Heterocyclic amines: evaluation of their role in diet associated human cancer

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- 1 Heterocyclic amines are formed in parts per billion levels when meat is cooked.
- 2 The heterocyclic amines MeIQx and PhIP are efficiently absorbed into the systemic circulation after ingestion of cooked food.
- 3 We have shown that MeIQx and PhIP, both *in vitro* and *in vivo*, are substrates for human hepatic CYP1A2, which exclusively and efficiently catalyses their conversion to genotoxic hydroxylamines.
- 4 MeIQx and PhIP are promutagens. MeIQx is a very powerful bacterial mutagen whereas PhIP is a more potent mammalian cell mutagen. Using a mammalian cell target gene, *hprt*, we have shown that PhIP induces a characteristic mutational 'fingerprint'.
- 5 MeIQx and PhIP are carcinogenic in bioassays. The PhIP mutational 'fingerprint' has been detected in the *Apc* gene of 5/8 colonic tumours induced by PhIP in rats.

Keywords heterocyclic amines MeIQx DiMeIQx PhIP CYP1A2 bioavailability metabolism mutagenicity carcinogenicity

Introduction

The causes of human cancer, although many and varied, can be broadly grouped into lifestyle, occupational, iatrogenic and cryptogenic [1, 2]. Cancers which arise due to occupational factors tend to affect a variety of tissues, but comprise probably less than 5% of total cancers. Iatrogenic cancers due to exposure to radiation and drugs also affect a number of different tissues but again only account for a very small percentage of total cancer incidence. Cancers which have a cryptogenic origin are most likely due to viruses, particularly certain forms of leukaemia, lymphoma, sarcoma and cancer of the cervix. The majority of human cancer, between 70 and 80%, is related to lifestyle [2, 3]. Two lifestyle factors in particular are prominent, these being tobacco and diet. Tobacco-related products are associated with about 30% of all cancer, especially tumours of the lungs, but also of the pancreas, bladder, kidneys, oral cavity and oesophagus. However, the single greatest contributor to human cancer, accounting for some 35–45% of the disease, is diet [2–4]. The realization that a sizeable

proportion of human cancer is associated with diet has led to a considerable effort to identify and quantify the agents responsible. The development of short-term genotoxicity assays has allowed dietary components to be screened and a number of compounds have been identified as candidate aetiological agents. Dietary factors which correlate most strongly with cancer are high fat, low fibre, consumption of well cooked meat, nitrite/nitrate intake, low vitamin C, alcohol and mycotoxins [1]. Sources of dietary genotoxins include those which are naturally occurring such as mycotoxins, hydrazines, pyrrolizidine alkaloids, and some flavenoids, food contaminants such as certain pesticides and solvents, and products which are generated during the preparation of food. This latter category comprises numerous genotoxic chemicals which include nitrosamines and nitrosoamides, polycyclic aromatic hydrocarbons and heterocyclic amines (HAs). Of particular interest to our group have been the heterocyclic amines which, based upon their extreme genotoxicity in short term mutagenicity tests and their mass in cooked meat foods, are the greatest contributors to the mutagenicity of cooked meat products [5].

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Heterocyclic amine formation and human exposure

The cooked food mutagenic amines comprise about twenty chemicals; all have a characteristic heterocyclic structure and an exocyclic amine function (Figure 1). They fall into several groups, pyrido-imidazoles, quinoxalines, quinoxalines, pyridines and furopyridines. They are formed only when food is cooked, particularly red meat or meat containing products [6, 7]. Heterocyclic amines formed at high temperatures are known as pyrolytic mutagens e.g. 3-amino-1-methyl-5H-pyrido [4,3-b]indole (Trp-P-2) and those formed at temperatures less than 300°C are known as thermic mutagens. Extensive analysis of cooked foods has shown that these compounds, especially the thermic mutagens, are generated at the parts per billion level [8]. Initially, confirmation of HA formation in food was achieved by isolation, purification and chemical characterisation and showed that three thermic mutagens, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (Di-MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), together accounted for about 60% of the mutagenicity of cooked beef [9]. We developed specific and highly sensitive assays for Trp-P-2, MeIQx, DiMeIQx and PhIP, based upon gas chromatography-mass spectrometry [10–12] and applied these to the analysis of foodstuffs (Table 1). Neither MeIQx, DiMeIQx nor

