Food derived carcinogenic amnoimidazoazaarenes
bioactivation and DNA adduct formation
Frandsen, Henrik

Publication date:
1996

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact rucforsk@ruc.dk providing details, and we will remove access to the work immediately and investigate your claim.
FOOD DERIVED CARCINOGENIC
AMINOIMIDAZOAZAARENES.
Bioactivation and DNA adduct formation

Ph.D. Thesis

Henrik Frandsen

Institute of Toxicology
Division of Biochemical and Molecular Toxicology
Laboratory of Biochemical and Genetic Toxicology
National Food Agency of Denmark

and

Institute for Life Sciences and Chemistry
Roskilde University Center
Denmark
Carcinogenic aminoimidazoazaarenes are formed during cooking of meat and fish. Important factors for the formation of these compounds are meat type, cooking temperature and time. The compounds are genotoxic in bacterial and mammalian cells. In animal feeding studies the compounds tested so far were found to be multiple organ carcinogens.

The aminoimidazoazaarenes are metabolically activated by hydroxylation of the exocyclic aminogroup to the N-hydroxyamino derivative. The resultant proximate mutagens often need further activation by phase II transferases for formation of reactive species that form adducts with DNA. Adducts with 2-deoxyguanosine have been characterized for a number of aminoimidazoazaarenes. Adducts with DNA have also been found in animals after exposure to these compounds.

**In vivo** major metabolic detoxification pathways are ring hydroxylation followed by conjugation and conjugation of the exocyclic amino group. Estimations of human cancer risk have indicated that ingestion of food containing aminoimidazoazaarenes are of importance.

**Key words:** Aminoimidazoazaarene, Metabolism, Activation, DNA adducts.


**ISBN:** 87-601-6161-2

**Printing:** Quickly Tryk A/S
SUMMARY

Several carcinogenic heterocyclic aromatic amines are formed during cooking of meat and fish. The important factors for the formation of these compounds are the type of meat being cooked, cooking temperature and duration of cooking. Under standard household cooking conditions, the aminooimidazoazaarenes (AIA) which are condensation products of amino acids and creatinine, seem to be the most important compounds, due to the amounts formed and their possible biological effects.

The AIA are genotoxic in a number of test systems such as those using Salmonella typhimurium and mammalian cells. In rodent feeding studies IQ, 4-MeIQ, 8-MeIQx and PhIP were found to be multiple organ carcinogens.

These procarcinogens require metabolic activation to exert their genotoxic effects. Activation has been shown to occur via hydroxylation, catalysed by CYP1A enzymes, of the exocyclic amino group to the N²-OH derivatives. These derivatives are proximate mutagens that to some extent bind covalently to DNA and other macromolecules. Further activation of the N²-OH group through esterification by e.g. acetyltransferase or sulfotransferase results in formation of highly reactive species that form adducts with DNA and 2'-deoxyguanosine, but not with 2'-deoxyadenosine, 2'-deoxycytidine or thymidine. Adducts from 2'-deoxyguanosine and IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx and PhIP have been characterized.

In rodents in vivo, major metabolic pathways, that result in detoxification products, are hydroxylation of the ring system followed by conjugation with sulphate or glucuronic acid. Also, conjugates of the exocyclic amino group with sulphate or glucuronic acid are major metabolites.

³²P-Postlabeling analyses of DNA from rodents given AIA compounds orally as well as studies using radiolabelled compounds showed that adducts with 2'-deoxyguanosine are also formed in vivo. Adduct levels seem to increase linearly with dose.

Risk estimates, based on extrapolations from results of long term animal cancer studies, have suggested that ingestion of AIA compound are important in human cancer etiology.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>6</td>
</tr>
<tr>
<td>SUMMARY IN DANISH (SAMMENDRAG)</td>
<td>7</td>
</tr>
<tr>
<td>LIST OF PAPERS</td>
<td>8</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Chemical carcinogenesis</td>
<td>12</td>
</tr>
<tr>
<td>DIET AND CANCER</td>
<td>13</td>
</tr>
<tr>
<td>HETEROCYCLIC AROMATIC AMINES IN FOOD</td>
<td></td>
</tr>
<tr>
<td>Formation and occurrence</td>
<td>14</td>
</tr>
<tr>
<td>MUTAGENICITY AND CARCINOGENICITY</td>
<td>18</td>
</tr>
<tr>
<td>METABOLISM</td>
<td>21</td>
</tr>
<tr>
<td>The quinolines</td>
<td>21</td>
</tr>
<tr>
<td>The quinoxalines</td>
<td>24</td>
</tr>
<tr>
<td>The pyridine</td>
<td>27</td>
</tr>
<tr>
<td>DNA ADDUCT FORMATION</td>
<td>29</td>
</tr>
<tr>
<td>MECHANISM OF ADDUCT FORMATION</td>
<td>33</td>
</tr>
<tr>
<td>DNA ADDUCT FORMATION IN VIVO</td>
<td>35</td>
</tr>
<tr>
<td>ESTIMATES OF HUMAN INTAKE AND CANCER RISK</td>
<td>37</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>38</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>41</td>
</tr>
<tr>
<td>PAPER I</td>
<td></td>
</tr>
<tr>
<td>PAPER II</td>
<td></td>
</tr>
<tr>
<td>PAPER III</td>
<td></td>
</tr>
<tr>
<td>PAPER IV</td>
<td></td>
</tr>
</tbody>
</table>
From the Department of Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden:
Dr. Spiros Grivas
Dr. Rolf Andersson

From Nestec Ltd, Research Centre, Lausanne, Switzerland:
Dr. Robert J. Turesky

From Medical Research Council, Carshalton, Surrey, UK:
Dr. Peter Farmer

I also wish to thank my tutors: Dr. John C. Larsen, Institute of Toxicology, Danish National Food Agency and Dr. Ole Andersen, Institute for Life Sciences and Chemistry, Roskilde University Center, Denmark for valuable comments to the manuscript.

I wish to thank Vivian Jørgensen for performing the Ames test and Bo Lund Jensen for assistance with graphical work.
Especially, I wish to thank Joan E. Gluver for supplying me with skilful technical assistance throughout the studies.

Thanks also to Dr. James Felton, Lawrence Livermore Natl. Laboratory, Livermore, CA and Dr. Errol Zeiger, Cellular and Genetic Toxicology Branch, NIEHS, North Carolina, for providing PhIP for the present studies.

Copenhagen, January 1996

Henrik Frandsen
PREFACE

The present study was initiated to provide knowledge about the mechanism of activation and DNA binding of the food derived carcinogens PhIP and 4,8-DiMeIQx. PhiP and 4,8-DiMeIQx were chosen as representatives of the AIAs that are formed at high and medium frying temperatures, respectively.

Due to the presence of these potentially deleterious compounds in the normal human diet, there is a need for developing biomarkers of exposure and effects. Research on the mechanism of activation and adduct formation of several AIAs and pyrolysate mutagens have been conducted by a number of research groups throughout the world. PhIP, in particular, has attracted attention, which has resulted in a strong competitive environment but also in the establishment of many collaborative studies. The results presented here form part of the contribution to these collaborative international efforts.

In paper I, the microsomal metabolism of PhIP and identification of the genotoxic metabolite was published.

Paper II describes the formation of DNA adducts of PhIP and the structural characterization of the adduct.

In paper III, the microsomal metabolism of 4,8-DiMeIQx and identification of the genotoxic metabolites was published.

Paper IV describes the formation of DNA adducts of 4,8-DiMeIQx and the structural characterization of the adduct.

The studies on PhIP and 4,8-DiMeIQx are planned to continue. The future aims are to develop methods for measuring adducts in samples from humans exposed to AIAs by ingestion of meat prepared under normal household conditions. The development of such biomarkers will be useful in the estimation of individual human cancer risk associated with AIA ingestion.

I wish to thank all of the collaborators for a fruitful corporation.

From the Institute of Toxicology, Danish National Food Agency:

Dr. Preben A. Nielsen
Dr. Eva S. Rasmussen
Dr. Lars O. Dragsted
Dr. John C. Larsen
SAMMENDRAG


AIA forbindelserne er genotoksiske i mange test systemer som *Salmonella typhimurium* og mammale celler. I langtids studier i gnavere er IQ, 4-MeIQ, 8-MeIQx og PhIP vist at medføre kræft i flere organer.

Disse prokarcinogener kræver metabolisk aktivering for at udviske genotoksisk effekt. Aktiveringen er vist at ske ved CYP1A katalysert hydroxylering af den exocykliske aminogruppe til et N²-OH derivat. Disse derivater bindes i nogen grad kovalent til DNA og andre makromolekyler. En yderligere aktivering af N²-OH gruppen kan ske ved esterifikation katalysert af acetyltransferase eller sulfotransferase, hvorved der dannes meget reaktive derivater, som danner addukter med DNA og 2'-deoxyguanosin, men ikke med 2'-deoxyadenosin, 2'-deoxycytidin eller thymidin. Addukter mellem 2'-deoxyguanosin og IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx og PhIP er blevet karakteriseret strukturelt.

I gnavere *in vivo* er det vist, at de vigtigste metaboliserings veje som fører til dannelse af detoksifiserings produkter, er hydroxylering af ringsystemet efterfulgt af konjugering med sulfat eller glucuronsyre. Konjugering af den exocykliske amino gruppe med sulfat eller glucuronsyre er også vigtige metaboliseringsveje.

Thirty-P-Postlabeling analyse af DNA fra gnavere, som er doseret oralt med AIA, såvel som undersøgelser hvor der anvendt radioaktivt mærkede stoffer har vist at addukter med 2'-deoxyguanosin også dannes *in vivo*. Addukt niveauet stiger lineært med dosis størrelsen.

Risiko extrapolering baseret på langtids studier i dyr indikerer at indlagelse af AIA forbindelser er af betydning for udvikling af kræft i mennesker.
LIST OF PAPERS

This thesis is based on the following papers. In the text they will be referred to by Roman numerals and author names.


Papers relating to aminoimidazoazaarene research not included in this thesis.


ABBREVIATIONS

AaC 2-amino-9H-pyrido[2,3-b]indole
acetyl-CoA acetyl coenzyme A
AIA aminoimidazoazaarene
ATP adenosine-5'-triphosphate
CYP cytochrome P-450
DiMeIQx or 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline
4,8-DiMeIQx 2-amino-3,4,7-trimethylimidazo[4,5-f]quinoxaline
4,7-DiMeIQx 2-amino-3,5,7-trimethylimidazo[4,5-f]quinoxaline
5,7-DiMeIQx 2-amino-3,5,8-trimethylimidazo[4,5-f]quinoxaline
dG deoxyguanosine
dG-C8-IQ $N^2$-(deoxyguanosin-8-yl)-IQ
dG-N$^2$-IQ 5-(deoxyguanosin-N$^2$-yl)-IQ
dG-C8-MeIQx $N^2$-(deoxyguanosin-8-yl)-8-MeIQx
dG-N$^2$-MeIQx 5-(deoxyguanosin-N$^2$-yl)-8-MeIQx
dG-C8-DiMeIQx $N^2$-(deoxyguanosin-8-yl)-4,8-DiMeIQx
dG-C8-PhIP $N^2$-(deoxyguanosin-8-yl)-PhIP
DNA deoxyribonucleic acid
Glu-P-1 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole
Glu-P-2 2-aminodipyrido[1,2-a:3',2'-d]imidazole
IQ 2-amino-3-methylimidazo[4,5-f]quinoline
IQx 2-amino-3-methylimidazo[4,5-f]quinoxaline
MeAaC 2-amino-3-methyl-9H-pyrido[2,3-b]indole
MeIQ or 2-amino-3,4-dimethylimidazo[4,5-f]quinoline
4-MeIQ 2-amino-3,5-dimethylimidazo[4,5-f]quinoline
5-MeIQ 2-amino-3,4-dimethylimidazo[4,5-f]quinoline
4-MeIQx 2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline
7-MeIQx 2-amino-3,7-dimethylimidazo[4,5-f]quinoxaline
MeIQx or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
8-MeIQx 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1</td>
<td>N-acetyltransferase type 1</td>
</tr>
<tr>
<td>NAT2</td>
<td>N-acetyltransferase type 2</td>
</tr>
<tr>
<td>2-nitro-PhIP</td>
<td>1-methyl-2-nitro-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>N²-OH-IQ</td>
<td>2-hydroxyamino-3-methylimidazo[4,5-f]quinoline</td>
</tr>
<tr>
<td>N²-OH-MeiQx</td>
<td>2-hydroxyamino-3,8-dimethylimidazo[4,5-f]quinoxaline</td>
</tr>
<tr>
<td>N²-OH-PhIP</td>
<td>2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>5-OH-IQ</td>
<td>2-amino-5-hydroxy-3-methylimidazo[4,5-f]quinoline</td>
</tr>
<tr>
<td>4'-OH-PhIP</td>
<td>2-amino-1-methyl-6-(4-hydroxy-phenyl)imidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>7-oxo-MeiQx</td>
<td>2-amino-3,6-dihydro-3,8-dimethyl[4,5-f]quinoxaline-7-one</td>
</tr>
<tr>
<td>PAPS</td>
<td>3'-phosphoadenosine-5'-phosphosulphate</td>
</tr>
<tr>
<td>PCB</td>
<td>aroclor 1254</td>
</tr>
<tr>
<td>pdGp-C8-MeiQ</td>
<td>N²-(deoxyguanosin-8-yl)4-MeIQ-3',5'-diphosphate</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Trp-P-1</td>
<td>3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole</td>
</tr>
<tr>
<td>Trp-P-2</td>
<td>3-amino-1-methyl-5H-pyrido[4,3-b]indole</td>
</tr>
</tbody>
</table>
INTRODUCTION

*Chemical Carcinogenesis*

During the past two centuries it has been recognized that humans occupationally exposed to mixtures of chemical compounds, in particular tars from combustion, were at high risk of developing cancer. In the beginning of the 20th century the development of cancer in experimental animals was reported following repeated application of coal tar onto rabbit skin (reviewed by Miller, 1994 and Pitot, 1990). After the introduction of animal models in experimental carcinogenesis Kennaway and Heiger in 1930 identified the first pure chemicals, dibenz[a,f]anthracene and dibenz[a,h]anthracene, that could induce cancer (Kennaway and Heiger, 1930).

Since then, several chemicals including polycyclic aromatic hydrocarbons, aromatic amines, N-nitroso-compounds, alkylating agents and several naturally occurring compounds have been shown to be carcinogens. These different chemical classes of carcinogens do not seem to share any structural or chemical similarities. However, in 1961 the Millers recognized that a metabolite of N-acetylaminofluorene was a more potent carcinogen than the parent compound. This indicated that chemical carcinogens were activated to proximate carcinogens by enzymatic processes taking place in the cell (Miller and Miller, 1961).

Indications that metabolism was involved in the activation of carcinogens was also obtained from observations that carcinogens were covalently bound to macromolecules in animal tissue. In 1964, Brooks and Lawley discovered, by use of a radioactively labelled carcinogen, that DNA was the target molecule for reactive metabolites and they found a correlation between carcinogenic potency and DNA binding (Brookes and Lawley, 1964).

Many of the procarcinogens are lipophilic substances which are subject to modifications by phase I and/or phase II enzymes in order to make them more hydrophillic to facilitate excretion via urine or faeces. For example, alkyl groups, aromatic rings and amino groups may be hydroxylated; nitro groups and azo groups may be reduced; phenolic groups, alcohols and amino groups may be conjugated with glucuronic acid, acetate, phosphate or sulphate.

These enzymatic processes usually result in formation of detoxified, water soluble metabolites which are readily excreted. However, some of these enzymatic modifications may result in metabolites that are more potent carcinogens than the parent compound.
Often more than one enzymatic activation reaction is necessary for the formation of ultimate carcinogenic metabolites, although, some carcinogens exist in their ultimate form (e.g. alkylating reagent) and do not need metabolic activation (Miller and Miller, 1966; Dipple, 1988). The ultimate carcinogenic metabolites of the various classes of chemical carcinogens have one common feature. They are reactive electrophiles, that can react with cellular macromolecules. Especially, the reaction of an electrophilic metabolite with nucleophilic positions on the DNA bases resulting in the formation of DNA adducts seems to be a critical step in the development of cancer.

It is now realised that the development of cancer is a multistage process where several steps are involved. The process has been divided into four stages: initiation, promotion, conversion and progression, which each are thought to contain several different events (reviewed by Harris, 1991). The first stage, initiation, involves exposure to a carcinogen, formation of DNA adducts that result in a mutation leading to an initiated cell. During the second stage, promotion, the initiated cell starts to proliferate due to decreased responsiveness to regulatory growth factors. In the third stage, conversion, growing initiated cells are susceptible to genetic damage by chemical carcinogens resulting in conversion to a malignant cell. In the fourth stage, progression, genetic changes in the cells result in increased growth rate, the ability to invasive growth and the ability to metastasize.

**Diet and cancer**

Dietary constituents are now considered important factors both in the causation and in the prevention of important diseases, including cancer (Willett, 1994, Rogers et al., 1993). The diet has been estimated to account for 10% of cancers in some organs and up to 90% of cancers in other organs. A mean estimate of 35% has been suggested for the dietary importance on the cancer rate in the U.S. population (Doll and Peto, 1981). An association between intake of animal fat and cancers in the breast, pancreas and colon has been reported. However, in some epidemiological studies colon cancer seems to correlate stronger with intake of red meat and with preference for heavily browned meat surface. (Giovannucci, 1994: Gerhardson de Verdier, 1991, Willett et al., 1990)
Suggested mechanisms for these associations include: (1) Increased tumour incidence due to excessive calorie intake. A lower tumour incidence is observed in rodents on a calorie-restricted diet compared to controls. (2) Proliferative effects of bile acids on the colonic mucosa. (3) Increased iron absorption due to meat ingestion. Iron contributes to the production of reactive oxygen species that can damage cellular macromolecules e.g. DNA. (4) Formation of carcinogenic chemicals such as N-nitrosamines, heterocyclic aromatic amines and polycyclic aromatic hydrocarbons during cooking (reviewed by Ames et al., 1995).

The formation of carcinogenic substances in fried meat was for the first time reported in 1939 by Widmark, who had found that ethanol or petroleum ether extracts of horse meat, heat treated at a temperature of 275 °C resulted in the formation of mammary tumours, when painted on female mice in the occipital region (Widmark, 1939). In the 1970ies Sugimura and coworkers used the newly developed Salmonella mutagenicity assay (Ames et al., 1973) to look for mutagenic substances in grilled or broiled fish and meat (Sugimura, 1992). This led to the discovery of a series of mutagenic compounds identified as heterocyclic aromatic amines.

Heterocyclic aromatic amines in food.

Formation and occurrence.

Since it was first discovered that the charred surface of fish and meat contained mutagenic activity corresponding to that of 130-450 µg benzo[a]pyrene/ 100 g of meat (Nagao et al., 1977), a large number of mutagenic compounds has been isolated from heat processed proteins and foods and structurally characterized. The first class of mutagens that was structurally characterized was the pyrolysate mutagens which are formed during treatment of amino acids or proteins at very high temperatures. Examples of structures of the pyrolysate mutagens are shown in Figure 1. Later a second, more abundant class of mutagens, the aminoimidazoazaaarenes (AIAs), was structurally characterized. The AIAs are formed during ordinary cooking conditions and are characterized by one or two heterocyclic rings fused to an aminoimidazo ring (Felton, 1986a). Figure 2 shows the structures of IQ (Kasia et al., 1980a); 4-MeIQ, (Kasia et al., 1980b); 8-MeIQx, (Kasia et
al., 1981): 4,8-DiMeIQx, (Felton et al., 1984) and PhIP (Felton et al., 1986a). The AIAs are found in higher concentrations compared to the pyrolysate mutagens when cooking is carried out under normal household conditions. Therefore, the following text will mainly concentrate on the AIAs.

The AIAs that until now have been characterized are subdivided into three structurally different groups: the quinolines, the quinoxalines and the pyridines (Figure 2). AIAs have been detected in a series of cooked foods including fish, beef, chicken, lamb and pork. The
formation of mutagenic substances does not seem to differ between the various sorts of
meat, but seems to be qualitatively and quantitatively comparable under similar cooking
conditions. Cooking time and cooking temperature are the most important factors that
determine the amount of mutagens formed (Skog, 1993).

Fig. 2 Structures of selected aminomidazoazaarene mutagens.

