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Titel der Diplomarbeit

„Impact of Xanthohumol on the DNA stability of human peripheral lymphocytes: Results of two human intervention trials“

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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>B(a)P</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>BPDE</td>
<td>Benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide</td>
</tr>
<tr>
<td>b.w.</td>
<td>Body weight</td>
</tr>
<tr>
<td>CA</td>
<td>Chromosomal aberration</td>
</tr>
<tr>
<td>CYP</td>
<td>Chytochrome</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Heterocyclic aromatic amine</td>
</tr>
<tr>
<td>HED</td>
<td>Human equivalent dose</td>
</tr>
<tr>
<td>HO</td>
<td>Haem oxygenase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IQ</td>
<td>2-amino-3-methylimidazo(4,5-f)quinoline</td>
</tr>
<tr>
<td>MN</td>
<td>Micronucleus</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferases</td>
</tr>
<tr>
<td>NDMA</td>
<td>N-nitrosodimethylamine</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>p.o.</td>
<td>Oral administration</td>
</tr>
<tr>
<td>SCE</td>
<td>Sister chromatid exchanges</td>
</tr>
<tr>
<td>SCGE</td>
<td>Single cell gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferases</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5'-diphospho-glucuronosyltransferase</td>
</tr>
<tr>
<td>XAN</td>
<td>Xanthohumol</td>
</tr>
</tbody>
</table>
Zusammenfassung

Abstract

Aim of the study was the investigation of potential DNA-protective effects of xanthohumol (XAN), a prenylated hop flavonoid, which is contained in beers. Therefore, protocols were developed for *ex vivo* assays with peripheral lymphocytes. The cells were exposed to representatives of three major groups of food specific genotoxic carcinogens, namely to benzo(a)pyrene (B(a)P), a polycyclic aromatic hydrocarbon, 2-amino-3-methyl-3H-imidazo(4,5f)-quinoline (IQ), a heterocyclic aromatic amine and to N-nitrosodimethylamine (NDMA), an aliphatic nitrosamine. The compounds were activated with human derived liver homogenate (S9-mix) and incubated with peripheral lymphocytes, which were used as indicator cells. The cells were collected before and after consumption of a XAN-containing drink (XAN-content/liter: 12 mg). In total, 22 individuals participated in a placebo-controlled intervention trial, which had a cross-over design; the beverage was consumed over a period of 14 days. The results of single cell gel electrophoresis assays (SCGE assays), which are based on the determination of DNA migration in an electric field, show that induction of DNA-migration by B(a)P and IQ was significantly reduced in the lymphocytes by 19.2% and 14.7% after a two week intervention period, while no significant effects were detected in experiments with the nitrosamine. In order to prove that XAN accounts for the protective effects, a follow-up study (n=10) with a parallel design was realized with the pure flavonoid which was given in capsules to the volunteers (uptake: 12 mg/person/day; duration: 2 weeks). The results which were obtained with the carcinogens show an identical pattern as that found with the XAN containing drink, i.e. evidence for a protective effect was seen in combination with the heterocyclic amine and with the polycyclic aromatic hydrocarbon. Furthermore, attempts were made to elucidate the molecular mechanisms which account for the protective effects. Results of experiments in which the overall (CDNB) activity of glutathione-S-transferase (GST) and of the isoenzymes GST-α and GST-π were monitored, show that the activity of the latter isoenzyme was significantly induced (by 58.4%) after the intervention. This observation may explain the protective effects of the flavonoid as it is known that GSTs inactivate electrophilic metabolites of IQ and B(a)P. Taken together the present findings indicate that extremely low levels of XAN protect humans against DNA-damage caused by dietary carcinogens.
1. Introduction

The introduction part comprises several chapters: the first part describes the occurrence and the biological effects of xanthohumol (XAN), the second chapter concerns the formation, the occurrence and the effects of the model carcinogens which were tested. In the last part, the development and principles of several methods which were used in the present thesis are described.

1.1. Xanthohumol

Xanthohumol is a prenylated flavonoid and the only natural source is the female inflorescence of the hop plant, *Humulus lupulus* L. (*Cannabaceae*) (figure 1) [1]. The chemical structure and molecular weight of XAN are depicted in figure 1.

