^2 guanine position (Turesky *et al.* 1992). HCA-induced DNA adducts have been characterized and detected both *in vitro* and *in vivo*, and the major adduct for each HCA is similar in all species examined. However, monitoring for HCA-DNA adducts in humans is difficult, and an efficient method for routine monitoring in humans is not yet available. It is known that HCA-DNA adducts are necessary for the mutagenesis and carcinogenesis of these compounds; however, their role in human cancer is unknown at this time. In recent years, measurement of adducts as low as 1 to 10 adducts/10^{12} nucleotides has been achieved using accelerator mass spectrometry (AMS). This technology should help to determine the relationship between adduct levels and human cancer (Schut and Snyderwine 1999). This section discusses adduct formation, their occurrence in human and animal cells and tissues, and their removal.



dG-C8-PhIP



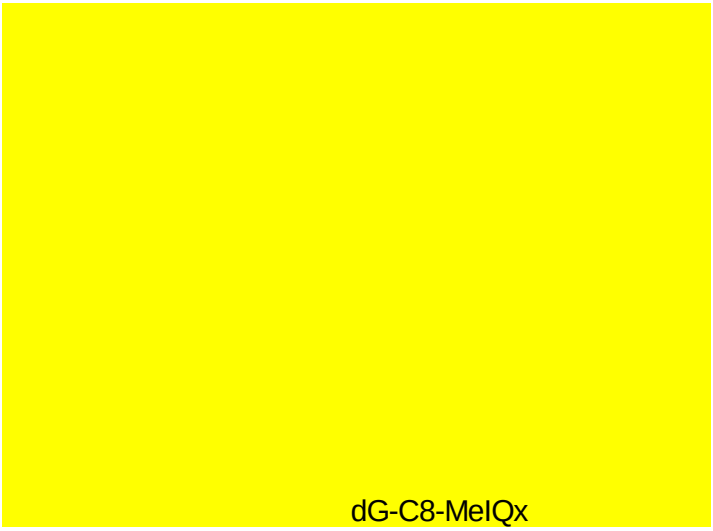


Figure 5-1. Predominant HCA-DNA adducts

5.1.1 Adduct formation

Activation of HCAs occurs through a two-step process involving both phase I and phase II enzymes (see Section 6.1.2). The CYP450s of the liver *N*-hydroxylate HCAs, which is then esterified by phase II enzymes of the liver or target tissues. The ester derivatives may include *N*-acetoxy, *N*-sulfonyloxy, *N*-prolyloxy, and *N*-phosphatyl derivatives. The loss of the ester moiety produces an electrophilic arylnitrenium ion, which is postulated to be the proximate reactive form adducting to DNA (Kerdar *et al.* 1993a, Schut and Snyderwine 1999).

In humans, DNA adducts occurred in tissues following exposure to dietary-relevant doses of HCAs (see Table 5-1). Human subjects undergoing surgery for colon or breast cancer were given gelatin capsules containing 20 to 84 μg PhIP to correspond to daily exposure levels (Dingley *et al.* 1999, Lightfoot *et al.* 2000). Adducts also occurred in isolated human prostate epithelial cells incubated with *N*-hydroxy-PhIP or *N*-hydroxy-MeIQx (Wang *et al.* 1999); isolated human mammary epithelial cells incubated with MeIQx or MeIQ (Pfau *et al.* 1992, Carmichael *et al.* 1996, Stone *et al.* 1998, Williams and Phillips 2000); and in colon, rectum, and kidney tissues collected during surgery or at autopsy (Totsuka *et al.* 1996). Dingley *et al.* (1999) reported that the number of adducts was significantly lower in normal tissue than in colon tumor tissue. In this study, five volunteers were administered a dose of 70 or 84 μg PhIP 48 to 72 hours before surgery to remove colon tumors. In normal colon tissue, 35 to 135 DNA adducts per 10^{12} nucleotides were identified, while in tumor colon tissue, 83 to 308 DNA adducts per 10^{12} nucleotides were noted.

Several investigators reported that the levels of DNA adducts in human tissues were generally greater than in rodents administered an equivalent dose (Turteltaub *et al.* 1999, Garner *et al.* 1999, Mauthe *et al.* 1999). In Turteltaub *et al*. (1999), human volunteers diagnosed with colon cancer were administered gelatin capsules containing 70 or 84 μg PhIP or 21 or 228 μg MeIQx. The mean adduct level per unit dose for colon DNA was 1.13 and 2.24 for PhIP and MeIQx, respectively. In comparison, rats administered PhIP and MeIQx by gavage (doses not reported) had mean adduct levels per unit dose of 0.11 and 0.94 for PhIP and MeIQx, respectively. In another comparative study (Garner *et al.* 1999), human DNA adduct levels per 10^{12} nucleotides for PhIP ranged from 1.57-143.7 in normal tissue and 6.63-167.9 in tumor tissue. In rats, a mean DNA adduct level of 1.93 per 10^{12} nucleotides was reported. For MeIQx, approximately four times more DNA adducts were formed in the colon of humans compared to rats. Mauthe *et al*. (1999) reported that colon samples from human volunteers administered 21 μg MeIQx had a mean of 27 DNA adducts per 10^{12} nucleotides. Rats and mice treated with equivalent body-weight doses had significantly lower (17 and 21 adducts per 10^{12} nucleotides, respectively) DNA adduct levels than humans.

Table 5-1 Analysis of HCA-DNA adducts in human tissues

Test system	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
5 Humans	PhIP	70-84 μg p.o. (gelatin capsule)	WBCs colon	35–135 adducts per 10^{12} nucleotides for normal colon tissue 83–308 adducts per 10^{12} nucleotides for tumor colon tissue adduct type = nr	Dingley *et al.* 1999
10 Humans	PhIP	20 μg p.o. (gelatin capsule)	breast	46–424 adducts per 10^{12} nucleotides for normal breast tissue 26–477 adducts per 10^{12} nucleotides for tumor breast tissue adduct type = nr	Lightfoot *et al.* 2000
5 Humans	PhIP	70 or 84 μg p.o. (gelatin capsule)	colon	0.03–0.13 adducts per 10^{9} nucleotides adduct type = nr	Turteltaub *et al.* 1999
Humans (number nr)	PhIP	20 μg p.o. (gelatin capsule)	colon	1.57–143.7 adducts per 10^{12} nucleotides (normal tissue) 6.63–167.9 adducts per 10^{12} nucleotides (tumor tissue) adduct type = nr	Garner *et al.* 1999
Human prostate epithelial cells	PhIP	10^{-5} M	prostate	13–22 RAL x 10^{7} adduct type = dG-C8-PhIP	Wang *et al.* 1999

Test system	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Human mammary epithelial cells	PhIP	500 μM	mammary	0.2–0.6 adducts per 10^8 nucleotides adduct type = nr	Carmichael *et al.* 1996
Human mammary epithelial cells	PhIP	500 μM	mammary	0.13–4.0 adducts per 10^8 nucleotides adduct type = nr	Stone *et al.* 1998
Human mammary epithelial cells	MeIQ	1 μM	mammary	number of adducts and type = nr numbers peaked at 55 minutes	Pfau *et al.* 1992
Human mammary epithelial cells	MeIQ	500 μM	mammary	1.3–35.3 adducts per 10^8 nucleotides adduct type = nr	Carmichael *et al.* 1996
Human mammary epithelial cells	MeIQ	500 μM	mammary	1.99–17.8 adducts per 10^8 nucleotides adduct type = nr	Stone *et al.* 1998
7 Humans	MeIQx	21 or 228 μg p.o. (gelatin capsule)	colon	0.02–0.04 adducts per 10^9 nucleotides (21μg dose) 0.9–1.2 adducts per 10^9 nucleotides (228 μg dose) adduct type = nr	Turteltaub *et al.* 1999
5 Humans	MeIQx	21 μg p.o. (gelatin capsule)	colon	25.6 adducts per 10^{12} nucleotides (normal tissue) 28.0 adducts per 10^{12} nucleotides (tumor tissue) adduct type = nr	Mauthe *et al.* 1999

Test system	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Humans (number nr)	MeIQx	20-200 µg p.o. (gelatin capsule)	colon	28 adducts per 10^{12} nucleotides (mean) adduct type = nr	Garner *et al.* 1999
Human prostate epithelial cells	MeIQx	10^{-5} M	prostate	0.06–0.6 RAL x 10^{7} adduct type = dG-C8-MeIQx	Wang *et al.* 1999
Human colon, rectum, and liver cells from tumor tissues; kidney cells from tissues from autopsy specimens	MeIQx	none administered	colon, rectum, kidney	14 adducts per 10^{10} nucleotides (colon) 18 adducts per 10^{10} nucleotides (rectum) 1.10 adducts per per 10^{10} nucleotides (kidney) adduct type = 5'-pdG-C8-MeIQx	Totsuka *et al.* 1996

nr = not reported

RAL = relative adduct labeling

In general, HCA-DNA adducts form in a dose-dependent manner in rodents (Turteltaub *et al.* 1999). The formation of PhIP-DNA adducts has been described in several models including *Drosophila*, rats, mice, and cynomolgus monkeys. The results of selected studies are summarized in Table 5-2. This table summarizes the number and types of adducts formed in various tissues in the studies. None of the studies measured levels of adducts formed in the control animals.

Table 5-2 Analysis of HCA-DNA adducts in animal models

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male and female cynomolgus monkeys	PhIP	50 μmol/kg; p.o.	WBCs	0.23 per 10^7 nucleotides (peak at 3 hours, adduct levels declined steadily thereafter) adduct type = C8-guanine-PhIP	Snyderwine *et al.* 1993
Male C57BL/6 mice	PhIP	1.0, 10.0, or 20.0 mg/kg; gavage	pancreas > thymus > heart > liver	The number of adducts increased in a dose-dependent manner adduct type = nr	Turteltaub *et al.* 1993
Male C57BL/6 mice	PhIP	150 μmol/kg; i.g.	pancreas > large intestine > lung > kidney > liver	adduct number and type = nr	Kerdar *et al.* 1993b
Male and female cynomolgus monkeys	PhIP	20 mg/kg; p.o., 9 doses	heart liver submandibular gland pancreas kidney	6.0 4.0 3.8 2.5 2.2 (adducts per 10^7 nucl.) adduct type = N-deoxyguanosin-8-yl-PhIP	Snyderwine *et al.* 1994

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male F344 rats	PhIP	220 μmol/kg; p.o.	pancreas colon lung heart liver	361 ± 72 201 ± 87 102 ± 38 99 ± 37 6 ± 3 (adducts per 10^8 nucl.) adduct type = N-deoxyguanosin-8-yl-PhIP	Kaderlik *et al.* 1994a
Male F344 rats	PhIP	10 μmol/kg; i.v.	pancreas colon lung heart liver	201 ± 22 28 ± 4 34 ± 3 31 ± 5 2 ± 1 (adducts per 10^8 nucl.) adduct type = N-deoxyguanosin-8-yl-PhIP	Kaderlik *et al.* 1994a
Male F344 rats	*N*-hydroxy-PhIP	10 μmol/kg; i.v.	pancreas colon lung heart liver	2.3-fold 4.0-fold 3.5-fold 4.0-fold 6.0-fold (increase compared to i.v. PhIP) adduct type = N-deoxyguanosin-8-yl-PhIP	Kaderlik *et al.* 1994a

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male F344 rats	*N*-acetoxy-PhIP	10 μmol/kg; i.v.	pancreas colon lung heart liver	4-fold 7-fold 28-fold 28-fold 20-fold (increase compared to i.v. PhIP) adduct type = N-deoxyguanosin-8-yl-PhIP	Kaderlik *et al.* 1994a
Male F344 rats	PhIP	50 mg/kg; gavage	pancreas > colon, lung, heart > liver	adducts found in 4/5 samples of each tissue adduct type = nr	Friesen *et al.* 1994
Male F344 rats	PhIP	100 mg/kg; 1 or 10 doses by gavage	liver	adduct number and type = nr	Davis *et al.* 1993a
Male F344 rats	PhIP	0.0001, 0.001, 0.01, 0.1, or 1.0 mg/kg; daily gavage for 23 days	pancreas > heart > kidneys > WBC, liver, cecum, spleen, colon, small intestine, stomach	adducts detectable only at the 0.1 and 1.0 mg/kg bw doses adduct type = nr	Friesen *et al.* 1996

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male and female F344 rats	PhIP	0.04%; diet for 1 to 8 weeks	colon	~4 adducts per 10^7 nucleotides (numbers tended to decline by 6–8 weeks) adduct types = deoxyguanosin-8-yl-PhIP 3',5'-diphosphate, dinucleotides and oligonucleotides derived from deoxyguanosin-8-yl-PhIP 3',5'-diphosphate, and N^2-(guanin-8-yl)-PhIP.	Ochiai *et al.* 1996
Male F344 rats	PhIP	400 ppm; diet for 2 weeks	liver pancreas	14.3 ± 1.0 508 ± 319 (adducts per 10^9 nucleotides) adduct type = N^2-(deoxyguanosin-8-yl)-PhIP-biphosphate	Pfau *et al.* 1997
Male Sprague-Dawley rats	PhIP (from cooked beef)	255 µg/rat (cumulative dose); diet for 27 weeks	liver, colon, stomach	nd	Shen *et al.* 1998
Male and female CDF_1 mice	MeIQ	3 µmol (single oral dose)	kidney > liver ≥ colon > forestomach > lung	~0.1 to 2.5 fmol/µg DNA (data presented in a bar graph) adduct type = nr	Hall *et al.* 1990

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male and female Big Blue® mice	MeIQ	300 ppm; diet for 1, 4, or 12 weeks	liver heart colon forestomach bone marrow	28.3 8.4 3.3 1.3 0.4 (adducts per 10^7 nucleotides at 12 weeks) adduct type = dG-C8-MeIQ-5'-monophosphate	Ochiai *et al.* 1998
Male F344 rats	MeIQx	0.4, 4, 40, or 400 ppm; diet for one week	liver	0.04, 0.28, 3.36, and 39 (low dose to high dose, adducts per 10^7 nucleotides) adduct type = nr	Yamashita *et al.* 1990
Male and female F344 rats	MeIQx	20 mg/kg; gavage 5 days/wk for two weeks	liver kidney heart colon WBC	19.64 (male), 10.2 (female) 14.27 (male), 3.28 (female) 3.32 (male), 3.08 (female) 2.06 (male), 1.31 (female) 1.34 (male), 0.18 (female) (adducts per 10^7 nucleotides) adduct type = nr	Davis *et al.* 1993b

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Male and female cynomolgus monkeys	MeIQx	20 mg/kg; gavage 5 days/wk for two weeks	liver kidney heart colon WBC	1.57 0.04 < 0.01 < 0.01 nd (adducts per 10^7 nucleotides) adduct type = nr	Davis *et al.* 1993b
Male Sprague-Dawley rats	MeIQx	acute: 0.001–34 µg/kg/day; gavage–single dose subchronic: 0.001–33 µg/kg/day; diet for 7 days 0.01–93 µg/kg/day; diet for 42 days	liver	acute: 0.006–1.7 subchronic: 0.03–2.7 (7 day) 0.019–53 (42 day) (adducts per 10^9 nucleotides, linear dose-response) adduct type = nr	Frantz *et al.* 1995
C57Bl/*lacZ* and *c-myc/lacZ* mice	MeIQx	20 µg/g; 10 daily doses by gavage	liver	0.5 ± 0.3 (C57Bl/*lacZ*) 1.6 ± 0.2 (*c-myc/lacZ*) (adducts per 10^7 nucleotides) adduct type = C8-guanine adduct	Davis *et al.* 1996

Test animal	Chemical	Dose and route of administration	Tissues examined	Number and type of adducts	Reference
Drosophila (3rd instar larvae)	MeIQx	1 mg/6.5 g-feed/bottle; with and without chlorophyllin (100, 200, or 300 mg)	whole larvae	6.5 ± 2.1 (no chlorophyllin) 3.4 ± 1.0 (100 mg) 3.4 ± 2.1 (200 mg) 1.7 ± 0.7 (300 mg) (adducts per 10^7 nucleotides) adduct type = dG-C8-MeIQx-5'-phosphate	Sugiyama *et al.* 1996

nd = not detected.
nr = not reported.
WBC = white blood cells.

5.1.2 Removal of DNA adducts

Transfected Chinese hamster ovary (CHO) cells, with a plasmid reporter gene for human growth hormone (hGH), were used to assay the effects of *N*-hydroxy-PhIP on hGH production (Fan and Snyderwine 1994). Both repair-deficient (UV5) and repair-proficient (AA8) cell lines were studied. PhIP inhibited hGH production, and the degree of inhibition was proportional to the level of PhIP-DNA adducts formed. Fewer adducts were needed to inhibit hGH production in repair-deficient cells compared to repair-proficient cells. At low PhIP-DNA adduct levels, recovery of gene expression was not greatly facilitated by repair proficiency, suggesting that the repair of PhIP-DNA adducts is not very efficient. A similar conclusion was reached by Stevnsner *et al.* (1995) who showed that PhIP-DNA adducts were removed with lower efficiency than adducts formed by *N*-acetyl-2-aminofluorene (AAF) or UV radiation.

Primary human mammary epithelial cells were incubated with PhIP or *N*-hydroxy-PhIP (1 to 50 μM), and the formation of DNA adducts was measured over 24 hours (Fan *et al.* 1995). Treatment with the parent compound did not result in detectable DNA adducts, while *N*-hydroxy-PhIP produced detectable adducts at a low concentration (1 μM) after 2 hours of incubation. The mammary cells removed 60% to 80% of adducts within 24 hours of exposure suggesting that mammary epithelial cells efficiently repair PhIP-DNA adducts.

The levels of PhIP-DNA adducts in liver, lungs, stomach, small intestine, cecum, colon, kidneys, WBC, heart, and spleen of male F344 rats were evaluated at 1, 2, 6, 12, 16, and 20 days after a single oral 50 mg/kg dose of PhIP (Cummings and Schut 1994). DNA adducts were highest in all tissues at 1 or 2 days after dosing, and adduct levels declined in all tissues through 20 days post-dose. The colon, followed by the spleen, contained the highest adduct levels at 24 hours after dosing; however, all tissues had similarly low levels of adduct concentrations by 20 days post-dose. In addition, the authors examined the cell turnover in various tissues. They suggested that the rapid rate of cell turnover in the intestines is more likely to explain the removal of PhIP-DNA adducts than does enzymatic repair of DNA.

DNA adduct formation and removal in rat liver following chronic dietary administration of MeIQx at 0.4, 8, or 400 ppm was investigated (Hirose *et al.* 1995b). Time-dependent increases in MeIQx-DNA adduct levels were observed until 16 and 8 weeks for the 0.4 and 8 ppm groups, respectively. For the high-dose group, adduct levels reached a maximum at week 12 and then gradually declined. When MeIQx administration stopped at week 20, a biphasic reduction in adduct levels was observed at all dose levels. Adduct removal was rapid over the first 4 to 8 weeks followed by a slower removal phase. The authors suggested that at least two types of damaged DNA, i.e., susceptible and resistant to MeIQx-DNA adduct removal, exist after chronic administration of MeIQx.

5.2 IARC review of genotoxicity studies

IARC (1993a, 1993b, 1993c) reviewed the genetic and related effects of PhIP, MeIQ, and MeIQx through 1992 (Table 5-3). PhIP was mutagenic in *Salmonella typhimurium* and in CHO cells. PhIP also induced DNA strand breaks and UDS in PCB-induced rat

hepatocytes and Chinese hamster V79 cells. CAs were observed in the bone marrow and peripheral blood cells isolated from exposed mice. In addition, bone marrow cells of PhIP-exposed mice and CHO cells showed increases in SCEs and MN.

MeIQx was mutagenic in *S. typhimurium* and induced somatic mutations and recombinations in *D. melanogaster*. The chemical also induced UDS in rats, mice, and Syrian hamster hepatocytes, and gene mutations in Chinese hamster V79 cells. It did not cause CAs, but it did result in an increase in SCEs in human lymphocytes. In *in vivo* mammalian studies, DNA damage in mice, increased SCEs and CAs in rat hepatocytes, and DNA adducts in various rat and mouse organs were observed.

MeIQ induced reverse mutations in *S. typhimurium* and somatic mutations and recombinations in *D. melanogaster*. In mammalian *in vitro* systems, MeIQ induced DNA strand breaks in rat hepatocytes, UDS in rat, hamster, and mouse hepatocytes, and gene mutations; further, an increase in SCEs and CAs in Chinese hamster lung or ovary cells was observed. Following i.p. or oral administration of MeIQ, DNA strand breaks and SCE were observed in rats and mice.