During 10 min an almost linear increase was observed in the amount of mutagenicity
formed in beef patties fried at 150, 190 and 230 °C, respectively (Knize, 1994). Several investigation have shown that the mutagenicity in fried meat increases with increasing frying temperature (reviewed by Skog, 1993). For each 50 °C rise in frying temperature a doubling of the mutagenic activity has been detected in pork (Nielsen et al., 1988). Increasing frying temperature also resulted in a different distribution among the different AIAs formed. At low frying temperatures, up to 200 °C, mainly the more polar AIAs, IQ and MeIQx are formed. At temperatures above 200 °C at shift towards more apolar AIAs, especially PhIP takes place (Skog, 1995, Felton et al., 1986b).

Table I. Concentrations of MeIQx, DiMeIQx and PhIP in meat samples and pan residues calculated as ng/g cooked product.

<table>
<thead>
<tr>
<th>Sample/Cooking temperature (°C)</th>
<th>MeIQx</th>
<th>DiMeIQx</th>
<th>PhIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork chop/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>175</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>200</td>
<td>0.2</td>
<td>1.9</td>
<td>0.3</td>
</tr>
<tr>
<td>225</td>
<td>2.6</td>
<td>1.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Bacon/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>ND</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>175</td>
<td>0.1</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>200</td>
<td>0.7</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td>225</td>
<td>23.7</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Minute steak/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>175</td>
<td>0.2</td>
<td>1.1</td>
<td>0.06</td>
</tr>
<tr>
<td>200</td>
<td>0.6</td>
<td>2.9</td>
<td>0.1</td>
</tr>
<tr>
<td>225</td>
<td>6.2</td>
<td>23.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Sirloin steak/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0.02</td>
<td>0.07</td>
<td>ND</td>
</tr>
<tr>
<td>175</td>
<td>0.2</td>
<td>0.8</td>
<td>ND</td>
</tr>
<tr>
<td>200</td>
<td>0.7</td>
<td>3.3</td>
<td>ND</td>
</tr>
<tr>
<td>225</td>
<td>1.6</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>Ground beef/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>ND</td>
<td>0.06</td>
<td>ND</td>
</tr>
<tr>
<td>175</td>
<td>0.2</td>
<td>1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>200</td>
<td>1.2</td>
<td>5.8</td>
<td>0.4</td>
</tr>
<tr>
<td>225</td>
<td>2.2</td>
<td>3.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

ND, not detected.
Data from Skog et al., 1995.
A1As are found not only in the surface of the fried meat, often the pan residue contains as high or higher levels. Examples of the content of MeIQx, DiMeIQx and PhIP in different meat samples and residues fried at different temperatures are shown in Table I. The important precursors for the formation of A1As in foods are creatinine or creatine, amino acids and carbohydrate, all compounds that are abundant in meat. Formation of A1As have been modelled by heating mixtures of creatinine and an amino acid or creatinine, an amino acid and a carbohydrate; IQ has been formed from creatinine/proline (Yoshida, 1984), 8-MeIQx from creatinine/glycine/glucose, (Jagerstadt et al., 1984); 4,8-DiMeIQx from creatinine/alanine/fructose (Grivas et al., 1985) and PhIP from creatinine/phenylalanine/glucose (Taylor et al., 1987). Microwave treatment of meat prior to cooking reduced the mutagenicity of cooked meat. Microwave treatment for 1 min leaves only 38% of the mutagenicity compared to controls, 2 min leaves 10% and 3 min leaves 6%. It was suggested that the juice that leaves the meat during the microwave treatment contains the precursors for the mutagen formation (Felton et al., 1992).

A1A mutagens and pyrolysate mutagens have also been identified in non meat sources, although in lower concentrations. MeIQ has been found in roasted coffee beans; AoC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2 have been found in cigarette smoke condensates, airborne particles or diesel exhaust (Kikugawa et al., 1989; Manabe et al., 1990; Kanai et al., 1990; Manabe et al., 1989; Manabe et al., 1991). PhIP related DNA adducts has been detected in urine from smokers of black tobacco. (Peluso et al., 1991). PhIP has also been detected in beer and wine (Manabe et al., 1993).

**Mutagenicity and Carcinogenicity**

The A1As are promutagens and require the presence of a metabolic activation system to show mutagenic activity in the Ames Salmonella assay. Two strains of Salmonella have usually been used in the detection of mutagenic activity, TA98 and TA1538. Table II lists the mutagenic potency of some of the A1As found in cooked food together with some structurally related compounds which have been produced in the search of structural proof of the A1As found in food. It can be seen that some of the A1As are very potent bacterial
mutagens, others are quite weak and that small structural changes have large effects on the mutagenic potency (Felton and Knize, 1990).

Studies on quantitative structure activity relationship has resolved some of the structural elements that are of importance for the mutagenic potency. In the IQ and IQx type of mutagens higher mutagenic potency as compared to e.g. PhIP is related to the presence of Ring 3 with double bonds in conjugation with Ring 2 (Figure 3) and with the number and position of nitrogen atoms in Ring 3, e.g. 4-MeIQx is more mutagenic than 4-MeIQ (Hatch et al. 1991, Vikse et al., 1993a)(For comparison see Figure 2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>TA98</th>
<th>TA1538</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ</td>
<td>433.000</td>
<td>200.000</td>
</tr>
<tr>
<td>4-MeIQ</td>
<td>660.000</td>
<td>750.000</td>
</tr>
<tr>
<td>5-MeIQ</td>
<td>142.000</td>
<td>NT</td>
</tr>
<tr>
<td>4-MeIQx</td>
<td>NT</td>
<td>875.000</td>
</tr>
<tr>
<td>7-MeIQx</td>
<td>233.000</td>
<td>528.000</td>
</tr>
<tr>
<td>8-MeIQx</td>
<td>110.000</td>
<td>93.000</td>
</tr>
<tr>
<td>4.8-DiMeIQx</td>
<td>206.000</td>
<td>320.000</td>
</tr>
<tr>
<td>4.7-DiMeIQx</td>
<td>351.000</td>
<td>38.700</td>
</tr>
<tr>
<td>5.8-DiMeIQx</td>
<td>74.000</td>
<td>3.100</td>
</tr>
<tr>
<td>5.7-DiMeIQx</td>
<td>243.000</td>
<td>NT</td>
</tr>
<tr>
<td>PhIP</td>
<td>2000</td>
<td>NT</td>
</tr>
</tbody>
</table>

Table II. Mutagenicity of AIA compounds in Salmonella typhimurium strain TA98 and TA1538 expressed as revertants/μg.

Also the presence and position of methyl groups has a strong effect on the mutagenic potency. A methyl group in position 4 of IQx results in a higher mutagenic potency than a methyl in position 5 and the addition of a methyl group in position 7 of 4-MeIQx results in a higher mutagenic potency than addition of a methyl group in position 8 (Vikse et al., 1993b)(Consult Fig. 2).

The proposed ultimate mutagenic metabolite of the AIAs is a nitrenium ion generated after O-esterification of the proximate mutagenic N²-hydroxylated metabolite (Figure 3). The presence of a long conjugated system of double bonds in the AIAs, that is able to delocalize the charge on the nitrenium ion will stabilize the ion and increase the probability that it will reach DNA before reacting with other cellular nucleophiles (Dipple et al., 1968). Recently, molecular orbital calculations on nitrenium ion stabilities of a series
of AIAs and pyrolysate mutagens have shown good correlation between mutagenic potency and nitrenium ion stability (Ford and Griffin, 1992).

In the Ames Salmonella assay the metabolic activation to the proximate mutagenic metabolite takes place outside the bacteria, and it has been proposed that the methyl groups in position 3 and 4 will provide a hydrophobic region that facilitates membrane transport. Also, methyl groups in certain positions will result in a different metabolic pattern by e.g. blocking a detoxification reaction and thereby increasing the mutagenic potency.

![Fig. 3 Pattern of resonance delocalization of the positive charge of a nitrenium ion of IQ through the \( \pi \) electron system, Hatch et al., 1991.]

The AIAs have been tested for genotoxicity in a large number of mammalian systems both in vitro and in vivo with both positive and negative results, possibly as a result of use of different protocols, different cell types, different metabolic activation system or none, and different doses (reviewed by Munro et al., 1993). It does, however, seem that the potency of the AIAs is reversed as compared to the potency in the Salmonella assay, with PhIP being more potent in inducing e.g. sister chromatid exchanges than MeIQ and MeIQx (Thompson et al., 1987).

Studies on carcinogenicity after oral treatment have been conducted with IQ, 4-MeIQ, 8-MeIQx and PhIP in mice and rats. All were found to be carcinogenic in various organs in rodents. A summary of selected oral carcinogenicity studies is shown in Table III.
Carcinogenicity studies in monkeys with oral dosing of IQ, MeIQx and PhIP are still ongoing. However, already after one-seventh of the expected life span of the monkeys IQ which was the first compound tested, turned out to be a liver carcinogen (Adamson et al., 1994; Table III).

Table III. Summary of carcinogenicity studies with AIA compounds.

<table>
<thead>
<tr>
<th>AIA</th>
<th>Animal Species</th>
<th>Dose mg/kg/d</th>
<th>Number of animals with tumours</th>
<th>Most sensitive tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ</td>
<td>CDF&lt;sub&gt;1&lt;/sub&gt; mouse</td>
<td>M 35.5</td>
<td>M 27/39</td>
<td>lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 31</td>
<td>F 27/36</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td>F344 rat</td>
<td>M 12</td>
<td>M 36/40</td>
<td>Zymbal gland</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 8.6</td>
<td>F 27/40</td>
<td>Zymbal gland</td>
</tr>
<tr>
<td></td>
<td>SD rat</td>
<td>F 69.3</td>
<td>14/32</td>
<td>mammary gland</td>
</tr>
<tr>
<td></td>
<td>monkey</td>
<td>M/F 10</td>
<td>11/20</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M/F 20</td>
<td>19/20</td>
<td>liver</td>
</tr>
<tr>
<td>MeIQ</td>
<td>CDF&lt;sub&gt;1&lt;/sub&gt; mouse</td>
<td>35</td>
<td>M 12/15</td>
<td>forestomach</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F 16/22</td>
<td>forestomach</td>
</tr>
<tr>
<td></td>
<td>F344 rat</td>
<td>M 16</td>
<td>M 19/20</td>
<td>Zymbal gland</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 10.9</td>
<td>F 17/20</td>
<td>Zymbal gland</td>
</tr>
<tr>
<td>MeIQx</td>
<td>CDF&lt;sub&gt;1&lt;/sub&gt; mouse</td>
<td>M 77</td>
<td>M 15/37</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 70</td>
<td>F 32/35</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td>F344 rat</td>
<td>M 19.4</td>
<td>M 20/20</td>
<td>liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 13.1</td>
<td>12/19</td>
<td>clitoral gland</td>
</tr>
<tr>
<td>PhIP</td>
<td>CDF&lt;sub&gt;1&lt;/sub&gt; mouse</td>
<td>M 86.7</td>
<td>M 11/35</td>
<td>lymphoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 53.3</td>
<td>F 26/38</td>
<td>lymphoma</td>
</tr>
<tr>
<td></td>
<td>F344 rat</td>
<td>20</td>
<td>M 16/29</td>
<td>colon</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F 14/30</td>
<td>mammary gland</td>
</tr>
</tbody>
</table>

Data from Munro et al., 1993 and Adamson et al., 1994.

Metabolism

The Quinolines: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ)
In vitro studies have indicated that IQ and MeIQ are activated to mutagenic metabolites by microsomes or S9 from various species including mouse, rat, rabbit, monkey and man (Yamazoe et al., 1983, Aune et al., 1986, Alldrick et al., 1985, Turesky et al., 1991a, Davies et al., 1993). Microsomes from monkeys, however, had a low capacity for metabolic activation of IQ. IQ and MeIQ induces unscheduled DNA synthesis in hepatocytes from PCB treated rats, and sister chromatid exchanges in V79 cells when co-cultured with hepatocytes (Brunborg et al., 1988) The structure of the proximate mutagenic metabolite of IQ has been determined as the 2-hydroxyamino derivative, N^2-OH-IQ (Yamazoe et al., 1983). By transfection of cells with murine or human P450 isozymes, it has been shown that the activation of IQ and MeIQ to the mutagenic metabolite is catalyzed by cytochrome P450 1A2 (CYP1A2). CYP1A1 only showed very low capacity to activate IQ and MeIQ (Snyderwine and Battula, 1989, McManus et al., 1990). Prostaglandin H synthase was also able to activate IQ by oxidation of the exocyclic amino group to a nitro group (Morrison et al., 1993, Wild and Degen, 1987).

Fig. 4 Pathways for metabolic activation of IQ. Snyderwine et al., 1992.
$N^2$-OH-IQ needs further activation by esterification of the hydroxylamino group to exert its genotoxic activity. Figure 4. Incubation of $N^2$-OH-IQ with rat hepatic cytosol and pentachlorophenol, an inhibitor of both O-acetyltransferase and sulphotransferase, resulted in reduced mutagenicity in *Salmonella* TA98. Incubation with 2,6-dichloro-4-nitrophenol, a more specific inhibitor of sulphotransferase, did not result in reduced mutagenicity. This indicates that $N^2$-OH-IQ is a substrate for rat acetyltransferase but not for sulphotransferase (Snyderwine et al., 1988a). Activation of $N^2$-OH-IQ with cytosol from various organs from rats and monkeys has shown that there is a large interspecies and interorgan variation in the ability to generate DNA binding metabolites by acetyl-CoA, PAPS, L-Proline and ATP- dependant activating enzymes (Davis et al., 1993). Studies with human transferases, expressed in *Salmonella* bacteria or by use of human liver cytosol, have shown that $N^2$-OH-IQ not is a substrate for human sulphotransferase, a poor substrate for acetyltransferase isozyme NAT1, but is a substrate for human acetyltransferase isozyme NAT2 (Turesky et al., 1991a, Chou et al., 1995, Wild et al., 1995).

![Fig. 5 Structures of metabolites of IQ identified in rodents.](image)

Gluc = Glucuronic acid.
In vivo studies in rats have shown that IQ and MeIQ are readily absorbed and excreted. Within 24 hours after dosing with radiolabelled compounds more than 90% of the radioactivity had left the animals. 45-65% via the urine and 37-49% via the faeces (Sjodin et al., 1984, Inamasu et al., 1989). The major water soluble metabolites of IQ were identified as the N²-sulfamate and the O-sulphate and glucuronide conjugates of a 5-hydroxylated metabolite. Minor metabolites were 5-OH-IQ, N²-acetylated IQ, IQ-N-glucuronide and demethylated IQ (Inamasu et al., 1989, Luks et al., 1989, Størmer et al., 1987, Alexander et al., 1989, Turesky et al., 1986), Figure 5.

The water soluble metabolites of MeIQ were primarily the O-sulphate and glucuronide conjugates of the 5-hydroxylated metabolite. The N²-sulfamate of MeIQ was much less abundant than the N²-sulfamate of IQ. A minor nonpolar metabolite of MeIQ was identified as the N²-acetyl derivative (Alexander et al., 1989a).

The Quinoxalines: 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2 amino 3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx).

In vitro studies have shown that microsomes from rats, humans and monkeys can metabolically activate MeIQx to mutagenic metabolites. Microsomes from monkeys showed the lowest capacity to activate MeIQx (Davis et al., 1993a). The proximate mutagenic metabolite from activation with rat hepatocytes and with human microsomes was isolated and identified as the N²-hydroxylated derivative (Turesky et al., 1990, Rich et al., 1992, Turesky et al., 1991a). Human CYP1A2 was estimated to account for at least 66% to more than 90% of the phase I metabolism of MeIQx through N²-hydroxylation (McManus et al., 1990, Rich et al., 1992). Murine CYP1A2 has also been shown to activate MeIQx, whereas CYP1A1 showed no activity (Snyderwine et al., 1989).

The phase II activation of N²-OH-MeIQx was studied by use of cytosol from various organs from rats and monkeys and a large variation was observed in the ability of acetyltransferase, sulphotransferase, aminoacyl-tRNA synthetase and phosphatase to activate N²-OH-MeIQx, depending on species and organ (Davis et al., 1993b). Studies with human transferases expressed in Salmonella or by use of human liver cytosol showed that N²-OH-MeIQx not is a substrate for human sulphotransferase. Human acetyltransferase isozyme NAT1 has a low capacity to activate N²-OH-MeIQx, whereas human
acetyltransferase isozyme NAT2 has a higher capacity for activation (Turesky et al., 1991a, Chou et al., 1995, Wild et al., 1995).

The metabolism of MeIQx was studied in hepatocytes from rats and ten metabolites were characterized. In hepatocytes from uninduced rats N²-sulfamate formation is a major metabolic pathway, whereas ringhydroxylation followed by sulphate or glucuronide conjugation are major metabolites in rats induced with Aroclor 1254, β-naphtoflavone or isosafrole. Formation of the mutagenic N²-hydroxylated metabolite as well as its N-glucuronide was also detected. The formation of a 2-nitro derivative is indicated by the presence of a conjugate where the 2nd nitrogen atom of MeIQx was displaced by the sulphur atom of glutathione (Turesky et al., 1990, Wallin et al., 1989).

Liver microsomes from PCB induced rats metabolized 4,8-DiMeIQx to two major and three minor metabolites. One major and one minor metabolite were identified as the N²-hydroxy and the 2-nitro derivatives of DiMeIQx, respectively. Both were mutagenic to Salmonella typhimurium TA98 without metabolic activation system. The other major and two minor metabolites were identified as the 8-hydroxymethyl derivative of DiMeIQx and its N²-hydroxy and 2-nitro derivatives. The last two metabolites were mutagenic to Salmonella typhimurium TA98 without metabolic activation system (III, Frandsen et al., 1994a), Figure 6. Murine CYP1A2 is the predominant isoform responsible for the activation of DiMeIQx to mutagenic species, whereas CYP1A1 shows only little activation (Snyderwine et al., 1989).

![Fig. 6 Structures of metabolites of 4,8-DiMeIQx formed by hepatic microsomes from PCB induced rats. III, Frandsen et al., 1994a.](image-url)
**In vivo** studies in rats dosed with $^{14}$C labelled MeIQx showed that 36-41% of the dose is excreted with the urine during the first 24 hours, and 1-3% during the next 24 hours period. The remaining radioactivity is excreted with the faeces with less than 1% retained in the tissues after 72 hours (Sjodin et al., 1989). Several metabolites from urine, bile and faeces from rats have been isolated and characterized as: $N^2$-sulphamates of MeIQx, an 8-hydroxymethyl derivative together with its $N^2$-sulfamate and 4 (or 5) sulphate conjugate (structure uncertain), a $N^2$-glucuronide conjugate, sulphate and glucuronide conjugates of a 4 (or 5) hydroxylated metabolite, and $N^2$-acetylated and N-demethylated metabolites (Sjodin et al., 1989, Turesky et al., 1988, Hayatsu et al., 1987). Both the dose and use of enzyme inducers influenced the metabolism of MeIQx, with a higher percentage being $N^2$-hydroxylated after PCB induction (Turesky et al., 1991b). The metabolic profile in monkeys is quite similar to the profile in rats except for the presence of two additional metabolites, a $N^1$-glucuronide and 7-oxo-MeIQx. The latter was ascribed as an enteric bacterial metabolite of MeIQx (Snyderwine et al., 1995), Figure 7.

![Fig. 7 Structures of MeIQx metabolites characterized in monkeys.](image)

Gluc = Glucuronic acid. Snyderwine et al., 1995.

An *in vivo* study in humans with and without the use of the CYP1A2 inhibitor furafylline
showed that CYP1A2, in humans, accounts for 91% of the metabolism of MeIQx, most likely via N²-hydroxylation (Boobis et al., 1994).

An in vivo study of the metabolism of DiMeIQx in conventional and germ free rats indicated that the intestinal flora does not influence metabolism. Major metabolites were identified as 8-hydroxymethyl-DiMeIQx and its N²-acetylated derivative (Knize et al., 1989).

The Pyridine: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

Several studies have shown that PhIP is activated to a proximately mutagenic metabolite by rat hepatocytes and liver microsomes from rabbit, rat, mouse (Holme et al., 1989, McManus et al., 1989, Wallin et al., 1990, I, Frandsen et al., 1991, Buonarati et al., 1990a). The activated metabolite was identified as the N²-hydroxylated metabolite N²-OH-PhIP. The other major microsomal metabolite of PhIP was identified as the 4'-hydroxylated product (4'-OH-PhIP), which is a detoxification product with thousand times lower mutagenic potency than PhIP (Kurosaka et al., 1992). Figure 8. N²-OH-PhIP has also been shown to induce sister chromatid exchanges in CHO cells and DNA strand breaks in V79 cells (I, Frandsen et al., 1991, Holme et al.,1989). Rat and mouse CYP1A2 N²-hydroxylated PhIP at a high rate compared to other isozymes, and CYP1A1 detoxificated PhIP at a comparatively high rate (Wallin et al., 1990, McManus et al., 1990, Buonarati et al., 1991). Also human liver microsomes have a high capacity for CYP1A2 dependent activation of PhIP to N²-OH-PhIP, however the capacity of human microsomes to detoxify PhIP to 4'-OH-PhIP is extremely low compared to rodents. The ratio of N²-hydroxylation to 4'-hydroxylation was estimated to 97:1, 3,3:1, 1,7:1 in man, rat and mouse respectively (Davis et al., 1993a, Zhao et al., 1994, Lin et al., 1995).