![Figure 1: Female hop cones (picture used with the kind permission of Marianna Jelinek) and chemical structure of XAN](image)

Hops contribute to a major extent to the taste of beer as it adds flavor and bitterness. The main dietary source of XAN is beer [2], which is one of the most popular alcoholic beverages and it is consumed worldwide. In Austria the annual beer consumption reaches levels of 106.9 liters per person per year on average [3]. The contents of XAN in different brands of beers are shown in table 1.
Table 1: XAN-contents in different beer brands

<table>
<thead>
<tr>
<th>Beer</th>
<th>XAN content (μg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>European beer</strong></td>
<td></td>
</tr>
<tr>
<td>Stout</td>
<td>340</td>
</tr>
<tr>
<td>Pilsner</td>
<td>12-28</td>
</tr>
<tr>
<td>Lager</td>
<td>2</td>
</tr>
<tr>
<td><strong>US major brand</strong></td>
<td></td>
</tr>
<tr>
<td>Lager/Pilsner</td>
<td>9-34</td>
</tr>
<tr>
<td><strong>US microbrews</strong></td>
<td></td>
</tr>
<tr>
<td>American porter</td>
<td>690</td>
</tr>
<tr>
<td>Strong ale</td>
<td>246</td>
</tr>
<tr>
<td>India pale ale</td>
<td>160</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Non-alcohol beer</td>
<td>3</td>
</tr>
</tbody>
</table>

1) (adapted from Stevens et al. [4])

1.1.1. Biological effects of XAN

The Japanese researcher Sakae Arimoto investigated lyophilisates of beer for anti-mutagenic properties [5]. Already in the 1980s, she detected protective effects against heterocyclic aromatic amines, which are formed during cooking in meats (see chapter 1.2.3.), in bacterial test systems and also in laboratory rodents. Clarissa Gerhäuser and her coworkers from the DKFZ Heidelberg used another approach. They examined the anti-oxidative effects of single compounds of beer, as well as the inhibition of COX2 and iNOS, induction of protective enzymes and the inactivation of phase I enzymes with high-throughput in vitro models [1]. Figure 2 shows the chemical structures of different phenolic constituents of beer.
The most interesting finding of the group’s experiments was the observation of the pronounced protective effects of XAN in several model systems. Protective effects were not only seen at the initiation level, but also in regard to tumor promotion and progression [6]. In subsequent investigations it was found that the compound possesses apart from its preventive effects of DNA damage and other properties associated with anti-carcinogenic and anti-inflammatory effects [7] also anti-viral [8] and anti-bacterial properties [9]. Figure 3 shows different modes of action of XAN which are related to cancer protection.
Figure 3: Tumor protective properties of XAN. ↓ - reduction, ↑ - increase, COX2 - cyclooxygenase 2, GST - glutathione-S-transferase, HAA - heterocyclic aromatic amine, iNOS – inducible nitric oxide synthetase, LP – lipid peroxidation, NO – nitric oxide, PAH – polycyclic aromatic hydrocarbons, PGE - prostaglandine E und VEGF- vascular endothelial growth factor. (adapted from [10])

The effects of XAN involve inhibition of the activation of carcinogens [11], as well as increase of detoxification by induction of phase II enzymes [6], anti-oxidative effects [6] and induction of enzymes, which are involved in radical scavenging [12]. As a consequence, lipid peroxidation is also inhibited. Furthermore, it was reported that XAN inhibits COX2 and has anti-inflammatory properties [1]. Different mechanisms contribute to the inhibition of cell division, for example the anti-estrogenic potential [6, 13], which is associated with the inhibition of the growth of breast tumors. In many in vitro experiments, biologically relevant effects were seen with low doses. However, these results were obtained under in vitro conditions with cultured cells; therefore the findings of these studies cannot be extrapolated to humans.
1.1.2. Animal experiments

The promising results with cell lines led to the realization of the first animal study, which concerned the effects of XAN at the Institute of Cancer Research, Vienna, Austria. The experiment was conducted by Franziska Ferk et al. [14] and the results indicate that XAN protects against DNA-damage induced by the heterocyclic aromatic amine IQ. It was found that the flavonoid prevents induction of DNA-damage in hepatic and colonic tissue of IQ-treated rats. Furthermore, reduction of the numbers of IQ-induced preneoplastic lesions was observed in both organs, which was paralleled by an increase of the activity of uridine 5'-diphospho-glucuronosyltransferase (UGT) [14].