Table 5-3. Genetic and related effects of HCA exposure reviewed by IARC (1993)

Test system	End point	Results (no. positive/no. studies)		
		PhIP[a]	MeIQ	MeIQx
Prokaryote				
S. typhimurium	reverse mutation	9/10[b]	6/9[c]	12/15[d]
S. typhimurium	SOS repair	nr	nr	2/2
S. typhimurium	umu expression	nr	nr	1/1
E. coli	differential toxicity	nr	nr	1/1
Nonmammalian eukaryotes				
D. melanogaster	somatic mutation and recombination	nr	1/1	1/1
Mammalian *in vitro*				
Rat hepatocytes	UDS, DNA strand breaks	1/1	2/2	1/1
Rat hepatocytes	covalent binding to DNA	nr	nr	1/1
Syrian hamster hepatocytes	UDS	nr	1/1	1/1
Mouse hepatocytes	UDS	nr	1/1	2/3
Chinese hamster V79 cells	SCEs, DNA strand breaks	2/2	1/2	nr
Chinese hamster V79 cells	gene mutations	nr	1/1	2/3
CHO cells	gene mutations	1/1	1/1	1/1
CHO cells	SCE	4/5	1/1	1/1
CHO cells	CA	1/4	1/1	1/2

Test system	End point	Results (no. positive/no. studies)		
		PhIP[a]	MeIQ	MeIQx
CHO cells	MN	4/5	nr	nr
Human lymphocytes	SCE	nr	nr	1/1
Human lymphocytes	CA	nr	nr	0/2
Mammalian *in vivo*				
Rat liver, kidney, large intestine	DNA strand breaks	nr	1/1	nr
Rat hepatocytes	SCE	nr	nr	1/1
Rat hepatocytes	CA	nr	nr	1/1
Rat liver	covalent binding to DNA	nr	nr	2/2
Rat heart, liver	covalent binding to DNA	nr	nr	1/1
E. coli in Swiss albino mice (host-mediated assay)	DNA damage	nr	1/1	1/1
Mouse colonic epithelial cells	SCE	nr	1/1	nr
Mouse bone marrow cells	SCE	1/1	nr	0/1
Mouse bone marrow cells	CA	1/1	nr	nr
Mouse bone marrow cells (males)	covalent binding to DNA	nr	nr	1/1
Mouse bone marrow erythrocytes	MN	1/1	nr	0/1
Mouse peripheral blood cells	CA	1/1	nr	nr
Mouse peripheral blood lymphocytes	MN	1/1	nr	nr
Mouse small intestinal cells	gene mutation	nr	nr	0/1
Mouse (various tissues)	covalent binding to DNA	nr	nr	1/1

[a]Includes *N*-hydroxy-PhIP, 1-MePhIP (PhIP), and 3-MePhIP.

[b]Negative study was with 3-MePhIP.

[c]Negative studies were with strains TA102 and TA98/1,8-DNP_6.

[d]Negative studies were with strains TA96, TA98/1,8-DNP_6, and TA104.

nr = not reported

CA = chromosomal aberrations, CHO = Chinese hamster ovary, MN= micronuclei, SCE = sister chromatid exchange, UDS = unscheduled DNA synthesis

5.3 Genotoxicity studies since the 1993 IARC review

The results and treatment concentrations for *in vivo* studies with PhIP, MeIQx, and MeIQ reported after the 1993 IARC monographs were published are summarized in Table 5-4. Similarly, Table 5-5 tabulates the results from *in vitro* studies since the IARC monographs and lists the lowest effective concentration (LEC) for positive studies or the highest ineffective concentration (HIC) for negative studies.

Almost all *in vivo* tests in rats and mice were positive for all three chemicals. PhIP was mutagenic in *in vitro* tests including *S. typhimurium*, CHO cells, and human cancer cell lines. The only *in vitro* test that did not show positive results with PhIP was for gene conversions and translocations *in Saccharomyces cerevisiae*. Positive results were also noted in *in vivo* tests for PhIP, including tests for DNA damage and gene mutations in CD-1 and transgenic mice, and in Big Blue® rats.

For MeIQx, positive results were noted in almost all of the *in vitro* tests, including tests in *S. typhimurium*, *S. cerevisiae*, and *D. melanogaster*. The only *in vitro* tests showing negative results were for MN in CHO cells, and tests for UDS in rat and mouse liver slices. DNA damage and gene mutations were reported in most of the *in vivo* tests in mice. The only reported negative results were for CA in C57BL/6 mice and one gene mutation test in Big Blue® mice.

MeIQ also showed mutagenic results in *S. typhimurium*, *E coli*, and human, rat, and mouse liver slices. As with PhIP, negative results were noted for gene conversions and translocations *in S. cerevisiae*. MeIQ was positive in all the *in vivo* tests, including tests for gene mutations in Big Blue® mice, and for DNA damage and CA in Swiss albino and CDF_1 mice. Detailed descriptions of these individual genotoxicity studies for all three HCAs are provided in Appendix A.

Table 5-4. Current *in vivo* studies on the genotoxicity of HCAs

Species and strain	Endpoint	Results	Concentration	Reference
PhIP				
C57BL/6 neonates	gene mutation	–	26.2 mg/kg, i.p.	Dass *et al.* 1998
Rats (strain not specified)	gene mutation	+	400 ppm, diet	Burnouf and Fuchs 2000
Swiss albino mice	DNA damage, host-mediated assay	+	2.5 mg/kg, i.p. or 200 mg/kg, oral	Knasmüller *et al.* 1992
C57BL/6N mice	CA SCE micronucleus	– + +	400 ppm, diet 100 ppm, diet 200 ppm, diet	Director *et al.* 1996
CD-1 mice	DNA damage	+	40 mg/kg, i.p.	Sasaki *et al.* 1997
CD-1 mice	DNA damage	+	40 mg/kg, i.p.	Sasaki *et al.* 1998
*gpt*Δ transgenic mice	gene mutation	+	400 ppm, diet	Masumura *et al.* 1999

Species and strain	Endpoint	Results	Concentration	Reference
*gpt*Δ transgenic mice	gene mutation	+	400 ppm, diet	Masumura *et al.* 2000
Big Blue® mice	gene mutation	+	100 ppm, diet	Zhang *et al.* 1996
Big Blue® rats	gene mutation	+	400 ppm, diet	Okonogi *et al.* 1997b
Big Blue® rats	gene mutation	+	65 mg/kg, gavage	Okochi *et al.* 1999
Big Blue® rats	gene mutation	+	200 ppm, diet	Stuart *et al.* 2000a
Big Blue® rats	gene mutation	+	400 ppm, diet	Stuart *et al.* 2000b
MeIQx				
BALB/c mice	reverse mutations host-mediated assay	+	1.5 mg/kg, oral	Alldrick *et al.* 1995
Swiss albino mice	DNA damage host-mediated assay	+	2.5 mg/kg, i.p. or 40 mg/kg, oral	Knasmüller *et al.* 1992
C57BL/6 mice	CA	-	400 ppm, diet	Breneman *et al.* 1996
C57BL/6 mice	SCE	+	400 ppm, diet	Breneman *et al.* 1996
C57BL/6 mice	micronucleus	-	400 ppm, diet	Breneman *et al.* 1996
C57BL/*lacZ* *c-myc*/*lacZ*	gene mutation	+	20 ppm, gavage	Davis *et al.* 1996
C57BL/*lacZ* *c-myc*/*lacZ*	gene mutation	+	0.06%, diet (600 ppm)	Ryu *et al.* 1999
C57BL/*lacZ* *c-myc*/*lacZ*	gene mutation	+	0.06% diet (600 ppm)	Thorgeirsson *et al.* 1999
Big Blue® mice	gene mutation micronucleus	– +	100 mg/kg, gavage or 300 ppm, diet	Nishikawa *et al.* 2001
MeIQ				
Swiss albino mice	DNA damage host-mediated assay	+	2.5 mg/kg, i.p, or 40 mg/kg, oral	Knasmüller *et al.* 1992
CDF_1 mice	CA	+	400 ppm,diet	Ramsey *et al.* 1998
Big Blue® mice	gene mutation	+	300 ppm, diet	Ushijima *et al.* 1994
Big Blue® mice	gene mutation	+	300 ppm, diet	Ushijima *et al.* 1995a
Big Blue® mice	gene mutation	+	300 ppm, diet	Okonogi *et al.* 1997c
Big Blue® mice	gene mutation micronucleus	+ +	300 ppm, diet	Suzuki *et al.* 1996

CA = chromosomal aberrations; SCE = sister chromosome exchanges

Table 5-5. Current *in vitro* studies on the genotoxicity of HCAs

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
PhIP					
S. typhimurium TA98 (uvrB$^-$) TA1978 (uvrB$^+$)	revertants	 + –		 1.25 μg/mL 250 μg/mL	Malfatti *et al.* 1995
S. typhimurium TA98	revertants	 +		 10 μM	Apostolides *et al.* 1996[a]
S. typhimurium TA98	revertants	 +		 500 ng/plate	Guyonnet *et al.* 2000[a]
S. typhimurium TA98	revertants	 +		 400 ng/plate	Yu *et al.* 2001[a]
S. typhimurium TA1538 YG1019	revertants	 + +		 10–300 pg/plate (3.6 revertants/ng) 10–300 pg/plate (5.0 revertants/ng)	Pfau *et al.* 1999
S. typhimurium TA1538 DJ460	revertants	 + +		 100 ng/plate 100 ng/plate	Wild *et al.* 1995
HepG2 cells	MN		+	25–50 μM	Knasmüller *et al.* 1999
CHO cells	MN		+	134 μM	

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
S. typhimurium	revertants				Koch *et al.* 1998
TA100		+		< 5 μg/plate	
TA1535		+		< 5 μg/plate (estimated from graph)	
S. typhimurium	revertants			100–100,000 pM	Suzuki *et al.* 1998
TA1538/ARO		+		650 revertants/nmol	
TA1538		+		370 revertants/nmol	
S. typhimurium	revertants			100–100,000 pM	Kamataki *et al.* 1999
TA1538/ARO		+		2,100 revertants/nmol	
TA1538		+		400 revertants/nmol	
S. typhimurium	revertants				Constable *et al.* 1999
TA98		+		704 revertants/μg	
DJ4309		+		32 revertants/μg	
E. coli	base substitution mutations				Garganta *et al.* 1999
CL101		+		0.15 μg/plate	
CL102		+		0.15 μg/plate	
CL104		+		0.15 μg/plate	
CL105		+		0.15 μg/plate	

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
E. coli	DNA repair assay	+		0.26–12.50 μg/mL (LEC not specified)	Knasmüller *et al.* 1992
S. cerevisiae					Paladino *et al.* 1999
YHE2	gene conversions	–		100 μg/mL	
YB110	chromosomal translocations	–		100 μg/mL	
YGP1B	forward mutations	–		100 μg/mL	
D. melanogaster	wing spot test			400 μg/g medium	Kasai *et al.* 1998
	DNA repair test		+	200 μg/g medium	
TK6 human lymphoblastoid cells	*hgprt* mutations	+	+	2.5 μg/mL	Morgenthaler and Holzhauser 1995
TK6 human lymphoblastoid cells	*hprt* mutations		+	2.5 μg/mL	Zhu *et al.* 2000
Human cancer cell lines	*hprt* mutations				Glaab and Skopek 1999
HCT116			+	10 μM	
DLD-1			+	10 μM	
HEC59			+	10 μM (estimated from graph)	

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
Human cancer cell lines	*hprt* mutations				Glaab *et al.* 2000
DLD-1			+	10 μM	
HCT116			+	10 μM	
CHO cells	*aprt* mutations		+	0.1 μg/mL	Thompson *et al.* 1991
CHO cells	*aprt* mutations		+	0.4 μg/mL	Wu *et al.* 1995 (samples from study by Thompson *et al.* 1991)
CHO cells	*hprt* mutations		+	2.5 μM	Yadollahi-Farsani *et al.* 1996
V79 Chinese hamster lung fibroblasts	6-thioguanine resistance			5 μM	Lawson and Kolar 1994
V79 Chinese hamster lung fibroblasts	6-thioguanine resistance			nr	Lawson *et al.* 1997
Human-hamster A_L cells	specific locus mutation test	+		12 μg/mL	Waldren *et al.* 1999
Human lymphocytes	CA	+	–	3.13–12.5 μg/mL	Otsuka *et al.* 1996a
TIG-7 human fibroblasts	CA	+		12.5 μg/mL	
Chinese hamster lung	MN	+			Otsuka *et al.* 1996b
CHL/IU		+	–	5 μg/mL	
		+	–	20 μg/mL	
Human lymphoblastoid cells	MN		+	2.0 ng/mL	Pfau *et al.* 1999
HepG2 cells	MN		+	25 μM	Knasmüller *et al.* 1999
CHO cells	MN		+	25 μM	
HepG2 cells	MN		+	0.6 mM	Sanyal *et al.* 1997
Human blood lymphocytes	SCE	+		1.56 μg/mL	Otsuka *et al.* 1996a

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
MCL-5 human lymphoblastoid cells	DNA single strand breaks (comet tail length)		+	90.9 μg/mL	Pfau *et al.* 1999
Human lymphoblastoid cells	DNA single strand breaks (comet tail length)		+	0.2 mM	Martin *et al.* 1999
Human colon cells Rat colon cells	DNA single strand breaks (comet tail length)		– +	1.5 mM 0.5 mM	Pool-Zobel and Leucht 1997
Human liver slices	UDS		+	0.005 mM	Beamand *et al.* 1996
Human liver slices	UDS		+	0.005 mM	Beamand *et al.* 1998a
Human liver slices Rat liver slices Mouse liver slices	UDS		+ – +	0.005 mM 200 mM 0.002 mM	Beamand *et al.* 1998b
Rat hepatocytes	UDS		+	0.1 mM	Kaderlik *et al.* 1994a
MeIQx					
S. typhimurium TA98	revertants	 +		 100 ng/plate	Edenharder *et al.* 1995[a]
S. typhimurium TA98	revertants	 +		 10 ng/plate	Hirose *et al.* 1998a[a]
S. typhimurium TA98	revertants	 +		 12 ng/plate	Yu *et al.* 2001[a]

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
S. typhimurium	revertants			1–100,000 pM	Suzuki *et al.* 1998
TA1538/ARO		+		97,000/nmol	
TA1538		+		31,200/nmol	
S. typhimurium	revertants			1–100,000 pM	Kamataki *et al.* 1999
TA1538/ARO		+		720,000/nmol	
TA98		+		31,000/nmol	
S. typhimurium					Pfau *et al.* 1999
TA1538	revertants	+		10–150 pg/plate	
YG1019	revertants	+		10–150 pg/plate	
MCL-5	MN		+	5 ng/mL	
MCL-5	DNA strand breaks (comet tail length)		+	454.5 μg/mL	
S. typhimurium	revertants				Aryal *et al.* 2000
OY1001/1A2		+		< 1.0 μM	
OY1002/1A2		+		~ 1.0 μM	
OY1003/1A3		–		> 10 μM	
				(data presented graphically only)	
E. coli	*LacZ* revertants	+ (1A1)		1000 pmol/plate	Josephy *et al.* 2001
		+ (1A2)		30 pmol/plate	
		– (1B1)		10,000 pmol/plate	

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
E. coli	DNA repair assay	+		0.26–12.50 μg/mL (LEC not specified)	Knasmüller *et al.* 1992
S. cerevisiae					Paladino *et al.* 1999
YHE2	gene conversions	+		100 μg/mL	
YB110	chromosomal translocations	+		100 μg/mL	
YGP1B	forward mutations	+		100 μg/mL	
D. melanogaster (3rd instar larvae)	DNA adduct formation			1 mg/6.5-g feed bottle	Sugiyama *et al.* 1996[a]
MCL-5	MN	+		5 ng/mL	Pfau *et al.* 1999
HepG2	MN		+	25–50 μM	Knasmüller *et al.* 1999
CHO	MN	+	–	900 μM	
HepG2	MN		+	0.6 mM	Sanyal *et al.* 1997[a]
MCL-5	Comet assay		–	2.13 mM	Martin *et al.* 1999
MCL-5	DNA strand breaks (comet tail length)	+		454.5 μg/mL	Pfau *et al.* 1999
Human liver slices	UDS			5 μM	Beamand *et al.* 1998b
Rat liver slices	UDS		–	200 μM	
Mouse liver slices	UDS		–	200 μM	

+

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
MeIQ					
S. typhimurium TA98 with tryptamine or tyramine	revertants	+		0.5 ng/plate	Abu-Shakra 1992
S. typhimurium TA98 TA100	revertants		+ +	7.5 ng/plate 160 ng/plate (dose resulting in ~2,000 revertants per plate)	Edenharder *et al.* 1994[a]
S. typhimurium	revertants	+		0.5 ng/plate	Sengstag *et al.* 1994
S. typhimurium DJ400 DJ460	revertants	+ +		< 10 pmol/plate > 100 pmol/plate (graphical presentation only)	Grant *et al.* 1992
S. typhimurium TA1538/ARO	revertants	+		0.25 ng/plate	Wild *et al.* 1995
S. typhimurium TA1538/ARO TA1538	revertants	+ +		0.1–10,000 pM 4,730,000/nmol 336,000/nmol	Suzuki *et al.* 1998
S. typhimurium TA1538/ARO TA98	revertants	+ +		0.1–10,000 pM 11,000,000/nmol 140,000/nmol	Kamataki *et al.* 1999

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
S. typhimurium	revertants				Josephy *et al.* 1995
YG1019		+		< 1 pmol/plate	
DJ4501A2		+		1 pmol/plate	
YG1019			–	10 pmol/plate	
HepG2	MN			25–50 μM	Knasmüller *et al.* 1999
CHO	MN		–	4,700 μM	
S. typhimurium	revertants				Aryal *et al.* 2000
OY1001/1A2		+		> 0.1 μM	
OY1002/1A2		+/+		< 0.1 μM	
OY1003/1A3		–		> 1 μM	
				(graphic representation only)	
E. coli	DNA repair assay	+		0.26–12.50 μg/mL (LEC not specified)	Knasmüller *et al.* 1992
E. coli	*LacZ* revertants	+		≥ 10 pmol/plate	Josephy *et al.* 2000
E. coli	*lacZ* revertants	+		> 1 pmol/plate	Josephy *et al.* 2001

Species (test system)	Endpoint	Results (with activation)	Results (without activation)	Concentration LEC or HIC	Reference
S. cerevisiae					Paladino *et al.* 1999
YHE2	gene conversions	–		100 μg/mL	
YB110	chromosomal translocations	–		100 μg/mL	
YGP1B	forward mutations	–		100 μg/mL	
V79 Chinese hamster lung fibroblasts	6-thioguanine resistantce	+		5 μM	Lawson and Kolar 1994
HepG2 cells	MN		+	0.9 mM	Sandhu *et al.* 2001[a]
HepG2 cells	MN		+	25–50 μM	Knasmüller *et al.* 1999
CHO	MN		–	4,700 μM	
Human livers slices	UDS			100 μM	Beamand *et al.* 1998b
Rat liver slices	UDS		+	5 μM	
Mouse liver slices	UDS		+	20 μM	

[a]HCA used as positive control for inhibition of mutagenicity

LEC = lowest effective concentration; HIC = highest ineffective concentration

CA = chromosomal aberrations; MN = micronuclei; SCE = sister chromatid exchanges; UDS = unscheduled DNA synthesis

nr = not reported

5.4 Comparative mutagenicity

All three compounds exhibit a high degree of mutagenicity in many test systems. In comparison to other well known mutagens, PhIP, MeIQ, and MeIQx show a high degree of potency. For example, in *S. typhimurium*, all three compounds are more potent than benzo[a]pyrene, and MeIQ and MeIQx are more potent than aflatoxin B1 (see Table 5-6). In transgenic mouse models, all three compounds have also exhibited a high degree of mutagenic potency. In one study, transgenic mice fed MeIQx showed a 40-fold higher mutant frequency than control mice (see Appendix A).

Table 5-6. Mutagenicity of HCAs and other carcinogens in *S. typhimurium* TA98 and TA100 with S9 mix

	Revertants (µg)	
Compound	**TA98**	**TA100**
PhIP	1,800	120
MeIQx	145,000	14,000
MeIQ	661,000	30,000
IQ	433,000	7,000
4,8-DiMeIQx	183,000	8,000
7,8-DiMeIQx	163,000	9,900
Trp-P-2	104,200	1,800
4-CH_2OH-8-MeIQx	99,000	3,000
IQx	75,000	1,500
Glu-P-1	49,000	3,200
Trp-P-1	39,000	1,700
Cre-P-1	19,000	400
AF-2[a]	6,500	42,000
Alfatoxin B_1	6,000	28,000
Glu-P-2	1,900	1,200
7,9-DiMeIQx	540	na
Benzo[a]pyrene	320	660
AαC	300	20
MeAαC	200	120
4'-hydroxy-PhIP	2	na

Source: Nagao 1999.

[a]AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, without S9 mix.

na = not available

Table 5-7 describes the mutagenicity of PhIP, MeIQx and MeIQ in several test systems. In *S. typhimurium* strain TA98, the relative potencies are: MeIQ > MeIQx > PhIP, PhIP > MeIQ > MeIQx in a Chinese hamster ovary DNA repair deficient cell line and PhIP =

MeIQ > MeIQx in a Chinese hamster lung cell line (Cappelli *et al.* 2000). The reasons for these varying potencies in different test systems have to do with differences in various activating, deactivating, and DNA repair enzymes. However, mutations. Metabolic activation occurs through CYP1A2-mediated *N*-hydroxylation followed by phase II esterification to reactive ester derivatives that bind to DNA.

Table 5-7. Comparative mutagenicity of HCAs within different test systems

HCA	*S. typhimurium* TA 98 (rev./mg)	CHL DT[a] rev./106/mg	CHO UV5 TG[b] mL/mg[c]
PhIP	1,800	40.0	500.0
MeIQx	145,000	5.7	3.3
MeIQ	661,000	38.0	13.0

Source: Nagao *et al.* 1994, Cappelli *et al.* 2000.

[a]Chinese hamster lung cells, diptheria toxin resistance.