PhIP could be detected in raw meat, but all three were present at parts per billion in cooked meat [11, 12]. Trp-P-2 was not detected in any cooked meat sample examined [10]. The levels of thermic mutagens generally depend upon the temperature, duration and method of cooking [8]. The formation of HAs could be reproduced in reactions involving mixtures of amino acids, carbohydrate and creatine or creatinine [13–15]. It has been suggested that the mechanism of their formation initially requires Maillard reactions between hexoses and amino acids resulting in Strecker degradation products, e.g. pyridines and pyrazines which subsequently react with creatinine (formed from creatine) in an aldol condensation to yield imidazoquinoxalines and imidazoquinoxalines [13].

The systemic bioavailability of heterocyclic amines

Although exposure to the HAs in food is lifelong, on a daily basis it has been estimated to be only about 0.1–10 µg day⁻¹ per person, thus detection of HAs in plasma is extremely difficult. In animal studies we have determined that, irrespective of the route of administration, HAs appear to be almost completely bioavailable and subject to extensive metabolism prior to excretion [16]. About 30% of the administered dose is excreted in

Figure 1 Structures of some of the heterocyclic amines formed when food is cooked or pyrolysed.

Table 1 Levels of MeIQx, DiMeIQx and PhIP in food, determined by GC-MS

Food	MeIQx (ng g ⁻¹)	DiMeIQx (ng g ⁻¹)	PhIP (ng g ⁻¹)
Fried beef patties	2.2	0.7	16.4
Fried beef steak	0.5	0.1	0.6
Fried fatty bacon	1.2	0.3	2.7
Barbecued pork	0.4	0.1	4.2
Barbecued chicken	0.3	0.1	int
Beef stock cube	0.6	0.3	0.3
Vegetable stock cube	<0.05	<0.1	<0.2
Condensed consomme	0.1	<0.1	<0.2
Hydrolysed yeast extract	<0.05	<0.1	<0.2

Limits of detection of the assay are 0.05, 0.1 and 0.2 ng g⁻¹ for MeIQx, DiMeIQx and PhIP, respectively.
int-interference.

Figure 2 Chromatograms for the analysis of a) MeIQx and b) PhIP in human urine after a fried beef meal. Selected ion monitoring traces of the di-bisTFMB derivatives of MeIQx (m/z 438), [$^{13}\text{C},^{15}\text{N}_2$]MeIQx (m/z 441), PhIP (m/z 449) and [$^2\text{H}_5$]PhIP (m/z 454) are shown.

the urine, but only a small percentage as unchanged amine [17], which is consistent with the HAs being extensively absorbed and bioavailable. We have studied, on over 100 separate occasions, normal human volunteers fed standard fried beef meals containing variable amounts of HAs (250–7500 ng of HA, determined by gas chromatography—mass spectrometry, GC-MS) and have shown that about 2% of the ingested MeIQx and about 1% of the PhIP is consistently and reproducibly excreted in the urine (Figure 2), in the 24 h post-meal [18–20].