Similar effects have been obtained with other flavonoids like epigallocatechin gallate (EGCG) and quercetin in in vitro and in vivo studies, however in the case of XAN, protective effects were obtained with extremely low doses, which correspond to uptake of 20 mg/person per day [15-18].

Further in vivo investigations with XAN were conducted by a Portuguese group, which found inhibition of angiogenesis in rats [19]. Another paper describes positive effects on the wound healing process, decreased release of vascular endothelial growth factor (VEGF) and nitric oxide (NO) and a reduction of oxidative stress [20].

1.1.3. Human studies

According to our knowledge, no human studies which concern the chemopreventive properties of XAN have been published so far.

1.2. Occurrence and biological properties of the model carcinogens used in the studies

The aim of the present study was to find out whether XAN protects humans against genotoxic carcinogens which are contained in the diet, namely N-nitrosodimethylamine (NDMA), benzo(a)pyrene (B(a)P) and 2-amino-3-methyl-3H-imidazo(4,5-f)quinoxaline (IQ). The chemical structures of the mutagens are depicted in figure 4. These compounds have been used in earlier studies aimed to identify chemopreventive agents in food [21-24]. The
metabolism of the substances has been studied intensely [25].

NDMA was chosen as representative for the nitrosamines, B(a)P for aromatic hydrocarbons and IQ as a representative of the heterocyclic amines. The chemical structures of the compounds are shown in figure 4; their occurrence and classification by the IARC is summarized in table 2.

![Figure 4: Chemical structures of A: N-nitrosodimethylamine (NDMA), B: benzo(a)pyrene (B(a)P) and C: 2-amino-3-methylimidazo(4,5-f)quinoline (IQ)](image)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Abbreviation</th>
<th>Substance group</th>
<th>Occurrence</th>
<th>Cancerogenic properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-nitrosodimethylamine</td>
<td>NDMA</td>
<td>Nitrosamines</td>
<td>Cured meat and tobacco smoke</td>
<td>Group 2A (probably carcinogenic to humans)</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>B(a)P</td>
<td>Polycyclic aromatic hydrocarbons</td>
<td>Barbecued meat, exhaust fumes and tobacco smoke</td>
<td>Group 1 (human carcinogen)</td>
</tr>
<tr>
<td>2-amino-3-methyl-3H-imidazo(4,5f)-quinoline</td>
<td>IQ</td>
<td>Heterocyclic aromatic amines</td>
<td>Barbecued meat and fish</td>
<td>Group 2A (probably carcinogenic to humans)</td>
</tr>
</tbody>
</table>

As structurally related compounds which belong to the same groups are activated and/or detoxified via similar metabolic pathways, it is likely that protective effects which were found in combination with the different individual model compounds are also relevant for other representatives of the same category.

1.2.1. Nitrosamines

In 1956, the British scientists John Barnes and Peter Magee reported that N-nitrosodimethylamine (NDMA) leads to formation of liver tumors in rats. Subsequent
research led to the isolation of other nitrosamines. To date approximately 300 representatives have been identified and more than 90% of them were found to be DNA-reactive [26-27].

1.2.1.1. Chemical structure and formation

Nitrosamines are compounds which have the general structure $R_1N(-R_2)-N=O$. $R_1$ and $R_2$ are either alkyl groups, aryl groups or benzene rings. The metabolites of these compounds are electrophilic and bind to DNA; most representatives are stable at room temperature [28]. Figure 5 depicts the chemical structures of different nitrosamines which were identified in human foods [29].