[b]Chinese hamster ovary cells, ultraviolet sensitive mutant-XPD mutant-deficient in nucleotide excision repair, 6-thioguanine resistance.

[c]Inverse of the lowest dose tested that produced an increase in mutation frequency.

5.5 Summary

HCA-induced DNA adducts have been characterized and detected both *in vitro* and *in vivo*, and the major adduct for each HCA is similar in all species examined. The predominant adduct forms at the C8 position of guanine; however, MeIQx also forms a minor adduct at the N^2 guanine position, which is a base-pairing site. In humans, DNA adducts occur at dietary relevant doses and usually occur at higher levels than those in rodents administered an equivalent dose. Adducts have been identified in human colon, breast tissue, and prostate cancer following exposure to HCA. While HCA-DNA adducts are necessary for the mutagenesis and carcinogenesis of these compounds, their role in human cancer is unknown at this time.

In comparison to other well known mutagens, PhIP, MeIQ, and MeIQx show a high degree of potency. For example, in *S. typhimurium*, all three compounds are more potent than benzo[a]pyrene, and MeIQ and MeIQx are more potent than aflatoxin B1. The relative mutagenic potencies of these three HCAs varies with the test system. In *S. typhimurium* strain TA98, the relative potencies were MeIQ > MeIQx > PhIP; however, PhIP ≥ MeIQ > MeIQx in a Chinese hamster ovary system.

PhIP was mutagenic in prokaryotes *(S. typhimurium, E. coli)*, insects, rodents (*in vitro* and *in vivo* systems) and humans (*in vitro*) but was not mutagenic in yeast (based on a single test). In nonmammalian cells, PhIP induced DNA damage in insects but did not induce mitotic recombination (insects) or gene conversion (yeast). PhIP also was genotoxic in both human and rodent *in vitro* systems and in rodent *in vivo* systems, inducing CA, DNA damage, MN, and SCE in all three systems. PhIP also induced UDS in humans and rodents (*in vitro)* and mitotic recombination in rodents (*in vitro*) (see Table 5-8).

MeIQ was mutagenic in prokaryotes *(S. typhimurium, E. coli)*, rodents (*in vitro* and *in vivo*) and humans (*in vitro).* MeIQ did not induce mutation, CA or gene conversion in yeast (*S. cerevisiae*), albeit only a single study was performed. MeIQ also was genotoxic in both human and rodent *in vitro* systems and rodent *in vivo* systems. In rodents, MeIQ induced DNA damage *(in vitro* and *in vivo*), MN (*in vitro*), SCE (mixed results *in vitro* and positive *in vivo*), UDS (*in vitro*), and chromosomal aberrations (*in vivo*). In human cells, MeIQ induced MN and UDS (see Table 5-8).

MeIQx was mutagenic in prokaryotes *(S. typhimurium, E. coli)* and in rodent *in vitro* and *in vivo* systems; no mutagenic tests were available in humans. MeIQx also induced other types of genetic damage in yeast, rodents, and humans. In yeast cells, MeIQx induced gene conversion. In rodents, MeIQx induced DNA damage (*in vivo* and *in vitro*) and SCE (positive *in vitro* and mixed results *in vivo*) but did not induce CA (*in vitro* and mixed results *in vivo*), UDS (*in vitro*), or MN (*in vivo*). In human *in vitro* systems, MeIQx induced DNA damage, MN, SCE, and UDS but did not induce CA (see Table 5-8).

Table 5-8. Summary of genetic effects of PhIP, MeIQx, and MeIQ exposures

Nonmammalian systems								Mammalian systems																	
Prokaryotes		Eukaryotes						*In vitro*														*In vivo*			
S. typhimurium	*E. coli*	*S. cerevisiae*			*D. melanogaster*			Rodent							Human							Rodent			
PhIP																									
G	G	C	G	GC	D	G	R	C	D	G	R	M	S	U	C	D	G	M	S	U	C	D	G	M	S
+	+	na	–[1]	[1]	[1]	+	–[1]	[1]	+	+	+[1]	[1]	+	+	+	+	+	+	+[1]	[1]	+-	+	+	+[1]	+-
MeIQx																									
+	+[1]	na	na	+[1]	+-	na	na	–[1]	+	+	na	na	+[1]	–	–	+	na	+	+[1]	[1]	+-	+	+	-	+-
MeIQ																									
+	+	–[1]	[1]	[1]	na	na	na	na	+	+	na	+[1]	+-	+	na	na	+[1]	[1]	na	+[1]	[1]	[1]	+	na	+[1]

Adapted from IARC 1993a, 1993b, 1993c.

\+ considered to be positive for the specific end-point and level of biological activity

+[1] considered to be positive, but only one valid study was available

+- both positive and negative responses

– only negative responses

–[1] negative response from a single study

na = not available

C = chromosomal aberrations, D = DNA damage, G = gene mutation, GC = gene conversion, M = micronuclei, R = mitotic recombination, S = sister chromatid exchange, T = cell transformation, U = unscheduled DNA synthesis

6 Other Relevant Data

As discussed in the previous sections, HCAs are widely present in the American diet, are potent mutagens in bacteria and mammalian cells, induce DNA damage, and cause cancer at multiple sites in rats and mice. This section reviews the toxicokinetics (absorption, distribution, metabolism, and excretion), DNA adduct formation, and mechanisms of carcinogenic action for PhIP, MeIQx, and MeIQ.

6.1 Toxicokinetics

All HCAs must be metabolically activated to induce mutagenic effects (Övervik and Gustafsson 1990, Aeschbacher and Turesky 1991). The IARC (1993a, 1993b, 1993c) reported that PhIP, MeIQx, and MeIQ are readily absorbed following oral administration and are rapidly distributed to most tissues, particularly the liver, kidneys, and intestines. These compounds are metabolically activated by hydroxylation of the exocyclic amine to form *N*-hydroxy-PhIP, -MeIQx, and -MeIQ. These compounds are reactive and can bind to DNA or other macromolecules. CYP450 1A2 is the major phase I enzyme catalyzing HCA activation with CYP1A1 being secondary. DNA binding is enhanced by further metabolism of the *N*-hydroxy forms by *O*-acetyltransferase sulfotransferase or amino-acetyl-tRNA synthetases. *N*-acetylation also occurs *in vivo* in rodents and humans, but metabolites derived from this pathway are generally not as mutagenic as the *N*-hydroxylated metabolites. Inactivation primarily occurs through ring hydroxylation with subsequent conjugation with glucuronic acid, sulfate, or glutathione (Övervik and Gustafsson 1990). Excretion occurs through the urine, bile, and feces. In experimental animals, as much as 60% to 100% of the HCAs and their metabolites may be eliminated within 24 hours (IARC 1993a, 1993b, 1993c).

The absorption, distribution, metabolism, and excretion of PhIP, MeIQ, and MeIQx have been the subject of numerous papers that were not reviewed by IARC or that were published after the IARC (1993a, 1993b, 1993c) reviews. Some of these are reviewed below.

6.1.1 Absorption and distribution

Both *in vitro* and *in vivo* studies indicate that HCAs are almost completely absorbed from the gastrointestinal tract in humans (Davies *et al.* 1996, Malfatti *et al.* 1999, Lang *et al.* 1999, Kulp *et al.* 2000, Krul *et al.* 2000) and experimental animals (Turteltaub *et al.* 1992, 1993). Volunteers given [2-^{14}C]PhIP (70 to 84 μg) by capsule excreted 50% to 90% of the administered dose in the urine during the first 24 hours (Malfatti *et al.* 1999, Lang *et al.* 1999). However, Kulp *et al.* (2000) found that the total urinary excretion of PhIP and PhIP metabolites varied widely (4% to 53% dose excreted within 24 hours) in volunteers that consumed 200 g of cooked chicken. The authors suggested that the absorption and bioavailability of PhIP might be different when consumed as part of the normal diet rather than in a gelatin capsule.

The absorption of several HCAs (IQ, PhIP, MeIQ, and MeIQx) given as a mixture was modeled using a computer-controlled *in vitro* system that mimicked the physiological conditions of the human stomach and small intestine (Krul *et al.* 2000). Comparable to an

in vivo human model, all four HCAs were readily absorbed in the *in vitro* system, with approximately 50% of the total dose recovered in dialysate after 2 hours and 95% after 6 hours. The recovery from the jejunal and ileal compartments represented 94% of the total recovery. The remaining 5 ± 1.5% of the starting material was recovered in the solution at the end of the small intestine segment.

Studies in experimental animals show that HCAs are rapidly distributed throughout the body and that tissue concentrations change rapidly with time (Watkins *et al.* 1991, Turteltaub *et al.* 1992, 1993, Snyderwine *et al.* 1994, Dragsted *et al.* 1995, Vikse *et al.* 1995, Mauthe *et al.* 1998). When male F344 rats were given [2-^{14}C]PhIP by gavage, the highest concentrations of radioactivity at 12 hours post-dose were found in the colon and cecum, while the highest concentrations at 24 hours and later were detected in the kidney and liver (Watkins *et al.* 1991). Turteltaub *et al.* (1992) administered [^{14}C]PhIP by intubation at a dose of 41 ng/kg, which was considered to be equivalent to a human dietary dose, to six- to eight-week old C57BL/6 male mice. At 30 minutes and 1 hour, tissue levels of radiolabel were highest in intestine, stomach, and adipose tissue, followed by liver, kidneys, pancreas, lung, and spleen. By 96 hours post-dose, only liver, pancreas, muscle, spleen, and lung contained a significant amount of radioactivity. In a follow-up study, peak tissue levels were reached within 3 hours, with the greatest concentration of radioactivity in the gastrointestinal tract (GI), liver, kidney, pancreas, and thymus (Turteltaub *et al.* 1993). Following a 1.0-mg/kg dose of [^{3}H]PhIP administered by oral intubation to male Wistar rats, the highest total radioactivity was found in the stomach, small intestines, and bladder after 2 hours. At 24 hours post-dose, the highest residual radiolabel concentrations were detected in the kidney and liver (Dragsted *et al.* 1995). Snyderwine *et al.* (1994) administered oral doses of PhIP (20 mg/kg) as either a single dose or nine daily doses to male and female cynomolgus monkeys (*Macaca fascicularis*). PhIP-DNA adducts were detected in all 28 tissues examined, with the exception of fat and bone marrow.

PhIP or PhIP metabolites may cross the placental barrier as demonstrated by the appearance of radiolabeled PhIP in fetal tissues (Brittebo *et al.* 1994, Hasegawa *et al.* 1995). MeIQ was able to pass across the placenta in pregnant NMRI mice (Bergman 1985). Hashimoto *et al.* (1995) demonstrated that MeIQx reaching the fetal liver through the placenta could be activated and further suggested that both human fetal CYP3A7 and polymorphic NAT2 were required for its mutagenic activation.

6.1.2 Metabolism

Widmark (1939) was the first to report the presence of carcinogenic substances in cooked meat. Later, extracts from cooked beef and fish were shown to be highly mutagenic in the Ames assay (Sugimura *et al.* 1977, Commoner *et al.* 1978). Subsequent studies showed that these mutagens were HCAs, particularly those with a pyridine (e.g., PhIP), quinoline (e.g., MeIQ), or quinoxaline (e.g., MeIQx) moiety. It is now known that metabolic activation of HCAs requires phase I hepatic CYP-mediated *N*-hydroxylation followed by esterification of the *N*-hydroxylamines by phase II pathways (Figure 6-1). These ester derivatives are highly reactive and can covalently bind to DNA. For phase I enzymes, a consensus opinion is that CYP1A2 is the primary pathway for *N*-hydroxylation; however, evidence has been reported of a possible role for CYP1A1, 2A6, 3A4, 2C9/10, 2A3, and

1B1, (Aoyama *et al.* 1990, McManus *et al.* 1990, McKinnon and McManus 1995, Pelkonen and Raunio 1995, Shimada *et al.* 1996, Hammons *et al.* 1997, Crofts *et al.* 1998, Schut and Snyderwine 1999, Williams *et al.* 2000, Josephy *et al.* 2001).

Phase I activation may occur in the same tissue as the ultimate activation of HCAs, or the metabolite may be transported to other tissues for the final phase II activation reaction. For example, human studies have documented PhIP metabolism in breast, prostate, and colon tissues (Turesky *et al.* 1991a, Dubuisson and Gaubatz 1998, Wang *et al.* 1999). Furthermore, some animal studies indicate that the phase II esterification activity in extrahepatic tissues can be as high or higher than the activity in the liver. Thus, both liver and extrahepatic tissues that are potential targets for HCA-induced carcinogenesis are known to metabolize HCAs. At least four mammalian cytosolic enzymes are likely involved in phase II activation of HCAs, and they all form the same DNA adducts (Schut and Snyderwine 1999).

The active and inactive metabolites of HCAs were identified through many *in vitro* and *in vivo* studies. This section describes the metabolic pathways that lead to bioactivation and those that lead to detoxification. Finally, interspecies differences in metabolism are discussed.

6.1.2.1 Phase I bioactivation

As mentioned above, CYP1A2 is the primary phase I enzyme responsible for *N*-hydroxylation of HCAs. Many studies have demonstrated that *N*-hydroxylation of HCAs by CYP1A2 is strongly associated with mutagenesis and DNA adduct formation (Kaderlik *et al.* 1994b, Ghoshal *et al.* 1995, Hammons *et al.* 1997, Schut and Snyderwine 1999). Other studies are briefly reviewed below.

Shimada *et al.* (1989) used *S. typhimurium* expressing human *CYP1A2* genes and reported that CYP1A2 had a primary role in the metabolic activation of MeIQ. Similar results were reported with yeast. MeIQ was activated by incubation with yeast microsomes prepared from *Saccharomyces cerevisiae* strains expressing human CYP1A1 or CYP1A2 (Sengstag *et al.* 1994). The number of revertants induced in *S. typhimurium* TA98 was increased by incubation with microsomes containing CYP1A2 but not by those with CYP1A1 activity. Josephy *et al.* (1995) later demonstrated that both CYP450 and *N*-acetyltransferase (NAT) enzyme activities were required for the mutagenicity of MeIQ. The *Salmonella* strain, DJ4501A2, containing plasmids bearing the genes for both CYP1A2 and NAT, had a mutagenic response to MeIQ while the parent strain did not. MeIQx was not a hepatocarcinogen in the cynomolgus monkey, presumably due to the lack of constitutive CYP1A2 (or CYP1A1) expression and the subsequent conversion of MeIQx to its *N*-hydroxylamine in the monkey (Lynch *et al.* 1995).

The metabolic pathways of MeIQx in human hepatocytes were dependent on MeIQx concentration. At 50 μM, MeIQx-N^2-SO_3^- was the predominant metabolite; at 1μM (an approximate human exposure level), IQx-8-COOH, a CYP1A2 oxidation product, was the major metabolite (representing 41% of the initial quantity of MeIQx added to human hepatocytes *in vitro*). The authors concluded that CYP1A2 is involved in both the metabolic activation and detoxification of MeIQx in humans (Langouët *et al.* 2001).

Despite the existence of this prominent pathway of detoxification of MeIQx in humans, other researchers have demonstrated the formation of MeIQx adducts to DNA in humans (see Table 5-1), including adducts in the colon after administration of MeIQx *in vivo* and in colon, rectum, kidney, and prostate cells *in vitro*. Due to the lack of evidence for a comparable pathway of metabolism for other HCAs, the formation of the carboxyl metabolite of MeIQx is not illustrated in Table 6-1, which summarizes pathways common to all three HCAs.

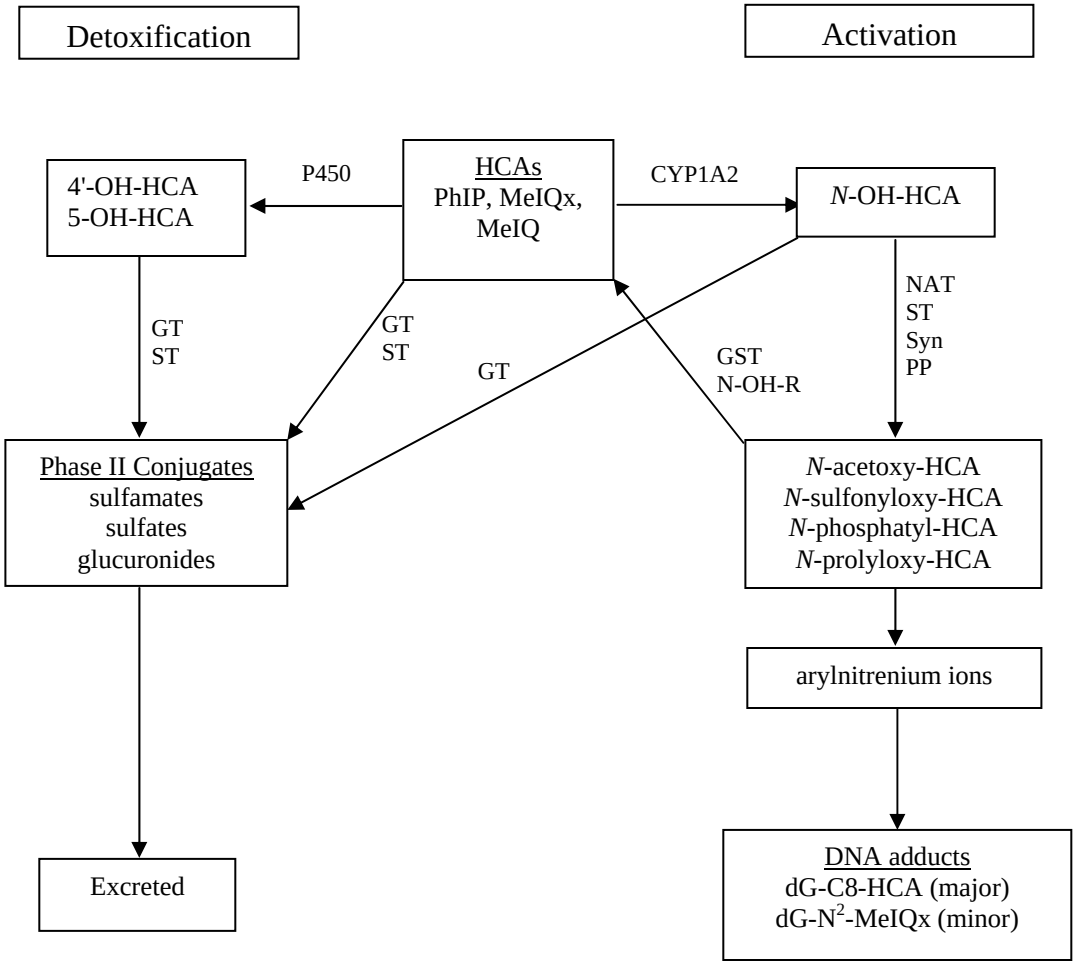


Figure 6-1. Metabolism of HCAs

Sources: Snyderwine 1999, King *et al.* 1999, Schut and Snyderwine 1999, Dashwood 2002.

Abbreviations: HCA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; OH, hydroxy; P450, cytochromes P450; CYP1A2, cytochrome P4501A2; GT, glucuronyltransferase; ST, sulfotransferases; GST, glutathione-*S*-transferase; *N*-OH-R, *N*-hydroxy-reductase; NAT, *N*-acetyltransferase; Syn, prolyl-tRNA synthetase; PP, phosphorylase; dG-C8, *N*-(deoxyguanosin-8-yl); dG-N^2, 5'-(deoxyguanosin- N^2-yl).

6.1.2.2 Phase II bioactivation

Four mammalian cytosolic enzymes (*N*-acetyltransferase, sulfotransferase, prolyl tRNA, or phosphorylase) have been implicated in the phase II activation reactions for HCAs (Buonarati *et al.* 1990, Boobis *et al.* 1996, Dubuisson and Gaubatz 1998, Schut and Snyderwine 1999). The *N*-hydroxy metabolites of HCAs are not highly reactive with DNA *in vivo* but become highly reactive when they are esterified during phase II reactions (Dubuisson and Gaubatz 1998). Some of the recent studies supporting the role of phase II bioactivation in the mutagenicity and carcinogenicity of HCAs are briefly reviewed below.

Dubuisson and Gaubatz (1998) investigated phase II activation of PhIP by cytosolic acetyltransferase (AT), sulfotransferase (ST), and tRNA synthetase/kinase enzymes from human breast tissue. In addition, activation by prostaglandin H synthetase present in microsomal fractions of mammary epithelial cells was investigated. All samples showed the capacity to metabolically activate *N*-hydroxy-PhIP via one or more pathways; however, not all individuals possessed all four enzyme systems. The AT and tRNA synthetase/kinase were the major activation pathways in the six individuals included in this study.

The relative roles of NAT1 and NAT2 in mutagenic activation of MeIQ were explored by Oda *et al.* (1999) using *S. typhimurium* strains expressing the human NAT enzymes. The genotoxic activity of MeIQ was undetectable to very low in the *O*-acetyltransferase-deficient parent strain (NM6000). In contrast, the same doses of MeIQ in an *S. typhimurium* strain expressing human NAT1 (NM6001) produced dose-dependent increases in genotoxicity. The results with the bacterial strain expressing human NAT2 (NM6002) were even more striking, with activity that was > 5-fold higher than that for NAT1. The authors concluded that NAT2 plays a key role in the genotoxicity of MeIQ.