Fig. 8 Structures of microsomal metabolites of PhIP. Wallin et al., 1990.
N'-OH-PhIP did not bind covalently to DNA, but addition of rat liver cytosol and PAPS, and to a much lesser extent acetyl-CoA, resulted in DNA adduct formation (Bounarati et al., 1990b). Human hepatic sulphotransferase activated N'-OH-PhIP to DNA binding metabolites by a factor of 3.3 to 12.9 more efficiently than rat liver cytosol (Ozawa et al., 1994). In one study with *Salmonella typhimurium* expressing human acetyltransferases it was concluded that neither NAT2 nor NAT1 could acetylate N'-OH-PhIP to an ultimate mutagenic metabolite (Wild et al., 1995). In other studies, however, using human microsomes and DNA binding as detection, NAT1 and to a larger extent NAT2 together with sulphotransferase and phosphotransferase were found to activate N'-OH-PhIP to a DNA binding metabolite (Turesky et al., 1991a, Chou et al., 1995, Lin et al., 1995).

The metabolism of PhIP *in vivo* has been studied in mice, rats and monkeys. Following oral administration to mice of 14C labelled PhIP, 31% of the radioactivity was recovered in the urine and 30% in the faeces after 24 hours. HPLC analysis of the urine demonstrated the presence of 11 metabolites (Turteltaub et al., 1989). In a study using doses equivalent to human dietary intakes, almost 100% of the dose was excreted during 96 hours, 90% in urine and 10% in faeces. Only 0.01 - 0.04% of the dose could be detected in the tissues 48 to 96 after exposure (Turteltaub et al., 1992). In the rat faeces is the major route of excretion, accounting for 78% of the dose after 24 hours, 51% was identified as unmetabolized PhIP (Watkins et al., 1991a). Several of the urinary and faecal metabolites have been isolated and characterized. 4'-PhIP sulphate and an N-glucuronide of N'-OH-PhIP are the major urinary metabolites (Alexander et al., 1989b, Alexander et al., 1991, Watkins et al., 1991b, Bounarati et al., 1992), Figure 9. One minor metabolite was identified as a conjugate of glutathione, where the sulphur atom of glutathione is attached directly to the imidazole ring in position 2. The presence of this conjugate indicates that PhIP has been oxidized to 2-nitro-PhIP, as 2-nitro-PhIP has the ability to react with the thiol groups of glutathione and rat serum albumin (Alexander et al., 1991, Reistad et al., 1994). A study in monkeys demonstrated the presence of 4 metabolites in urine, PhIP-4'-glucuronide, PhIP-4'-sulphate, 4'-hydroxy-PhIP and an N-glucuronide of N'-OH-PhIP. In faeces 4'-hydroxy-PhIP and PhIP were found. PhIP-4'-sulphate was the major urinary metabolite accounting for 64-72% of the dose (Snyderwine et al., 1993a).

*An recent in vivo* study in humans with and without the use of the CYP1A2 inhibitor
furafylline showed that CYP1A2 accounts for 70% of the metabolism of PhIP in humans, most likely via $N^2$-hydroxylation (Boobis et al., 1994).

Fig. 9 Structures of metabolites of PhIP identified in rat urine. Gluc = Glucuronic acid. Kaderlik et al., 1994.

**DNA adduct formation**

Adduct formation of $N^2$-hydroxylated metabolites of AIAs with deoxynucleosides and DNA *in vitro* has been studied for a number of compounds including IQ, MeIQ, MeIQx, DiMeIQx and PhIP. $N^2$-OH-IQ to a small extent binds covalently to calf thymus DNA at neutral pH. By reducing pH to 5 a minor increase in binding is observed. Addition of excess acetic anhydride to the reaction mixture, resulting in formation of the putative $N^2$-acetoxy derivative of IQ, dramatically increases DNA binding. $N^2$-acetoxy-IQ also reacts with
deoxyguanosine forming one major and one minor adduct. No reactions with deoxycytidine, deoxyadenosine or thymidine were observed. The major adduct was structurally characterized as $N^2$-(deoxyguanosin-8-yl)-IQ (dG-C8-IQ) (Snyderwine et al., 1988b, Turesky et al., 1992) and the minor adduct as 5-(deoxyguanosin-N$^2$-yl)-IQ (dG-N$^2$-IQ) (Turesky et al., 1992). The level of the dG-C8 adduct was approximately 8-10 times higher than the level of dG-N$^2$ adduct. HPLC analysis of DNA enzymatically digested after reaction with $^{14}$C-N$^2$-acetoxy-IQ showed that both adducts had been formed. dG-C8-IQ accounted for 70% of bound radioactivity and dG-N$^2$-IQ accounted for 4% of the bound radioactivity (Turesky et al., 1992). $^1$H-NMR spectroscopic analysis of the adducts revealed that the preferred conformation around the glycosidic bond was the anti form of the dG-N$^2$-IQ adduct and the syn form of the dG-C8-IQ adduct. It was suggested that this conformational difference may influence the persistence of the two adducts, as the dG-C8-IQ adduct is expected to induce greater distortion of the DNA structure than the dG-N$^2$-IQ adduct (Turesky et al., 1992), Figure 10.

![Fig. 10 Structures of DNA adducts of IQ. Turesky et al., 1992.](image)

Adduct formation of 4-MeIQ was studied by use of the $^{32}$P-postlabeling assay. $N^2$-OH-MeIQ was reacted with the four 2'-deoxynucleoside-3'-monophosphates in the presence of acetic anhydride and postlabeling analysis showed that only the guanine nucleotide had reacted forming one adduct. The adduct was structurally characterized as $N^2$-(deoxyguanosin-8-yl)-MeIQ-3',5'-diphosphate (pdGp-C8-MeIQ). Postlabeling analysis of DNA from livers of mice fed MeIQ, showed a single spot co-eluting with pdGp-C8-MeIQ demonstrating that MeIQ also binds to C-8 of guanine in vivo (Tada et al., 1994).
Like IQ, 8-MeIQx produced one major and one minor adduct with deoxyguanosine when \( N^2\)-OH-MeIQx was reacted with deoxynucleosides or DNA in the presence of acetic anhydride. The major adduct which was formed in 8-10 times higher amounts than the minor was characterized as \( N^2\)-deoxyguanosin-8-yl(MeIQx) (dG-C8-MeIQx). The minor adduct was characterized as 5-(deoxyguanosin-N\(^2\)-yl(MeIQx) (dG-N\(^2\)-MeIQx). \(^1\)H-NMR studies revealed that dG-C8-MeIQx has acquired a syn conformation around the glycosidic bond, whereas dG-N\(^2\)-MeIQx is in the anti conformation (Turesky et al., 1992), Figure 11. In a \(^32\)P-postlabeling study dG-C8-MeIQx was also detected as the major adduct formed when 3'-deoxyguanosine monophosphate reacted with \( N^2\)-OH-MeIQx in the presence of acetic anhydride. In liver DNA from rats given MeIQx intragastrically, dG-C8-MeIQx, was also the major adduct formed, together with some minor unidentified adducts (Ochiai et al., 1993).

Reaction of the putative \( N^2\)-acetoxy derivative of 4,8-DiMeIQx, formed by acetylation of \( N^2\)-OH-DiMeIQx, with deoxynucleosides resulted in the formation of only one adduct. The structure was characterized as \( N^2\)-(deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-DiMeIQx) and NMR studies showed that the adduct has a syn conformation around the glucosidic bond, Figure 12. Absence of formation of a minor adduct of 4,8-DiMeIQx, although, structurally similar to MeIQx was ascribed to the presence of the 4-methyl group, which will sterically hinder attack of C-5 of 4,8-DiMeIQx on guanine-N\(^2\). \( N^2\)-acetoxy-4,8-DiMeIQx also formed dG-C8-DiMeIQx as the major adduct with calf thymus DNA, together with some minor adducts. The minor adducts had UV spectra similar to dG-C8-4,8-DiMeIQx and could be hydrolysed to dG-C8-4,8-DiMeIQx by nuclease P1, indicating
that the minor adducts was incompletely digested oligomers. *In vivo*, dG-C8-DiMeIQx was also the major adduct formed in liver DNA from rats dosed with 4,8-DiMeIQx (IV, Frandsen et al., 1994b).

![dG-C8-DiMeIQx](image)

**Fig. 12** Structure of the DNA adduct of 4,8-DiMeIQx. IV, Frandsen et al., 1994b.

$N^2$-OH-PhIP, in contrast to $N^2$-hydroxylated metabolites of other AIA, does not react to any extent with DNA or nucleosides. Acetylation with acetic anhydride to form the $N^2$-acetoxy derivative increases the reactivity. However, this compound is sufficiently stable to allow isolation and characterization (II, Frandsen et al., 1992). $N^2$-acetoxy-PhIP reacts with deoxyguanosine and its 3’-phosphate, but not with other deoxynucleosides, forming one adduct that has been identified as $N^2$-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) and its 3’-phosphate (II, Frandsen et al., 1992, Lin et al., 1992, Nagaoka et al., 1992), Figure 13.

![dG-C8-PhIP](image)

**Fig. 13** Structure of the DNA adduct of PhIP. II, Frandsen et al., 1992.

$N^2$-acetoxy-PhIP also reacts with DNA forming an adduct that by HPLC following
enzymatic hydrolysis was identified as dG-C8-PhIP (II, Frandsen et al., 1992). $^{32}$P-postlabeling analysis of DNA modified in vitro also showed dG-C8-PhIP as the major adduct, together with some minor adducts (Lin et al., 1992). Analysis of DNA from rats given PhIP orally revealed the presence of dG-C8-PhIP as the major adduct formed in vivo (II, Frandsen et al., 1992, Lin et al., 1992).

**Mechanism of adduct formation**

The electrophilic metabolites of the AIAs generally formed major adducts at the C-8 position of guanine, only minor adducts of a few of the AIAs are found at the N2 position of guanine. The C-8 of guanine is only weekly nucleophilic and other alkylating or aralkylating agents tend to react with position N-7, O6 or N2 of guanine. It has been suggested that the formation of C-8 adducts of aromatic amines is a rearrangement product of an initially formed adduct with the more nucleophilic N-7 position of guanine. Evidence for this theory has been obtained by reacting N2-acetoxy-2-aminofluorene with deoxyguanosine, methylated in position C-8 to prevent rearrangement.

![Fig. 14 Sites of substitution of 2'-deoxyguanosine. I: Sites modified by alkylating agents. II: Sites modified by arylaminating agents. III: Sites modified by aralkylating agents. Dipple, 1995.](image-url)
The adduct obtained from this reaction was shown to have a structure where the N-7 position of guanine was attached to the exocyclic amino group of 2-aminofluorene (Humphreys et al., 1992). The evidence for initial adduct formation at the N-7 position of guanine, a common position for reaction with electrophilic alkylating agents, led to an attempt to make a general mechanistic explanation of the selectivity of adduct formation by alkylating, aralkylating and arylaminating ultimate electrophilic metabolites (Dipple, 1995).

Alkylating agents tend to react at the O6 and N-7 positions of guanine, arylaminating agent form major adducts at position C-8 of guanine (rearranged from N-7) and minor adducts at position N2 and aralkylating agents react almost exclusively at the N2 position of guanine, Figure 14.

The theory is based on model studies of the benzylation of guanosine, and the difference in product distribution was ascribed to differences in the reaction character, S1 or SN2, and ability of the developing ion to delocalize charge, Figure 15 (Moschel et al., 1979, Moschel et al., 1980, Moschel et al., 1986). The theory suggests that reaction through a SN2 mechanism results in formation of a N-7 guanine adduct, reaction through a SN1 mechanism will result in O6 adduct formation if the charge on the ionized intermediate is localized and in N2 adduct formation if the charge is delocalized.

\[
\begin{align*}
\text{SN2} & \quad RX + Y^- \rightarrow RY + X^- \\
\text{SN1} & \quad RX \rightarrow R^+ + X^- \rightarrow RY + X^- 
\end{align*}
\]

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig15.png}
\caption{Fig. 15.}
\end{figure}

However, the evidence for the character of the reactions is limited. The theory has recently been questioned as theoretical basis was provided that some of the carbonium ions which in the above theory are assumed to react by an SN1 mechanism can not possibly do so, because they are too unstable to exist (Loechler 1994).

The selectivity in adduct formation on guanine may instead be hypothesized by use of the concept of hard and soft nucleophiles/electrophiles, according to which hard electrophiles...
tend to react with hard nucleophiles and soft electrophiles tend to react with soft nucleophiles (Carey and Sundberg, 1990). Other factors that have to be taken into consideration are that the free energy of the products has to be lower than the free energy of the reactants and that steric hindrance may prevent formation of certain adducts. Using the hard-soft/nucleophile-electrophile concept, alkylating agents of the RCH₂X type, which are soft electrophiles, will react with soft nucleophiles, O⁶ and N-7 of guanine. Also, nitrenium ions derived from arylaminating agents are soft electrophiles and tend to react at positions O⁶ and N-7 of guanine (rearranging to a C-8 adduct). Some arylaminating agents also to a lesser extent react with N² of guanine, which is borderline between hardness and softness. Reaction at the N² position affords that the electrophile is able to delocalize charge into the aromatic ring system so reaction takes place via a harder carbon electrophile.

Carbonium ions derived from aralkylating agents are borderline between hard and soft electrophiles and will react with N² of guanine, Figure 14.

**DNA adduct formation in vivo**

Numerous studies have been conducted to study DNA adduct formation of heterocyclic aromatic amines *in vivo*.

IQ-DNA adducts have been detected by ³²P-postlabeling analysis in liver, heart, kidney, colon, stomach and bladder in rats after oral administration of IQ (Yamashita et al., 1988,Overvik et al., 1991, Snyderwine et al., 1988c). Several adducts were detected in each organ, including a dG-C8-IQ adduct. Total adduct levels were highest in liver followed by kidney, colon, stomach and bladder (Snyderwine et al., 1988c). Studies using multiple dosing (5 or 50 mg/kg/day) for up to 15 days have shown that accumulation of adducts in several organs occurred in a dose and time dependant manner (Schut et al., 1994). Also, ³²P-postlabeling analysis of liver DNA from monkeys dosed with IQ revealed that dG-C8-IQ is the major adduct formed in monkeys (Snyderwine et al., 1993b).

Several DNA adducts of 4-MeIQ have been detected in the liver of CDF₁ female mice fed 0.04% MeIQ in the diet. However, addition of nuclease P1 and phosphodiesterase digestion
to the $^{32}$P-postlabeling procedure reduced the number of adducts to a single spot, identified as the dG-C8-MeIQ adduct (Tada et al., 1994).

DNA-adduct formation was studied in mice given a single oral dose of $^{14}$C labelled 8-MeIQx. Adduct levels were higher after 6 hours than after 24 hours, with the liver having the highest adduct level followed by large intestine, stomach, spleen, small intestine, kidney and lung (Alldrick and Lutz, 1989). By use of accelerator mass spectrometry, it was shown that the adduct level in mouse liver was linearly related to the dose down to an exposure as low as 500 ng/kg bw, the lowest dose administered (Turteltaub et al., 1990).

$^{32}$P-Postlabeling analysis of DNA from various organs of rats treated with 20 mg/kg bw/day, 5 days per week for two weeks, showed highest adduct level in the liver followed by kidney, heart and colon (Davis et al., 1993a). A study of the dose-response relationship in adduct formation in liver DNA of rats receiving 0.04, 0.4, 4, 40 and 400 ppm in the diet for one week revealed a linear response in adduct formation. Continued dosing at the highest level for 61 weeks resulted in induction of hepatocellular carcinomas (Yamashita et al., 1990). The structure of the major adduct formed in the rat in vivo was identical to the major adduct formed in vitro, dG-C8-MeIQx (Ochiai et al., 1993). Also in monkeys dG-C8-MeIQx was the major adduct found in liver DNA following treatment with MeIQx. The adduct level, however, was much lower than the adduct level found in rats treated at a comparable dose level (Snyderwine et al., 1993b, Davis et al., 1993a).

Several DNA adducts of 4,8-DiMeIQx were detected by $^{32}$P-postlabeling analysis in rat liver DNA following oral administration of 4,8-DiMeIQx (Yamashita et al., 1988). By comparison with a synthetic standard the major adduct formed in vivo, accounting for 60-70% of the bound carcinogen, was identified as dG-C8-DiMeIQx (IV, Frandsen et al., 1994b).

DNA adduct formation of PhIP has been studied in various organs of mice, rats, monkeys and humans.

$^{32}$P-Postlabeling analysis of DNA from mice given a single oral dose of PhIP at 0, 1, 10, and 20 mg/kg bw showed highest adduct level in pancreas followed by thymus, heart and liver. The adduct level increased linearly with dose (Turteltaub et al., 1993). Also in rats...
given a single oral dose of 0, 0.5, 5 and 50 mg/kg bw a dose related increase in adduct level was observed. The highest adduct levels were found in the large intestine followed by white blood cells, stomach, small intestine, kidney, liver and lung (Schut and Herzog, 1992). Studies in monkeys receiving either a single or multiple oral doses of 20 mg/kg bw showed that, upon multiple dosing, DNA adducts tend to accumulate in certain organs. Adduct levels in liver, pancreas, kidney, small intestine and colon increased 1.5 to 2.4 fold after multiple dosing for 10 days, adduct level in brain increased 5 fold, heart 10 fold and aorta 31 fold (Snyderwine et al., 1994).

The structure of the major adduct found in rat liver DNA after a single oral dose of PhIP was shown by chromatographic comparison to be identical to the adduct formed in vitro, dG-C8-PhIP (II, Frandsen et al., 1992, Lin et al., 1992).

In a pilot study with human DNA samples, using both GC/MS and 32P-postlabeling analysis, evidence for PhIP-DNA adducts was found in two of six colon samples. PhIP-DNA adducts were not detected in samples from pancreas or urinary bladder (Friesen et al., 1994).

32P-Postlabeling analyses of DNA adducted with AIA compounds have resulted in considerable interlaboratory variation, with up to six adduct spots being detected for each compound. However, inclusion of nuclease P1 digestion in the postlabeling analysis was recently shown to reduce the number of adduct spots to one or two, in accordance with the adducts characterized after in vitro formation. The additional adduct spots usually detected in 32P-postlabeling analysis are suggested to be adducted oligonucleosides that are resistant to hydrolysis by micrococcal nuclease or spleen phosphodiesterase (Pfau et al., 1994, Fukutome et al., 1994).

**Estimates of human intake and cancer risk**

The concentration of heterocyclic aromatic amines (HAA) in cooked meat and fish is in the ppb range and large variations in concentration depending on meat type, frying temperature and frying time have been observed. This together with interindividual consumption habits will markedly influence the daily exposure of individual humans to heterocyclic aromatic amines.
An average daily intake has been estimated to 0.4 - 16 µg for the Japanese population (Wakabayashi et al., 1992). For the German population the average daily intake was estimated to 0.8 - 8.4 µg based on an annual consumption of 56 kg of meat and 5 kg of fish (Eisenbrand and Tang, 1993). Based on an annual consumption of 56 kg of meat and 9 kg of fish the intake was calculated to be 0 - 16.5 µg per day in the Danish population (Dragsted, 1994). By combining the concentrations of heterocyclic aromatic amines found in foods cooked under normal household conditions with a dietary survey of the U.S. population, daily intakes of PhIP, MeIQx, DiMeIQx and IQ have been estimated to 16.64, 2.61, 0.81 and 0.28 µg respectively (Layton et al., 1995).