Figure 5: Chemical structures of nitrosamines in foods (from [29])

Nitrosamines are formed as a consequence of the reaction of a nitrosating compound with organic nitrogen in food [30-31]. They are produced from nitrite and secondary amines which are contained in proteins. Their formation depends on the pH of the environment, the alkalinity of the amine and the temperature [30]. Figure 6 shows the reactions which lead to the formation of nitrosamines.
Humans are exposed to nitrosamines either via consumption of meat products which contain these compounds or via endogenous formation in the stomach. The main sources are cured meat products and fish [32]. Furthermore, they were also found in smoked, pickled and salty preserved foods and in certain alcoholic beverages such as beers and whiskey [32-33]. Endogenous formation, which takes place in the stomach in particular, occurs via the consumption of nitrate in vegetables and drinking water. Numerous food components influence the formation of nitrosamines in the digestive tract, for example phenolic compounds have a strong impact on the nitrosation reactions [34].

1.2.1.2. Metabolism and carcinogenic effects

Nitrosamines are potent carcinogens in human foods. They are activated in the liver via cytochrome P450 CYP2E1 which catalyzes the hydroxylation of the α-carbon atom [25, 35]. This reaction leads to the formation of an instable product decomposing to electrophilic DNA-reactive carbenium ions. The reactions are schematically depicted in figure 7.
1.2.1.3. Animal experiments

The carcinogenic properties of nitrosamines have been studied in numerous animal species. Most, but not all of the compounds, are potent carcinogens. In general, the carcinogenic potency of aliphatic and cyclic nitrosamines decreases with their molecular weights [28]. One of the most potent compounds in foods is NDMA which induces tumors in liver, nose and kidneys of rats [37]. Results of animal experiments show that nitrosamines are more potent carcinogens than most PAHs and HAs. Whereas these compounds have median toxic doses (TD$_{50}$) up to several milligrams per day per kg body weight [38-39], nitrosamines have much lower values in the range of micrograms per day per kg body weight [37].
1.2.1.4. Human studies

There is evidence for endogenous formation of nitrosamines during digestion [40]. Evidence from case-control studies showed an association between the intake of nitrates and nitrosoamines with gastric cancer; however, the evidence was insufficient in regard to oesophageal cancer [41]. It was repeatedly claimed that the contamination of drinking water with nitrates might increase the risk of gastric cancer [42-43]. Other investigations were conducted in regions of China and Southeast Asia, in which the consumption of salted fish is common. The rates of nasopharyngeal carcinomas, which is a rare type of tumors in most areas of the world (<1 per 100,000 persons per year), is highly increased in these regions (20-40 per 100,000 persons per year) [44]. High levels of NDMA have been detected in some samples of salted fish [45].

1.2.1.5. Protective effects of dietary constituents against nitrosoamines

In the 1970s and 1980s numerous studies were conducted in order to identify protective effects of dietary constituents which protect against the carcinogenic and DNA-damaging properties of nitrosamines. Animals were fed with amines and nitrosating agents; subsequently, the excretion of nitrosoamines in the urine was monitored [46]. Protective effects were found for example with vitamin E [47] and beverages like coffee and tea [48-49]. Furthermore, researchers from the IARC developed a human model in which individuals consumed foods with high nitrite contents and the amino acid proline under controlled conditions. Subsequently, the nitrosoproline (NPRO) concentrations were measured in the urine [50]. NPRO does not induce cancer and can be used for human studies. The results of these experiments show that nitrosamines are formed endogenously. Furthermore, protective effects of foods and/or certain constituents such as vitamin C and E were observed which inhibited endogenous formation of NPRO [51].

1.2.2. Polycyclic aromatic hydrocarbons

The first polycyclic aromatic hydrocarbon (PAH) dibenz(a,h)anthracene was isolated in 1930 by Kennaway und Hieger [52]; subsequently, it was shown that dermal application of the compound on the skin of laboratory rodents causes papillomas [52]. Three years later,
benzo(a)pyrene (B(a)P) was isolated from coal tar and characterized [53]. This compound became the most intensely studied model carcinogen among the PAHs [54-56].

1.2.2.1. Chemical structure and formation

PAHs consist of several aromatic benzene rings and are formed by incomplete combustion of organic matter [57] and are found in industrial and traffic exhaust fumes [58-59]. At present, several hundred substances are known [60].

PAHs have in general planar structures and almost all representatives are lipophilic. Figure 8 shows the chemical structures of different carcinogenic compounds among this group.