The metabolism of heterocyclic amines

Our experiments with both animals and man indicated that MeIQx and PhIP are extensively metabolised prior

to excretion. Studies with rodents have demonstrated that both MeIQx and PhIP are C- and N-oxidised, glucuronidated, acetylated and form sulphamates [16, 21–26]. The pathway by which the HAs are activated to genotoxins involves N-oxidation of the exocyclic amine function to the N-hydroxylamine, whereas ring C-hydroxylation is a detoxication reaction. The N-hydroxy derivatives can be further activated by esterification. We devised high performance liquid chromatography (h.p.l.c.) assays to measure the metabolites of MeIQx and PhIP and using liver microsomal fractions showed that humans efficiently N-hydroxylate both amines (Table 2) but do not produce the C-oxidation detoxication products [27, 28]. Animal studies using chemical modifiers of enzyme activity and specific antibodies raised against CYP enzymes showed that CYP1A sub-family enzymes were involved in the

Table 2 Human CYP1A2 catalysed metabolism of MeIQx and PhIP

	<i>MeIQx</i>		<i>PhIP</i>	
	in vitro <i>N</i> -hydroxylation by human liver microsomal fraction [@]			
	<i>Control</i>	<i>+furafylline</i>	<i>Control</i>	<i>+furafylline</i>
Mean $\pm$ s.d.	76.6 $\pm$ 21.1	6.5 $\pm$ 5.3	55.5 $\pm$ 15.6	0.85 $\pm$ 0.90
	in vivo excretion of unchanged amine in the urine*			
<i>Subject</i>	<i>Placebo</i>	<i>Furafylline</i>	<i>Placebo</i>	<i>Furafylline</i>
1	<4.2	30.6	1.0	3.3
2	2.6	36.9	2.6	7.7
3	2.6	57.9	1.1	5.0
4	1.1	23.2	1.0	3.3
5	1.2	18.8	0.5	1.9
6	3.7	19.9	1.4	2.5
Mean $\pm$ s.d.	2.2 $\pm$ 1.1	31.2 $\pm$ 14.8	1.2 $\pm$ 0.7	4.2 $\pm$ 2.1

[@] values are pmol N-hydroxy HA min⁻¹ mg⁻¹ microsomal protein.

^{*} % of the ingested dose excreted as unchanged amine in urine.

oxidative metabolism of MeIQx and PhIP and that the activation reaction (N-hydroxylation) was catalysed by CYP1A2 [29, 30], an enzyme constitutively expressed in liver. Human microsomal enzymes were particularly efficient at catalysing the N-hydroxylation of MeIQx and PhIP (Table 2). Human liver CYP1A2 can be specifically inhibited by the xanthine derivative furafylline [31]. Indeed in the presence of furafylline, human liver microsomal N-hydroxylation of both MeIQx and PhIP were almost completely abolished (Table 2) [27, 28]. We therefore used this compound to inhibit CYP1A2 activity in human volunteers and then examined their urinary excretion of MeIQx and PhIP. Since elimination of MeIQx and PhIP in urine is first order and very rapid, alterations in the level of metabolism are reflected in the urinary excretion of unchanged amine. Pretreatment of subjects with furafylline (125 mg, sufficient to cause >99% inhibition of CYP1A2 activity *in vivo* as measured by effects upon phenacetin *O*-deethylation) prior to consumption of a standard beef meal, caused a 14-fold and fourfold increase in the excretion of unchanged MeIQx and PhIP, respectively, in urine (Table 2) [19]. This corresponds to about >90% and 70% inhibition of the metabolic clearance of MeIQx and PhIP, respectively, and demonstrates that metabolism of MeIQx and PhIP is predominantly catalysed by CYP1A2 in humans, presumably to their N-hydroxy derivatives.

The mutagenicity of heterocyclic amines

The heterocyclic amines are very powerful mutagens in short term tests such as the Ames *Salmonella typhimurium* assay [5]. In order to express their mutagenic potential, the HAs require metabolic activation catalysed by liver enzymes. In all cases, the primary genotoxic metabolite

is the N-hydroxylamine generated by N-oxidation of the exocyclic amino function. Both MeIQx and PhIP are converted to their respective N-hydroxy derivatives by hepatic microsomal oxidative metabolism and humans are particularly efficient at catalysing these CYP1A2 mediated reactions [27, 28]. Of the two compounds, MeIQx is by far the more powerful bacterial mutagen and PhIP is the more effective mammalian cell mutagen. This observation is emphasised in experiments with Chinese hamster ovary cells [32]. A similar response is also seen in the *dlb-1* locus assay in C57/SWR f₁ mice *in vivo*, in which mutations in small intestinal stem cells are detected by loss of the binding site of the lectin *Dolichos bifloris* agglutinin on the cell surface of clonally derived villus epithelial cells [33]. In this assay PhIP is a powerful mutagen inducing mutation with a single 20 mg kg⁻¹ dose [34] whereas MeIQx does not induce any detectable increase with doses up to 200 mg kg⁻¹ and using a variety of regimens [33].