Cancer risks associated with intake of HAAs have been estimated by combining the above intake estimates with the TD$_{50}$ from animal tumour studies, mostly in rats, assuming a linear dose-response relationship. The assumption of linear dose-response relationship has been supported by linearity in DNA binding over a dose range covering 5 orders of magnitude down to a single dose of 500 ng/kg of MeIQx (Felton et al., 1992). The published cancer risk estimates associated with HAA ingestion vary from 1 in 1000 (Felton et al., 1992) to approximately 1 in 10,000 (Gaylor and Kadlubar, 1991, Layton et al., 1995). A total lifetime cancer risk interval of 0.2 - 2300 per million individuals has been estimated for the danish population (Dragsted, 1994). In a risk assessment covering five HAA's: PhIP, MeIQx, DiMeIQx, IQ and AcC, ingestion of PhIP accounted for almost 50% of the cancer risk (Layton et al., 1995).

**Conclusion**

Several heterocyclic aromatic amines of the AIA type are formed during cooking of meat and are found in amounts ranging from "not detected" to more than 80 µg/kg. The most important factors for the formation of the these compounds, both on the meat surface and in the pan residue, are the type of meat being cooked, cooking temperature and duration of cooking.

The most important of the AIA compounds, both due to the amount formed and due to possible biological effects, seem to be IQ, 4-MeIQ, 8-MeIQx, 4,8-DiMeIQx and PhIP. These AIA compounds are genotoxic in a number of test systems including bacterial and
mammalian systems. Metabolic activation is required for these compounds to exert their mutagenic effect and hydroxylation of the exocyclic amino group has been shown to generate the proximate mutagenic metabolite. Further activation by transferases such as acetyltransferase, sulphotransferase, phosphotransferase or aminoacyltransferase to generate DNA binding metabolites are usually required.

IQ, MeIQ, MeIQx and PhIP were found to be multiple organ carcinogens in oral feeding studies in rodents. Also in monkeys IQ was found to be a liver carcinogen. In most of these studies, however, adequate data to provide a dose response relationship were not obtained (Munro et al., 1993). Furthermore, many of the experiments were terminated before the expected life span of the animals (Gold et al., 1994). Risk extrapolation to low dose human exposure, which is a factor of 100,000 less than the doses used in animal experiments, therefore is associated with great uncertainty.

Based on animal carcinogenicity data and estimated exposures the human lifetime cancer risk from ingestion of AIA compounds has recently been estimated within the range of 1-38 per 100,000 (Layton et al., 1995). In animals, however, metabolic transformation to non-genotoxic metabolites seems to be the major metabolic pathway, whereas in humans CYP1A2 mediated transformation to genotoxic metabolites is the major metabolic pathway. Assessment of human cancer risk based on animal bioassays may therefore result in an underestimation.

Large interindividual variations are found among humans in the activity of enzymes involved in activation and detoxification of chemical carcinogens. This results in differences in DNA adduct formation between individuals. Also rates of repair of DNA damage caused by chemical carcinogens vary among individuals (Harris, 1989). Large interspecies differences in metabolism are found, e.g. the ratio of phase I activation (N\(^2\)-hydroxylation) of PhIP to phase I detoxification (4\(^{\prime}\)-hydroxylation) has been estimated to 97:1 in man, whereas in the rat, which is the species often used in cancer bioassay studies, the ratio has been estimated to 3.3:1. Also large interspecies variations in phase II activation are observed, the degree of activation depending on the substrate, tissue and species (Lin et al., 1995; Davis et al., 1993b).

Future developments of molecular biomarkers reflecting the exposure or the biological response to dietary exposures to AIA compounds, e.g. DNA adducts in urine, may provide
the means of identifying the most sensitive individuals. Also information obtained from studies on molecular biomarkers in humans and in animals from cancer bioassay studies can provide data for more accurate estimations of human cancer risk (Shuker and Farmer, 1992, Strickland and Groopman, 1995).

The results from own investigations have elucidated the structures of genotoxic metabolites and DNA adducts of PhIP and 4,8-DiMeIQx. These results provide a good basis for future development of molecular biomarkers based on measurement of DNA adducts, that can improve risk assessment of human exposure to these chemicals.
References


Boobis, A.R., Lynch, A.M., Murray, S., de la Torre, R., Solans, A., Farre', M., Segura, J., Gooderham, N.J., and Davies, D.S. (1994) CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate...
carcinogens is their major route of metabolism in humans. *Cancer Research*, 54, 89-94.


Frandsen, H., Grivas, S., Andersson, R., Dragsted, L. and Larsen, J.C. (1992) Reaction of the N\textsuperscript{2}-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N\textsuperscript{2}-(2'-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis*, 13, 629-635.


Kanai, Y., Wada, O. and Manabe, S. (1990) Detection of carcinogenic glutamic acid pyrolysis products in


the liver of rats fed MeIQx. *Carcinogenesis*. 14, 2165-2170.


51


PAPER I
Metabolic formation, synthesis and genotoxicity of the N-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP)

Henrik Frandsen, Eva S. Rasmussen, Preben A. Nielsen, Peter Farmer, Lars Dragsted and John C. Larsen

Institute of Toxicology, National Food Agency, Markvej Bygade 19, DK-2860 Søborg, Denmark and 1The Medical Research Council, Carshalton, Surrey, UK

Hepatic microsomes from rats pretreated with PCB were found to metabolize the food mutagen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) to two major metabolites, one of which was identified as the N-hydroxy derivative, 2-hydroxy-2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (N-OH-PhIP). This identification was based on mass spectral (MS), UV and HPLC data by comparison with chemically synthesized N-OH-PhIP, including mass spectral (MS), UV and HPLC data as well as the specific mutagenic activity in the Ames test. Moreover the NMR spectrum of synthetic N-OH-PhIP as well as its specific mutagenicity in the SCE assay are presented.

Materials and methods

Chemicals and apparatus

PhIP was kindly donated by Dr James Felton and Mark Knize, Lawrence Livermore National Laboratory, USA. NO₂-PhIP was 90% pure by HPLC (t₁/₂ 136 min). 2-Amino-4'-hydroxy-1-methyl-6-phenylimidazo(4,5-b)pyridine (4'-hydroxy-PhIP) was kindly donated by Dr Ian Alexander, National Institute of Public Health, Oslo, Norway. Nicotinamide-adenine dinucleotide-phosphate, Trisodium-iso-isoctate and Tris (hydroxymethyl)aminomethane were purchased from Sigma, St Louis, MO, USA. HPLC grade methanol was obtained from Rathburn, Walkerburn, UK. C-18 Sep-Pak columns were obtained from Waters, Milford, MA, USA. All other chemicals were obtained from Merck, Darmstadt, FRG, and were of analytical purity, except palladium on carbon and hydrazine hydrate which were of reagent grade. The Salmonella tester strain TA98 was kindly provided by Professor Bruce N. Ames, University of California, Berkeley, CA, USA. CHO cells (CHO Ki, no. 03-403-B) were from Flow, Rockmansworth, UK. UV spectra were recorded on a Shimadzu UV-160 double beam spectrophotometer. Mass spectra of the electron impact (EI) mode were recorded on a VG 70-250 at an electron energy of 70 eV. Positive fast atom bombardment (FAB) mass spectra were recorded at 8 kV using xenon as neutral beam and glycerol as matrix. HPLC was performed on a Hewlett-Packard model 1084 B liquid chromatograph. For analytical purposes a 250 × 4.6 mm Lichrosorb RP-18, 5 μm column (Merck, Darmstadt, FRG). flow 1 ml/min was used. For preparative purposes a 300 × 7.6 mm Lichrosorb RP-18, 5 μm column, flow 2.8 ml/min was used. Solvents: A 50 mM ammonium acetate; B, methanol. For both analytical and preparative purposes the following solvent programme was used: 10% B for 5 min followed by a linear gradient to 60% B at 25 min, isocratic from 25 to 35 min and a linear gradient to 100% B at 40 min. Plasma spray liquid chromatography (LC) MS was performed on a VG Trios-3 coupled with a Waters 600 multisolvent delivery system. The solvents were 20 mM ammonium acetate and 20 mM ammonium acetate in 90/10 acetonitrile. The same column, flow and solvent programming as in analytical HPLC were used. NMR spectra were obtained in the solvents specified with a Varian VXR-400 FT NMR spectrometer. The oxygen electrode was from pHOX Systems Ltd, Ivel Road, Shefford, UK.

Synthesis of 2-nitro-1-methyl-6-phenylimidazo[4,5-b]pyridine (NO₂-PhIP)

PhIP (5.2 mg) was dissolved in a mixture of 0.5 ml phosphoric acid (85%) and 0.2 ml sulphuric acid. The solution was cooled on ice-water and four 5 μl portions of 2 M sodium nitrite were added during a 2 min period under vigorous stirring. The mixture was held at room temperature and after stirring for 25 min extracted twice with 25 ml of ethylacetate. The organic phase was washed with water, dried with magnesium sulphate and evaporated to dryness. The residue was dissolved in 0.5 ml of methanol and NO₂-PhIP was obtained as yellow crystals after addition of 1 ml of water and cooling. Yield 2.6 mg (44.5%). The NO₂-PhIP was judged > 95% pure by HPLC (254 nm). Mass spectrum (EI) m/z 254 (25%), M+ 225 (100%). UV absorption maximum in methanol was at 350 nm. The molar extinction coefficient was estimated to 11200 M⁻¹ cm⁻¹.

Synthesis of N-OH-PhIP

A reaction tube containing 5 mg of 10% Pd/C was evacuated for 5 min and filled with argon. After addition of 2 ml of methanol the suspension was purged with oxygen free argon for 5 min. The suspension was cooled on ice-water and 10 μl of hydrazine hydrate (80%) was added under stirring. Stirring was continued for 30 min at 0°C before a solution of 2.6 mg of NO₂-PhIP in 2 ml of argon-purged

© Oxford University Press

Introduction

During cooking of meat several hetero-aromatic amine mutagens, imidazoazarenes (AIA), are formed (Felton et al., 1986a,b; Nielsen et al., 1988). When frying at high temperatures, PhIP on a weight basis accounts for ~80% of the mass mutagenic activity in the Ames Salmonella test. Moreover the NMR spectrum of synthetic N-OH-PhIP as well as its specific mutagenicity in the SCE assay are presented.

Materials and methods

Chemicals and apparatus

PhIP was kindly donated by Dr James Felton and Mark Knize, Lawrence Livermore National Laboratory, USA. NO₂-PhIP was 90% pure by HPLC (t₁/₂ 136 min). 2-Amino-4'-hydroxy-1-methyl-6-phenylimidazo(4,5-b)pyridine (4'-hydroxy-PhIP) was kindly donated by Dr Ian Alexander, National Institute of Public Health, Oslo, Norway. Nicotinamide-adenine dinucleotide-phosphate, Trisodium-iso-isoctate and Tris (hydroxymethyl)aminomethane were purchased from Sigma, St Louis, MO, USA. HPLC grade methanol was obtained from Rathburn, Walkerburn, UK. C-18 Sep-Pak columns were obtained from Waters, Milford, MA, USA. All other chemicals were obtained from Merck, Darmstadt, FRG, and were of analytical purity, except palladium on carbon and hydrazine hydrate which were of reagent grade. The Salmonella tester strain TA98 was kindly provided by Professor Bruce N. Ames, University of California, Berkeley, CA, USA. CHO cells (CHO Ki, no. 03-403-B) were from Flow, Rockmansworth, UK. UV spectra were recorded on a Shimadzu UV-160 double beam spectrophotometer. Mass spectra of the electron impact (EI) mode were recorded on a VG 70-250 at an electron energy of 70 eV. Positive fast atom bombardment (FAB) mass spectra were recorded at 8 kV using xenon as neutral beam and glycerol as matrix. HPLC was performed on a Hewlett-Packard model 1084 B liquid chromatograph. For analytical purposes a 250 × 4.6 mm Lichrosorb RP-18, 5 μm column (Merck, Darmstadt, FRG). flow 1 ml/min was used. For preparative purposes a 300 × 7.6 mm Lichrosorb RP-18, 5 μm column, flow 2.8 ml/min was used. Solvents: A 50 mM ammonium acetate; B, methanol. For both analytical and preparative purposes the following solvent programme was used: 10% B for 5 min followed by a linear gradient to 60% B at 25 min, isocratic from 25 to 35 min and a linear gradient to 100% B at 40 min. Plasma spray liquid chromatography (LC) MS was performed on a VG Trios-3 coupled with a Waters 600 multisolvent delivery system. The solvents were 20 mM ammonium acetate and 20 mM ammonium acetate in 90/10 acetonitrile. The same column, flow and solvent programming as in analytical HPLC were used. NMR spectra were obtained in the solvents specified with a Varian VXR-400 FT NMR spectrometer. The oxygen electrode was from pHOX Systems Ltd, Ivel Road, Shefford, UK.

Synthesis of 2-nitro-1-methyl-6-phenylimidazo[4,5-b]pyridine (NO₂-PhIP)

PhIP (5.2 mg) was dissolved in a mixture of 0.5 ml phosphoric acid (85%) and 0.2 ml sulphuric acid. The solution was cooled on ice-water and four 5 μl portions of 2 M sodium nitrite were added during a 2 min period under vigorous stirring. The mixture was held at room temperature and after stirring for 25 min extracted twice with 25 ml of ethylacetate. The organic phase was washed with water, dried with magnesium sulphate and evaporated to dryness. The residue was dissolved in 0.5 ml of methanol and NO₂-PhIP was obtained as yellow crystals after addition of 1 ml of water and cooling. Yield 2.6 mg (44.5%). The NO₂-PhIP was judged > 95% pure by HPLC (254 nm). Mass spectrum (EI) m/z 254 (25%), M+ 225 (100%). UV absorption maximum in methanol was at 350 nm. The molar extinction coefficient was estimated to 11200 M⁻¹ cm⁻¹.

Synthesis of N-OH-PhIP

A reaction tube containing 5 mg of 10% Pd/C was evacuated for 5 min and filled with argon. After addition of 2 ml of methanol the suspension was purged with oxygen free argon for 5 min. The suspension was cooled on ice-water and 10 μl of hydrazine hydrate (80%) was added under stirring. Stirring was continued for 30 min at 0°C before a solution of 2.6 mg of NO₂-PhIP in 2 ml of argon-purged

© Oxford University Press

93
methanol was added. After 10 min stirring at 0°C, 8 ml of cold, argon-purged methanol was added, and after centrifugation the supernatant was isolated and evaporated to dryness. The oily residue was dissolved in 0.3 ml of methanol and crystals of 
N-OH-PhIP were obtained after addition of 0.7 ml of water and cooling. Yield 1.4 mg (57%). The 1H-NMR spectrum (270 MHz) contained peaks at δ 7.41 (M+H)− and 125. UV absorption maximum in methanol at 310 nm. The molar extinction coefficient was estimated to 1500 M−1 cm−1.

**Synthesis of 2-(phenoxyl)aminomethyl-6-(phenoxyl)aminomethyl-5-(hydroxylamino) derivative**

To a solution of 100 μg 
N-OH-PhIP in 0.5 ml argon-purged dimethylformamide was added 1 mg nitrosoanisole and 20 μl acetic acid. After 18 h at room temperature, the solution was evaporated to dryness and dissolved in 0.5 ml acetonitrile and analysed by LC-MS.

**Preparation of microsomes**

Adult male Wistar rats (age 7–8 weeks, weight >200 g), delivered from Møllebøg Breeding Center Ltd., Lille Skensved, Denmark, were used to prepare PCB-induced hepatic microsomes. PCB (Aroclor 1254, 500 mg/kg, dissolved in corn oil) was injected i.p. 3 days before sacrifice and microsomes were prepared essentially as described by Lake (1987). The microsomes were stored in liquid nitrogen until use. The microsomes were characterized by measuring the concentration of protein (Sedmark and Grossberg, 1977), of P450 (Lake, 1987): 3.1 nmol/mg protein and of benzpyrene hydroxylase activity (Yamazoe et al., 1981): 2-hydroxy-biphenyl: 3.8 nmole formed/mg protein min, 4-hydroxy-biphenyl: 1.5 nmol formed/mg protein min.

**Microsomal activation of PhIP**

The incubation mixture consisted of: 1 mg/ml microsomal protein, 1 unit/ml 5% methanol was added. After 10 min stirring at 0°C, 8 ml of cold, argon-purged dimethylformamide was added. After 2 min the test compound, dissolved in 0.1 ml of methanol, was added. After 2 min the test compound, dissolved in 0.1 ml of methanol, and 0.1 ml of a 7 h nutrient broth (Ovoid) culture of the test bacteria (1.0 × 109 CFU/ml) as negative. 2-Nitrofluorene was tested without S9 mix at a concentration of 0.1 μg/plate giving 176 ± 76 revertants/plate and 2-aminoanthraquinone was tested at a concentration of 0.1 μg/plate with S9 mix giving 114 ± 32 revertants/plate. The specific mutagenic activity (revertants/nmol) was calculated by linear regression analysis.

**Results**

Nitro- and hydroxyamino derivatives of PhIP were synthesized from PhIP (Figure 1) by modifications of the procedures used by Yamazoe et al. (1988) for the synthesis of the corresponding derivatives of 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQ). Based on HPLC areas at 254 nm the nitro-derivative (NO2-PhIP) was obtained in ~80% yield. The structure of NO2-PhIP was verified by 254 and ions at 225, M+ -NCH3 and 209, M+ -O-NCH3. The 1H-NMR spectrum of NO2-PhIP in D2O dimethyl sulfoxide (DMSO) showed a singlet at 5.42 (N CH3), a triplet at δ 7.5 (H-4'), a triplet at δ 7.6 (H-3' and H-5'), a doublet at δ 7.9 (H-2' and H-6'), a singlet at δ 8.7 (H-7) and a singlet at δ 9.0 (H-5).

In the crude reaction mixture a by-product was detected in 20%
yield on HPLC. The by-product was purified by preparative HPLC and assumed to be 2-hydroxy-1-methyl-6-phenylimidazo(4,5-b)pyridine (OH-PhIP) (MS, EI m/z =225, M⁺ not shown) formed by attack on water on the intermediate diazonium ion or/and by hydrolysis of NO₂-PhIP. NO₂-PhIP was unstable and was, especially in slightly acidic solution, converted to OH-PhIP (Figure 1).

The NO₂-PhIP was reduced to N-OH-PhIP (Figure 1) as described in Materials and methods. This compound was very sensitive to oxygen especially when dissolved in organic solvents. A methanolic solution slowly turned red, possibly due to formation of the azoxy-derivative of PhIP.

The positive FAB mass spectrum of N-OH-PhIP showed the expected molecular ion at 241, (M + H)⁺ and an ion at 225 (not shown). The ¹H-NMR spectrum (Figure 4) showed a singlet at δ 3.7 (N-CH₃), a doublet δ 7.5 (H-4'), a triplet at δ 7.6 (H-3' and H-5'), a doublet at δ 7.7 (H-2' and H-6'), and two doublets at δ 8.3 and 8.4 (H-7 and H-5). NH and OH protons are not seen due to exchange with deuterium.

Further confirmation that the N-OH-PhIP had been correctly synthesized was achieved through the synthesis of the phenylazoxy derivative (Hashimoto et al., 1982). N-OH-PhIP reacted slowly with nitrosobenzene in acetic DMF. The reaction was followed on HPLC and as the N-OH-PhIP peak diminished a new peak arose. This product was purified by HPLC but a mass spectrum containing the molecular ion could not be obtained neither by EI nor FAB MS. However analysis of the crude reaction mixture by plasma spray LC MS showed the expected molecular ion at 330, (M + H)⁺ for the phenylazoxy derivative and an ion at 314, (M + H)⁺-O (Figure 5).

Two major metabolites were observed when PhIP was incubated with hepatic microsomes from rats pretreated with PCB, see Figure 6. As seen from Figure 7, the time course of the formation of the two metabolites was almost linear for 30 min when ~80% of the added PhIP had been consumed. The two metabolites were purified by preparative HPLC. One of the metabolites (peak b) was identified as N-OH-PhIP, as it gave the same mass and UV spectra as synthetic N-OH-PhIP and coeluted with this in HPLC. The fraction containing the N-OH-PhIP peak was the only fraction showing reactivity in the assay for N-hydroxylated amines by Belanger et al. (1981) and it was mutagenic in the Ames tester strain TA98 without metabolic activation system. Mutagenic activity without metabolic activation system was not detected when the remainder HPLC fractions were pooled and subjected to analysis. The other metabolite (peak a) was characterised by its MS spectrum showing 241, (M + H)⁺, but no 225 peak as N-OH-PhIP. On HPLC it co-eluted with a reference sample of 4'-hydroxy-PhIP kindly donated.
Fig. 4. 400 MHz \textsuperscript{1}H-NMR spectrum of N-OH-PhIP, dissolved in D$_2$O made acidic to pH 4 with DCl. Signals at \(\delta\) 2.1, 4.8 and 8.2 originate from the solvent. Inset: the aromatic region enlarged. Proton assignment: singlet at \(\delta\) 3.7 (N-CH$_3$), doublet \(\delta\) 7.5 (H-1''), triplet at \(\delta\) 7.6 (H-3' and H-5''), doublet at \(\delta\) 7.7 (H-2' and H-6''), and two doublets at \(\delta\) 8.3 and 8.4 (H-7 and H-5). NH and OH protons are not seen due to exchange with deuterium.

by Alexander et al. (1989), indicating the identity of this compound.