Several exposure routes are relevant for humans. Fruits and vegetables can be contaminated by airborne particles which are “loaded” with PAHs [61]. Atmospheric particle bound PAHs are destroyed gradually by light, but they persist in soils for many decades. Therefore, the concentrations in primary plant products can be quite high when the fields are located near contaminated areas. Other important sources of formation are high temperature
cooking processes. Fat, which drips into the flames during barbecuing, causes contaminations on the surface of the meat. The PAHs can reach levels of $\geq 900$ µg/kg in grilled meat products [62]. Also smoking of foods can be a source of the development of PAHs, for example smoked sausages can have levels $\geq 10$ µg/kg [63].

1.2.2.2. Metabolism and carcinogenic effects

The metabolism of PAHs is complex and is shown for the model compound B(a)P in figure 9.

![Metabolic pathways of B(a)P](adapted from [64])

Figure 9: Metabolic pathways of B(a)P (adapted from [64])

The first reaction is an epoxidation step which is catalysed by CYP1A1 and CYP1A2 [65], subsequently the metabolites are converted by epoxide hydrolase to diol-epoxides and then again to epoxides [65]. Some of the reaction products, for example benzo(a)pyrene-7,8-dihydriodiol-9,10-epoxide (BPDE) are extremely electrophilic and bind to DNA-bases like
guanine [65]. These lesions are repaired predominantly by nucleotide excision repair and to a minor extent by base excision repair [66]; unrepaired lesions may lead to base mutations [67].

Epoxides can be formed at different positions of the molecules; initially it was assumed that the so called “K-regions” epoxides are of particular importance. However, it was found in later studies that these metabolites are easily detoxified and it was demonstrated that “Bay-regions” play a key role in regard to formation of DNA adducts [68]. The location of these regions is depicted in figure 10.

![Bay-region and K-region](adapted from [10])

The cancer inducing potency of the PAHs depends primarily on their chemical structures. Bicyclic compounds do not possess mutagenic and carcinogenic properties [69], while substances consisting of three benzene rings like 9,10-dimethylbenzanthracene are DNA-reactive carcinogens [70]. Benz(a)anthracene is a representative of the tetracyclic derivatives and its mutagenic potency is increased due to its methyl groups. 5-Ring compounds are for example benzo(a)pyrene and dibenz(a,h)anthracene. 6-Ring compounds are highly DNA-reactive, but are found in foods in minor concentrations. Not all PAHs are carcinogenic, for example pyrene, anthracene and perylene are not mutagenic [71].

The reactions which catalyse the detoxification of the metabolites have been intensely studied [65, 72-73]. Electrophilic metabolites like epoxides and quinones, which cause DNA-damage and cancer, are primarily conjugated by GSTs [65]. Other enzymes like uridine-5'-diphospho-glucuronyltransferase (UGT), glucuronyl- and sulfotransferases are also involved in the detoxification [65].
1.2.2.3. Animal experiments

B(a)P causes positive results in almost all genotoxicity tests [71]. In bacterial test systems addition of liver homogenate leads to base change mutations; investigations with mammalian cells showed that not only gene mutations, but also chromosomal aberrations are induced by these compounds. Since the 1960s certain PAHs were used as model compounds in experimental cancer research. Apart from dermal application on laboratory rodents, these compounds were also administered orally or intraperitoneal, which causes the formation of tumors in several inner organs. Benzo(a)anthracene causes the induction of cancer in liver and lungs of mice [74] while benzo(a)pyrene causes formation of papillomas and/or carcinomas of the esophagus, forestomach and tongue of mice [75].

1.2.2.4. Human studies

Many occupational studies show that workers which are exposed to PAHs have increased cancer rates [76]. Typical examples are coke oven workers and pavement workers [76].

The contribution of PAH-exposure via the diet to human cancer risks is difficult to estimate. Most data come from skin painting experiments with rodents and therefore they cannot be extrapolated to oral uptake by humans. Furthermore, it is notable in this context that the absorption rates of the compounds via the intestinal tract differ strongly in laboratory animals and humans [77].