Expression of both human CYP1A2 and *O*-acetyltransferase activity by *S. typhimurium* increased the number of revertants following exposure to MeIQ by up to 79-fold over the TA98 parent strain (Kamataki *et al.* 1999). When compared to strains expressing CYP1A2 alone, the highest levels of genotoxicity and cytotoxicity were demonstrated for a *S. typhimurium* strain expressing both human CYP1A2 and *O*-acetyltransferase activity (Aryal *et al.* 2000).

Human prostate tissue was transplanted into nude mice, and the mice were subsequently exposed to 200 mg/kg of PhIP intragastrically (i.g.) (Cui *et al.* 2000). CYP1A2 mRNA was undetectable in human prostate fibroblast cultured cells, present at low levels in normal human prostate tissues, and present at higher levels in normal human prostate epithelial cultured cells. NAT2 was detected in all samples of prostate tissue and prostate cells. The authors proposed that PhIP may be activated initially by liver CYP1A2 with final activation in the prostate by NAT2.

6.1.2.3 Detoxification

HCAs also are metabolically detoxified through various phase I and phase II pathways (Figure 6-1). Many studies indicate that the organ specificities for DNA binding, mutagenesis, and carcinogenesis observed with HCAs are related to the inter- and intra-

species differences in the balance between detoxification and activation pathways (Davis *et al.* 1993b, Snyderwine *et al.* 1993, 1995, Lin *et al.* 1994, Kaderlik *et al.* 1994a, 1994b, Ghoshal *et al.* 1995). Some of these studies are reviewed below.

Incubation of *N*-acetoxy-PhIP with human liver and colon cytosol preparations in the presence of calf thymus DNA was used to measure binding of PhIP to DNA (Lin *et al.* 1994). When glutathione (GSH) and purified human glutathione S-transferase (GST) A1-1 were added, binding was inhibited by 90%. Examination of human hepatic and colon mucosal cytosols demonstrated GST activity toward *N*-acetoxy-PhIP in all livers, but not in any of the colons tested.

Kaderlik *et al.* (1994b) incubated PhIP with rat hepatocytes with and without pretreatment to deplete GSH and to prevent its resynthesis. In GSH-depleted cells, a 15-fold increase in adduct formation was detected, but there was no change in the formation of PhIP metabolites. An inhibitor of glucuronidation also increased the formation of adducts by 2-fold. In addition, pentachlorophenol, an inhibitor of sulfotransferases, decreased adduct formation slightly without having any effect on formation of PhIP metabolites. The authors concluded that GSH and glucuronidation may play a protective role against the carcinogenicity of PhIP and may explain the low level of PhIP-DNA adducts in rat liver. They suggested that this finding may explain why the liver is not a target tissue for PhIP-induced carcinogenesis.

The capacity for *N*-sulfation of MeIQx using liver cytosols of rats and humans along with cDNA-expressed sulfotransferases (rat ST1A1 and ST1C1 and human ST1A2 and ST1A3) was examined (Ozawa *et al.* 1995). MeIQx was *N*-sulfated in rats; however, sulfamation of MeIQx was not detected in human liver cytosol. Cells expressing human ST1A2 and ST1A3 failed to effectively biotransform MeIQx to sulfamate. The authors concluded that rat ST1A1, a phenol(aryl)sulfotransferase, is capable of detoxifying MeIQx through sulfamate formation in the rat liver; in contrast, ST1C1, a major *N*-hydroxyarylamine(amide) activating enzyme in rat liver, showed only marginal sulfamate-forming activity for MeIQx. The authors suggested that the formation of MeIQx-sulfamate was only a minor contributor to the detoxification of MeIQx in humans.

Nowell *et al.* (1999) characterized the ability of human UDP-glucuronyltransferases to detoxify PhIP by glucuronidation. They found that glucuronidation activity toward *N*-hydroxy-PhIP was present in every section of the intestine, with the highest level detected in the colon.

King *et al.* (1999) reported an enzymatic mechanism by which *N*-hydroxy-PhIP, often described as the proximate carcinogenic metabolite of PhIP, was converted by human and rat liver microsomes back to PhIP. A similar finding was observed for MeIQx. The mechanism of detoxification uses a microsomal NADH-dependent reductase that rapidly converts the *N*-hydroxy arylamine back to the parent amine. The ability of cells to reduce *N*-hydroxy-amines may be protective against formation of mutagenic metabolites.

6.1.2.4 Interspecies differences in metabolism

Understanding the interspecies differences in metabolism is vital for understanding differences in response to chemical exposures and for extrapolating the results from one species to another. In general, the available data indicate that humans have a higher capacity for activating HCAs than nonhuman primates and rodents (Davis *et al.* 1993b, Ozawa *et al.* 1994, Boobis *et al.* 1996, Turesky *et al.* 1998a, Langouët *et al.* 2001). Studies show that the catalytic efficiencies of human microsomes and CYP1A2 are greater than that for the rat or monkey (Davis *et al.* 1993b, Boobis *et al.* 1996, Turesky *et al.* 1998b, Turesky *et al.* 2001); human livers express CYP1A2 at higher concentrations than uninduced rat livers (5 to 245 and 5 to 35 pmol/mg protein, respectively) (Turesky *et al.* 1999); humans metabolize almost the entire dose using the CYP1A2 pathway while rats metabolize much of the dose through ring-hydroxylation (Boobis *et al.* 1996); and human albumin and hemoglobin adduct levels per unit dose are higher than the levels found in rats (Dingley *et al.* 1998). Therefore, humans may be more sensitive to HCAs than are laboratory animals.

A comparison of PhIP metabolism by human, rat, and mouse hepatic microsomes showed that all three species generated two metabolites, identified as *N*-hydroxy-PhIP and 4'-hydroxy-PhIP (Lin *et al.* 1995). However, human liver microsomes had a capacity for formation of *N*-hydroxy-PhIP that was 1.8- and 1.4-fold higher that that of rats and mice, respectively. The ratio of *N*-hydroxylation to 4'-hydroxylation was 97:1, 3.3:1, and 1.7:1 for humans, rats, and mice, respectively. *N*-hydroxy-PhIP formation is believed to represent the activating pathway for genotoxicity, while formation of 4'-hydroxy-PhIP is representative of detoxification (Figure 6-1). The capacities of the three species for further metabolic activation by cytosol-dependent pathways of acetyltransferase, sulfotransferase, amino-acyl-tRNA synthetase, and ATP-dependent kinases also were examined. The levels of activity of these enzymes varied among human, rat, and mouse, but cytosol derived from human liver was able to metabolize *N*-hydroxy-PhIP via all pathways.

Garner *et al.* (1999) administered [^{14}C]-labeled PhIP and MeIQx to rats and humans to research the metabolism and macromolecule binding in human colon tissue compared to the rat. Interspecies differences were observed in the urinary excretion and the metabolic profiles. Human colon tissue contained more PhIP- and MeIQx-DNA adducts than rat colon tissue (DNA adducts are discussed further in Section 6.2). However, the doses given to rats and humans were not equivalent on a body weight basis and the time of tissue collection differed considerably (4 to 6 hours post-dose in humans versus 48 hours post-dose in rats).

6.1.3 Excretion

In laboratory animals, HCAs and their metabolites are excreted in the urine (Turteltaub *et al.* 1989, 1992, Watkins *et al.* 1991, Buonarati *et al.* 1992, Kaderlik *et al.* 1994a, Snyderwine *et al.* 1993, 1995), feces and bile (Turteltaub *et al.* 1989, 1992, Alexander *et al.* 1991, Watkins *et al.* 1991, Snyderwine *et al.* 1993, 1994, 1995), and breast milk (Ghoshal and Snyderwine 1993, Davis *et al.* 1994a, Brittebo *et al.* 1994, Hasegawa *et al.* 1995). Studies using human volunteers have focused on urinary excretion (Lynch *et al.*

1992, Ji *et al.* 1994, Stillwell *et al.* 1997, 1999a, 1999b, Reistad *et al.* 1997, Turesky *et al.* 1998a, Kulp *et al.* 2000). Some of these studies are reviewed below.

At normal dietary exposure levels, significant inter-individual variability in the urinary excretion of MeIQx was observed (Lynch *et al.* 1992). Ten male volunteers consumed 240 ± 9 (SEM) g fried beef patties, which contained 2.2 ± 0.2 ng MeIQx/g meat. The amount of parent MeIQx excreted in the urine was directly proportional to the amount consumed.

In human volunteers who consumed well-done cooked beef that contained PhIP, the median total intake of PhIP was approximately 8.1 μg (range, 5.9 to 10.4 μg) (Stillwell *et al.* 1997). Urine collected over 0 to 12 hours post-dose contained 4.3 ± 1.7% (mean ± SD) of the total PhIP dose, while the total amount of PhIP excreted over the 12 to 24 hour period was 0.9 ± 0.4%. Reistad *et al.* (1997) reported similar results for volunteers who ate a meal of fried ground beef (295 g of cooked meat, mean PhIP intake of 1.18 μg) together with boiled potatoes and a green salad. Total excretion of PhIP by 24 hours post-meal was 6 to 23 ng (0.5% to 2% of dose), while hydrolyzed urine samples contained 24 to 100 ng of PhIP and hydrolyzed PhIP metabolites (2% to 8.5% of dose).

Four major PhIP metabolites were detected in urine collected 24 hours after volunteers had consumed 200 g of cooked chicken containing a total of 27 μg of PhIP (Kulp *et al.* 2000). No metabolites were detected in pre-meal urine collection. The metabolites were N^2-hydroxy-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4'-PhIP-sulfate, and N^2-hydroxy-PhIP-N^3-glucuronide, which cumulatively represented 4% to 53% of the ingested dose.

In laboratory animals, the total percentage of radiolabeled compound excreted in the urine and feces was variable (Table 6-1). The number or identification of metabolites and the amount of unmetabolized parent compound were not always identified; however, the major urinary metabolites included 4'-hydroxy-PhIP, 4'-PhIP-sulfate, 4'-hydroxy-PhIP-glucuronide, *N*-hydroxy-PhIP-glucuronide, 4'-hydroxy-PhIP-sulfate, *N*-hydroxy-PhIP-N^2-glucuronide, *N*-hydroxy-PhIP-N^3-glucuronide, and MeIQx-N^1-glucuronide. The major fecal metabolite identified was *N*-hydroxy-PhIP-N^3-glucuronide.

The major PhIP metabolites present in bile of monkeys after a single oral dose of [^{3}H]PhIP were PhIP-4'-*O*-glucuronide, PhIP-4'-sulfate, 4'-hydroxy-PhIP, N^2-hydroxy-PhIP-N^3-glucuronide, and unmetabolized PhIP (Snyderwine *et al.* 1993). Following exposure to MeIQx, 7-oxo-MeIQx accounted for approximately 40% to 80% of the biliary metabolites in monkeys. Other compounds detected in the bile included MeIQx-5-*O*-glucuronide, MeIQx-N^2-glucuronide, MeIQx-N^1-glucuronide, MeIQx-5-sulfate, MeIQx-sulfamate, and unchanged MeIQx (Snyderwine *et al.* 1995).

Table 6-1. Excretion of HCAs in urine and feces of experimental animals

Chemical	Test animal	Route	Time (h)	Urine (%)		Feces (%)		# Metabolites		Reference
				Total	Parent compound	Total	Parent compound	Urine	Feces	
PhIP	C57BL/6	i.p.	24	39	4–14	12	small amount[a]	11	2	Turteltaub *et al.* 1989
		gavage		31		30		11	2	
PhIP	C57BL/6	i.p.	24	30	2	46.5	nr	> 10	nr	Buonarati *et al.* 1992
N-OH PhIP	C57BL/6	i.p.	24	17.9	nr	53.6	nr	nr	nr	Buonarati *et al.* 1992
4'-OH-PhIP	C57BL/6	i.p.	24	37.6	nr	41.6	nr	nr	nr	Buonarati *et al.* 1992
PhIP	C57BL/6	gavage	96	90	nr	10	nr	nr	nr	Turteltaub *et al.* 1992
PhIP	F344	gavage	24	15	14	78	66	4	2	Watkins *et al.* 1991
		i.p.		14	nr	36	65	nr	2	
PhIP	F344	gavage	24	39–41	< 1	36–43	71–74	5	nr	Kaderlik *et al.* 1994a
PhIP	monkey	gavage	48	37	1	23	nr	5	1	Snyderwine *et al.* 1993
MeIQx	Sprague-Dawley	gavage	72	20–45	6–20	45–70	nr	5	5	Turesky *et al.* 1991b
MeIQx	monkey	gavage	72	50-70	15–25	15–20	nr	8	1	Snyderwine *et al.* 1995a
MeIQ	Sprague-Dawley	gavage	24	37.7–45.3	nr	39.5–60.8	nr	nr	nr	Sjödin and Jägerstad 1984
			72	41.7–49.2		55.9–66.2				
MeIQ	Wistar	i.p.	24	1.3[b]	71	3.8[b]	71	nr[b]	nr[b]	Størmer *et al.* 1987

[a]Percentage not reported.
[b]Only included data for the parent compound and one dichloromethane-extractable metabolite (*N*-acetylated derivative).
nr = not reported

6.2 Mechanisms of action

Acetylation of the *N*-hydroxy metabolites of HCAs is thought to be the proximate activation event. Therefore, the rate at which the acetylation reaction occurs is an important factor in the accumulation of DNA adducts, mutations, and tumors. Researchers also have attempted to identify mutational fingerprints for mutagenic chemicals by analyzing similarities in mutations found in different tumors and in different species. A similar pattern of mutations appearing in animal tumors and in human tumors resulting from exposure to the same agent indicates a common mechanism. Finally, many studies have investigated various modulators of HCA-induced mutagenesis and carcinogenesis to provide a better understanding of the underlying mechanisms. The following sections discuss how studies of acetylator genotypes, DNA adducts, mutations in protooncogenes and tumor suppressor genes, and other factors, including chemical modulation, have added to our understanding of HCA-induced carcinogenesis.

6.2.1 Acetylator genotypes

The phase II reaction catalyzed by *N*-acetyltransferases is a major metabolic activation step for HCAs. Therefore, polymorphisms in the acetylator genotype may affect susceptibility to HCA-induced tumorigenesis. Several researchers who examined the role of acetylator genotypes on HCA-DNA adduct formation in mammalian systems concluded that the increased formation of these adducts by rapid acetylators may translate to a higher risk for tumor development (Minchin *et al.* 1992, Stone *et al.* 1998, Purewal *et al.* 2000b). In contrast, the numbers of intestinal PhIP-DNA adducts detected in Syrian hamster lines congenic at the *NAT2* locus were not different between rapid and slow acetylators. This finding may be explained by data showing that aromatic amine carcinogens have a higher affinity for NAT1 than NAT2, and at low substrate concentrations, NAT1 has a larger role in *N*-acetylation and *N,O*-acyltransfer. Further, no preneoplastic lesions or tumors occurred in these animals in experiments lasting from 10 to 56 weeks (Steffensen *et al.* 2000). For additional information on genetic susceptibility, please see Section 3.4.

6.2.2 DNA Adducts

As discussed in Section 5.1, phase I and phase II activation of HCAs leads to formation of arylnitrenium ions, which have been proposed as the proximate reactive form in formation of DNA adducts. PhIP, MeIQ, and MeIQx have all been reported to form a predominant adduct at the C8 position of guanine bases (see Figure 5-1); MeIQx also forms a minor adduct at the N^2 position of guanine bases.

Kerdar *et al.* (1993a) observed linear relationships between the DNA-binding potencies and the mutagenic potencies of the arylnitrenium ions; further, the study authors concluded that differences in mutagenicity among the HCAs was primarily due to differences in adduct levels rather than differences in the chemical structure of the arylamine residues bound to DNA. Furthermore, animal studies indicate that DNA adduct levels are associated with carcinogenesis. For example, MeIQx induces high adduct levels and tumors in the livers of rats and mice; whereas, PhIP does not induce high DNA adduct levels in rats and mice and is not a liver carcinogen. However, the relationship

between adduct level and carcinogenesis is not a simple matter of cause and effect because high adduct levels may occur in tissues that do not form tumors. Several studies show that guanine-base mutations frequently occur in oncogenes and tumor suppressor genes in HCA-induced tumors, suggesting that HCA-DNA adducts are involved (Schut and Snyderwine 1999). Oncogene activation and inactivation of tumor suppressor genes are discussed in Section 6.2.3.

DNA adduct formation has been shown in a variety of animal models, including cynomolgus monkeys, rats, mice, and *Drosophila* larvae (See Table 5-6). Fewer data are available for HCA adduct formation in human DNA; however, as shown in Table 5-5 DNA adduct formation has been demonstrated for human colon, rectum, kidney, prostate epithelial, and mammary epithelial cells either *in vivo* or *in vitro*. The reports by several investigators (Turteltaub *et al.* 1999, Garner *et al.* 1999, Mauthe *et al.* 1999) that the levels of DNA adducts in human tissues were generally greater than in rodents administered an equivalent dose of HCAs support extrapolating results from animal models to humans.

6.2.3 HCA-induced changes in protooncogenes and tumor suppressor genes

Based on the hypothesis that tumorigenesis results from an accumulation of genetic alterations that activate oncogenes and inactivate tumor suppressor genes, the identification of specific genes that are altered in PhIP-induced tumors may provide a direct link between the presence of DNA adducts and the initiation of tumorigenesis. This section summarizes the potential effects of HCAs on protooncogenes and tumor suppressor genes according to target organ in rodent models.

6.2.3.1 Mammary tumors

No mutations in Ki-*ras* or N-*ras* were detected in female F344 rats fed a diet containing either 100 or 400 ppm PhIP for 104 and 52 weeks (Ushijima *et al.* 1995a). Ha-*ras* and *p53* mutations were detected in only 3 of 17 and 1 of 10 tumors, respectively. The authors concluded that HCA-induced rat mammary tumors were unique in that they were negative for *ras* and *p53* mutations. In another study, 30 rat mammary carcinomas were examined for genetic alterations induced by oral administration of PhIP (Yu *et al.* 2000). None of the genes associated with human breast cancer (i.e., *p53, $p21^{waf1}$, APC, β-catenin, E-cadherin, Bcl-x, Bax, IGFIIR,* and *TGFBIIR*) were found to contain mutations.

The genetic alterations in PhIP-derived mammary tumors (n = 23) of female Sprague-Dawley rats were examined to determine the expression of several genes implicated in the development and progression of human breast cancer (e.g., c-*erbB*-1 ([epidermal growth factor receptor {EGFR} gene], c-*erbB*-2 [neu], TGF-α, and c-*myc*) (Davis and Snyderwine 1995). Of these, 43%, 57%, and 74% had increased expression of EGFR, TGF-α, and neu mRNAs, respectively. The authors suggested the increased expression of these genes, especially in tumors demonstrating papillomatosis, may be associated with PhIP-induced mammary gland cancer in rats.

6.2.3.2 Colon tumors

Candidate genes for a role in PhIP-induced colon carcinogenesis have included *APC*, Ki-*ras*, *p53*, and *β-catenin*, which may play a critical role in the tumor suppressor activity of *APC*. However, *ras* mutations were not detected in colon tumors of F344 rats given 0.04% PhIP in the diet (Kakiuchi *et al.* 1995) or 0.01% MeIQx in the diet (Tanakamaru *et al.* 2001). Makino *et al.* (1994) reported that no mutations were found in the *p53* gene in colon tumors from PhIP-treated rats.

Mutations of the *APC* gene and microsatellite sequences in rat tumor DNA were examined (Toyota *et al.* 1996, Kakiuchi *et al.* 1995). DNA was isolated from colon tumors formed in male F344 rats fed a diet containing 0.04% PhIP for more than 43 weeks. A total of five *APC* gene mutations (two mutations in one tumor, a single mutation with loss of the normal allele in two tumors, and a single mutation in a fourth tumor) were found in four of the eight adenocarcinomas examined. All five mutations involved deletions of a guanine base from a 5'-GGGA-3' sequence (see Section 5), and four of the mutations shared an identical seven nucleotide sequence of 5'-GTGGGAT-3' around the mutation. Four of the five mutations in which a guanine base was deleted were located within only two of the 26 5'-GGGA-3' sequences present in the rat *APC* gene. The authors concluded that the observed pattern of mutations was strong evidence for a mutational profile or "fingerprint" for PhIP-induced tumors.

Mutations in the *β-catenin* gene, which regulates the tumor suppressor activity of *APC*, have been associated with rat colon tumors, but not with mammary tumors (Dashwood *et al.* 1998, Tsukamoto *et al.* 2000). *β-catenin* was mutated in four of seven PhIP-induced colon tumors, and two of these mutations involved amino acid substitutions at a potential phosphorylation site (Dashwood *et al.* 1998). Tsukamoto *et al.* (2000) compared the incidence of *β-catenin* mutations in adenomas, adenocarcinomas, and ACF from colons of male F344 rats treated with PhIP. Mutations were identified in 7 of 7 (100%) adenomas, 6 of 12 (50%) adenocarcinomas, and 14 of 46 (30.4%) ACF. Similar to the findings of Dashwood *et al.* (1998), all of the *β-catenin* mutations in adenomas and adenocarcinomas and 78% of those in ACF were in codons 32 through 34.