N-OH-PhIP purified from either microsomal incubations or prepared by chemical synthesis was tested separately for mutagenic activity in the Ames tester strain TA98 without metabolic activation, and almost linear increases in the formation of mutant colonies with dose were observed. Two different preparations of synthesized and metabolically formed N-OH-PhIP were tested in duplicates and the specific mutagenic activities were calculated by linear regression analysis and found to be 2796 ± 237 (mean ± SD, \(r = 0.96\)) revertants induced per nanomol of the metabolically formed N-OH-PhIP and 2638 ± 158 (mean ± SD, \(r = 0.98\)) revertants induced per nanomol of synthetic N-OH-PhIP.

These results were obtained with the addition of 1 mM dithiothreitol prior to the addition of the test compound. If dithiothreitol was omitted from the incubation mixture N-OH-PhIP induced only 180 ± 52 (mean ± SD) revertants/nmol. If dithiothreitol was substituted with a metabolic activation system the specific mutagenic activity was 1970 ± 100 (mean ± SD) revertants/nmol.

The results of the SCE assays are presented in Figure 8. No significant differences between replica cultures and between experiments were found for PhIP with metabolic activation. The same apply to N-OH-PhIP without activation. For both substances the lowest concentration tested caused a significant increase in SCE (N-OH-PhIP: 0.51 nmol/ml, \(P < 0.01\); PhIP: 2.67 nmol/ml, \(P < 0.01\)). Significant dose dependent increases in SCE (linear trend, \(P < 0.01\)) were found in all experiments. N-OH-PhIP and PhIP induced 0.062 and 0.022 SCE/ chromosome/nmol added to the media respectively. At the highest doses tested N-OH-PhIP and PhIP induced 13 and 19% mitotic inhibition, respectively. The positive controls benzof[al]pyrene with metabolic activation and Mitomycin C without also significantly increasing the number of SCE.

The oxygen concentrations in the SCE assay incubations mixtures was measured to 8.3 mg/ml without metabolic activation system, to 6.1 mg/ml with metabolic activation system and to 3.3 mg/ml with the addition of 0.5 mM dithiothreitol.

Discussion

Several of the heteroaromatic amines formed during frying of meat have been shown to be biotransformed into direct acting bacterial mutagens by hydroxylation of the exocyclic aminogroup (Kadlubar and Beland, 1985; Snyderwine et al., 1987; Yamazoe...
Formation, synthesis and genotoxicity of N-OH-PhIP

Fig. 6. HPLC analysis of microsomal PhIP metabolites after 30 min incubation. Peak a is identified as 4'-hydroxy-PhIP. Peak b is N-OH-PhIP. Peak c is PhIP. Peak d was also present in a control incubation not containing PhIP.

Fig. 7. Time course of formation of the two major microsomal PhIP metabolites. Each point represents the peak area, to the various incubations times (minutes), measured at 254 nm in HPLC analysis. - - N-OH-PhIP. *- - 4'-hydroxy-PhIP.

et al., 1988; Holme et al., 1989; Wallin et al., 1990). Holme et al. (1989) and Turteltaub et al. (1990) have recently reported that PhIP is transformed by hepatocytes from PCB-treated rats or by microsomes from 3-methylcholanthrene treated mice to the directly mutagenic N-hydroxy derivative. Furthermore PhIP induced SCE in V79 cells when co-cultured with hepatocytes from PCB treated rats. The N-hydroxy derivative was tentatively identified by its reactivity in a colorimetric assay for N-hydroxylated amines and by co-elution in HPLC and identity of UV spectra with N-OH-PhIP (Holme et al., 1989) or by co-elution in HPLC with synthetic N-OH-PhIP (Turteltaub et al., 1990).

The present study describes a detailed method for synthesis of the nitro and N-hydroxy derivatives of PhIP and provides final evidence that PhIP, in accordance with the previously published results by Wallin et al. (1990) and Turteltaub et al. (1990), is metabolized by PCB-induced hepatic microsomes to N-OH-PhIP, and that this compound is mutagenic in S. typhimurium TA98 without metabolic activation. The structure is confirmed by comparing MS, UV and HPLC data on the N-OH-PhIP purified from microsomal incubations with data on the synthetic N-OH-PhIP. Furthermore we have characterized the synthetic N-OH-

PhIP by NMR spectroscopy and by synthesis of the phenylazoxy derivative. The HPLC fraction containing the microsomal formed N-OH-PhIP shows, as the only fraction, reactivity in the assay for N-hydroxylated amines, in analogy with the results obtained with synthetic N-OH-PhIP. This further confirms the structure and we are also able to show that the specific mutagenic activities in S. typhimurium of microsomal formed and synthetic N-OH-PhIP are identical. The synthetic N-OH-PhIP was found to be directly genotoxic to CHO cells. In this assay N-OH-PhIP induced SCE with a specific activity three times higher than PhIP, the latter with metabolic activation. This result clearly indicates that N-OH-PhIP is a proximate mutagenic metabolite in eukaryotic cells as well as in bacteria.

As N-OH-PhIP is readily oxidized, we found it necessary to add an antioxidant to the test mixtures for genotoxic activity, in order to obtain reproducible results. If the antioxidant was omitted from the incubation mixture, large variations were observed in the specific mutagenic/genotoxic activities obtained from different experiments. N-OH-PhIP has about four times higher specific mutagenic activity in the Ames Salmonella assay than PhIP, after addition of a metabolic activation system. This is in agreement with that PhIP is only partially metabolized to N-OH-PhIP. When the metabolic activation system is substituted with 1 mM diethiothreitol the specific mutagenic activity of N-OH-PhIP is six times higher than the specific mutagenic activity of PhIP after metabolic activation. The lower oxygen content in assay mixtures containing diethiothreitol than in assay mixtures containing a microsomal activation system can, by improving the stability of N-OH-PhIP, account for this higher mutagenic activity of N-OH-PhIP. Addition of antioxidants has previously been shown to be beneficial in the testing of readily oxidized compounds (Yamazoe et al., 1983). Addition of diethiothreitol to a solution of N-OH-PhIP did not change the HPLC retention time of N-OH-PhIP and diethiothreitol did not induce SCE in CHO cells or His" revertants in the Ames test. Thus diethiothreitol does not seem to react with N-OH-PhIP but only to exert a protective action toward oxidation.

Mutagens/carcinogens exert their action through reactions with DNA. In a preliminary experiment, we were not able to detect any reaction between DNA and N-OH-PhIP, when incubated for several days at room temperature under oxygen free conditions. Although N-hydroxylation seems to be a necessary metabolic step in the mutagenicity of PhIP, further activation is apparently
needed for reaction with DNA. Studies have shown that aryl hydroxylamines are very weak mutagens in an acetyl-CoA-dependent O-acetyltransferase-deficient strain of S. typhimurium (TA98 1.8-DNP) (Saito et al., 1983). Other studies indicate that sulphonylation or acylation of 2-hydroxymamine-3-methylimidazo[4.5-f]quinoline (N-hydroxy-IQ) by bacterial O-acetyltransferase or sulphotransferase enhance the reactivity of N-hydroxy-IQ towards DNA 5- to 30-fold (Snyderwine et al., 1988). Therefore, N-OH-PhIP may need further activation by sulphonation of the N-hydroxy group, improving the leaving group capability of the hydroxy group and facilitating the formation of a reactive nitrene or nitrenium ion. Such reactive intermediates have been generated by photolysis of the arylamide of 2-amino-3-methylimidazo[4.5-f]quinoline (IQ) and was shown to react with DNA (Wild et al., 1989).

Acknowledgements

The authors wish to thank Rolf Andersson and Kjell Olsson, Swedish University of Agricultural Sciences for performing 1H-NMR analysis. Ahmed Biichen for EI technical assistance. This work was in part supported by grant no. 75 from Gluver, Bo Lund Jensen, Jonna Madsen and Vivian Jorgensen for excellent Carlshalton, Surrey, UK, for assistance with FAB mass spectrometry. and Joan Acknowledgements

References


Received on August 15, 1990; accepted on October 8, 1990.
PAPER II
Reaction of the N²-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N²(2'-deoxyguanosin-8-yl)-PhIP

Henrik Frandsen, Spiros Grivas, Rolf Andersson, Lars Dragsted and John C. Larsen

Institute of Toxicology, National Food Agency, Markvej Bygade 19, DK-2860 Seborg, Denmark and 1Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, S-750 07 Uppsala, Sweden

The direct acting mutagenic N²-hydroxylated metabolite of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) does not react with DNA. Upon acetylation of the N²-hydroxy-PhIP with acetic anhydride two products could be detected. Mass spectrometric analysis showed that both products were monoacetyl derivatives of N²-hydroxy-PhIP. One of the products did not show any reactivity towards DNA and is probably the N-acetyl derivative of N²-hydroxy-PhIP. The other product which is most likely to be N²-acetoxy-PhIP reacted with DNA and 2'-deoxyguanosine but not with 2'-deoxycytidine, 2'-deoxyadenosine or 2'-deoxymethylidine. The PhIP-2'-deoxyguanosine adduct was purified and characterized by mass spectrometric (H and [¹³C]NMR analysis, showing that PhIP like the other cooked food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline, had reacted with C-8 of guanine forming N²(2'-deoxyguanosin-8-yl)-PhIP. HPLC analysis of enzymatically hydrolyzed calf thymus DNA that had been reacted with N²-acetoxy-PhIP showed one adduct which was chromatographically and spectroscopically identical to N²(2'-deoxyguanosin-8-yl)-PhIP. HPLC separation followed by liquid scintillation counting of hydrolyzed liver DNA from a rat dosed with [³H]PhIP showed that radioactivity coeluted with C-8 of guanine forming N²(2'-deoxyguanosin-8-yl)-PhIP, indicating that PhIP in vivo also forms an N²(2'-deoxyguanosin-8-yl)-PhIP adduct.

Introduction

The heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP*) is an indirect mutagenic compound formed during frying of meat (1-3). PhIP, which constitutes ~80% of the mass of mutagenic material in beef fried at high temperatures, has been shown to induce sister chromatid exchanges (SCES) in mammalian cells (4-6). PhIP has also been shown to induce lymphomas in mice (7). to induce intestinal adenocarcinomas in rats (8), to produce DNA adducts in various organs (9) and has recently been found to induce aberrant crypt foci in the large intestine of rats (10).

*Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SCES, sister chromatid exchanges; N²-OH-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine; N²-OAc-PhIP, 3-acectoxyamino-PhIP; N²(2'-deoxyguanosin-8-yl)-PhIP; N²(2'-deoxyguanosin-8-yl)-PhIP; PCE, polychlorinated biphenyl; FAB, fast atom bombardment; BEs, N,N-bis(2-hydroxyethyl)ethanol sallonic acid; DMF, dimethylformamide; DMSO, dimethylsulfoxide; N,N'-Ac,N,N'-OAc-PhIP, N,N-acetyldihydroxyamino-PhIP, 2-OIH-PhIP, 2-hydroxy-1-methyl-6-phenylimidazo[4,5-b]pyridine; DEPT, distortionless enhancement by polarization transfer; N²-OH-Iq, 2-hydroxyimidazo[4,5-b]imidazo[4,5-f]quinoline; Glu-P1, 2-amino-6-methylimidazo[4,5-f]quinoline; TTP-P2, 3-amino-1-methyl-5H-pyrido[1,2,3-]indole

PhIP is metabolically activated by hepatocytes or hepatic microsomes, from rat, mouse and rabbit, to the proximate mutagenic N²-hydroxy derivative (5,6,11-13), but further activation is apparently needed for the formation of an ultimate mutagenic metabolite, which is reactive towards DNA (6,12). Previous studies have shown that 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N²-OH-PhIP) can bind covalently to DNA after further activation with cytosolic O-acetyltransferase or O-sulfotransferase or acetic anhydride (14,15). While PhIP, by [³H]postlabeling, has been shown to form adducts with DNA in vivo and in vitro, the DNA adducts have not previously been characterized. In this study N²(2'-deoxyguanosin-8-yl)-PhIP (N²(2'-deoxyguanosin-8-yl)-PhIP) was synthesized and was shown by HPLC, combined with diode array detection or liquid scintillation counting, to be the major adduct formed, in vitro upon reaction between DNA and 2-acetylaminoimidazo[4,5-b]pyridine (N²-OAc-PhIP) and in vivo in the rat after dosing with [³H]PhIP.

Materials and methods

Chemicals and instrumentation

PhIP was kindly donated by Dr Torfi Ljeger, Cellular and Genetic Toxicology Branch, NIEHS, North Carolina. Non-specifically labeled [³H]PhIP was made from PhIP by Amersham. UK. The exact position of tritium in the [³H]PhIP is not known. The [³H]PhIP was adsorbed on a SEP-PAK and washed with 0.1 M HCl to remove exchangeable tritium, and eluted with acetic anhydride for purification on HPLC. The sp. act. was 21.1 Ci/mmol and the radiochemical purity was >99%. N²-OH-PhIP was synthesized as previously described (6). Calf thymus DNA, 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxymethylidine and nuclease P1 (EC 3.1.30.2) from Penicilium citrinum were obtained from Sigma, St Louis, MO. Alkaline phosphatase (Escherichia coli C75) was obtained from Amersham, UK. Polychlorinated biphenyl (PCB) (Anocor 1254) was obtained from Monsanto Industrial Chemical Co., St Louis, MO. C₁₃ SEP-PAK columns were obtained from Waters, Milford, MA. HPLC grade acetonitrile was obtained from Rathburn, Walkerburn, UK. silicone gel 60 (70-230 mesh), silicone TLC plates and all other chemicals were obtained from Merck, Darmstadt, Germany.

Evaporation of solvents under reduced pressure was accomplished with a Büchi rotary evaporator. HPLC analyses were performed with either a Hewlett Packard model 1090 B liquid chromatograph or a Hewlett Packard 1000 M liquid chromatograph equipped with a photo-diode array detector. Nuclear HPLC columns were obtained from HPLC technology, Macclesfield Cheshire, UK. Spectrophotometer HPLC columns were obtained from Phase Separation, Desselde Ind. Est., Queensferry, Clwyd, UK. Positive fast atom bombardment (FAB) mass spectra were recorded at 8 keV using xenon as neutral beam and glycerol or glycerol/4-toluenesulfonic acid as matrix. Positive ion thermospray LC-MS was performed on a Kratos Profile mass spectrometer equipped with a thermospray interface. The solvent was 50% 0.1 M ammonium acetate pH 4.5 in acetonitrile, flow 1 ml/min, source temperature 73°C. NMR spectra were recorded with a Varian VXR-400 FT spectrometer with DMSO-d₆ as solvent. Radioactivity was measured with a Packard Tri-Carb 460 CD liquid scintillation counter with external standardization.

Acetylation of 2'-deoxyguanosine

660 µg of N²-OH-PhIP (2.75 amol) was dissolved in 0.5 ml argon purged 50% dimethylformamide in water and cooled to 0°C. Three times 1 µl of acetic anhydride was added during 10 min with stirring. After a further 10 min stirring at 0°C, acetylation of the DNA adducts have not previous
and evaporated to dryness without heating on a rotary evaporator and analyzed by preparative TLC mass spectrometry. One major acetylation product, peak B, tentatively identified as N'-OAc-PhIP. (Figure 1) was obtained when 5 μl acetic acid was added prior to the addition of acetic anhydride. This methodology was used in the rest of this work.

**Reaction of N'-OAc-PhIP with 2'-deoxynucleosides and purification of N'-[(2'-O-acetyl-β-D-ribofuranosyl)-N'-nitroso]pyrrolo[2,3-b]indole (N'-OAc-PhIP)

2'-Deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and 2'-deoxythymidine were dissolved at a concentration of 1 mg/ml in 0.1 M Na-acetate (pH 4.5) and 0.1 M BES/NaOH pH 7.4. Two ml of each solution and a control not containing 2'-deoxyguanosine were placed in test tubes and heated to 37°C. One hundred μl of the crude acetylation mixture were added dropwise, during 2 min, to each of the vigorously stirred test tubes. After stirring for a further 15 min the reaction mixtures were analyzed by HPLC.

Since only reaction between N'-OAc-PhIP and 2'-deoxyguanosine gave a product which was not present in the control incubation, this reaction was scaled up by a factor of 5.

The crude product from acetylation of 660 μg of N'-OH-PhIP was added, during 10 min, to a well-stirred solution of 5 mg 2'-deoxyguanosine in 10 ml 0.1 M BES/NaOH pH 7.4 at 37°C. In the crude reaction product it was found that ~50% of the N'-OAc-PhIP had reacted with 2'-deoxyguanosine. After a further 10 min of stirring the precipitate was collected, washed with water and dried. The residue was taken up in 5 ml 20% DMF in chloroform and applied on a 1.6 x 9 cm column of silica gel equilibrated with DMF:chloroform, 1:4 v/v. After washing with this solvent system the adduct was eluted with DMF:chloroform, 1:1 v/v and evaporated to dryness. The yield of the adduct from N'-OH-PhIP was 31%. The yield of adduct was calculated on the basis of experiments where [3H]-N'-OH-PhIP was used.

The synthesis was repeated several times until enough material for mass spectral and NMR analyses had been collected.

**Binding of N'-OAc-PhIP to DNA

The crude product from acetylation of 500 μg of N'-OH-PhIP was added, during 10 min, to a well-stirred solution of 4 mg calf thymus DNA in 4 ml 0.1 M BES/NaOH pH 7.4. After a further 10 min of stirring the DNA solution was precipitated by sodium acetate addition of 2 vol ice-cold ethanol, collected, dried and redissolved in 4 ml water. The DNA solution was extracted twice with 4 ml of dichloromethane, to remove non-covalently bound reaction products and the DNA was precipitated by addition of 100 μl 3 M sodium acetate and 2 vol ice-cold ethanol.

**Enzymatic hydrolysis of PhIP-DNA

The synthesis was repeated several times until enough material for mass spectral and NMR analyses had been collected.

**Modification of DNA in vivo

One adult male Wistar rat (8 weeks, weight 250 g), delivered from Møllegård Breeding Center Ltd, Lille Skensved, Denmark, was injected i.p. with 200 mg PCB/kg (Aroclor 1254, dissolved in corn oil). After 5 days the rat was starved overnight and dosed by gavage with [3H]-PhIP (1 μCi/μg, 21 Ci/mol, dissolved in 70% ethanol). The animal was killed after 2 h and the liver removed. The liver was homogenized and the DNA was isolated by the phenol/chloroform extraction procedure previously described (17). The 30 μg DNA obtained was dissolved in 15 ml of 0.1 M hydrochloric acid and hydrolyzed by heating to 100°C for 1 h. The DNA hydrolysate was loaded onto a C18 SEP-PAK, which had been washed with acetonitrile and water. After washing with 3 vol of water, the SEP-PAK was eluted with 3 ml of 10% ammonium formate pH 3.5 in acetonitrile. The eluate was evaporated to dryness and the residue was resuspended in 250 μl of 80% acetonitrile and separated by HPLC from which one fraction was collected and analyzed by liquid scintillation counting. Synthetic N'-[(2'-O-acetyl-β-D-ribofuranosyl)-N'-nitroso(pyrazol-5-yl)]pyrrolo[2,3-b]indole, N'-OAc-PhIP, and calf thymus DNA reacted with N'-OAc-PhIP were likewise hydrolyzed in 0.1 M hydrochloric acid for comparison with the rat liver DNA. The hydrolysate product from [3H]-PhIP-DNA was purified on a SEP-PAK. Liquid scintillation analysis showed that only background level of radioactivity was present in the water wash. 98% of the applied radioactivity was recovered in the acetonitrile eluate. The eluate was analyzed by HPLC and TLC (15% acetic acid, 5% methanol in chloroform).
quite unstable and rapidly decomposed when stored at room temperature at neutral pH. The stability of product B was improved at acidic pH. Both products were purified by HPLC and analyzed by positive FAB mass spectrometry (spectra not shown). Both peaks A and B showed a molecular ion, (M+H)\(^+\), at m/z 283 and a daughter ion at 241 (loss of ketene). The mass spectra shows that both products are monoacetyl derivatives of N\(^2\)-OH-PhIP. The chemical reactivities of the two compounds, relative to N\(^2\)-OH-PhIP, indicate that A, which does not react with DNA or 2'-deoxynucleosides, is N\(_2\),N\(_2\)-acetylhydroxyamino-PhIP (N\(^2\)-Ac-N\(^2\)-OH-PhIP) and that peak B, which does react with both DNA and 2'-deoxynucleosines, is N\(^2\)-OAc PhIP (18). The relative yields of the two products could be varied by changing the reaction conditions. Product A was exclusively observed when pyridine was added to the reaction mixture, while addition of acetic acid resulted in formation of product B, tentatively identified as N\(^2\)-OAc-PhIP, as the major product.