The threshold doses for PAH-uptake which were defined by the European Union in 2006 are 1.0 µg/kg for foods which are consumed by children and infants, 2.0 µg/kg for oils, fats and non smoked fish and 5.0 µg/kg for meat products [78].

1.2.2.5. Protective effects of dietary constituents against PAHs

Lee Wattenberg was one of the first researchers, who examined diverse compounds for their cancer protective properties systematically [79-81]. His findings indicate that feeding of plant constituents of cruciferous vegetables and leek are protective against PAHs like B(a)P or 7,12-DMBA in animal experiments. The compounds which are present in these plants (glucosinolates) increase the activity of GSTs, which play a major role in the detoxification of the electrophilic metabolites of PAHs [49]. GSTs can also be induced by catechins present in green tea and coffee [82-83] and compounds from spices like cardamom and cinnamon [84].
1.2.3. **Heterocyclic aromatic amines**

Heterocyclic aromatic amines (HAs) were discovered by the Japanese cancer researcher Takashi Sugimura and his coworkers in the early 1970s [85]. They examined the crust of fried fish and discovered that the extracts had a high mutagenic activity in bacterial tests. Subsequently, they identified 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) as the active principle of the extract. In collaboration with John Weisburger from New York and other scientists, structurally similar compounds were identified in the following years [86]. HAs are almost solely found in barbecued meat [87]. This finding may explain the higher incidences of colon cancer in countries with high meat consumption [88].

1.2.3.1. **Chemical structure and formation**

HAs are characterized by heterocyclic structures, i.e. they consist of aromatic and non-aromatic rings and an amino group. Today more than 20 compounds have been identified [89]. The chemical structures of several HAs contained in food are shown in figure 11.

![Chemical structures of different HAs](adapted from [10])

- **R = -H (IQ)**
  - **R = -CH₃ (MeIQ)**

- **R₁, R₂, R₃ = -H (IQx)**
  - **R₃ = -CH₃, R₂, R₃ = -H (8-MeIQ)**
  - **R₁, R₃ = CH₃, R₂ = -H (4,8-DiMeIQx)**
  - **R₁, R₂ = -CH₃, R₃ = -H (7,8-DiMeIQx)**

- **R = -H (ααC)**
  - **R = -CH₃ (MeααC)**

- **R = -H (7-MeIQx)**
  - **R = -CH₃ (7,9-DiMeIQx)**

**Figure 11: Chemical structures of different HAs** (adapted from [10])
Margareta Jägerstad from the Swedish University of Agricultural Sciences (Uppsala, Sweden) clarified the reactions which lead to the formation of the HAs. Generally they are formed in specific types of Maillard reactions from sugars, amino acids and creatine [90]. The latter compound is contained in muscle tissue and plays a role in the energy metabolism [91]. IQ is formed due to formation of glycol-aldehyde-alkylimine, which is oxidized and leads to formation of glyoxal monoalkylimine. Subsequently, the latter compound can be reduced to glyoxal. Both molecules (glyoxal and glyoxal monoalkylimine) condense and form pyridine radicals. The free radicals react with the aldehyde and creatinine and as a consequence IQ is formed as final reaction product [92]. Figure 12 depicts the chemical reactions which lead to the formation of different HAs.

\[ \text{CHO} \quad \text{CHOH} \quad \text{CHOH} \quad \text{R}^- \]
\[ \text{CHO} \quad \text{CHOH} \quad \text{CHOH} \quad \text{R}^- \]
\[ \text{Carbohydrate} \]

\[ \text{CH}=\text{O} \quad \text{CH}=\text{O} \quad \text{CH}=\text{O} \quad \text{CH}=\text{O} \]
\[ \text{Glyoxal} \quad \text{Glyoxal monoalkylimine} \quad \text{Schiff Base} \]

\[ \text{CH}=\text{NR} \quad \text{CH}=\text{NR} \quad \text{CH}=\text{OH} \quad \text{CH}=\text{OH} \]
\[ \text{Reverse-aldol reaction} \quad \text{Glyoxal-aldehyde alkylimine (enol type)} \]

\[ \text{HCHO} \quad \text{CH}_2\text{CHO} \quad \text{HCHO} \quad \text{CH}_2\text{CHO} \]

\[ \text{Pyridine radical} \]

\[ \text{IQ} \quad \text{MeIQ} \quad \text{MeIQx} \quad 4,8\text{-DiMeIQx} \]

\[ \text{Creatine} \quad \text{Creatinine} \]

\[ \text{Dialkyl-dihydro pyrazine} \quad \text{Dialkyl-pyrazine radical} \]