6.2.3.3 Forestomach tumors

Mutations of Ha-*ras* and *p53* were identified in tumors induced by feeding 0.04% MeIQ to CDF_1 mice (Makino *et al.* 1992, Ushijima *et al.* 1995b, 1995c, Nagao *et al.* 1997). Ha-*ras* mutations, all G:C→T:A transversions at the second nucleotide of codon 13, were identified in two of three forestomach squamous cell carcinomas and in two of four cell lines derived from other carcinomas (Makino *et al.* 1992); further, these mutations were identified in 22 of 64 forestomach tumors (Nagao *et al.* 1997). In contrast to the findings for Ha-*ras* mutations, the mutations in *p53* varied greatly (Ushijima *et al.* 1995c). Mutations in *p53* were identified in two of the four tumor samples and in all four cell lines. In another study, Ushimima *et al.* (1995b) reported that four of eight forestomach tumors (one papilloma and seven squamous cell carcinomas) contained Ha-*ras* mutations and two of four tumors (one papilloma and three squamous cell carcinomas) contained *p53* mutations. All four mutations in Ha-*ras* involved G:C→T:A transversions, while mutations in *p53* included one G:C→T:A transversion and one G:C→A:T transition. The

authors cautioned that the limited number of activating mutations for *ras* genes may limit their potential as specific markers for MeIQ-induced mutations across species.

6.2.3.4 Zymbal gland tumors

Rat Zymbal gland tumors, induced by feeding 0.03% MeIQ or 0.04% MeIQx in the diet, were examined for mutations in the c-Ha-*ras* gene (Kudo *et al.* 1991). For MeIQ, a G:C→T:A transversion was detected in 9 of 14 (64.3%) squamous cell carcinomas and 1 of 1 papilloma. c-Ha-*ras* mutations, induced by MeIQx, were detected in 2 of 6 (33.3%) Zymbal gland squamous cell carcinomas; these two tumors were composed of a G:C→T:A transversion and an A:T→T:A transversion, each in 1 of 6 (16.7%) squamous cell carcinomas. Nagao *et al.* (1997) reported similar results for MeIQ and also detected mutations at a different Ha-*ras* site in two other Zymbal gland tumors.

6.2.3.5 Liver tumors

Ushijima *et al*. (1995b) evaluated alterations in the *p53* gene as induced by dietary MeIQx (400 ppm) treatment in CDF1 mice. Two poorly-differentiated and one of two moderately differentiated hepatocellular carcinomas showed mutations in *p53*; however, no mutations of *p53* were exhibited in nine well-differentiated hepatocellular carcinomas, 13 neoplastic nodules, or five cholangiocarcinomas. The authors concluded that the mutation in *p53* is only involved in more malignant types of hepatocellular carcinomas. Mutations were found in G:C base pairs and three of four were G:C→T:A transversions.

6.2.4 Other factors

6.2.4.1 Chemical modulators

Dashwood (2002) recently reviewed modulation of HCA-induced mutagenicity and carcinogenicity and divided the inhibitory or promotional mechanisms of action into various categories. These included blocking agents, suppressing agents, bioantimutagens, and tumor promoters. Blocking agents include interceptor molecules (e.g., chlorophyllin, arctiin, dietary fiber), HCA activation inhibitors (e.g., tea polyphenols, indole-3-carbinol), CYP1A1 inducers (e.g., indole-3-carbinol), phase II enzyme modulators (e.g., inducers of GST, glucuronyltransferases, or sulfotransferases), and electrophile scavengers (e.g., tea polyphenols). Suppressing agents are believed to work by altering signaling pathways that control apoptosis or cell proliferation and include many dietary chemicals (e.g., polyphenols, retinoids, carotenoids, and vitamins). The bioantimutagens (e.g., vanillin, caffeine, coumarin) are agents that act on repair and replication processes of damaged DNA and reduce mutation frequency (Sanyal *et al.* 1997).

Interceptor molecules may form a molecular complex with HCAs or with their activated forms, and the binding strength of the complex is correlated to antimutagenic potency in experiments with HCAs (Dashwood and Guo 1993). Chlorophyllin given continuously or during the post-initiation period to F344 rats exposed to PhIP resulted in a reduced number of large ACF (Guo *et al.* 1995) and arctiin administration reduced the number of ACF and mammary tumor multiplicity in female Sprague-Dawley rats given PhIP (Hirose *et al.* 2000) (see Sections 4.4.1.2 and 4.4.1.3).

Bioactivation of HCAs was discussed in Sections 6.1.2.1 and 6.1.2.2. Agents that inhibit one or more of the phase I and II activation enzymes or induce enzymes involved in detoxification pathways may reduce the mutagenic and carcinogenic effects of HCAs (Guo *et al.* 1995, Schut and Snyderwine 1999, Huber *et al.* 1997, Xu and Dashwood 1999, Nelson *et al.* 2001, Dashwood 2002). Although CYP1A1 can activate HCAs, induction of this isozyme can result in inhibition by increasing ring hydroxylation. Ring hydroxylation generates the 4'- or 5-hydroxy metabolites, which are conjugated by phase II enzymes and excreted (see Figure 6-1). For example, relatively high concentrations (> 100 ppm) of indole-3-carbinol preferentially induced CYP1A1 over CYP1A2 and increased excretion of ring-position conjugates (Guo *et al.* 1995, Dashwood 2002).

6.2.4.2 Oxidative damage

In several studies, antioxidants were shown to significantly inhibit HCA-induced carcinogenesis in rats (Hirose *et al.* 1995c, 1998a). Kato *et al.* (1996) determined that the 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) level in rat liver increased dose dependently with the dietary concentration of MeIQx. The authors suggested that the findings indicate that in addition to the formation of DNA adducts, DNA modifications due to oxidative damage may be important in MeIQx-induced liver carcinogenesis in rats. Similar results were found in subsequent studies of antioxidants and MeIQx (Hirose *et al.* 1998b, 1999a, 1995b). In these studies, the authors concluded that synthetic antioxidants, especially HTHQ, were very strong chemopreventors of HCA-induced carcinogenesis; however, depressed metabolic activation rather than antioxidant activity was responsible for the observed effect.

Using ^{32}P-labeled DNA fragments, Murata *et al*. (1999) researched the ability of *N*-hydroxy-MeIQx [MeIQx(NHOH)] to induce oxidative DNA damage. Formation of 8-oxodG, by MeIQx(NHOH) in the presence of Cu(II), and β-nicotinamide adenine dinucleotide (NADH), was measured in calf thymus DNA. MeIQx(NHOH) induced DNA damage in the presence of Cu(II) and the intensity of the damage increased with increasing MeIQx(NHOH) concentration. The amount of 8-oxodG increased with the concentration of MeIQx(NHOH) in the presence of NADH and Cu(II); when NADH was added, a substantial increase in 8-oxodG formation was observed. The authors suggested that *N*-hydroxy-MeIQx induces Cu(II)-mediated DNA damage through reactive oxygen species, and in the presence of NADH, the damage occurs at a very low concentration of *N*-hydroxy-MeIQx through a cycling redox reaction.

A possible role for generation of superoxide radical ($O_2^{\cdot -}$) in HCA carcinogenicity was proposed by Sato *et al.* (1992) and Maeda (1995). When MeIQ and other HCAs were incubated with NADPH and CYP450 reductase, the greatest amount of $O_2^{\cdot -}$ was generated in the presence of MeIQ. The ability of HCAs to generate $O_2^{\cdot -}$ correlated well ($r = 0.88$) with their mutagenicity in the Ames test.

In a subsequent study, $O_2^{\cdot -}$ was generated when recombinant human cytochrome b5 reductase (rh-Cytb5Rd) was incubated with HCAs including MeIQ in the presence of NADH *in vitro* (Maeda *et al.* 1999). The authors concluded that HCA-induced DNA

damage may occur via these reactive intermediates, suggestive of another possible mechanism of action in addition to adduct formation.

6.2.4.3 Cell proliferation, apoptosis, and cell-cycle control

In addition to the initial event of PhIP-DNA adduct formation, PhIP-induced mammary carcinogenesis in rats likely results from other mechanisms (Snyderwine 1999). These include increased proliferation of epithelial cells of terminal end buds (TEBs), retarded differentiation of TEBs to alveolar buds, and increased levels of serum prolactin, a promoter of mammary gland cancer.

Venugopal *et al.* (1999b) examined the effect of PhIP and *N*-hydroxy-PhIP on apoptosis in human mammary epithelial MCF-10A cells. Cell death (trypan blue exclusion) was significantly inhibited by 1 and 5 μM *N*-hydroxy-PhIP and by 100 μM PhIP. Both PhIP and *N*-hydroxy-PhIP increased the levels of expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L. The authors also examined the MAP kinase pathway, including expression of ERK1 and ERK2 proteins, and concluded that inhibition of cell death occurs independently of PhIP-DNA adduct formation. In another study, Venugopal *et al.* (1999a) administered PhIP (150 mg/kg, p.o., 4x) to lactating Sprague-Dawley rats separated from their 10-day-old pups. PhIP administration was associated with a significant decrease in the apoptotic index and an increase in the number of larger alveoli. The expression of Bcl-2, an inhibitor of apoptosis, was increased in PhIP-exposed rats while the expression of Bax, a stimulator of apoptosis, was decreased in exposed rats. PhIP also significantly ($P < 0.05$) increased the serum concentrations of prolactin and thereby inhibited mammary gland involution. The authors proposed that the ability of PhIP to increase prolactin levels likely plays a role in PhIP-induced mammary carcinogenesis.

Snyderwine *et al*. (1998b) examined the effects of orally administered PhIP (75 mg/kg, p.o. for 10 days) and dietary fat on the percentage of proliferating cells in TEB epithelial structures of mammary glands in female Sprague-Dawley rats. The percentage of proliferating cells in TEBs was significantly increased after 10 days of treatment with PhIP. Rats fed either high- or low-fat diets for 6 weeks after PhIP administration showed significant differences in proliferating cells in TEBs only between PhIP + high-fat diet and control + low-fat diet. PhIP administration for 10 days also significantly decreased the number of alveolar buds compared to the number of buds in control rats. The authors suggested that the effects of PhIP on proliferation and differentiation of mammary gland structures may contribute to the targeting and carcinogenicity of PhIP in rats.

Zhu *et al.* (2000) investigated the effects of PhIP on cell cycle and gene mutations in human lymphoblastoid (TK6) cells incubated in coculture with Chinese hamster XEMh1A2-MZ cells genetically engineered to express human CYP1A2. PhIP induced S-phase arrest, but eluded the G_1 and G_2-M checkpoints. PhIP exposure increased apoptosis and the mutation frequency at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus. The authors suggested that the activation of the S-phase checkpoint may allow the cell to accumulate genetic damage and replicate damaged templates, although the possibility of activation of DNA repair mechanisms during S phase could not be excluded. They further suggested that tissues with higher numbers of cells actively undergoing replication would be more

susceptible to PhIP-induced genetic damage. This finding would be consistent with the induction of tumors of the colon, mammary gland, and bone marrow in rats treated with PhIP.

6.3 Summary

HCAs are readily absorbed and rapidly distributed following oral administration. Excretion occurs by a combination of urinary and fecal routes. HCAs are distributed to most tissues; especially to the liver, GI tract, and kidneys. Tissue concentrations generally peak within 12 to 24 hours and decline rapidly thereafter. The relative order of concentration of PhIP in tissues may depend on the route of administration and the length of exposure. Once HCAs are absorbed from the GI tract, they may be subject to recirculation by the enterohepatic system in the bile. HCAs and their metabolites have also been detected in fetuses and in the milk of lactating rodents.

HCAs are metabolized by both phase I and phase II enzymes. *N*-hydroxylation, by liver CYP1A2, is the major phase I activation pathway. However, there is some evidence that CYP1A1, 1B1, and a few other isozymes may have a role in bioactivation. Further activation by phase II enzymes is necessary for the formation of the arylnitrenium ion that ultimately binds to DNA. Four phase II cytosolic enzymes (*N*-acetyltransferase, sulfotransferase, prolyl tRNA, and phosphorylase) can catalyze the ultimate activation step. The phase II activation reactions may take place in the liver or in other tissues. The data indicate that the activation steps are more efficient in human tissues than in experimental animals and that rapid acetylators may be at higher risk.

PhIP, MeIQ, and MeIQx adduct to the guanine base, with the predominant adduct formed at the C8 position. MeIQx also forms a minor adduct at the N^2 guanine position. DNA adducts have been identified in human colon and mammary tissue following exposure to dietary-relevant concentrations of HCAs. In animal studies, adducts are formed in a dose-dependent manner and are associated with carcinogenesis. In fact, all HCAs that are carcinogenic in animals also induce DNA adducts. However, the relationship is not a simple matter of cause and effect. High adduct levels do not necessarily mean that tumors will occur. Therefore, tissues from HCA-induced tumors have been examined for specific mutations in genes that are associated with human cancers. These include protooncogenes and tumor suppressor genes such as Ki-*ras*, Ha-*ras*, *Apc*, *p53*, *β-catenin*, and others. Guanine-base mutations occurred with the greatest frequency in these genes, particularly G:C→T:A transversions, and suggest that the HCA-induced adducts are involved. The observed mutation patterns provide evidence for a mutational profile, or "fingerprint," for PhIP-induced colon tumors and MeIQ-induced forestomach and Zymbal gland tumors.

Studies investigating chemical modulators have identified several inhibitory or promotional mechanisms that influence HCA-induced mutagenesis and carcinogenesis. Many potential chemical modulators have been studied, including blocking agents, suppressing agents, bioantimutagens, and tumor promoters. Many of the modulators act by more than one mechanism, which underscores the complexity of the interactions among HCAs and other components of the diet. These studies have increased our understanding of HCAs and their potential role in human cancers. Other potential factors

in HCA-induced carcinogenesis include oxidative damage from 8-oxodG or superoxide radicals and effects on cell proliferation, apoptosis, and cell-cycle control.

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Appendix A: Individual Genotoxicity Studies

A Individual Genotoxicity Studies

A.1 PhIP

A.1.1 Prokaryotic systems

A.1.1.1 Induction of mutations in Salmonella typhimurium

In the presence of induced rat liver S9 mix, PhIP was mutagenic in *S. typhimurium* strain TA98 (Johansson *et al.* 1995b, Malfatti *et al.* 1995, Wakabayashi *et al.* 1995, Apostolides *et al.* 1996, Guyonnet *et al.* 2000, Yu *et al.* 2001), TA1538 (1995), and a strain that expressed *N*, *O*-acetyltransferase, YG1019 (Pfau *et al.* 1999). Wild *et al*. (1995) utilized strains TA1538, TA1538 1,8-DNP, DJ400, and DJ460 because each exhibited a different acetylator phenotype (*S. typhimurium NAT* $^{+}$, *NAT* $^{-}$, human *NAT1*, and human *NAT2*, respectively). PhIP induced revertants in a dose-dependent manner regardless of acetylator status. Malfatti *et al*. (1994) showed that *N*-hydroxy-PhIP induced revertants in exposed *S. typhimurium* strain TA98 1,8-DNP_6 without metabolic activation. Another study showed that human hepatocyte (HepG2) cell homogenate activated PhIP resulting in the induction of revertants of strain YG1024 (Knasmüller *et al.* 1999). However, the concentration of PhIP required to induce the same or greater number of revertants was 1000-fold higher when HepG2 homogenate was used in place of rat S9. The authors suggested that these differences in effective dose might be related to the activities of the HepG2 and the rat liver enzymes that catalyze the *N*-hydroxylation of PhIP.

Koch *et al*. (1998) examined the mutagenicity and mutational specificity of PhIP as well as the effect of DNA-repair status on PhIP mutability in *S. typhimurium* strains TA100 and TA1535. Both strains were deletion *uvrB*; however, TA100 contains plasmid pKM101 that carries the *MucAB* genes, homologues to *E. coli umuDC*. PhIP increased the number of revertants in a dose-dependent manner over control levels in both strains TA100 and TA1535. The mutagenic potencies were 17.3 and 71.7 revertants per mg in TA1535 and TA100, respectively. Eighty-eight PhIP-induced TA100 mutants and 217 PhIP-induced TA1535 mutants were sequenced to determine the specificity of the induced mutations. PhIP induced predominantly G:C→T:A transversion mutations in strain TA100 and G:C→A:T transition mutations in strain TA1535. Of the total mutants analyzed, 72% of the TA100 and 96% to 99% of the TA1535 contained mutations at a CCC sequence with the mutation at the second C. The differences in the mutational specificity observed in the two strains were explained by the capacity of strain TA100 to perform error-prone lesion bypass (promoted by the MucAB proteins) of PhIP DNA lesions.

The role of DNA repair status in the removal of PhIP-induced DNA-adducts and PhIP-induced mutations was examined by Malfatti *et al*. (1995). *S. typhimurium* strains TA98 (*uvrB* deficient) and TA1978 (*uvrB* proficient) were treated with PhIP in the presence of S9. Adducts were detected in both strains; however, the TA1978 strain required a larger dose of PhIP to achieve the same level of DNA adducts as that detected in the PhIP-exposed TA98 strain. The authors hypothesized that nucleotide excision repair (NER) removes adducts from the TA1978 (*uvrB*$^{+}$) strain, resulting in decreased adduct levels in the PhIP-exposed TA1978 strain. Revertants were induced only in the PhIP-exposed

TA98 strain. Using the number of PhIP-induced TA98 revertants and the number of DNA adducts (as detected by ^{32}P-postlabeling), the authors calculated that only one mutation was induced for every 25 PhIP adducts.

Suzuki *et al.* (1998) and Kamataki *et al.* (1999) developed a new *Salmonella* tester strain that was much more sensitive to PhIP and other HCAs than the parent tester strain TA1538. This tester strain, designated TA1538/ARO, contained plasmids expressing human CYP1A2 and NADPH-cytochrome reductase cDNAs and *Salmonella O*-acetyltransferase (OAT). Preincubation with S9 was not needed for this strain. PhIP induced 650 revertants/nmol in the TA1538/ARO strain compared to 370 revertants/nmol in the TA1538 strain with rat liver S9 activation (Suzuki *et al.* 1998). (see Table A-1 for a comparison of the potency of PhIP in various *Salmonella typhimurium* strains).

Salmon *et al*. (1997) showed that the method of meat preparation affected the concentration of PhIP in the cooked meat and therefore affected the mutagenicity of the meat extracts. *S. typhimurium* strain TA98 (in the presence of induced-rat liver S9) produced fewer revertants when exposed to marinated chicken extracts than to extracts from unmarinated chicken. The study showed that the levels of PhIP were 92% to 99% (P = 0.00001) lower in extracts from marinated chicken when compared to extracts from unmarinated chicken. In a similar study, PhIP levels were compared among unmarinated and marinated (barbecue sauce, teriyaki sauce, and turmeric-garlic sauce) beef extracts (Nerurkar *et al.* 1999). The extracts induced revertants in *S. typhimurium* strains TA98 in the presence of induced-rat liver S9. An increase in the number of revertants was observed after exposure to extracts of cooked beef that was marinated in barbecue sauce. Conversely, the number of revertants was decreased in strains exposed to extracts of cooked meat that was marinated in teriyaki or turmeric-garlic sauce. Marinating in teriyaki or turmeric-garlic decreased the formation of PhIP in cooked beef. All cooked beef extracts were mutagenic (127 to 312 revertants per g cooked meat) when compared to the negative control (DMSO, 13 revertants per plate). Tikkenen *et al*. (1996) showed that one marinade decreased the formation of PhIP in grilled chicken extracts and resulted in a five-fold decrease in the number of TA98 revertants (in the presence of induced-rat liver S9). However, three other marinades had no consistent effect on the mutagenicity of cooked-chicken extracts. Increases in the cooking temperature corresponded with increased mutagenicity of the cooked-chicken extracts. Extracts from chicken cooked at 110ºC were not mutagenic, but extracts of chicken cooked at 170ºC had revertants in strain TA98. The greatest induction of revertants occurred when strains were exposed to extracts from chicken cooked at 220ºC.

Thiébaud *et al*. (1995) collected the airborne cooking by-products generated when ground beef, bacon, or soybean-based (tempeh) burgers were fried. PhIP was the most abundant HCA present in all the fried meat extracts, with a relative amount representing 47% to 65% of the total HCAs. The airborne by-products were mutagenic when assayed in *S. typhimurium* strain TA98 in the presence of induced-rat liver S9. The mutagenic potencies of the airborne by-products ranged from 1,300 revertants per gram of cooked food (beef patties fried at 198ºC) to 10,400 revertants per gram of cooked food (beef patties fried at 277ºC). The airborne cooking by-products accounted for 24% to 34% of the mutagenic activity of the fried beef samples.

A.1.1.2 Induction of mutations in Escherichia coli

In *E. coli*, two recent studies examined the mutagenicity and mutational specificity of PhIP. Constable *et al*. (1999) showed that PhIP was mutagenic in *E. coli* strain DJ4309 without S9 metabolic activation. *E. coli* strain DJ4309 carries two plasmids, one containing the *S. typhimurium NAT* gene and the other carrying the human *P4501A2* gene and human NADPH-cytochrome P450 reductase. PhIP was not mutagenic in an *E. coli* strain that carried similar plasmids but did not express human P4501A2. In *E. coli* strain DJ4309, the mutagenic potency of PhIP was 32 revertants per ng (or 0.032 revertants per μg), and the mutagenic potency of *N*-hydroxy-PhIP was 424 revertants per μg.