Reaction of N\(^2\)-OAc-PhIP with 2'-deoxynucleosides. Synthesis of N\(^2\)-OAc-PhIP reacted with 2'-deoxygenosine in neutral solution, whereas addition of N\(^2\)-OAc-PhIP to solutions of 2'-deoxyadenosine, 2'-deoxycytidine, and 2'-deoxythymidine only resulted in products which were also present in a control incubation without containing 2'-deoxynucleoside. Figure 2 shows a chromatogram of the crude product from reaction of N\(^2\)-OAc-PhIP and 2'-deoxyguanosine. Compound B eluting at 11.5 min is the putative N\(^2\)-Ac-N\(^2\)-OH-PhIP and compound D eluting at 14 min is 2-OH-PhIP, which is a minor impurity originating from the synthesis of the N\(^2\)-OH-PhIP. An unidentified compound (peak, A), with high absorption at 360 nm, eluted at ~11 min. The area of this peak was much larger when 2'-deoxyguanosine was not present in the reaction mixture, and this compound might be a decomposition product of N\(^2\)-OAc-PhIP. The adduct, which eluted at 12.5 min, precipitated quantitatively out of the solution and was further purified by silica gel chromatography, which removed a red colored impurity, that did not elute from the HPLC column. The UV spectrum of the adduct shows maxima at 364.5, 270.5 and 238.5 nm (Figure 3). The positive ion thermospray mass spectrum of the purified product showed a molecular ion at 490 (M+H)\(^+\) (Figure 4), which is consistent with an adduct between PhIP and 2'-deoxyguanosine. As shown in Figure 5, its [\(^1\)H]NMR spectrum in DMSO-d\(_6\) revealed the presence of all sugar protons, the two pyridine protons (H-5 and H-7), the five benzene protons (H-2', H-6'), the 2'-amino group of the base and of the N-Me group of PhIP. However, the characteristic C-8 proton of guanine was not present. This observation is a strong indication that the nitrogen of the 2-amino group of PhIP was attached to the C-8 of 2'-deoxyguanosine. That was confirmed through \([\^13\)C\]NMR spectroscopy where all 21 carbon atoms could be observed, and those with a single H bound to them were enhanced in the distortionless enhancement by polarization transfer (DEPT) experiment (see Table I). Again, the absence of a \([\^13\)C\]signal from guanines C-9 when the DEPT technique was employed confirms that PhIP is bound to this carbon atom. These results from the [\(\^1\)H\] and [\(\^13\)C\]NMR spectroscopy taken together with the thermospray mass spectrum corroborate that the structure of the adduct is N\(^2\)-(2'-dG-8-yl)-PhIP. The reaction scheme is depicted in Figure 6.

![Fig. 3. UV spectrum of N\(^2\)-(2'-dG-8-yl)-PhIP obtained with a photodiode array detector at the elution conditions from the HPLC column (see Figure 2).](image)

![Fig. 4. Positive ion thermospray mass spectrum of N\(^2\)-(2'-dG-8-yl)-PhIP showing the molecular ion (M+H)\(^+\) at m/z = 490 and a daughter ion at m/z = 374, loss of deoxyribose. Solvent: 50% 0.1 M ammonium acetate pH 4.5 in acetonitrile, flow 1 ml/min, source temperature 73°C.](image)

**Table I.** \([\^13\)C\]NMR chemical shifts of N\(^2\)-(2'-dG-8-yl)-PhIP and PhIP

<table>
<thead>
<tr>
<th>N(^2)-(2'-dG-8-yl)-PhIP</th>
<th>PhIP</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>154.29</td>
<td>157.98</td>
<td>C-3a</td>
</tr>
<tr>
<td>152.89</td>
<td>156.29</td>
<td>C-2</td>
</tr>
<tr>
<td>152.74</td>
<td></td>
<td>C-6(^a)</td>
</tr>
<tr>
<td>149.74</td>
<td></td>
<td>C-2(^a)</td>
</tr>
<tr>
<td>148.95</td>
<td></td>
<td>C-4(^a)</td>
</tr>
<tr>
<td>146.11</td>
<td></td>
<td>C-8(^a)</td>
</tr>
<tr>
<td>139.48</td>
<td>139.29</td>
<td>C-5</td>
</tr>
<tr>
<td>137.98</td>
<td>139.03</td>
<td>C-1(^i)</td>
</tr>
<tr>
<td>129.93</td>
<td>127.87</td>
<td>C-7a</td>
</tr>
<tr>
<td>128.97(^*)</td>
<td>128.86</td>
<td>C-3(^i),5(^i)</td>
</tr>
<tr>
<td>127.34(^*)</td>
<td>126.56</td>
<td>C-4(^i)</td>
</tr>
<tr>
<td>126.79(^*)</td>
<td>126.43</td>
<td>C-2(^i),6</td>
</tr>
<tr>
<td>125.88</td>
<td>126.22</td>
<td>C-6</td>
</tr>
<tr>
<td>113.42(^*)</td>
<td>111.63</td>
<td>C-7(^i)</td>
</tr>
<tr>
<td>108.66</td>
<td></td>
<td>C-5(^i)</td>
</tr>
<tr>
<td>87.37(^*)</td>
<td></td>
<td>C-4(^i),7</td>
</tr>
<tr>
<td>82.05(^*)</td>
<td></td>
<td>C-1(^i),7</td>
</tr>
<tr>
<td>71.32(^*)</td>
<td></td>
<td>C-3(^i),8</td>
</tr>
<tr>
<td>62.34</td>
<td></td>
<td>C-5(^i),7</td>
</tr>
<tr>
<td>38.87</td>
<td></td>
<td>C-2(^i),6</td>
</tr>
<tr>
<td>28.11</td>
<td>28.40</td>
<td>N-Me</td>
</tr>
</tbody>
</table>

\([\^13\)C\]NMR chemical shifts of N\(^2\)-(2'-dG-8-yl)-PhIP at 20°C, referenced to the solvent \([\^6\)DMSO-d\(_6\) 39.50). Assignments for PhIP are based on two-dimensional experiments and for N\(^2\)-(2'-dG-8-yl)-PhIP on comparisons to the chemical shifts of 2'-deoxyguanosine in DMSO-d\(_6\), and to those of PhIP. Values marked with an asterisk are those peaks enhanced in the DEPT experiment.
H.Frandsen et al.

Reaction scheme.

Reaction of N\textsuperscript{2}-OAc-PhIP with calf thymus DNA

N\textsuperscript{2}-OAc-PhIP reacted with calf thymus DNA giving a covalently bound PhIP residue, which could not be removed by repeated precipitations, solvent extractions or dialysis against 20% ethanol in water. The PhIP–DNA adduct was hydrolyzed enzymatically with nuclease Pl and analyzed by HPLC. Figure 7 shows the chromatograms monitored at 260 and 360 nm of the hydrolyzed PhIP–DNA adduct. Peaks A–D are the four nucleosides: 2′-deoxycytidine, 2′-deoxyguanosine, 2′-deoxythymidine and 2′-deoxyadenosine respectively. Peak E coeluted with synthetic N\textsuperscript{2}-(2′-dG-8-yl)-PhIP and had an identical UV spectrum. Any other possible adducts between PhIP and nucleosides were not detected to any appreciable extent, when the HPLC was monitored at 260 nm with enhanced sensitivity. HPLC analysis of an enzyme digest of [3H]PhIP–DNA followed by liquid scintillation counting of collected fractions showed that 50% of the applied radioactivity coeluted with N\textsuperscript{2}-(2′-dG-8-yl)-PhIP. The remaining 50% of the applied radioactivity did not elute from the HPLC column. The small bump eluting at ~19.5 min may represent incompletely hydrolyzed DNA fragments bound to PhIP, as this bump was much larger and the adduct peak much smaller when hydrolysis was carried out at lower temperature (37°C). The adduct was stable under the hydrolysis conditions. Attempts to hydrolyze the PhIP–DNA and liberate the adducts with DNase 1, phosphodiesterase and alkaline phosphatase (37°C) were not successful.

Modification of DNA in vivo

Liver DNA from a rat dosed with [3H]PhIP was isolated and hydrolyzed in dilute hydrochloric acid. The hydrolysate was separated by HPLC and fractions were collected and analyzed by liquid scintillation counting. N\textsuperscript{2}-(2′-dG-8-yl)-PhIP and the PhIP–DNA adduct, made in vitro, were hydrolyzed similarly for comparison. The results are shown in Figure 8. Panel 1 shows the HPLC profile of the acid hydrolysate of the N\textsuperscript{2}-(2′-dG-8-yl)-PhIP adduct. The hydrolyzed adduct eluted at 12 min. Panels 2 and 3 show the HPLC profile of the acid hydrolyzed PhIP–DNA adduct formed in vitro, monitored at 360 and 254 nm respectively. A product eluting at the same retention time as in panel 1 and showing an identical UV spectrum confirms the identity of the adduct formed from N\textsuperscript{2}-OAc-PhIP and DNA to be N\textsuperscript{2}-(2′-dG-8-yl)-PhIP. The small peak eluting just before the hydrolyzed adduct peak is also present, but to a lesser extent in panel 1. This peak grew larger when acid hydrolysis was carried out for a prolonged time. This early eluting peak may thus represent a further degradation product of the adduct. Panel 3 also shows that N\textsuperscript{2}-(2′-dG-8-yl)-PhIP is the major adduct formed in DNA. This is further confirmed in panel 4 showing...
PhIP reactions with 2′-deoxyguanosine

**Fig. 7.** Chromatograms of the enzymatically hydrolyzed PhIP–DNA adduct analyzed on a Spherisorb C18, 3 μm, 150 x 4.6 mm. Flow rate: 0.8 ml/min. Solvents: I, 50 mM ammonium acetate pH 4.5; II, acetonitrile. Elution programming: isocratic 2% II by 1 min, linear gradient to 10% II by 13 min, linear gradient to 40% II by 17 min, isocratic 40% II by 20 min and a linear gradient to 80% II by 25 min. Peaks A–D are 2′-deoxycytidine, 2′-deoxyguanosine, 2′-deoxythymidine and 2′-deoxyadenosine respectively. Peak E is N2-(2′-deoxy-8-yl)-PhIP.

**Fig. 8.** HPLC profiles of N2-(2′-deoxy-8-yl)-PhIP and the PhIP–DNA adduct subjected to acid hydrolysis. Panel 1: hydrolysate of N2-(2′-deoxy-8-yl)-PhIP. Panel 2 and 3: hydrolysate of the in vitro formed PhIP–DNA adduct monitored at 360 and 254 nm. Peaks at 4 and 6 min in panel 3 originate from the calf thymus DNA. Panel 4: hydrolysate of [3H]PhIP–DNA formed in vivo. Fractions were collected at 1 min intervals and radioactivity was measured by liquid scintillation counting. Panel 5: hydrolysate of liver DNA from a rat given [3H]PhIP p.o. Fractions were collected by 1 min intervals and radioactivity was measured by liquid scintillation counting.

**Fig. 9.** Decay of N2-acetoxy-PhIP at 0°C versus time. +: 2′-deoxyguanosine present in the incubation mixture. ■: 2′-deoxyguanosine not present in the incubation mixture.

The results from the liquid scintillation counting on fractions from the HPLC separation of the SEP-PAK eluate of hydrolyzed [3H]PhIP–DNA. 60% of the applied radioactivity coeluted with the hydrolysis product of N2-(2′-deoxy-8-yl)-PhIP. The remaining 40% of the radioactivity did not elute from the HPLC column. TLC analysis of the SEP-PAK eluate followed by liquid scintillation counting of collected spots showed that the radioactivity that did not elute from the HPLC column coeluted with a product which was also formed when N2-acetoxy-PhIP was added to a buffer not containing 2′-deoxyguanosine. This byproduct from the reaction is probably azo-PhIP, formed by dimerization of two nitrenes. This byproduct seems to intercalate strongly into the DNA. Panel 5 shows the results from the liquid scintillation counting on fractions from the HPLC separation of hydrolyzed liver DNA from a rat dosed with [3H]PhIP. The radioactivity eluting at 12 min indicates that PhIP, in vivo, also binds covalently to C-8 of guanine.

**Kinetics**

Results from the investigations of the reaction kinetics of 0°C, where the reaction between N2-OAc-PhIP and 2′-deoxyguanosine was sufficiently slow to be followed by HPLC is shown in Figure 9. The decay of N2-OAc-PhIP showed a linear relation of −ln(C/C0) versus time, regardless of whether 2′-deoxyguanosine was present or not. As no solvolysis product (N2-acetoxy-PhIP) was detected, this linear relation indicates that the rate was first order with respect to N2-OAc-PhIP and zero order with respect to 2′-deoxyguanosine.

**Discussion**

The proximate mutagenic N2-hydroxylated metabolite of the food mutagen PhIP (N2-OH-PhIP) did not bind covalently to DNA or 2′-deoxyguanosine, neither at neutral pH nor at slightly acidic pH. Acidic pH usually increases the binding of arylhydroxylamines e.g. 2-hydroxyamino-3-methylimidazo[4,5-f]quinoline (N2-OH-IQ) to DNA, since protonation of the hydroxyl group facilitates the formation of a reactive nitrene. The fact that acidic pH does not increase the degree of binding of N2-OH-PhIP to DNA, indicates that the heterolytic cleavage of the N2-O bond proceeds less readily in N2-OH-PhIP than in N2-OH-IQ.

Covalent binding of N2-hydroxylated amines to DNA is generally considered to proceed via activation by cytosolic acyl- or sulfo transferases and the subsequent formation of reactive nitrenes or nitrenium ions (20). Formation of the presumed N-acetoxy derivatives of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (21), 2-amino-6-methylidipyrido[1,2-a:3',2'-d]imidazole (Glut-P-1) (22) and 3-amino-1-methyl-SH-pyrido[4,3-b]indole (Trp-P-2) (23), formed by reaction with acetic anhydride or ketene, have previously been shown to lead to reaction with DNA.
and 2'-deoxyguanosine. After acetylation of N²-OH-PhIP to N²-OAc-PhIP, which enhances the heterolytic cleavage of the N-O bond, N²-OH-PhIP reacted with both DNA and 2'-deoxyguanosine in high yields. Isolation of N-acetoxy-arylamines are usually precluded by their chemical instability (18), but the relative stability of the N-O bond in N²-OAc-PhIP made possible its isolation and mass spectrometric characterization of this still rather reactive compound.

The highest yields of adducts were obtained when N²-OAc-PhIP was added slowly to a 37°C solution of DNA or 2'-deoxyguanosine. The slow addition of N²-OAc-PhIP combined with its rate of reaction keeps the ratio between nucleophile DNA or nucleoside and electrophile high, whereby formation of side reaction products from the putative nitrene intermediate is kept low. One of these side reaction products is probably azo-PhIP, formed by dimerization of two nitrenes, which is a common reaction of nitrenes (24).

Analysis of the hydrolysis product of DNA, modified by N²-OAc-PhIP in vitro, indicated that N²-OH-PhIP had mainly bound to the guanine of DNA, and since the nucleoside – PhIP adduct, on HPLC, coeluted with and had an identical UV spectrum as synthetic N²-(2'-dG-yl)-PhIP, the major adduct formed in vitro thus appeared to be N²-(2'-dG-8-yl)-PhIP.

PhIP also bound to liver DNA in vivo, upon dosing a rat with [³H]PhIP. Since part of the eluted radioactivity which had bound covalently to DNA, upon acid hydrolysis coeluted with the hydrolysis product of synthetic N²-(2'-dG-8-yl)-PhIP and PhIP–DNA made in vitro, N²-(2'-dG-8-yl)-PhIP also appeared to be formed in vivo. It cannot be excluded that other nucleoside – PhIP adducts are formed in vivo.

Binding of the exocyclic amino group to the C-8 position of guanine seems to be the most common site of reaction of activated heterocyclic aromatic amines with DNA. N²-OH-PhIP, N²-OH-Glu-P-1 and N²-OH-Trp-P-2 have formerly been shown to bind to DNA in a similar way, after activation with acetic anhydride or ketene.

The yield of adduct from N²-OH-PhIP was 2.5% when N²-OAc-PhIP was reacted with DNA and 50% when N²-OAc-PhIP was reacted with 2'-deoxyguanosine. This suggests that intercalation of N²-OAc-PhIP into the DNA strand is not an important step in the binding of this N-acetoxy derivative to DNA. This is in contrast to findings with N²-OH-PhIP and N²-OH-Glu-P-1, where intercalation was suggested to be of importance in the DNA binding of these substances (21, 22). In these studies N²-OAc-PhIP was generated in situ, by addition of acetic anhydride to a mixture of DNA and N²-OH-PhIP (21), and N²-OAc-Glu-P-1 was reacted with DNA at low temperature (22). The relatively low yield of adduct obtained when N²-OAc-PhIP was reacted with DNA compared to that with 2'-deoxyguanosine indicates that the DNA structures reduces binding of N²-OAc-PhIP to guanine, either by steric hindrance or by reduction of the nucleophilicity of guanine, due to hydrogen bonding.

The fast decomposition of N²-OAc-PhIP at physiological conditions, 37°C and pH 7.4, prevented kinetic investigations of the reaction between N²-OAc-PhIP and 2'-deoxyguanosine. Investigations of the kinetics at 0°C, where the reaction between N²-OAc-PhIP and 2'-deoxyguanosine was sufficiently slow to be followed by HPLC, indicated that the rate was first order with respect to N²-OAc-PhIP and zero order with respect to 2'-deoxyguanosine. This is in accordance with an SN1 mechanism, where heterolytic fission of the N-O bond in N²-OAc-PhIP leads to the formation of a reactive nitrene or nitrenium ion.

From the results of this study it can be concluded that the proximate mutagenic N²-hydroxylated metabolite of PhIP can be activated by acetylation and that the resulting N²-OAc-PhIP reacts effectively with 2'-deoxyguanosine and DNA, resulting in the formation of N²-(2'-dG-8-yl)-PhIP.

[³H]Postlabeling analyses of DNA from various organs from rats dosed with PhIP, have recently been shown to result in high adduct levels in lung, pancreas and heart, but lower levels in the liver (9). The liver is usually the primary target organ for DNA-binding of heterocyclic amines with an exocyclic amino group. It can be speculated that the relatively higher stability of N²-OH-PhIP, as compared to N-hydroxy derivatives of other heterocyclic aromatic amines, may explain this different mode of action, as PhIP upon phase I activation to N²-OH-PhIP is sufficiently stable to allow its distribution throughout the whole organism. Adduct levels in the various organs may thus depend on their activity of phase II enzymes, and their DNA repair capacity in the various organs.

Acknowledgements

The authors wish to thank Peter Farmer, the Medical Research Council, Carshalton, Surrey, UK, for FAB mass spectrometric measurements. Katsa analytical, Manchester, UK, for thermospray mass spectrometric measurements. Joan Oliver and Bettina Lyngsoe Hansen for excellent technical assistance and Bo Lund Jensen for graphical work. This work was in part supported by grant no. 75 from Nor- Diska kontaktorganet for fornidskraftsning.

References


Received on September 16, 1991; revised on December 19, 1991; accepted on January 6, 1992.
Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline to mutagenic metabolites

Henrik Frandsen, Preben A. Nielsen, Spiros Grivas and John C. Larsen

Institute of Toxicology, National Food Agency, Markhaug Bygade 19, DK-2860 Seborg, Denmark and Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7016, Uppsala, Sweden

Heterocyclic aromatic amines are formed in the crust of meat during ordinary cooking. These aromatic amines are potent bacterial mutagens and also potent rodent carcinogens. 2-Amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (DiMeIQx) is one of the more abundant heterocyclic aromatic amines, accounting for ~20% of the mutagenic material found in cooked meat. DiMeIQx is metabolically activated, by hepatic microsomes from PCB treated rats, to two major and three minor metabolites. One major and one minor metabolite were identified as 2-hydroxyamino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline and 3,4,8-trimethyl-2-nitro-3H-imidazo[4,5-f]quinoline, respectively, confirmed by comparison of HPLC retention times, and UV and mass spectra of synthetic standards. Both metabolites were mutagenic in Salmonella typhimurium TA98 without metabolic activation. The other major metabolite was identified as 2-amino-8-hydroxymethyl-3,4-dimethyl-3H-imidazo[4,5-f]quinoline by mass and NMR spectral analysis. The two remaining minor metabolites were identified as the 2-hydroxyamino- and 2-nitro- derivatives of 2-amino-8-hydroxymethyl-3,4-dimethyl-3H-imidazo[4,5-f]quinoline by UV and mass spectral analysis. Both of these metabolites were mutagenic in S. typhimurium TA98 without metabolic activation.