The first step in a chemically induced mutational event is thought to be interaction of the reactive chemical, in this case derived from the N-hydroxy or an esterified derivative of N-hydroxy HA, with DNA [35] to form an adduct. This can either be repaired or misrepaired or causes replication errors during mitosis thereby inducing mutation. Activated derivatives of both MeIQx and PhIP preferentially form DNA adducts with guanine bases, primarily at the C-8 position [36–38]. It is therefore not surprising that HA-induced mutations frequently involves guanine bases.

To determine if a characteristic mutation pattern or 'fingerprint' is induced by PhIP, we investigated mutations in a selectable marker gene, *hprt*, of a Chinese hamster cell line, XEMh1A2-MZ, which had been genetically engineered to express stably the human CYP1A2 gene [39]. PhIP treatment (1–200 μ M) induced a significant dose dependent increase in the frequency

of *hprt* mutations in these cells as measured by acquired resistance to 6-thioguanine [40]. The majority of PhIP-induced mutations (Table 3) involved simple base substitutions nearly all of which were at guanine cytosine (G.C) pairs suggesting that the premutagenic lesions involve adduction to cytosine, or more likely, to guanine [41]. PhIP-induced G.C→tyrosine adenosine (T.A) transversions were the most frequent base substitutions at G.C sites. However, significant numbers of G.C→A.T transitions and G.C→C.G transversions were also observed. Ten per cent of the mutations observed were frameshifts involving the loss of a single G.C base pair, the majority of which occurred at a 5'-GGG sequence in exon 9. Co-incidentally, in a study of PhIP mutations at the *hprt* locus of human lymphoblastoid cells [42] a run of six contiguous guanine residues at position 207–212 in exon 3 was found to be a 'hotspot' sequence for single guanine base deletions. We found three GC→TA and one GC→CG transversions, but no frameshift mutations, in four of the guanines in the sequence. Any cells carrying substitutions at the other two guanine sites in this sequence would have escaped

selection for 6-TG^r phenotype since they are the third letter of codons and hence their alteration does not lead to a change of phenotype.

We suggest that in this Chinese hamster cell line the premutagenic lesions associated with PhIP mutagenesis occur at guanine bases which implies that nearly all such mutations must have arisen from the non-transcribed strand. Such strand-specific bias for mutagenesis has been shown to be due to preferential repair of DNA damage on the transcribed strand [43]. The DNA sequence context of PhIP-induced mutations reveals a disproportionately high number of base substitutions at sites flanked by a guanine on the 5' side and/or an adenine on the 3' side of the putatively adducted guanine base. These results have effectively defined a mutational 'fingerprint' for PhIP which may be useful when analysing mutations in cancer-related genes associated with dietary related tumours. Such information will be valuable in assessing what role, if any, PhIP may have in dietary related carcinogenesis.

Table 3 PhIP-induced mutation of *hprt* gene of XEMh1A2 cells

	Mutation	Number of mutants
Base substitution	GC→TA	25
	GC→AT	6
	GC→CG	2
	AT→GC	0
	AT→TA	0
	AT→CG	0
Frame shifts	−1 G.C base pair	5
	+1 G.C base pair	0
	−1 A.T base pair	0
	+1 A.T base pair	0
Complex mutation		1
Gene disruption		1 ^a
Splice mutant	−exon 2	2
	−exons 2, 3 and 4	1
	−exon 4	1
	−exon 5	1
	−exon 7	3
	−17 bp of exon 9	1

Mutations were determined by RT/PCR of total RNA.