Garganta *et al*. (1999) utilized a set of *E. coli* tester strains (Δ*uvrB* pKM101); each strain carried an F' plasmid with a unique mutation in the *lacZ* gene. Each F' *lacZ* gene required a different, but specific, mutation to restore β-galactosidase activity. In the presence of rat liver S9 mix, 59% of the mutants induced by PhIP-exposure were G:C→T:A transversion mutations with a mutagenic potency of 662 revertants per μg of PhIP.

A.1.2 Eukaryotic systems

A.1.2.1 Induction of mutations in Saccharomyces cerevisiae

Metabolically competent yeast strains were used to assess the recombinogenic potential (gene conversions, chromosomal translocations, and forward mutations) of PhIP and other HCAs (Paladino *et al.* 1999). The strains contained vectors that expressed human CYP1A2, human NADPH-cytochrome P450 oxidoreductase, and NAT2. PhIP-induced gene conversion and translocation were too weak to be scored as positive. PhIP did not induce mutations at the URA3 locus but did induce a small, but insignificant, NAT2-dependent increase in recombination frequency. The authors proposed that chemically induced mitotic recombination may contribute to loss of heterozygosity frequently observed in PhIP-induced rodent tumors.

A.1.2.2 Mutagenicity in Drosophila melanogaster

Kasai *et al*. (1998) used the wing-spot test to determine the mutagenicity of PhIP in *D. melanogaster*. Single spots arise primarily by point mutations, deletions, or mitotic recombination. Twin spots result from mitotic recombination. Over the narrow dose range of 50 to 80 μg/g medium, PhIP induced a dose-dependent increase in small single spots and moderately induced large single spots, but the frequency of twin spots was not different from controls. The authors concluded that PhIP induced DNA deletions but not recombination. Using the DNA repair test, Kasai *et al*. (1998) showed that PhIP preferentially killed repair double meiotic recombination-deficient Rec $^{-}$ male larvae resulting in a decreased Rec $^{-}$ male to Rec $^{+}$ female ratio. The authors concluded that the results of the DNA repair test suggested that PhIP induced DNA damage.

A.1.3 Mammalian systems

A.1.3.1 In vitro *assays*

Specific locus (*aprt, hprt, ouabain*) forward mutation test

Using human lymphoblastoid TK6 cells and metabolic activation (induced-rat liver S9 or co-culture with irradiated Chinese hamster cells that expressed human CYP1A2),

Morgenthaler and Holzhauser (1995) and Zhu *et al.* (2000) showed that *hprt* mutant frequencies increased after treatment with 5 μg/mL of PhIP. A dose-dependent increase in mutations also was observed at the *tk* locus (Morgenthaler and Holzhauser 1995). DNA sequence analysis of 54 *hprt* mutants revealed that all mutations occurred at G:C base pairs, consistent with the observation that PhIP reacts primarily with 2'-deoxyguanosine (see Section 6). Except for a group of single base-pair deletions in a run of six guanines in exon 3, most of the mutations were G:C→T:A transversions. Mutations were preferentially induced on the nontranscribed strand. Sequences that contained the triplet sequences GGA or AGG were preferred sites of mutation induction.

Glaab and Skopek (1999) examined the cytotoxicity and mutagenicity of PhIP in mismatch repair (MMR)-defective cells. Cells without MMR were less sensitive to the cytotoxic effects of PhIP than were cells with functional MMR. In the presence of induced-mouse liver S9 mix, PhIP induced mutations at the *hprt* locus in a dose-dependent manner in both the MMR-defective and MMR-proficient cell lines. However, the increase in mutant frequency was 3-fold greater in the MMR-defective cell lines. Since persons with hereditary nonpolyposis colorectal cancer syndrome (HNPCC) carry defects in MMR, the authors proposed that these individuals might be more sensitive to dietary carcinogens such as PhIP. In a follow-up study, Glaab *et al*. (2000) determined the mutational specificity of PhIP in MMR-deficient cell lines. DNA sequence analysis of PhIP-induced *hprt* mutants showed a significant increase ($P < 0.05$) in the number of transversion mutations. There was an increase in the number of mutations (transitions, transversions, and frameshifts) in the run of six guanines in exon 3; 31% of the PhIP-induced mutations, but only 20% of the spontaneous mutations, occurred in this run of six guanines. The authors suggested that MMR-defective cells might be at increased risk for PhIP-induced mutations in homopolymeric runs of guanines (such as those contained in the *BAX*, *IGFIIR*, or *hMSH6* genes).

UA21 cells, CHO cells, are hemizygous for the dihydrofolate reductase (*dhfr*) gene. UA21 cells were exposed to 20 μM *N*-hydroxy-PhIP for one hour to induce *dhfr* $^-$ mutants (Carothers *et al.* 1994). The predominant mutation, detected in 75% of the mutants analyzed, was G:C→T:A (single and tandem-double) transversions. All but one mutation occurred at guanines on the nontranscribed strand.

Thompson *et al*. (1991) determined the mutagenicity of PhIP at the *aprt* locus in NER-deficient CHO cells expressing the mouse *P4501A2* gene (UV5P3), or in NER-proficient CHO cells expressing the mouse *P4501A2* gene (5P3R2). In UV5P3 cells, PhIP-induced *aprt* mutations at a dose 1/40 of that in repair-proficient cells (Thompson *et al.* 1991). The authors concluded that PhIP-adducts were efficiently removed in the NER-proficient cells. Wu *et al*. (1995) determined the mutagenic potential and mutational specificity of PhIP in UV5P3 cells. Exposure to 0.4 μg per mL of PhIP for 48 hours resulted in a 7-fold increase in the *aprt* mutant frequency. DNA sequence analysis of PhIP-induced *aprt* mutants revealed that transversion mutations occurred in 31 of the 32 mutants analyzed, and 24 of the transversions occurred at G:C base pairs. Seventy-five percent of the mutations occurred at three hotspots located in exons 2 and 3. The authors suggested that DNA sequences, similar in sequence to the hotspot sequences, may occur in important

genes such as those that control cell replication and survival, and these genes may be more susceptible to PhIP-induced mutations.

Chinese hamster V79 cells expressing human CYP1A2 were exposed to various concentrations of PhIP for 24 hours (Yadollahi-Farsani *et al.* 1996). PhIP induced *hprt* mutants in a dose-dependent manner. Examination of 45 *hprt* mutants revealed a predominance of G:C→T:A transversion mutations. A one-base deletion hotspot was observed in a 5' GGGA 3' sequence. All of the mutations, except for a single complex mutation, occurred at G:C base pairs on the nontranscribed strand. The abundance of mutations on the nontranscribed strand may indicate preferential repair of DNA adducts from the transcribed strand. The authors noted that PhIP-induced colon tumors from rats (see Section 4) contained an identical mutation in the *Apc* tumor suppressor gene, a -1G deletion in a 5' GGGA 3' sequence (see Section 6).

Lawson and Kolar (1994) co-cultured Chinese hamster V79 cells with hamster pancreas duct epithelial cells (DEC), human DEC, pancreas duct tissue homogenates from hamsters fed a control or high-fat diet, or hamster hepatocytes. Exposure of V79 and DEC co-cultures to 10 μM PhIP for 16 hours resulted in a 30-fold or a 17-fold increase in *hprt* mutant frequencies in co-cultures with hamster or human DECs, respectively. The mutagenicity of PhIP was 12,200 *hprt* mutants per 10^6 survivors per μmol in the presence of hamster DEC and 7,000 mutants per 10^6 survivors per μmol in the presence of human DECs.

In another study, immortal DEC cells (CK cells) were used to determine the effect of GSH on PhIP-induced mutagenicity (Lawson *et al.* 1997). Before the addition of PhIP and co-culture with CK cells, V79 cells were treated with buthionine sulfoximine (BSO), a GSH reducing agent. The *hprt* mutant frequency in the BSO-treated V79 cells was two-fold greater than the mutant frequency in V79 cells without BSO treatment. Conversely, co-cultures of V79 and CK cells were treated with sulfite and then exposed to PhIP. Sulfite treatment increased the levels of GSH in V79 cells but more than in CK cells. PhIP-exposed cells without sulfite treatment had 15 *hprt* mutants per 10^6 survivors but the PhIP-exposed, sulfite-treated cultures had only 7 mutants per 10^6 survivors. The mutant frequency dropped to 2 mutants per 10^6 survivors (the same as cultures not treated with PhIP) when both CK and V79 cells were treated with sulfite. The authors concluded that GSH reacts with, detoxifies, and metabolizes PhIP, thereby decreasing the genotoxicity of PhIP.

Wild-type A_L cells express S1 and are lysed by complement + anti-S1 antibody. $S1^-$ cells have mutant S1 expression, and instead of dying in the presence of complement and antibody they grow into colonies. Induced chick embryonic liver cultures (CELC) were co-cultured with human-hamster A_L cells (Waldren *et al.* 1999). Overnight exposure to 20 μg per mL of PhIP induced mutations at the $S1^-$ locus in A_L human-hamster cells. Over 65% of the PhIP-induced mutations were deletions 4.2 to 133 Mbp in size, and more than 50% of these deletions were larger than 21 Mbp. The authors concluded that PhIP is a clastogen in repair-proficient mammalian cells.

Kitazawa *et al.* (1994) exposed clones of the mouse tumor cell lines BMT11 and FM3A to *N*-hydroxy-PhIP. Genomic DNA was isolated from PhIP-exposed clones and analyzed by Southern blot. When compared to untreated controls, treatment with *N*-hydroxy-PhIP resulted in extra or shifted bands when the Southern blots were hybridized with mouse genome hypervariable minisatellite DNA sequences (Pc-1 and Pc-2). When minisatellite DNA sequences are used as probes, changes in the Southern blot pattern are probably due to homologous recombination between different repeat units of the microsatellite. The authors concluded that the PhIP-induced band changes suggest that PhIP induces recombinational mutations.

Cytogenetic tests

Chromosomal aberrations

Human peripheral blood lymphocytes were exposed for two hours to 0 to 25 μg per mL of PhIP in the presence and absence of rat liver induced S9 mix (Otsuka *et al.* 1996a). There was no significant induction of CAs in the absence of S9 mix. However, CAs were significantly induced ($P < 0.001$) after exposure to ≥ 10 μg of PhIP per mL in the presence of S9 and were primarily chromatid-type breaks or exchanges.

Diploid human fibroblast (TIG-7) cells were treated for two hours with PhIP in the presence of induced-rat liver S9 or *N*-hydroxy-PhIP in the absence of rat S9 mix (Otsuka *et al.* 1996a). CAs, primarily chromatid-type breaks and exchanges, were significantly induced ($P < 0.001$) after exposure to 12.5 to 20 μg per mL of PhIP or 1.25 to 2.5 μg of *N*-hydroxy-PhIP per mL, which also induced chromosome-type breaks. Consequently, the authors concluded that PhIP required metabolic activation to induce cytogenetic effects and that *N*-hydroxy-PhIP is clastogenic in the absence of S9.

Micronucleus test

Chinese hamster lung (CHL/IU) cells and sublines YG10003, YG10006, and YG10007 carrying the *O*-acetyltransferase gene from *S. typhimurium*, human *NAT2*, and human *NAT1*, respectively, were each exposed to 0, 5, or 10 μg of PhIP per mL for six hours in the presence of induced-rat liver S9 (Otsuka *et al.* 1996b). In the absence of S9 mix, little change in the frequency of MN was observed even after exposure to 20 μg of PhIP per mL. The incidence of micronucleated cells was elevated in all cells exposed to PhIP in the presence of S9. CHL/IU cells showed a 14.5% incidence of MN after treatment with 10 μg per mL of PhIP, whereas unexposed cells only had 0.8% of cells with MN. The incidences of MN in cell lines YG10003, YG10006 and YG10007 were significantly higher than the untreated CHL/IU cell line ($P < 0.001$). The authors suggested that these findings support the possibility that the acetyltransferase mediates the clastogenicity of PhIP.

Pfau *et al*. (1999) assessed the ability of PhIP to induce MN in MCL-5 cells, human lymphoblastoid cells that express five human cytochromes P450 and microsomal epoxide hydrolase. Exposure of MCL-5 cells to 2.0 to 22.5 ng/mL of PhIP for 24 hours induced a significant dose-dependent trend ($P < 0.002$) for MN formation. In another study, MN were formed when HepG2 hepatoctyes or CHO cells (in the presence of HepG2 homogenate) were exposed to 25 to 900 μM PhIP for one to two hours (Knasmüller *et al.* 1999).

Sanyal *et al.* (1997) and Knasmüller *et al.* (1999) investigated the genotoxic effects of HCAs in HepG2 cells. HepG2 cells exposed to 0.6 mM PhIP for four hours developed a 3-fold increase in the number of MN compared to controls. However, groups of cells exposed to 0.6 mM PhIP and 5 μg/mL of caffeine, vanillin, or coumarin showed about a 40% to 50% decrease in MN compared to cells exposed to PhIP alone (Sanyal *et al.* 1997). Knasmüller *et al.* (1999) tested PhIP at 25 to 900 μM, also in a HepG2 cell line, and reported that it produced a dose-dependent increase in MN, with the LEC in the range of 25 to 50 μM. The authors concluded that the MN/HepG2 tests might give more accurate results with HCAs than other *in vitro* mammalian cell systems because it includes enzymes involved in the metabolic conversion of amines.

Sister chromatid exchanges

Otsuka *et al*. (1996a) determined the frequency of SCEs in human blood lymphocytes exposed to PhIP. In the presence of 2.5% or 5.0% induced-rat liver S9, the number of SCEs per metaphase increased in a dose-dependent manner. The number of SCEs doubled after exposure to 1.56 μg of PhIP per mL.

DNA damage/repair test

DNA single strand breaks

The single cell gel electrophoresis (Comet) assay was used to determine the DNA strand-breaking activity of PhIP in the human lymphoblastoid cell line MCL-5 (Pfau *et al.* 1999). MCL-5 cells express human CYP1A1, CYP1A2, CYP2A6, CYP3A4, and CYP2E1 as well as epoxide hydrolase. In the presence of hydroxyurea (HU) and arabinose C (ara-C), compounds that inhibit DNA resynthesis during nucleotide excision repair, the median comet tail length in untreated control cell populations ranged from 6.0 μm to 10.0 μm. In cells treated with 90.9 μg of PhIP per mL for 30 minutes, the median tail length was increased to 83.5 μm. Martin *et al*. (1999) used the Comet assay to investigate DNA damage from PhIP in MCL-5 cells, both with and without HU and ara-C. PhIP (0.20, 0.41, or 2.03 mM) resulted in significantly increased comet tail length, both with and without HU and ara-C; however, a 7.3-fold increase in tail length was seen with HU and ara-C, as compared to the tail increase without the compounds. In a dose-response study, PhIP induced an increase in comet tail length even at the lowest dose (45.5 μg per mL). Pool-Zobel *et al*. (1997) showed that PhIP-exposure (0.5, 1, or 1.5 mM for 30 min) did not increase comet tail length in primary human colon mucosa cells. However, at the same doses, the comet tail length was increased in PhIP-exposed rat colon mucosa cells. The authors suggested that in rats the first pathway of activation of PhIP may occur in extrahepatic tissue such as the colon. They further suggested that the human colon mucosa cells were at a decreased risk for genotoxicity induced by unmetabolized PhIP. In addition, the authors noted that cells isolated from other human donors may contain more metabolic activation enzymes.

Human mammary epithelial cells were exposed to 2 mM *N*-hydroxy-PhIP for two hours (Fan *et al.* 1995). After exposure, ^{32}P-postlabeling detected 10 to 11 PhIP-DNA adducts per 10^7 nucleotides. PhIP-DNA adducts were quickly removed so that only 18% of adducts remained after 24 hours. The authors suggested that human mammary epithelial cells were sufficiently able to repair the PhIP-DNA adducts.

Unscheduled DNA synthesis

Human (Beamand *et al.* 1996, 1998a, 1998b) and rat and mouse liver slices (Beamand *et al.* 1998b) were used to determine if HCAs induced UDS. Treatment with 5 μM or 50 μM PhIP induced UDS in 24 hour cultured human liver slices, particularly in the centrilobular hepatocytes. The increase in UDS was significant ($P < 0.001$). All the HCAs tested induced dose-related increases in UDS in human liver slices, and PhIP was the most potent HCA tested in human liver tissue. UDS significantly increased in mouse liver slices at 2 μM, but did not increase in rat liver slices at 5 μM to 200 μM (Beamand *et al.* 1998b). Kaderlik *et al*. (1994a) showed similar results in primary rat hepatocytes isolated from Arochlor 1254 treated rats. PhIP-induced UDS increased 15-fold when the GSH pools were depleted by 1-bromo-heptane (BH) and BSO. Inhibition of glucuronidation by D-galactosamine decreased the formation of the *N*-glucuronides of *N*-hydroxy-PhIP and resulted in a two-fold increase in the level of UDS. The authors proposed that glutathione and glucuronidation might play important roles in protecting against PhIP-induced carcinogenesis in the liver.

A.1.3.2 In vivo *assays*

Specific locus test (mouse)

PhIP exposure did not induce mutations in mouse splenic lymphocytes (Dass *et al.* 1998). Neonatal mice, eight-day-old pups, were treated with a total dose of 6.5 mg/kg or 26.2 mg/kg of PhIP. Even after an expression time of 24 weeks, no treatment-related increase in the *hprt* mutant frequency was observed. The authors suggested that this lack of mutagenicity was caused by the inability of PhIP to form adducts in lymphocytes or of mouse neonates to sufficiently metabolize PhIP to bioactive metabolites.

Using an unselected mutagenesis approach, Burnouf and Fuchs (2000) showed that genomic DNA isolated from PhIP-exposed mice contained mutations in one GTGGGAT sequence of the *Apc* gene. Rats were fed PhIP (400 ppm) for 1, 2, 4, or 6 weeks. Genomic DNA was isolated from the colon epithelia in each group. Primers were designed to preferentially amplify mutant DNA in the presence of wild-type DNA; the special primers produced more mispairs when hybridized to the wild-type sequence and fewer mispairs when hybridized to the mutant sequence. Mutant *Apc* DNA was preferentially amplified, and an amplification factor was calculated for each group. Control, 1-week treated group, and 6-week treated group showed mutant signals of 1, 3.3, and 12.8, respectively. The increase in the mutant signal was a function of PhIP treatment. The strengths of the 1-week and 6-week groups were significantly different from the control group, $P < 0.01$ and $P < 0.001$, respectively.

Host-mediated assay

The organ-specific genotoxic effects of five HCAs, including PhIP, MeIQ, and MeIQx, were investigated in Swiss albino mice using a microbial animal-mediated assay (Knasmüller *et al.* 1992). Two *E. coli* K12 strains were used. One was DNA-repair deficient (*uvrB*/*recA*), and the other was the wild-type strain (uvr^+/rec^+). Cultures of the repair-deficient strain were mixed 10:1 with the wild-type strain and concentrated. A portion (0.2 mL) of this suspension was injected into the tail vein of male Swiss albino mice. Test compounds were dissolved in distilled water containing 10% DMSO and were administered either i.p. or orally immediately after treatment with the *E. coli* suspension.

The mice were sacrificed after two hours, and liver, spleen, kidneys, testes, and blood samples were collected. Blood and tissue homogenates were plated on NR-S agar plates, and survival for each of the bacterial strains was determined. All test compounds induced significant dose-related genotoxic effects at i.p. doses ranging from 2.3 to 40 mg/kg; however, PhIP exhibited the lowest genotoxic activity. With all the HCAs, effects were more pronounced in the liver, lungs, and blood, than in the kidneys, spleen, or testes. Results were similar following oral administration; however, higher concentrations were required to induce an effect. The authors compared these results with those from the *in vitro* experiments (see Section A.1.2). Based on the *in vitro* results, doses required to induce an effect in living animals were higher than expected. Although the rank order of genotoxic potencies for different compounds was similar in both experiments, the effects were much more uniform in the *in vivo* experiments. These data suggest that the genotoxicity of HCAs may be overestimated by *in vitro* experiments.

Sister chromatid exchanges

Beginning at eight weeks of age, C57BL/6N female mice were fed a powdered diet of rodent chow spiked with 0, 100, 250, or 400 ppm of PhIP (Director *et al.* 1996). Approximately five mice per group were sacrificed after 4, 16, 25, 31, and 38 weeks of exposure. After 25 or 33 weeks on the PhIP-spiked diet, the mice were fed control rodent chow. After five or six weeks of a diet of control rodent chow, the mice were euthanized. Fluorescence *in situ* hybridization (FISH) analysis showed that neither dicentrics nor translocations were significantly increased in either the peripheral blood lymphocytes or bone marrow in PhIP-exposed mice. Analysis of SCEs in peripheral blood lymphocytes showed a significant ($P < 0.003$) increase in the average number of SCEs per cell in mice exposed to PhIP for 25 weeks (no return to the control diet). The average number of SCEs in cells from control mice was 6.4. In mice exposed to 100, 250, or 400 ppm of PhIP (with no return to the non-PhIP diet), the average number of SCEs was 12.6, 13.8, and 16.2 SCEs, respectively. In mice fed the control diet for five or six weeks after PhIP-exposure, the frequency of SCEs dropped and was not significantly different from control levels. The authors concluded that chronic ingestion of PhIP does not result in persistent chromosomal damage in peripheral blood or bone marrow cells in exposed mice.