Introduction

During frying of meat several mutagenic heteroaromatic amines of the aminimidazoazacarrene (AIA) type are formed in the meat crust. Several of these AIA compounds have been shown to be carcinogenic in rats and mice when administered in the diet, producing tumors in a variety of organs (Wakabayashi et al., 1992).

A recent epidemiological investigation has indicated an increased risk of colorectal cancer associated with high meat intake and in particular with preference for browning of the meat surface (Gerhardsson de Verdier et al., 1991). As AIA compounds are present in the meat surface they may be involved in the etiology of human cancer.

2-Amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (DiMeIQx) is a potent bacterial mutagen which has been identified in beef extracts (Takahashi et al., 1985), in smoked dried bonito (Kikugawa et al., 1986), in fried ground beef (Knize et al., 1989). In order to further clarify the mutagenic activation of DiMeIQx we have studied the in vitro metabolism and identified five metabolites four of which were found to be mutagenic in the Salmonella strain TA98.

Materials and methods

Chemicals

DiMeIQx and [2-14C]DiMeIQx were synthesized as previously described (Grivas, 1985). DiMeIQx was determined to have >95% purity by HPLC with detection at 250 nm. [2-14C]DiMeIQx was determined to have >92% radiochemical purity by HPLC with radioactivity detection. Isocitric dehydrogenase (from porcine heart), nicotinamide-adenine-dinucleotide-phosphate, trisodium-dl-isocitrate and 3-(N-morpholino)propanesulfonic acid (MOPS) were obtained from Sigma (St Louis, MO), PCB (Aroclor 1254) was obtained from Monsanto (St Louis, MO). HPLC grade acetonitrile was obtained from Rathburn (Aberdeen, FRG) and were of analytical purity, except palladium on carbon (Pd/C) and hydrazine hydrate which were of reagent grade. The Salmonella tester strain TA98 and TA98NR were kindly provided by Professor Bruce N.Ames (University of California, Berkeley, CA) and Professor Herbert S.Rosenkranz (University of Fribourg, PA), respectively.

Synthesis of 3,4,8-trimethyl-2-nitro-3H-imidazo[4,5-f]quinoline (DiMeNOQx)

DiMeIQx, 11 mg, was suspended in 1 ml of 10% tetrafluoroboric acid, cooled on ice-water and 30 ml of 2 M sodium nitrite was added dropwise under stirring. The solution was stirred for 20 min at 0°C. The diazotization mixture was added dropwise to an ice-water cooled solution of 2.3 g sodium nitrite in 15 ml of water. The pH of the reaction mixture was maintained at 4.5 ± 0.1 by addition of first tetrafluoroboric acid and then 2 M sodium hydroxide. The mixture was allowed to warm to room temperature and, after stirring for 25 min, the mixture was neutralized with 2 M sodium hydroxide and extracted twice with 25 ml of ethyl acetate. The organic phase was dried with magnesium sulfate and evaporated to dryness. The product was purified by silica gel chromatography (Kieselgel 60, 1.6 × 9 cm, 2% methanol in ethyl acetate) and evaporated to dryness. Yield 10 mg (80%). The purity was shown to be >98% by HPLC (323 nm).

Synthesis of 2-hydroxyamino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (DiMeAOQx)

A reaction tube containing 20 mg of 10% Pd/C was evacuated for 5 min and filled with argon. After addition of 3 ml of tert-butylhydroperoxide (THF) the suspension was purged with argon for 2 min. The suspension was cooled on ice-water and
Fig. 1. [2-14C]DiMeIQx and DiMeIQx were incubated for 40 minutes with hepatic microsomes from Wistar rats pretreated with PCB followed by HPLC analysis. Panel 1 and 2: HPLC profiles of metabolites of [2-14C]DiMeIQx obtained with both an on-line radioactivity detector and a UV detector showing that five metabolites could be detected. Panel 3–5 Metabolites of DiMeIQx were separated on HPLC, and 0.5 min fractions were collected and tested for mutagenic activity in TA98 without metabolic activation (panel 3), TA98 with S9 (panel 4) and in the nitroreductase deficient strain TA98NR without metabolic activation (panel 5). The experiment was conducted twice with identical results. There was a time delay of ~0.5 min to both radioactivity detector and fraction collector.

10 µl of hydrazine hydrate (80%) was added followed by addition of a solution of 2.6 mg of NO2-DiMeIQx in 3 ml of argon purged THF. After 30 min of stirring at 0°C the suspension was centrifuged, the supernatant isolated, evaporated to dryness under vacuum and stored at −20°C until use.

Synthesis of 3,4,8-trimethyl-2-phenylazoxy-3H-imidazo[4,5-f]quinazoline (phenylazoxy derivative of DiMeIQx)

Nitrosobenzene, 1 mg, dissolved in 0.1 ml dimethylformamide (DMF), was added to a solution of 200 µg N⁴-OH-DiMeIQx in 0.5 ml argon purged DMF. After
standing for 1 h at room temperature, the solution was analyzed by HPLC/thermospray mass spectrometry.

Preparation of microsomes

Adult male Wistar rats (age 7-8 weeks, weight ~200 g), delivered from Metykred Dreecon Centre (Lille, Skensved, Denmark), were used to prepare PC PB induced hepatic microsomes. PCB (500 mg/kg, dissolved in corn oil) was injected i.p. 5 days before sacrifice and microsomes were prepared essentially as described by Lake (1987). The microsomes were stored in liquid nitrogen until use. The protein content was determined by the method of Sedmark and Grossberg (1977). The concentration of P450 (Lake, 1987) was 2.3 nmol P450/mg protein. The biphenyl hydroxylase activities (Yamazoe et al., 1981) were 3.0 nmol 2-hydroxy-biphenyl formed/(mg protein min) and 4.4 nmol 4-hydroxy-biphenyl formed/(mg protein min).

In vitro metabolism

The incubation mixture consisted of: 1 mg microsomal protein/ml, 1 unit DL-isocitrate-dehydrogenase/ml, 0.5 mM NADP^+, 10 mM sodium-β-citrate and 5 mM magnesium chloride in 50 mM MOPS, 0.15 mM KCl, pH 7.4. The mixture was preincubated for 2 min at 37°C before addition of 10 μg test substance (dissolved in 5 μl of DMF)/ml incubation mixture. The mixture was incubated at 37°C. The reaction was terminated by the addition of two volumes of ice-cold argon purged ethanol. After centrifugation the supernatant was isolated and analyzed by HPLC.

Analytical

HPLC analysis was performed on a Hewlett Packard model 1090 B liquid chromatograph equipped with a photo-diode array detector. Metabolites were separated on a Nucleosil C8, 5 μm, 250 × 4.6 mm column obtained from HPLC Technology (Macclesfield Cheshire, UK). Flow rate 0.8 ml/min. Solvents: A, 50 mM ammonium formate; B, acetonitrile. Solvent programming: 0% B by 1 min, a linear gradient to 25% B by 5 min, isocratic 25% B by 8 min, a linear gradient to 100% B by 13 min, isocratic 100% B by 15 min and a linear gradient to 10% B by 17 min.

In experiments with ^3C-labelled substrate the effluent from the column was monitored by both a photo-diode array detector and an on-line radioactivity detector (Radiomatic, Flo-one beta, Tampa, FL) with Packhard Pica aqua scintillation liquid (Radiomatic, CT). The flow rate of the scintillation liquid was four times the flow rate of the effluent.

In experiments with unlabelled substrate the HPLC fractions were collected in argon filled test tubes at 0.5 min intervals and analyzed for mutagenic activity.

Positive ion thermospray mass spectra were obtained at a resolution of 600 on a Profile mass spectrometer (MACS, Manchester, UK). The effluents from the HPLC was introduced into the thermospray interface at a flow rate of 0.8 ml/min. Both vaporizer and source temperature were 170°C. The same solvent programming as above was used; however, in order to avoid excessive fragmentation the ammonium formate was substituted by 0.5% formic acid.

NMR spectra were obtained with a Varian VXR400 spectrometer with DMSO-d_6 as solvent.

Results

Incubation of DiMeIQx with hepatic microsomes from Wistar rats pretreated with PCB produced five metabolites. Figure 1 (panels 1 and 2) shows the radiochromatogram and the UV profile, respectively, after 40 min of incubation with [2-^14C]DiMeIQx. After 20 min of incubation only two major metabolites (A1 and B) could be detected but longer incubation times resulted in increasing amounts of metabolites A2, B1 and B2.

To assess the mutagenic activity of the metabolites, fractions were collected at 0.5 min intervals and tested for mutagenicity in S.typhimurium TA98 both with and without the addition of N-Methyldiethlyammonium chloride. The effluents were analyzed by HPLC/thermospray mass spectrometry and found to contain the following substances: 258, 228, and 244.
metabolic activation system (S9) and in the nitroreductase deficient strain TA98NR without addition of S9. The mutagenic activity of the HPLC fractions without S9 is shown in Figure 1 (panel 3). Fractions 21, 22, 24–26 and 30–32 all contained mutagenic activity. Addition of S9 resulted in increased mutagenicity in fractions 21–23 and in a highly increased mutagenicity in fractions 26–27, originating from the parent compound. The mutagenicity in fraction 25 was still present at the same level as without added activation system, whereas mutagenicity in fractions 30–31 had almost disappeared (Figure 1, panel 4). In the nitroreductase deficient strain TA98NR without S9 activation (Figure 1, panel 5) the mutagenicity of fractions 21, 22 and 24–26 was at the same level as in TA98. However, the mutagenicity in fractions 30–31 was reduced by >50% as compared with TA98.

Identification of the microsomal metabolites

2-Nitro and N²-hydroxyamino derivatives of DiMeIQx were synthesized by the two step procedure shown in Figure 2, and the compounds were characterized by UV spectroscopy and thermospray mass spectrometry. The positive ion thermospray mass spectrum of NO₂-DiMeIQx showed a molecular ion \((M+H)^+\) at \(m/z\) 238 (Figure 2A) and the mass spectrum of \(N²-OH-DiMeIQx\) showed a molecular ion \((M+H)^+\) at \(m/z\) 244 (Figure 2B), which confirms the structure of the two compounds. Further evidence that the \(N²-OH-DiMeIQx\) had been formed was obtained by synthesis of the phenylazoxy derivative which by HPLC/thermospray mass spectrometric analysis showed the correct molecular ion \((M+H)^+\) at \(m/z\) 333 (not shown) (Hashimoto et al., 1982). The identity of metabolites A1 and A2 was determined as \(N²-OH-DiMeIQx\) and NO₂-DiMeIQx, respectively, by comparison of retention times in HPLC, UV spectra and mass spectra with synthetic standards.

Metabolite B was purified by preparative HPLC, and characterized by thermospray mass spectrometry and \(^1\)H-NMR spectroscopy. The mass spectrum showed a molecular ion \((M+H)^+\) at \(m/z\) 244, which the mass spectrum of \(N²-OH-DiMeIQx\) also did, but no daughter ion at \(m/z\) 228 (Figure 3). This indicates that metabolite B is hydroxylated in a different position of the molecule than \(N²-OH-DiMeIQx\). As shown in Figure 4 the \(^1\)H-NMR spectrum revealed the presence of all protons. The assignments are based on comparisons with literature values of the chemical shifts of DiMeIQx (Grivas, 1985) and on irradiation experiments. Irradiation at the 4-methyl group at 2.8 p.p.m. resulted in an increased signal height of the 5-H proton due to elimination of long range coupling between the 4-methyl group and the 5-H proton. Similarly, irradiation at the \(\text{CH}_2\) group at 4.76 p.p.m. resulted in an increased signal height of the 7-H proton. This indicates that the 8-methyl group is...
oxidized to a hydroxymethyl group. The results from NMR experiments together with the mass spectrum corroborate that metabolite B is 2-amino-8-hydroxymethyl-3,4-dimethyl-3H-imidazo[4,5-f]quinoline (8-OH-DiMeIQx). The NMR and mass spectroscopy data on 8-OH-DiMeIQx are in accordance with data published by Knize et al. (1989) on a similar compound isolated from urine of rats dosed with DiMeIQx.

Incubation of purified metabolite B, 8-OH-DiMeIQx, with hepatic microsomes from PCB treated rats resulted in a transformation of 8-OH-DiMeIQx to metabolites B1 and B2. The time course of this transformation is shown in Figure 5. The initial disappearance of 8-OH-DiMeIQx is followed by a concomitant rise in the content of metabolite B1 in the incubation mixture. After 20 min, the levels of metabolite B1 began to plateau, whereas the amount of metabolite B2 increased. This time course indicates that 8-OH-DiMeIQx is biotransformed to metabolite B1, which is further transformed to B2.

A comparison of the UV spectra of metabolites B1 and B2 with the UV spectra of N2-OH-DiMeIQx and NO2-DiMeIQx is shown in Figure 6. The spectral similarity between metabolite B1 and N2-OH-DiMeIQx and between metabolite B2 and NO2-DiMeIQx together with the time course of their formation indicates that metabolite B1 in the N2-OH derivative of 8-OH-DiMeIQx, 2-hydroxyamino-8-hydroxymethyl-3,4-dimethyl-3H-imidazo[4,5-f]quinoline, and that metabolite B2 is the 2-nitro derivative of 8-OH-DiMeIQx, 8-hydroxymethyl-3,4-dimethyl-2-nitro-3H-imidazo[4,5-f]quinoline. Further evidence for the proposed structures of metabolites B1 and B2 was obtained by analyses of the metabolites by HPLC/thermospray mass spectrometry.

Figure 7 (panel A) shows the mass spectrum of metabolite B1 having a molecular ion (M+H)+ at m/z 260. This is in
Fig. 8. Reaction scheme showing the proposed microsomal metabolism of DiMeIQx.

accordance with B1 being 2-hydroxyamino-8-hydroxymethyl-3,4-dimethyl-3H-imidazo[4,5-f]quinoline. The mass spectrum of metabolite B2 is shown in Figure 7 (panel B). The presence of a small molecular ion (M-H)+ at m/z 274 is in accordance with B2 being 8-hydroxymethyl-3,4-dimethyl-2-nitro-3H-imidazo[4,5-f]quinoline.

The proposed reaction scheme for the microsomal metabolism of DiMeIQx is depicted in Figure 8. Oxidation of the exocyclic amino group results in the formation of two direct acting mutagenic metabolites (N2-OH-DiMeIQx and N2-acetyl-8-OH-DiMeIQx). Hydroxylation of the methyl group in position 8 results in the formation of an indirect mutagenic metabolite (8-OH-DiMeIQx), which is further biotransformed to the two direct acting mutagenic metabolites N2-OH-8-OH-DiMeIQx and NO2-8-OH-DiMeIQx.

Discussion

It is well established that heterocyclic aromatic amines of the AL4 type are metabolically activated to proximate mutagenic metabolites by hydroxylation of the exocyclic amino group (Yarnazoe et al., 1983, 1988; Snyderwine et al., 1987; Holme et al., 1989; Buonarati and Felton, 1990; Turteltaub et al., 1990; Frandsen et al., 1991). Incubation of DiMeIQx with hepatic microsomes from Aroclor induced rats for 20 min resulted in two major metabolites, N2-OH-DiMeIQx and 8-OH-DiMeIQx, whereas longer incubation times resulted in three additional metabolites. It was thus shown that DiMeIQx was metabolized to a mutagenic N2-hydroxy derivative and that this metabolite can be further oxidized to a mutagenic nitro derivative. This further oxidation is an enzymatically catalyzed reaction, as incubation of N2-OH-DiMeIQx with microsomes resulted in the formation of the nitro derivatives, whereas incubation with heat inactivated microsomes did not result in formation of the nitro derivatives (results not shown). This secondary reaction has not usually been observed for ALA type compounds. However, it has recently been reported that IQ can be biotransformed to a direct-acting mutagenic nitro derivative by prostaglandin-H-synthase (Morrison, 1993).

Microsomal hydroxylation of DiMeIQx at the 8-methyl group resulted in an indirect mutagenic metabolite with a specific mutagenic activity comparable with the specific mutagenic activity of the parent compound (results not shown). This is in accordance with observations published by Knize et al. (1989). 2-Amino-3,8-dimethylimidazo[4,5-f]quinoline (MelQx), a compound of similar structure to DiMeIQx, also retains its mutagenicity after 8-hydroxylation (Hayasu et al., 1987). We have also shown that the indirect mutagen 8-OH-DiMeIQx can be oxidized by microsomes to direct-acting mutagenic N2-hydroxy and nitro derivatives. The N2-hydroxy derivatives retained their mutagenic activity in the Salmonella strain TA98 with addition of S9 and in the TA98NR strain. However, the nitro derivatives almost completely lost their mutagenic activity with addition of S9 and lost ~50% of their mutagenic activity in the nitro reductase deficient strain TA98NR. A reduction in the mutagenic activity of nitro compounds with the addition of a metabolic activation system is a well known phenomenon (Rosenkranz and Mermelstein, 1983). As can be seen from the radiochromatograms in Figure 1 (panel 1), the two nitro derivatives of DiMeIQx (A2 and B2) were produced in smaller amounts than the corresponding N2-hydroxy derivatives. However, the mutagenic activity without addition of S9 was much higher in the fractions containing the nitro derivatives than the fractions containing the N2-hydroxy derivatives. The N2-hydroxy derivatives are very reactive compounds, whereas the nitro-derivatives are quite stable. Decomposition of part of the N2-hydroxy derivative during fraction collection and pipetting may explain the lower specific mutagenic potency of the N2-hydroxy derivatives compared with the nitro derivatives.

Knize et al. (1989) isolated a putative 4-OH-DiMeIQx from rat urine and feces as a minor metabolite compared with 8-OH-DiMeIQx and N2-acetyl-8-OH-DiMeIQx. In this in vitro experiment we did not observe any 4-OH-DiMeIQx. This may be due to the use of a different rat strain than Knize et al., Wistar versus Sprague-Dawley, or the use of different inducers, Aroclor versus β-naphthoflavone or none. Furthermore we have investigated the microsomal metabolism, therefore hepatic enzymes located in the cytosol, which may affect the in vivo metabolism, were not considered.

In conclusion DiMeIQx is metabolically transformed to several metabolites that still retain mutagenic activity. This seems to be in contrast to most other AIAs where the predominant mutagenic metabolite found has been the N2-hydroxy derivative of the parent compound.

N2-OH-DiMeIQx is a very unstable compound and preliminary studies have shown that it reacts with 2′-deoxyguanosine to a small extent. However, acetylation of N2-OH-DiMeIQx results in an extremely reactive compound which gives adducts with 2′-deoxyguanosine in higher yields. Structural elucidation of this adduct is in progress.
Acknowledgements

The authors wish to thank Rolf Andersson (Swedish University of Agricultural Sciences) for recording the NMR spectra, and Joan Oliver, Bo Lund Jensen and Viggo Jensen for skilled technical assistance. This work was in part supported by The Danish Council for Agricultural Research as part of a Nordel collaborative project under the Swedish Agricultural Research Council for Agricultural Research.