Splice mutations were determined by PCR of genomic DNA.

^aNo PCR product recovered using RNA or genomic DNA template.

The carcinogenicity of heterocyclic amines

All of the heterocyclic amines tested thus far, including MeIQx and PhIP, have been shown to be carcinogenic in rodent bioassays [44–47]. MeIQx and PhIP induce tumours in a variety of tissues (Table 4) including, in the case of PhIP, the colon and mammary gland of rats [46]. However, animal carcinogenicity data are limited in their application to evaluating the risk of the HAs to man. For example, the structurally related HA, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), is a potent hepatocarcinogen in the cynomolgus monkey, *Macaca fascicularis* [48]. In contrast, MeIQx has, to date, proven negative in this species [48]. But *Macaca fascicularis* does not express CYP1A2 [or CYP1A1] constitutively and therefore is unable to convert MeIQx to its N-hydroxylamine [49]. Clearly, since CYP1A2 activity is essential for the bioactivation of HAs, data obtained in *Macaca fascicularis* cannot be extrapolated to man. In these bioassays, carcinogen dosage is considerably higher than the calculated daily human exposure; but at high doses there is evidence of saturation of amine activation [21, 22]. Furthermore HAs are potently tumorigenic in neonatal B6C3F₁ mice at cumulative doses which are 5–10 000-fold less than those used in standard bioassays [50]. This may be related to the greater efficiency of young animals, compared with

Table 4 Carcinogenicity of MeIQx and PhIP

Compound	Species	Concentration in diet (ppm)	Target	Reference
MeIQx	Mouse	600	Liver, lung, lymphoma, leukaemia	41
	Rat	400	Liver, skin, Zymbal gland, clitoral gland	42
PhIP	Mouse	400	Lymphoma	44
	Rat	400	Colon (male) Breast (female)	43

adults, to activate HAs [51]. It is therefore likely that the carcinogenic potency of HAs determined by conventional rodent bioassay is underestimated.

Recently, molecular analysis of mutations present in colonic tumours induced by PhIP in F344 rats has been reported [52]. Although no mutations of *Ki-ras* or *p53* were detected, mutations of the tumour suppressor gene *Apc* were found in five of eight PhIP-induced colon tumours and all involved deletion of a guanine base in a 5'-GGGA sequence [52]. Mutation of the *APC* gene is observed in about 60% of sporadic human colon cancers and is considered an initial or very early event in human colon carcinogenesis [53]. It is intriguing that the 5'-GGG sequence for single guanine base deletion found in our experiments reported above is also followed by an adenine base and hence is identical to the specific 'hotspot' in the *Apc* gene of PhIP-induced colon tumours in F344 rats (52).

Discussion

Given that the information obtained from animal studies does not always reflect the situation in humans, these studies emphasise how important it is to study the fate of dietary carcinogens, such as HAs, in man whenever possible.

Over the last few years, we and others have shown that humans are exposed to HAs in diet at a level of about 0.1–10 µg person⁻¹ day⁻¹, that these compounds are bioavailable, that there is good evidence for their extensive metabolic activation to N-hydroxylamines catalysed by CYP1A2 *in vivo* and that both liver and colon can further catalyse the esterification of HAs to ultimate genotoxic metabolites. Epidemiological studies have revealed that the combination of high levels of CYP1A2, NAT2 rapid acetylator phenotype and consumption of diets rich in 'well-done' meat and fish is associated with increased relative risk of developing colorectal cancer [54, 55]. Such observations provide strong circumstantial evidence for a biological association between HA exposure and diet-related tumours such as colorectal cancer. The important objectives for the future are to investigate HA induced mutagenicity/carcinogenicity, particularly with regard to the genetic changes associated with the mutation of critical gene targets in colorectal cancer [56] and other dietary related tumours.

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