DNA damage/repair test

Comet assay

Eight-week-old male CD-1 mice were injected (i.p.) with 40 mg of PhIP per kg (Sasaki *et al.* 1997). The mice were sacrificed at 1, 3, or 24 hours after exposure. The stomach, liver, kidneys, lungs, brain, spleen, and bone marrow were recovered from each mouse. Nuclei were isolated from the tissues and analyzed using a modified Comet assay. PhIP induced a significant amount of DNA damage (as determined by increased comet tail length) in the liver within one hour after treatment ($P < 0.001$). DNA damage was detected in the kidney and brain three hours after dosing ($0.01 < P < 0.05$ and $0.001 < P < 0.01$, respectively). In another study, Sasaki *et al.* (1998), specifically examined PhIP-induced DNA damage in the mucosa of the gastrointestinal and urinary tracts. PhIP was injected (i.p.) into eight-week-old CD-1 mice. Mice were euthanized 1, 3, or 8 hours after treatment and the stomach, duodenum, jejunum, ileum, colon and bladder were removed, minced, and homogenized. Using a modified Comet assay, comet tail lengths were measured in nuclei isolated from the minced tissues. PhIP induced a significant amount

of DNA damage (as determined by increased comet tail length) in the colon one to eight hours after treatment ($P < 0.05$).

Micronucleus test

Director *et al.* (1996) fed C57Bl/6N female mice a powdered diet with 0, 100, 250, or 400 ppm PhIP added (see Section A.3.4). There was a significant ($P < 0.0001$) dose-response increase in MN formation in normochromatic erythrocytes isolated from mice exposed to 250 or 400 ppm of PhIP for 16 weeks. Although the frequency of MN decreased in mice that were fed a control diet for five or six weeks after the initial 25 or 33 week exposure, the frequency of MN remained slightly, but not significantly, elevated.

Mutation tests in transgenic mice and rats

A transgenic mouse mutagenesis system detects both point mutations and deletion mutations *in vivo* (Nohmi *et al.* 1996). The *gpt*Δ mice carry lambda EG10 DNA as a transgene. Rescued phages are transfected into *E. coli* and converted into plasmid pYG142 carrying a chloramphenicol resistance gene, the *gpt* gene of *E. coli* and a chi site along with the *red* and *gam* genes. The *gpt* mutants are detected by growth in 6-thioguanine. Spi ⁻ mutants, missing the *red* and *gam* genes, are insensitive to phage P2 interference. The *gpt* allows the detection of point mutations whereas Spi allows detection of deletion mutations.

Seven-week old, homozygous *gpt*Δ C57BL/6J transgenic mice were fed a diet that contained 400 ppm of PhIP (Masumura *et al.* 1999). After 13 weeks on the PhIP diet followed by two weeks on a control (non-PhIP) diet, mice were sacrificed, and DNA was extracted from the colon, spleen, liver, testis, brain, and bone marrow. PhIP-induced *gpt* mutant frequencies were 19-, 10-, and 3-fold higher in the colon, spleen, and liver, respectively, and were statistically significant ($P < 0.05$) compared to control animals. In addition, PhIP treatment induced Spi ⁻ mutant frequencies in the colon, spleen, and liver were 10-, 4-, and 3-fold higher, respectively, than frequencies measured in control animals. There were no significant increases in *gpt* or Spi ⁻ mutant frequencies in the testis, brain, or bone marrow of PhIP-exposed mice. Similar results were obtained in PhIP-exposed female mice. In an extension of the 1999 study, Masumura *et al*. (2000) performed DNA sequence analysis of individual *gpt* and Spi ⁻ mutants isolated from the colons of exposed mice. Eighty-one percent of *gpt* mutants contained single base-pair substitutions with G:C→T:A transversion mutations predominating; 16% of the mutants contained frameshift mutations with single base-pair deletions at G:C base pairs predominating. In contrast, 90% of the *gpt* mutants from untreated mice contained base-pair substitution mutations, primarily G:C→A:T transitions; only four percent contained frameshift mutations. Spi ⁻ mutants isolated from PhIP-treated mice contained G:C base pair deletions (76% of mutants analyzed) with more than half of these occurring in monotonic G or C runs, while the remaining mutations occurred at G:C base pairs adjacent to run sequences. The authors suggested that PhIP primarily induces point mutations such as base-pair substitutions and single base-pair deletions, but not larger deletions. The authors also hypothesized that run sequences are important in the formation of PhIP-induced mutations.

Zhang *et al*. (1996) fed Big Blue® transgenic mice 100 ppm or 400 ppm PhIP for 30, 60, or 90 days. Big Blue® mice carry approximately 40 copies of the *lacI* gene. In addition, PhIP was fed at 250 ppm for 30 days. Mutant frequencies (*lacI*) increased in a dose- and time-dependent manner in DNA extracted from colonic epithelial cells of PhIP-exposed mice.

Big Blue® male and female rats were exposed to 400 ppm of PhIP for 60 days (Okonogi *et al.* 1997b). PhIP-induced *lacI* mutant frequencies in the colon mucosa were 20- and 25-fold higher than those measured in untreated control male and female rats, respectively. DNA sequence analysis of *lacI* mutants recovered from male and female rats showed that G:C→A:T transitions were the most frequent mutations in the untreated rats. However, in PhIP-treated rats, G:C base pair deletions were the most frequent mutations found, followed by G:C→T:A transversion mutations. A PhIP-induced *lacI* mutation hotspot occurred at a 5'-GGGA-3' sequence. The authors noted that this sequence has high homology with a PhIP-induced hotspot sequence in the *Apc* gene in rat colon tumors (see Sections 4 and 6).

In follow-up studies, Okochi *et al*. (1999) looked specifically at the effects of PhIP on *lacI* mutant frequency in the mammary glands of rats. Big Blue® female rats were mated to male Sprague-Dawley rats, and the F1 female rats were used for the PhIP experiments. Thirty-three F1 rats were given 65 mg/kg per day of PhIP by gavage. Doses were administered five times a week for two weeks. The control group received distilled water. Mammary carcinomas were observed in 15 of the 33 PhIP-treated rats and in three of the 14 control rats. The increased incidence of mammary carcinomas in PhIP-exposed rats correlated with a 13-fold higher *lacI* mutant frequency in the treated group than in the control group. In PhIP-treated rats, 43% of the mutations were G:C→T:A transversions. In contrast, 50% of the mutations in the control animals were G:C→A:T transition mutations. Single base-pair deletions (G:C) accounted for 21% of mutations in PhIP-treated rats, and 29% of these occurred at 5'-GGGA-3' sequences.

The *lacI* mutant frequency was measured in the prostates of Big Blue® F344 rats that were fed 200 ppm of PhIP for 61 days (Stuart *et al.* 2000a). PhIP was highly mutagenic in the prostate of Big Blue® rats and induced a *lacI* mutant frequency 20-fold higher than that measured in control rats. The mutational spectrum from the control rats revealed that 55% of the mutations were G:C→A:T transition mutations. The predominant mutations in PhIP-treated rats were -1 frameshifts and almost all of these were due to deletions of G:C base pairs (39% of independent mutations) and G:C→T:A transversions (32%). The authors suggested that the mutation data and prostate tumor data (see Section 4) are evidence that PhIP is a genotoxic carcinogen for rat prostate cancer. Similar results were reported for *lacI* mutant frequencies in colon tissues from Big Blue® F344 rats (Stuart *et al.* 2000b).

Studies utilized the Big Blue® transgenic rat to determine the mutational specificity of PhIP in the *cII* transgene (Stuart *et al.* 2000b). For sixty days, rats were fed rodent chow spiked with 400 ppm of PhIP. DNA was extracted from the colons of rats and used for the *cII* mutational assay. *cII* mutant frequencies were approximately 27-fold higher in

treated mice than that measured in control rats. *cII* mutations in control rats were G:C→A:T transitions (28% of total mutations), G:C→T:A transversions (26%), A:T→G:C transitions (12%) and -1 deletions (10%). The *cII* mutational spectra from PhIP-treated rats consisted primarily of G:C→T:A transversions (42%), G:C→C:G transversions (23%), G:C→A:T transitions (16%), and single base-pair deletions (12%). The *cII* gene contains a single 5'-GGGA-3' sequence (homologous to the hotspot in the *Apc* gene), which was not a hotspot for PhIP mutations.

A.1.4 Other tests

A *supF* shuttle vector was used to determine the mutagenic specificity of PhIP (Endo *et al.* 1994). Vector pSP189 was treated with *N*-hydroxy-PhIP and transfected into NER-proficient (GM0637 SV40) and NER-deficient (XP12Be SV40) fibroblasts. Plasmids were recovered from the fibroblasts and used to transform the bacterial indicator strain MBM7070. Mutation of the *supF* gene resulted in white and light blue colonies, while unmutated colonies were blue. The *supF* mutant frequency increased in an adduct-dependent manner. At maximal PhIP adduct levels, the GM and XP cells lines showed a 16- and 33-fold increase in mutant frequency over the background frequency, respectively. Regardless of repair status, base substitution mutations were the most commonly induced mutations with G:C→T:A transversions predominating (50% of total independent mutations). ^{32}P-postlabeling analysis showed that PhIP formed adducts at guanine bases with formation of the major adduct at the C8-guanine position (see Section 6). The mutation data correlated with adduct formation at guanines, as a majority of the base substitution mutations (97%) occurred at guanines. In a follow-up study, Endo *et al*. (1995) used a polymerase arrest assay to assess the distribution of PhIP adducts in *supF*. The results showed that the location of PhIP-induced mutations in the *supF* gene correlated with the sites of PhIP adduct formation in *supF*. However, there was no correlation between the intensity of an arrest site and the level of adducts at that site.

Shibutani *et al*. (1999) determined the mutagenic specificity and frequency of PhIP adducts in simian kidney (COS-7) cells. An oligodeoxynucleotide containing a single dG-C8-PhIP adduct was inserted into a single-stranded shuttle vector that was then transfected into COS-7 cells. The primary mutations detected were G:C→T:A transversions and to a lesser extent G:C→A:T transitions and G:C→C:G transversions. The mutant frequency depended on the neighboring sequence context at the lesion. The authors suggested that the mutations that occur at G:C base pairs in PhIP-exposed animal cells are probably caused by the dG-C8-PhIP adduct and that this adduct is a mutagenic lesion in mammalian cells.

Ushijima *et al*. (1995b) analyzed mutations in HCA-induced tumors in rodent studies. For PhIP, all of the mutations detected in rat mammary gland tumors (3/3) were G→A transversions.

A.2 MeIQx

A.2.1 Prokaryotic systems

A.2.1.1 Induction of mutations in Salmonella typhimurium

MeIQx was mutagenic in *S. typhimurium* strain TA98 in the presence of S9 (Lee *et al.* 1995, Edenharder *et al.* 1995, Hirose *et al.* 1998a, Yu *et al.* 2001). A variety of food products and chemicals have been tested for protective effects against the compound's mutagenicity: Edenharder *et al*. (1995) noted a decrease in mutagenic revertants with the addition of a variety of fruits and vegetables; Yu *et al*. (2001) observed a decrease in mutagenicity with the addition of three forms of wheat: whole, refined, and bran; and Hirose *et al*. (1998a) reported that the addition of antioxidants lowered the number of mutagenic revertants in a dose-dependent manner.

Suzuki *et al.* (1998) and Kamataki *et al.* (1999) developed a new *Salmonella* tester strain, designated TA1538/ARO, which is highly sensitive to mutagenic HCAs. MeIQx induced 9.7×10^4 revertants/nmol in the TA1538/ARO strain compared to 3.12×10^4 revertants/nmol in the TA1538 strain with rat liver S9 activation (Suzuki *et al.* 1998).

In a *S. typhimurium* strain that expressed *N, O*-acetyltransferase (YG1019) (see PhIP, Section A.1.1.1), MeIQx was shown to be 13.3 times more potent than strain TA1538 (Pfau *et al.* 1999). MeIQx was tested in the genetically engineered *S. typhimurium* tester strains OY 1001/1A2, OY1002/1A2, and OY1003/1A2. It was mutagenic in the two strains that coexpressed human CYP1A2 and NADPH-P450 reductase and overexpressed OAT (OY1001/1A2 and OY1002/1A2) and was not mutagenic in the OAT-deficient strain (OY1003/1A2) (Aryal *et al.* 2000) (see Table A-1 for a comparison of the potency of MeIQx in various *S. typhimurium* strains).

As discussed above (see PhIP Section A.1.1.1), Thiébaud *et al*. (1995) collected the airborne cooking byproducts formed from frying ground beef, bacon, or soybean-based burgers. MeIQx represented 15% to 38% of the total HCAs detected in smoke condensates from beef patties fried at 198ºC and 277ºC, respectively. MeIQx was not detected in smoke condensate from bacon or soy-based burgers. The airborne byproducts were mutagenic in *S. typhimurium* strain TA98 with S9 activation.

A.2.1.2 Induction of mutations in Escherichia coli

Josephy *et al.* (2001) examined recombinant human CYP1A1, 1A2, and 1B1 in several *E. coli* strains. MeIQx was about one-tenth as potent a mutagen as MeIQ (see Section A.3.1.2); however, the compound displayed the same P450 activation profile as MeIQ, showing the most potent mutagenicity and activation by CYP1A2, with a lesser degree of mutagenicity in a strain that indicated activation by CYP1A1.

A.2.2 Eukaryotic systems

A.2.2.1 Induction of mutations in Saccharomyces cerevisiae

Paladino *et al*. (1999) assessed the recombinogenic potential for HCAs, using metabolically competent yeast strains (see PhIP, Section A.1.2.1 and MeIQ, Section A.3.2.1). MeIQx (100 µg/mL) produced a 9-fold increase in mutations at the *URA*3 locus

and a 10-fold and a 6-fold increase in gene conversion and translocation frequency, respectively, also in the presence of NAT2. MeIQx had the highest recombinogenic potential of the three HCAs in this report; however, IQ was the most potent of the HCAs tested.

A.2.2.2 Mutagenicity in Drosophila melanogaster

The effects of chlorophyllin on DNA-adduct formation from MeIQx exposure in *D. melanogaster* was studied (Sugiyama *et al.* 1996). Third-instar larvae consisting of approximately equal numbers of DNA repair-deficient males and DNA repair-proficient females were fed a diet containing MeIQx (1 mg/6.5 g diet/bottle) for 6 hours, both with and without chlorophyllin (0, 100, 200, or 300 mg). A high degree of DNA-adduct formation was found in the *D. melanogaster* larvae without the addition of chlorophyllin; chlorophyllin (300 mg) significantly lowered the number of DNA adducts. In the DNA repair test, no significant cytotoxic effects against females were detected, whereas effects were noted in the males, indicating that damage done in the larval stage had been repaired during growth in the females. The DNA-repair test also showed a protective effect of chlorophyllin when administered at 200 or 300 mg.

A.2.3 Mammalian systems

A.2.3.1 In vitro *assays*

Cytogenetic tests

Micronucleus test

As discussed above for PhIP, Pfau *et al*. (1999) used MCL-5 cells to test HCA induced-MN formation. For MeIQx, concentrations of 2.0 to 22.5 ng/mL resulted in a significant ($P < 0.0003$) increase in MN formation. Knasmüller *et al*. (1999) (see PhIP Section A.1.3.1 and MeIQ Section A.3.3.1) reported similar results to the other HCAs when MeIQx was tested at 25 to 900 μM in a HepG2 cell line. A dose-dependent increase in MN, with the lowest effective concentrations in the range of 25 to 50 μM, was reported. Sanyal *et al*. (1997) also investigated the genotoxic effects of HCAs in HepG2 cells (see PhIP Section A.1.3.1 and MeIQ Section A.3.3.1), and reported that MeIQx exposure (0.6 mM) and exposure to 5 μg/mL of caffeine, vanillin, or coumarin resulted in a greater than 90% decrease in MN compared to cells exposed to MeIQx alone.

DNA damage/repair test

DNA single strand breaks

As discussed above (see PhIP Section A.1.3.1), Martin *et al*. (1999) investigated DNA damage from MeIQx, using the Comet assay in MCL-5 cells, both with and without HU and ara-C. MeIQx (0.21, 0.43, or 2.13 mM) did not result in significantly increased comet tail lengths, with or without HU and ara-C; however, a 1.5-fold increase in tail length was seen with HU and ara-C, as compared to the tail increase without the compounds. Pfau *et al*. (1999) also used the Comet assay in MCL5 cells to assess the DNA strand breaking activity of HCAs (see PhIP Section A.1.3.1). In the presence of HU and ara-C, the median comet tail length in untreated control cell populations ranged from 6.0 μm to 10.0 μm. In cells treated with 90.9 μg/mL of MeIQx for 30 minutes, the median tail length was 12.0 μm and was not significantly different from controls. At 454.5 μg/mL, comet tail length was significantly increased ($P < 0.001$).

Unscheduled DNA synthesis

As presented above for PhIP (see Section A.1.3.1), Beamand *et al*. (1996, 1998a, 1998b) used human, rat, and mouse liver slices to measure UDS in HCAs. In cultured human liver slices, MeIQx (5 to 200 µM) significantly induced UDS; MeIQx, at concentrations of 0.5 to 200 µM, did not induce UDS in cultured rat liver slices or mouse slices (Beamand *et al.* 1998b).

A.2.3.2 In vivo *assays*

Host-mediated assay

Alldrick *et al*. (1995) investigated the effects of dietary caffeine on the genotoxicity of MeIQx in a host-mediated assay in mice. Female BALB/c mice were fed a caffeine-free diet for 2 weeks; they were then maintained on a control diet or fed the same diet supplemented with 0.01% caffeine. Mice were then treated intravenously with 0.1 mL *S. typhimurium* TA98, and then orally dosed with 1.5 mg/kg MeIQx or with 10mM sodium acetate buffer as a control. The livers of the mice were removed and analyzed for his^+ revertants in *S. typhimurium*. An increase in the number of revertants was seen in the liver of the mice, and caffeine consumption resulted in a 47% reduction in the number of mutants induced by MeIQx ($P < 0.001$).

Knasmüller *et al*. (1992) tested five HCAs in the microbial animal-mediated assay (see PhIP, Section A.1.3.2). All compounds induced genotoxic effects when injected i.p. Oral administration required higher doses to induce respirable DNA damage.

Chromosomal aberrations

Breneman *et al*. (1996) studied CAs in the blood and bone marrow resulting from feeding C57BL/6 mice a diet containing 0, 100, 250, and 400 ppm MeIQx for 6 months. MeIQx did not result in a clear dose response in CAs in the blood, although a slight increase in aneuploidy was seen with increasing dose. Fewer aberrations were seen in the bone marrow than in the blood.

Sister chromatid exchanges

SCEs in peripheral lymphocytes were examined in C57B7/6 mice fed a diet containing 0, 100, 240, and 400 ppm MeIQx for 6 months (Breneman *et al.* 1996). The results were compared with the results from another group of mice that were fed MeIQx for 6 months followed by a control diet for 1 month. In the mice fed MeIQx for 6 months, no clear dose response was seen; however, there were slightly more SCEs in the 400-ppm group than in the other groups. In the mice fed MeIQx and then removed from it and fed a control diet, there was a significant decrease ($P < 0.005$) in SCEs only for those mice that had previously received MeIQx at 400 ppm.

DNA damage/repair test

Micronucleus test

Breneman *et al*. (1996) studied micronuclei formation in the erythrocytes resulting from feeding C57BL/6 mice a diet containing 0, 100, 250, and 400 ppm MeIQx for 6 months (see sister chromatid exchange section, above). The results were compared with the results from another group of mice that were fed MeIQx for 6 months followed by a control diet for 1 month. No increase in MN formation was noted at any of the concentrations in the MeIQx-fed mice or in the MeIQx-fed mice followed by the control diet.

Mutation tests in transgenic mice and rats

Davis *et al*. (1996) investigated the mutagenicity of MeIQx on transgenic mice. Male Muta™ mice were crossbred with C57BL/6J mice or female homozygous c-*myc* transgenic mice containing a chimeric gene consisting of the mouse albumin enhancer/promoter and mouse *cmyc* cDNA. C57BL/*lac*Z or c-*myc*/*lacZ* mice were obtained from the cross-breeding; the c-*myc*/*lacZ* mice were double transgenic mice that were heterozygous for both the *lacZ* and *c-myc* transgenes. The mice were administered 10 daily doses of 20 μg/g MeIQx via gavage in corn oil, and the animals were sacrificed 4 weeks after the last dose. The *Salmonella* mutagenicity assay showed significant ($P < 0.005$) increases in the mutant frequencies in the *lacZ* gene compared to control animals in both C57BL/*lacZ* and *cmyc/lacZ* mice. In addition, the frequency of mutations in the livers of the *cmyc/lacZ* mice was significantly ($P < 0.0005$) greater than in the livers of the C57BL/*lacZ* mice. DNA adducts, identified primarily on the guanine base, were identified in this study from MeIQx exposure.

Ryu *et al*. (1999) and Thorgeirsson *et al*. (1999) used the same transgenic mouse model used by Davis *et al*. (1996) to investigate further the mutagenicity of MeIQx. The mutant frequency in the *lacZ* gene of hepatic DNA was measured in both strains of mice on a diet containing MeIQx (0.06%) or a control diet for 30 and 40 weeks. In both strains and at 30 and 40 weeks of age, MeIQx-fed mice showed at least a 40-fold higher *lacZ* mutant frequency than mice on the control diet. In addition, in the MeIQx-fed mice, mutant frequency was 1.4- to 2.6-fold higher in the *c-myc* mice than in the C57BL strain. These differences were statistically significant ($P < 0.05$) in female mice after 30 weeks on the control diet and in both males and females after 40 weeks on the control diet. In addition, mutant frequencies and DNA adduct levels in MeIQx-fed mice were significantly higher in female mice than in males.