References


Received on July 20, 1993; accepted on October 6, 1993

Microsomal metabolism of DiMeIQx
PAPER IV
Formation of DNA adducts by the food mutagen 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (4,8-DiMeIQx) in vitro and in vivo. Identification of a N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx adduct

Henrik Frandsen, Spiros Grivas¹, Robert J. Turesky², Rolf Andersson¹, Lars O. Dragsted and John C. Larsen

Institute of Toxicology. National Food Agency. Morkhoj Bygade 19, DK-2800 Sabborg, Denmark and ¹Nestec Ltd. Research Centre, CH-1000 Lausanne 26, Switzerland

The covalent binding of the mutagenic N²-hydroxy meta-bolite of the food mutagen 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (4,8-DiMeIQx) to 2'-deoxy-nucleosides and DNA was investigated in vitro and in vivo. N²-Hydroxy-4,8-DiMeIQx reacted to a small extent spontaneously with 2-deoxyguanosine. However, acetylation of N²-hydroxy-4,8-DiMeIQx with acetic anhydride to form the N²-acetoxy derivative prior to reaction with 2-deoxyguanosine resulted in much higher yield of adduct. N²-Acetoxy-4,8-DiMeIQx did not form adducts with 2'-deoxyadenosine, 2'-deoxyctydine or 2'-deoxythymidine. The adduct formed between the N²-OH metabolite of 4,8-DiMeIQx and 2'-deoxyguanosine was analysed by mass spectrometry and NMR spectroscopy and the structure of the adduct was shown to be N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx. N²-Acetoxy-4,8-DiMeIQx reacted with calf thymus DNA and formed a covalently bound 4,8-DiMeIQx residue, which could not be removed by repeated precipitations or solvent extractions. The 4,8-DiMeIQx-DNA was hydrolysed enzymatically with nuclease PI/acid phosphatase and HPLC analysis showed that 70% of the bound mutagen was recovered as N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx. An additional minor adduct accounting for ~15% of the bound mutagen showed UV spectral characteristics similar to N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx and is probably an undigested oligomer. HPLC-autoanalysis of calf thymus DNA modified with 4,8-DiMeIQx in vitro and liver DNA from rats dosed with 50 mg/kg 4,8-DiMeIQx showed a similar adduct pattern. In both samples N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx accounted for 60-70% of the bound mutagen. Thus, these results show that 4,8-DiMeIQx similar to other heterocyclic amines form adducts with C-8 of guanine both in vitro and in vivo via its N²-OH metabolite.

Introduction

Heterocyclic aromatic amines (HAAs*) formed in the meat crust during ordinary cooking are potent mutagens and carcinogens (1). Daily exposure to these HAAs through the diet may be associated with diet related cancers. e.g. colorectal cancer (2).

2-Amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline (4,8-DiMeIQx) is a potent bacterial mutagen found in a variety of cooked food products at levels of 0.5-1.2 µg/kg. In fried meat it accounts for ~20% of the mutagenic activity (3-10).

Covalent binding of carcinogens to nucleic acids of DNA is recognized as an important step in the initiation of cancer, and carcinogen-DNA adducts are considered to be promising biomarkers for DNA damage caused by chemical carcinogens (11).

HAAs must undergo bioactivation through N-oxidation of the exocyclic amino group to reactive N²-hydroxy derivatives. These derivatives can either react directly with cellular macromolecules such as DNA and proteins, or prior to reaction they can be further activated by formation of reactive derivatives, such as acetate or sulfate esters (12).

The HAAs 2-amino-6-methylpyrido[1,2-a:3,2'-d]imidazole (Glu-P) (13), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) (14), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (15), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (16) and 2-amino 3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (19) have been reported to form adducts with 2'-deoxyguanosine (dG), by binding of the exocyclic amino group of the HAA to the C-8 of guanine. In addition to the major C-8 adducts, IQ and MeIQx have also been shown to form adducts with N² of guanine to a lesser extent (20).

In the present investigation we have examined the adduct formation of 4,8-DiMeIQx, and identified N²-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-[4,8-DiMeIQx]) as the major adduct formed both in vitro upon treatment of DNA with N²-acetoxy-4,8-DiMeIQx and in vivo after dosing rats with 4,8-DiMeIQx.

Materials and methods

Chemicals

4,8-DiMeIQx and [2-¹⁴C]4,8-DiMeIQx were synthesized as previously described (21,22). 4,8-DiMeIQx was determined to have >95% purity by HPLC with detection at 260 nm. The sp. act. of [2-¹⁴C]4,8-DiMeIQx was 48.7 mCi/mmol and the radiochemical purity was determined to be >92% by HPLC with radioactivity detection. The [2-¹⁴C]4,8-DiMeIQx was diluted with unlabeled 4,8-DiMeIQx to a sp. act. of 2.4 mCi/mmol prior to use. N²-OH-4,8-DiMeIQx and [2-¹⁴C]N²-OH-4,8-DiMeIQx were synthesized as previously described (23). Calf thymus DNA, 2'-deoxyadenosine, 2'-deoxyctydine, 2'-deoxyguanosine, 2'-deoxythymidine, micrococal nuclease (N-3755), phosphodiesterase I (P-6877), alkaline phosphatase (P-4377) and nuclease P1 (N-8630) from penicillium citrinum were obtained from Sigma, St Louis, MO. Acid phosphatase (from potato) and DNase I were obtained from Boehringer, Mannheim, Germany. [³²P]ATP (1000 Ci/mmol) was obtained from ICN Chemicals. T4 polynucleotide kinase were obtained from New England Biolabs. Soluene 350 was obtained from Packard, Meriden, CT. PEI-cellulose membranes were obtained from Machery Nagel, Düren, Germany. HPLC-grade acetonitrile was obtained from Rathburn, Walkerburn, Scotland. All other chemicals were obtained from Merck, Darmstadt, Germany, and were of analytical purity.

Analytical

HPLC analysis was performed on a Hewlett-Packard model 1090 B liquid chromatograph, Waldbronn, Germany, equipped with a photodiode array detector. Nucleosil C18, 5 µm. 250x4 mm column was obtained from Machery

*Abbreviations: HAAs, heterocyclic aromatic amines; 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline; Glu-P-I, 2-amino-6-methylpyrido[1,2-a:3,2'-d]imidazole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline; dG, 2'-deoxyguanosine; DMF, dimethylformamide; BES, N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; <RAL>, relative adduct level.
Acetylation of N'-OH-4,8-DiMeIQx and reaction with 2'-deoxynucleosides The N'-acetoxy derivative of N'-OH-4,8-DiMeIQx was prepared immediately prior to reaction with 2'-deoxynucleosides. N'-OH-4,8-DiMeIQx (1 mg, 4.1 μmol) was dissolved in 1 ml 75% dimethylformamide (DMF) containing 5 μl acetic acid and cooled to −50 °C. 12 μl of acetic anhydride was added with stirring over 10 min. Acetic acid was included in order to favour O-glycosidation reactions and suppress formation of the N'-acetyl derivative (16).

Since only reaction between acetylated N'-OH-4,8-DiMeIQx and 2'-deoxy-2'-deoxynucleosides yielded a product which was not present in the control incubation, this reaction was scaled up by a factor of 5. The synthesis was repeated twice.

The product precipitated and was collected by centrifugation, redissolved in 25% DMF and purified by HPLC as described above. The sample was injected onto the HPLC column several times and fractions were collected and evaporated to dryness. The overall yield was 494 μg, 10 μmol (12%), which was sufficient for structural characterization of the adduct.

DNA binding of 12-13C-labeled acetoxy-4,8-DiMeIQx

The product from acetylation of [2-13C]-OH-4,8-DiMeIQx and 2'-deoxynucleosides which was not present in the control incubation was a product that co-eluted with and showed identical UV absorption spectra as those of adducts formed in control incubations. The product is 3'-phospho-dG-(4&DiMeIQx). Since only reaction between acetylated N'-OH-4,8-DiMeIQx and 2'-deoxyguanosine gave a product which was not present in the control incubation, this reaction was scaled up by a factor of 5. The synthesis was repeated twice.

DNA binding of 12-13C-labeled acetoxy-4,8-DiMeIQx

The product from acetylation of [2-13C]-OH-4,8-DiMeIQx (200 μg, 0.82 μmol) was added dropwise to a vigorously stirred solution of calf thymus DNA (1 mg/ml) in 20 ml of 0.1 M, N,N-bis(2-hydroxyethyl) 2-aminohexane sulfonic acid (BES)/NaOH, pH 7.4. Two milliliters of each solution and a control not containing 2'-deoxynucleosides were placed in test tubes and cooled in ice/water. Two hundred milliliters of 2 M acetic acid was added dropwise, over 5 min, to each of the vigorously stirred test tubes. After stirring for a further 5 min the reaction mixtures were allowed to reach room temperature and analysed by HPLC.

Results

Reaction of acetylated N'-OH-4,8-DiMeIQx with 2'-deoxynucleosides

Room temperature incubation of N'-OH-4,8-DiMeIQx with dG at neutral pH resulted in the formation of small amounts of adduct (not shown). However, acetylation of N'-OH-4,8-DiMeIQx to the putative N'-acetoxy derivative prior to reaction with dG gave a much higher yield of adduct. Addition of N'-acetoxy-4,8-DiMeIQx to solutions containing 2'-deoxycytidine, 2'-deoxyadenosine or 2'-deoxythymidine only resulted in decomposition products which were also present in a control incubation not containing 2'-deoxynucleoside. Figure 1 shows the chromatogram of the crude product from reaction of N'-acetoxy-4,8-DiMeIQx with dG. The only product, not seen in control incubations, eluted at 21.3 min, and a UV spectrum recorded at the elution conditions from the column showed maxima at 326 and 258 nm, and a valley at 282 nm (Figure 1, inset). The product, which >90% precipitated from solution, was further purified by HPLC and characterized by mass spectrometry and NMR spectroscopy. The positive ion electrospray mass spectrum of the purified product showed a molecular ion [M + H]+ at 493.5 (Figure 2), which is in accordance with an adduct between 4,8-DiMeIQx and dG.
Formation of 4,8-DiMeIQx–DNA adducts

As shown in Table I, the 1HNMR spectrum revealed the presence of all non-exchangeable protons: all sugar protons, the two aromatic protons of 4,8-DiMeIQx (H-5 and H-7) and the three methyl groups at N-3, C-4 and C-8 of 4,8-DiMeIQx. However, the characteristic signal of the C-8 proton of guanine at δ 7.85 p.p.m. was absent. This indicates that the exocyclic amino group of 4,8-DiMeIQx is attached to the C-8 of guanine. On the basis of the mass spectrometry and NMR analysis it was concluded that the structure of the adduct was N2-(deoxyguanosin-8-yl)-4,8-DiMeIQx (dG-C8-[4,8-DiMeIQx]) (Figure 3).

The resonance for H-2'cb is somewhat downfield compared to what is observed for 2'-deoxyguanosine. Such a downfield shift is considered to be due to a deshielding effect of the nearby guanine N-3 atom and suggests that the adduct is in a syn conformation (31). A similar syn conformation of C-8 adducts has previously been observed for other heterocyclic amines (20).

The conformation of the glycosyl bond is in the anti conformation in unmodified DNA. A change to syn conformation in adducted DNA may have an influence on the structure of the DNA and on adduct persistence in vivo.

Reaction of N'-acetoxy-4,8-DiMeIQx with calf thymus DNA

[2-14C]N'-acetoxy-4,8-DiMeIQx reacted with calf thymus DNA, giving a covalent bound 4,8-DiMeIQx residue, which could not be removed by repeated solvent extractions or precipitations. The adduct level was 2.3 nmol/mg DNA, corresponding to one out of 300 guanine residues forming adducts. Higher adduct levels could be obtained by using a higher ratio of N'-acetoxy-4,8-DiMeIQx/DNA; however, this resulted in increasing difficulties in successful enzymatic hydrolysis of the modified DNA. HPLC analysis of heat denatured [2-14C]4,8-DiMeIQx–DNA subjected to enzymatic hydrolysis with nuclease P1/acid phosphatase showed one major and a few minor peaks (Figure 4). The major peak, eluting at 21.3 min accounted for 70% of the total eluted radioactivity. This peak co-eluted with dG-C8-[4,8-DiMeIQx] and had an identical UV spectrum which corroborated its identity. The most important of the minor peaks, eluting at 20.4 min, accounted for 14% of the radioactivity and this peak also had a UV spectrum that was identical to the spectrum of dG-C8-[4,8-DiMeIQx] (Figure 5).

HPLC analysis of [2-14C]4,8-DiMeIQx–DNA subjected to enzymatic hydrolysis with DNase, phosphodiesterase and alkaline phosphatase, showed one major and at least four minor peaks (Figure 6). The major peak, which accounted for

---

**Fig. 1.** Chromatogram monitored at 323 nm of the crude product from reaction of acetylated N2-hydroxy-4,8-DiMeIQx with 2'-deoxyguanosine. Inset, UV spectrum at the elution conditions from the column of the adduct, dG-C8-[4,8-DiMeIQx], eluting to 21.4 min.

---

**Fig. 2.** Positive-ion electrospray mass spectrum of purified dG-C8-[4,8-DiMeIQx] showing the molecular ion [M + H]+ at m/z 493.5.

**Table I.** 1HNMR chemical shifts of N'-tdG-X-4,8-DiMeIQx referenced to the solvent. DMSO-d6 = 2.19 p.p.m.

<table>
<thead>
<tr>
<th>Chemical shift (p.p.m.)</th>
<th>Multiplicity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.74</td>
<td>s</td>
<td>H-7</td>
</tr>
<tr>
<td>7.69</td>
<td>s</td>
<td>H-5</td>
</tr>
<tr>
<td>6.56</td>
<td>t</td>
<td>H-1'cb</td>
</tr>
<tr>
<td>4.46</td>
<td>m</td>
<td>H-3'cb</td>
</tr>
<tr>
<td>3.97</td>
<td>s</td>
<td>N-CH3</td>
</tr>
<tr>
<td>3.82</td>
<td>m</td>
<td>H-4'cb</td>
</tr>
<tr>
<td>3.66</td>
<td>dd</td>
<td>H-5'cb</td>
</tr>
<tr>
<td>3.54</td>
<td>dd</td>
<td>H-5'ca</td>
</tr>
<tr>
<td>3.25</td>
<td>m</td>
<td>H-2'cb</td>
</tr>
<tr>
<td>2.89</td>
<td>s</td>
<td>4-CH3</td>
</tr>
<tr>
<td>2.76</td>
<td>s</td>
<td>8-CH3</td>
</tr>
<tr>
<td>2.08</td>
<td>m</td>
<td>H-2'ca</td>
</tr>
</tbody>
</table>

**Fig. 3.** Structure of dG-C8-[4,8-DiMeIQx].

---

2555
32P-Postlabelling of 4,8-DiMeIQx-DNA formed in vitro and in vivo

Three adducts were detected by 32P-postlabelling under adduct intensification conditions both in calf thymus DNA modified with N'-acetoxy-4,8-DiMeIQx and in vivo. The major adduct co-migrated with synthetic 3'P-dG-C8-[4,8-DiMeIQx] (Figure 7) and the two minor adducts formed in vitro also co-migrated with those seen in vivo, Figure 7. Thus, all adducts appear to be derived from the N2-hydroxy metabolite of 4,8-DiMeIQx. The relative adduct level (<RAL>) values under adduct intensification conditions from calf thymus DNA modified with 4,8-DiMeIQx at a level of 1.0 µg/mg DNA and from rat liver DNA from an animal dosed at 50 mg/kg and analysis 72 h post-exposure are shown in Table II.

Therefore, the dG-C8 adduct accounts for ~70% of adducts in vitro and ~60% of adducts in vivo under adduct intensification conditions. Note that adduct intensification conditions preferentially label adducts over non-modified nucleotides and provide an "overestimation" of actual adduct levels (25). It is probable that these two other adducts may be incompletely digested oligomers, based upon the relative amount of dG-C8 in postlabelling versus adduct digested to the deoxynucleoside which was analysed by HPLC. The amount of dG-C8-[4,8-DiMeIQx] measured relative to other uncharacterized adducts or oligomers is comparable to what has been reported for other heterocyclic amines assayed by 32P-postlabelling (18,27,28).

Discussion

4,8-DiMeIQx is, like other HAAs, biotransformed to a mutagenic metabolite by oxidation of the exocyclic amino group
Formation of 4,8-DiMeIQx–DNA adducts

Adducts. Although 4,8-DiMeIQx, MeIQx and IQ are compounds of similar structure, we did not observe indications of a minor adduct in addition to the dG-C8-[4,8-DiMeIQx] adduct. 4,8-DiMeIQx, in contrast to IQ and MeIQx, has a methyl group in position 4 of the aromatic ring system. Steric hindrance by this methyl group probably prevents nucleophilic attack by the 2-amino group of guanine on the C-5 of 4,8-DiMeIQx.

N\textsuperscript{2}-Acetoxy 4,8-DiMeIQx also readily reacted with calf thymus DNA. HPLC analysis of the modified DNA after heat denaturation and enzymatic hydrolysis showed that 70% of the bound radioactivity co-eluted with dG-C8-[4,8-DiMeIQx]. However, one minor peak eluting earlier and accounting for 14% of the bound radioactivity was also present. This earlier eluting compound had a UV spectrum which was identical to the UV spectrum of dG-C8-[4,8-DiMeIQx]. Some additional minor peaks were too small for UV spectral analysis. However, digestion of the modified DNA with DNase, phosphodiesterase and alkaline phosphatase resulted in a hydrolysate where dG-C8-[4,8-DiMeIQx] only accounted for 44% of the radioactivity. In addition, four earlier eluting peaks with UV spectra similar to dG-C8-[4,8-DiMeIQx] could be identified. The radioactivity associated with these three peaks was added to the dG-C8-[4,8-DiMeIQx] peak after further hydrolysis with nuclease P1 and acid phosphatase (Figure 6). This indicates that enzymatic hydrolysis is incomplete and results in the presence of oligomers. Furthermore, increasing the degree of modification of the DNA with this bulky adduct resulted in less efficient enzymatic hydrolysis.

\textsuperscript{32}P-Postlabelling analysis of modified calf thymus DNA and liver DNA from rats dosed with 4,8-DiMeIQx showed a similar adduct pattern. dG C8-[4,8-DiMeIQx] accounted for 60–70% of the measured radioactivity, and two minor adducts, probably undegraded oligomers, accounted for 15–30% and 5–20% of the radioactivity respectively. These results are comparable with the results from HPLC analysis of DNA modified in vitro. 4,8-DiMeIQx DNA adduct formation in vivo analysed by the \textsuperscript{32}P-postlabelling technique have previously been described, resulting in three major and two minor adducts in liver DNA of rats fed 4,8-DiMeIQx (30). It has, however, recently been shown that the number of adduct spots found in the postlabelling assay of DNA adducted with HAAs are reduced when an additional nuclease P1 digestion is included in the assay (32). It is suggested that many of the additional adduct spots observed are oligomers. This is in accordance with our observation that additional nuclease digestion or nuclease digestion of heat-denatured DNA results in a reduced number of adduct peaks.

We have recently reported that 4,8-DiMeIQx is metabolized in vivo to the direct-acting mutagenic N\textsuperscript{2}-hydroxy-4,8-DiMeIQx and an indirect-acting mutagenic 8-hydroxymethyl derivative (8-CH\textsubscript{2}OH-4,8-DiMeIQx). Moreover, prolonged incubation times also resulted in formation of a direct-acting mutagenic N\textsuperscript{2}-hydroxy derivative of the 8-CH\textsubscript{2}OH-4,8-DiMeIQx (22). The adduct patterns, as measured by \textsuperscript{32}P-postlabelling analysis, between DNA modified with N\textsuperscript{2}-acetoxy-4,8-DiMeIQx and DNA modified in vivo were similar. This indicates that activation of 8-CH\textsubscript{2}OH-4,8-DiMeIQx to a DNA-reactive species does not take place in vivo to any appreciable extent, because additional adduct spots, originating from 8-CH\textsubscript{2}OH-4,8-DiMeIQx, would have been expected to be present in \textsuperscript{32}P-postlabelling analysis of DNA modified in vivo as compared to DNA modified with N\textsuperscript{2}-acetoxy-4,8-DiMeIQx.

![Fig. 7. \textsuperscript{32}P-Postlabelling of calf thymus DNA modified with N\textsuperscript{2}-acetoxy-4,8-DiMeIQx (upper), rat liver DNA 72 h following exposure to 4,8-DiMeIQx (50 mg/kg) (middle) and 3\textsuperscript{'}-phospho-dG-C8-[4,8-DiMeIQx] (lower). 0 = origin; adduct 1 = dG-C8-[4,8-DiMeIQx].](image-url)
In conclusion, metabolic activation of 4,8-DiMeIQx to the N²-hydroxy derivative results in formation of DNA adducts in vivo as well as in vitro. The N²-hydroxy derivative either directly reacts with dG of DNA or reacts after esterification to a more reactive species, resulting in formation of dG-C-[4,8-DiMeIQx] as the major adduct.

Acknowledgements

The authors wish to thank Joan Olaver for skilful technical assistance. This work was in part supported by The Danish Council for Agricultural Research as part of a Nordic collaborative project under the Scandinavian Contact Agency for Agricultural Research.

References


Received on April 28, 1994, revised on July 20, 1994, accepted on August 1, 1994.