Nishikawa *et al*. (2001) investigated the mutagenicity of MeIQx in Big Blue® mice. Mice received a single intragastric dose of MeIQx at 0, 0.1, 1.0, 10, or 100 mg/kg or were fed 0 or 300 ppm MeIQx in their diets for up to 12 weeks. Two days after treatment, a micronucleus assay was performed, and the animals were sacrificed after 4 or 12 weeks to analyze their tissues for *lacI* or *gtp* mutation and cell proliferation. No increase in mutant frequency was seen in *lacI*, nor was there cell proliferation in any of the major organs, including the liver and colon, from the intragastic administration. However, an increase in micronucleus formation in peripheral erythrocytes was observed at 100 mg/kg from the intragastric administration, and dietary administration of MeIQx for 12 weeks significantly increased the mutant frequency of both *lacI* and *gpt* in the liver and colon in a dose-dependent manner. Dietary administration of MeIQx for 4 weeks did not increase the *lac*I or *gpt* mutant frequency in any organs.

A.2.4 Other tests

Kudo *et al*. (1991) reported finding c-Ha-*ras*-activating mutations in rat Zymbal gland squamous cell carcinomas induced by MeIQx. Two of 6 squamous cell carcinomas had c-Ha-*ras* mutations. One was a G:C→T:A transversion at the second nucleotide of codon 13 and the other was a A:T→T:A transversion at codon 61. Ushijima *et al*. (1995b) analyzed mutations in HCA-induced tumors in rodent studies (see PhIP Section A.1.4 and

MeIQ Section A.3.4). For MeIQx, three of four of the rat liver tumors analyzed had G:C→T:A transversions, while the remaining tumor contained a C:G→G:C transversion.

A.3 MeIQ

A.3.1 Prokaryotic systems

A.3.1.1 Induction of mutations in Salmonella typhimurium

In the presence of rat induced S9 mix, MeIQ was mutagenic in *S. typhimurium* strain TA98 (Abu-Shakra 1992, Edenharder *et al.* 1994, Lee *et al.* 1994) and TA100 (Edenharder *et al.* 1994). Other studies with strain TA98 investigated mutagenic effects when an alternative activating system or a reactive metabolite of MeIQ was used. Sengstag *et al.* (1994) prepared microsomal fractions from yeast strains that were constructed to co-express cDNAs coding for human CYP1A1 or CYP1A2 in combination with human NADPH-cytochrome P-450 reductase. These microsomal fractions were used as an alternative activating system in the Ames test with *S. typhimurium* strain TA98. MeIQ was activated by CYP1A2 but not by CYP1A1. Kerdar *et al.* (1993a) investigated the mutagenicity and DNA binding of nitrenium ions derived from MeIQ and other HCAs. *S. typhimurium* strain TA98 was used to determine mutagenic potency. Azido-MeIQ was the second most potent out of 26 compounds tested with a mean of 39,200 revertants/nmol. A good correlation was observed between the DNA-binding potencies and mutagenic potencies of the arylnitrenium ions.

The relevance of the Ames assay using tester strains with endogenous bacterial *N*-acetyltransferase/*O*-acetyltransferase was questioned because the bacterial and human enzymes were likely to have different substrate specificities. Therefore, new tester strains of *S. typhimurium* were constructed that expressed high levels of human arylamine *N*-acetyltransferases (NAT1 and NAT2) (Grant *et al.* 1992). These new strains were designated DJ400 (TA1538/1,8-DNP pNAT1) and DJ460 (TA1538/1,8-DNP pNAT2). Rat S9 was used as the activating system. MeIQ was highly mutagenic in strain DJ460, but the response in DJ400 was only slightly more than that observed in the NAT-deficient parent strain (TA1538/1,8-DNP). In a similar study, Wild *et al*. (1995) (see PhIP Section) utilized strains TA1538; TA1538/1,8-DNP; DJ400; and DJ460, as each exhibited a different acetylator phenotype (*S. typhimurium NAT* $^{+}$, *NAT* $^{-}$, human *NAT1*, and human *NAT2,* respectively). MeIQ induced revertants in a dose-dependent manner in strains TA1538 and DJ460. The authors concluded that human NAT2 but not NAT1 can *O*-acetylate heterocyclic hydroxylamines.

As discussed above (see PhIP, Section A.1.1.1, and MeIQx, Section A.2.1.1), Suzuki *et al.* (1998) and Kamataki *et al.* (1999) developed a new *Salmonella* tester strain designated TA1538/ARO that does not require metabolic S9 activation. MeIQ induced 4.73×10^6 revertants/nmol in the TA1538/ARO strain compared to 3.36×10^5 revertants/nmol in the TA1538 strain with rat liver S9 activation (Suzuki *et al.* 1998). In a separate study, MeIQ induced 1.1×10^7 revertants/nmol in the TA1538/ARO strain compared to 1.4×10^5 revertants/nmol in the TA98 strain with rat liver S9 activation (Kamataki *et al.* 1999). Josephy *et al*. (1995) also developed a new strain of *S. typhimurium* (DJ4501A2) that does not require exogenous metabolic activation from S9.

MeIQ induced a dose-dependent increase in mutant reversions in this strain. (See Table A-1 for a comparison of the potency of MeIQ in various *S. typhimurium* strains.)

Another study showed that HepG2 cell homogenate activated MeIQ resulting in the induction of revertants of strain YG1024 (Knasmüller *et al.* 1999). MeIQ was a potent mutagen in this system; however, the concentration required to induce the same or a greater number of revertants was 13-fold higher when HepG2 homogenate was used in place of rat S9. The authors suggested that these differences in effective dose might be related to the activities of the HepG2 and the rat liver enzymes that catalyze the *N*-hydroxylation by cytochromes CYP1A1 and CYP1A2.

Aryal *et al.* (2000) used genetically engineered *S. typhimurium* tester strains in the SOS/*umu* assay. They developed a new *umu* tester strain (OY1002/1A2) that coexpressed human CYP1A2 and NADPH-P450 reductase and overexpressed OAT and compared the sensitivity of this strain to a previously developed strain (OY1001/1A2) and an OAT-deficient strain (OY1003/1A2). Different concentrations of MeIQ were incubated with the various tester strains, and induction of *umuC* gene expression was measured. Both tester strains OY1001/1A2 and OY1002/1A2 showed a dose-dependent increase in *umuC* gene expression with a greater response in OY1002/1A2. In contrast, gene expression was not induced above background levels in the OAT-deficient strain. MeIQ had the greatest genotoxic potential of all the compounds tested. These studies demonstrated that the genotoxicity of MeIQ and other HCAs depend on both the CYP1A2 enzyme and the OAT enzyme.

A.3.1.2 Induction of mutations in Escherichia coli

Two *E. coli* K12 strains (343/753 and 343/765) with different capacities for DNA repair (*uvrB*/*recA* [repair deficient] and uvr^+/rec^+ [repair proficient, wild-type], respectively) were exposed to HCAs in the presence and absence of liver S9 (Knasmüller *et al.* 1992). Test concentrations of MeIQ ranged from 0.07 to 12.5 µg/mL. Dose-dependent genotoxic effects were observed with S9, and exposure to MeIQ *in vitro* resulted in the lowest relative survival rates (repair-deficient strain compared to wild-type strain). These results were compared with *in vivo* data from a concurrent host-mediated assay (see Section A.3.2.2).

Kosakarn *et al.* (1993) studied the mutational specificity of NO_2-MeIQ in the *lacI* system of *E. coli* strain EE125 harboring the plasmid pKM101. This plasmid enhances SOS-associated error-prone repair and mutagenesis. A dose-dependent increase in mutation frequency occurred over the range of 10 to 1,000 µM NO_2-MeIQ. The mutation frequency was twice the spontaneous frequency at 100 µM and increased to more than seven times the background at 1,000 µM (1mM). The spectrum of mutations also differed considerably from the spontaneous spectrum. Base substitutions predominated, occurring at 30 sites and including all six possible types. However, G:C→T:A transversions were the most common. Single-base frameshifts, deletions, and complex mutations also were observed. NO_2-MeIQ was a direct-acting mutagen in this system but required higher concentrations than those required to induce reversions in *S. typhimurium* strains with the *hisD3052* mutation. The authors suggested that the different sensitivity to mutagenesis might result from differences either in DNA targets or in metabolic capacities.

MeIQ induced reverse mutations in *E. coli* strain DJ4309 that expresses recombinant human P450 form CYP1A2. Josephy *et al.* (2000) measured *lacZ* reversions in the wild-type strain and in isogenic strains expressing CYP1A2 variants. From a beginning pool of several thousand clones, 25 distinct CYP1A2 variants with altered activities were identified. All but two strains, except two, showed dose-dependent MeIQ-induced reversions; however, none of the variant clones were significantly more active than the wild-type strain. In an additional study, Josephy *et al*. (2001) examined recombinant human P450 forms CYP1A1, 1A2, and 1B1 in *E. coli* strains DJ701, DJ702, DJ711, and DJ712, strains which were developed specifically to express three P450 forms. MeIQ was a potent mutagen in strain DJ702, showing activation by CYP1A2; it also was mutagenic (but not as potent) in strain DJ712, indicating activation by CYP1A1.

A.3.2 Eukaryotic systems

A.3.2.1 Induction of mutations in Saccharomyces cerevisiae

As presented above for PhIP (see Section A.1.2.1) and MeIQ (see Section A.2.2.1), Paladino *et al*. (1999) used metabolically competent yeast strains to assess the recombinogenic potential for HCAs. As with PhIP, the results with MeIQ were weak. At 100 µg/mL, MeIQ induced a 4-fold increase in gene conversion and translocation in the presence of CYP1A2 and NAT2; however, the increase was not statistically significant. MeIQ also did not induce a significant increase in mutations at the *URA3* locus.

A.3.3 Mammalian systems

A.3.3.1 In vitro assays

Specific locus (*arpt, hprt, ouabain*) locus forward mutation test

Mutations in the *hprt* gene were measured in Chinese hamster V79 cells following exposure to electrophilic arylnitrenium ions derived from MeIQ (azido-MeIQ) and other HCAs (Kerdar *et al.* 1993a). Mutagenic potency was measured as the number of *hprt* mutants per 10^5 cells per µM. Statistically significant dose-dependent increases were observed for all five heterocyclic arylazides tested. Azido-MeIQ was the second most potent compound tested.

As discussed in Section A.1.3.1 for PhIP, Lawson and Kolar (1994) co-cultured Chinese hamster V79 cells with hamster pancreas DEC, human DEC, pancreas duct tissue homogenates from hamsters fed a control or high-fat diet, or hamster hepatocytes. V79 cells were exposed to 5, 10, 20, or 50 µM MeIQ for 16 hours. The maximum response occurred at 10 µM with about a 15-fold increase in *hprt* mutant frequencies in co-cultures with either hamster or human DEC. The mutagenicity of MeIQ was 5,600 *hprt* mutants per 10^6 survivors per µmol in the presence of hamster DEC and 6,400 mutants per 10^6 survivors per µmol in the presence of human DECs. The lowest mutagenicity, 700 *hprt* mutants per 10^6 survivors per µmol, occurred in co-cultures with hamster hepatocytes.

Cytogenetic tests

Micronucleus test

As discussed above for PhIP and MeIQx (see Sections A.1.3.1, and A.2.3.1), Sanyal *et al*. (1997) and Knasmüller *et al*. (1999) investigated the genotoxic effects of HCAs in HepG2 cells. HepG2 cells exposed to 0.9 mM MeIQ for four hours developed a 4-fold increase in the number of MN compared to controls. However, groups of cells exposed to

0.9 mM MeIQ and 5 µg/mL of caffeine, vanillin, or coumarin showed an approximate 40% to 50% decrease in MN compared to cells exposed to MeIQ alone (Sanyal *et al.* 1997). Knasmüller *et al*. (1999) (see PhIP, Section A.1.3.1 and MeIQx, Section A.2.3.1) reported a dose-dependent increase in MN, with the LEC in the range of 25 to 50 µM, when MeIQ was tested at 25 to 900 µM in a HepG2 cell line.

Sister chromatid exchanges

SCEs were measured in Chinese hamster V79 cells following exposure to electrophilic arylnitrenium ions derived from MeIQ (azido-MeIQ) and other HCAs (Kerdar *et al.* 1993a). SCE-inducing potency was measured as the number of SCE per metaphase per µM. The SCE-inducing potencies were highly correlated with the mutagenic potencies in *Salmonella*. Azido-MeIQ was the second most potent compound tested with an average of 7.93 SCE per metaphase per µM.

DNA damage/repair test

Unscheduled DNA synthesis

As discussed above for PhIP and MeIQx (see Sections A.1.3.1 and A.2.3.1), Beamand *et al*. (1996, 1998a, 1998b) tested human, rat, and mouse liver slices to determine if exposure to HCAs induced UDS. MeIQ was not as potent as PhIP or MeIQx in this system (Beamand *et al.* 1998b). Treatment with 100, 200, and 500 µM MeIQ induced UDS in 24-hour cultured human liver slices, primarily in the centrilobular hepatocytes. The increase in UDS was significant at all three concentrations, but was highest at 200 µM. MeIQ concentrations used with mouse and rat liver slices were 0.5 to 50 µM and 5 to 500 µM, respectively. UDS was significantly increased in mice liver slices at 20 and 50 µM and in rat liver slices at 5 and 20 µM. MeIQ was the only test compound that induced a significant response in rat liver slices.

A.3.3.2 In vivo *assays*

Host-mediated assay

As discussed above for PhIP aand MeIQx (see Sections A.1.3.2 and A.2.3.2), Knasmüller *et al*. (1992) tested five HCAs in the microbial animal-mediated assay. In this test, MeIQ was the most potent compound tested; it showed genotoxic effects primarily in the liver, lungs, and blood.

Chromosomal aberrations

Female CDF_1 mice were fed MeIQ at 400 ppm in the diet for 70 weeks (Ramsey *et al.* 1998). Control mice were maintained on a basal diet. Blood and bone marrow samples were collected from five control and eight exposed mice, and the cells were arrested in metaphase. CAs were observed in blood cells but not in bone marrow cells. Approximately a 2-fold increase in both abnormal cells ($P = 0.016$) and translocations ($P = 0.043$) and a 16-fold increase in acentric fragments ($P < 0.001$) were detected in peripheral blood lymphocytes compared to controls.

Mutation tests in transgenic mice and rats

Ushijima *et al*. (1994) fed three Big Blue® mice a diet containing 300 ppm of MeIQ for 12 weeks, while another four mice were fed a normal diet. Eighty-one mutations were identified from the liver, and 61 mutations were identified from the bone marrow of the three mice treated with MeIQ. The types and distributions of mutations in the *lacI* gene

were different in the liver and bone marrow. In the liver, G:C →T:A transversions were the predominant mutation (46%). Other mutations included G:C→A:T transitions (25%), complex mutations (15%), and one-base deletions (7%). Mutational hotspots were identified at nucleotides 56, 92, 186, and 201. In the bone marrow, the frequency of G:C→T:A transversions (23%), G:C→A:T transitions (21%), complex mutations (21%), and one-base deletions (23%) was similar. Hotspots were identified at nucleotides 92, 222, and 458. In control mice, 28 and 13 spontaneous mutations were found in the liver and bone marrow, respectively. About half of these were G:C→A:T transitions. Nucleotides 329 and 93 were identified as hotspots for spontaneous mutations in the liver, and nucleotide 93 was identified as a hotspot for spontaneous mutations in the bone marrow.

In a similar experiment, Ushijima *et al.* (1995a) fed six Big Blue® mice a diet containing 300 ppm MeIQ for 12 weeks, and four mice received a control diet. DNA was extracted from the liver and bone marrow. PCR-single-strand conformational polymorphism analysis was used as a rapid method to detect mutations in the *lacI* gene. Among the MeIQ-treated mice, 121 mutants were identified compared to 39 from untreated mice.

Okonogi *et al.* (1997c) showed that the mutational characteristics of MeIQ in the *lacI* gene coincided well with Ha-*ras* mutations detected in MeIQ-induced mouse forestomach tumors and rat Zymbal gland tumors. This study included 90 *lacI* mutants obtained from the colon of three Big Blue® mice fed a diet containing 300 ppm MeIQ for 84 days. G:C→T:A transversions were the predominant type of mutation.

Suzuki *et al.* (1996) and Ochiai *et al.* (1998) studied the mutagenicity of MeIQ in various organs, the relationship between mutational types and DNA adduct molecular species, and the relationship between adduct level and mutant frequency in Big Blue® mice. One group of mice was fed 300 ppm MeIQ in the diet, and another was maintained on a basal diet. Treated mice were sacrificed at 1, 4, and 12 weeks, and control mice were sacrificed after 1 or 12 weeks. DNA samples were collected from the liver, heart, colon, and bone marrow of three mice at each interval and from the forestomach of 8 to 16 mice. The forestomach samples were combined into pools of four each, and DNA was extracted from each pool. Blood samples were collected at 1, 4, 8, and 12 weeks. Mutant frequencies increased with feeding time, and an increased incidence ($P = 0.014$) of MN in peripheral blood reticulocytes was observed at 8 weeks (Suzuki *et al.* 1996). N^2-(deoxyguanosin-8-yl)MeIQ 5'-monophosphate was the only MeIQ-DNA adduct molecular species identified (Ochiai *et al.* 1998). This adduct was detected in DNA from all tissues at all time points in treated mice but was not detected in controls. Therefore, the differences in mutation type between bone marrow and other tissues were not explained by differences in adduct molecular species. No direct relationship was found between mutant frequency and adduct level. Adduct levels were highest in the liver and heart and lowest in the bone marrow; however, mutant frequencies were highest in the colon and lowest in the heart. In tissues with a low cell proliferation rate (i.e., heart and liver), adduct levels increased linearly with time. In contrast, adduct levels rapidly rose to a steady-state level in tissues with high cell proliferation rates. The authors concluded that mutant frequency was a product of DNA adduct levels and cell proliferation rates.

A.3.4 Other tests

Kudo *et al.* (1991) reported finding c-Ha-*ras* activating mutations in rat Zymbal gland squamous cell carcinomas induced by MeIQ. Nine of 14 squamous cell carcinomas and one of one papillomas had a G:C→T:A transversion at the second nucleotide of codon 13. In addition, mutations occurred in two of the squamous cell carcinomas at codon 12. One was a G:C→A:T transition and the other was a G:C→T:A transversion.

Mutations, loss of heterozygosity (LOH) and recombination of the *p53* gene were examined in forestomach tumors taken from CDF_1 mice exposed to MeIQ (Makino *et al.* 1992, Ushijima *et al.* 1995c). Two squamous cell carcinomas, one papilloma, and one metastatic tumor to a regional lymph node were included in these studies. The squamous cell carcinomas and papilloma were taken from different mice, but the metastatic tumor was taken from one of the mice with a squamous cell carcinoma. Samples from normal livers were used as controls. In addition, four cell lines were established from three forestomach tumors and a liver metastasis. Each of these was taken from a different mouse. Ha-*ras* mutations occurred in the papilloma and one squamous cell carcinoma and in two of the cell lines. All four mutations were G:C→T:A transversions at the second position of codon 13 (Makino *et al.* 1992). Mutations in the *p53* gene occurred in two of the four tumors and all four cell lines. The papilloma had a G:C→A:T transition at the second position of codon 171, and one of the squamous cell carcinomas had a G:C→T:A transversion at the second position of codon 113 and LOH. Two of the cell lines had a base substitution and LOH, and the two other cell lines had double mutations (a base substitution and a deletion) (Ushijima *et al.* 1995c).

As discussed above for PhIP (see Section A.1.4) and MeIQx (see Section A.2.4), Ushijima *et al*. (1995b) analyzed mutations in HCA-induced tumors in rodent studies. For MeIQ, all of the mouse forestomach tumors analyzed (four of four) were G:C→T:A transversions, while 10 of 11 rat Zymbal gland mutations were also G:C→T:A transversions, and the remaining tumor was a G:C→A:T transition.

Table A-1. Comparison of potency* of HCAs in different *Salmonella typhimurium* strains

S. typhimurium strain	PhIP	MeIQx	MeIQ	Reference
TA1538	370 rev./nmol	31,200 rev./nmol	336,000 rev./nmole	Suzuki *et al.* 1998
TA1538	396 rev./nmol	43,100 rev./nmol	396,000 rev./nmole	Wild *et al.* 1995
DJ400	195 rev./nmol	260 rev./nmol	< 2000 rev./nmol	Wild *et al.* 1995
DJ460	273 rev./nmol	21,3000 rev./nmol	196,000 rev./nmol	Wild *et al.* 1995
YG1019	5.0 rev./ng	3,800 rev./ng	NA	Pfau *et al.* 1999
TA1538/1,8-DNP	329 rev./nmol	< 200 rev./nmol	< 1000 rev./nmol	Wild *et al.* 1995
TA1538/ARO	650 rev./nmol	97,000 rev./nmol	4,730,000 rev./nmol	Suzuki *et al.* 1998

*Potency is expressed as the number of revertants per the concentration of the chemical, as provided in the individual references.

na = not available