**Figure 12: Formation and chemical structures of HAs** (adapted from [93])

The amounts which are contained in fried products differ over a wide range. IQ and MeIQx are usually found in the range between 0 and 8.3 ng/g [94] while PhIP and AαC are
more abundant [95-96]. The formation of HAs increases with temperature and frying time [97-98]. It is also notable, that small amounts are present in smoked fish [99] and alcoholic beverages [100].

1.2.3.2. Metabolism and carcinogenic effects

Approximately 90% of the HAs are absorbed in the human digestive tract. The compounds are metabolized in inner organs, predominantly in the liver by cytochrome P450 isoenzyme CYP1A2, which catalyses the N-oxidation of the exocyclic amine groups and therefore leads to the formation of N-hydroxy-HAs, which are DNA-reactive. Phase II enzymes such as N-acetyltransferases (NATs), UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) can either directly detoxify HAs, or on the other hand further metabolize the N-hydroxy-HAs to DNA reactive compounds. Furthermore, UGTs may also form isomeric N-glucuronide conjugates from N-hydroxy-HAs and therefore detoxify HA-metabolites. GSTs can also inactivate reactive N-hydroxy-HA reaction products and reduce these compounds back to the original amines, which are further metabolized by other phase II enzymes to a readily excretable form [101-102].

It is notable, that also a number of other reactions may play a role in the metabolism of certain HAs, for example it was shown that conjugation with sulfonic acid plays an important role in the activation of compounds such as PhIP and AαC [103-104]. The metabolism of HAs is depicted in figure 13.
The electrophilic metabolites of the HAs cause the formation of DNA adducts in particular with guanosine [105]. As a consequence mutations may occur, which may lead to malignant transformation of the cells [105]. HAs have a high mutagenic activity in bacterial mutagenicity tests [106]. Induction of DNA-damage and the formation of DNA-adducts were also found in laboratory rodents [107-108] and in humans [109].

1.2.3.3. Animal experiments

The carcinogenic properties of different HAs were studied in laboratory rodents and also in other species, for example in monkeys [110]. It was found that these compounds cause mainly tumors in the large intestine, but additionally also in other organs. PhIP causes apart from colon tumors also neoplasms in the mammary gland of rats [111]. In experiments with mice it was found that IQ targets mainly lungs, liver and forestomach, but causes tumor formation also in other organs like kidneys, small and large intestines [112-113].
1.2.3.4. Human studies

As the exposure of humans to HAs via foods is quite low (i.e. in the ng range/person/day) [114], DNA-adducts cannot be detected under normal exposure conditions and it is not possible to monitor the plasma levels of HAs in human biomonitoring studies [110]. Table 3 lists the IQ-content of different foods.

Table 3: IQ contents of different foods and tobacco smoke condensate

<table>
<thead>
<tr>
<th>Food</th>
<th>IQ (ng/g)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground beef, fried (275°C)</td>
<td>0.3-1.9</td>
<td>[115]</td>
</tr>
<tr>
<td>Walleye pollack, fried (260°C)</td>
<td>0.16</td>
<td>[116]</td>
</tr>
<tr>
<td>Ground beef, broiled</td>
<td>0.5</td>
<td>[117]</td>
</tr>
<tr>
<td>Salmon, broiled</td>
<td>0.3-1.8</td>
<td>[117]</td>
</tr>
<tr>
<td>Tobacco smoke condensate</td>
<td>0.26 per cigarette</td>
<td>[118]</td>
</tr>
</tbody>
</table>

1) (adapted from [94])

Nevertheless, some findings indicate, that human exposure to HAs may lead to induction of tumors [98]. In this context it is notable that a report of the U.S. National Toxicology Program (NTP) states “HAs are reasonably anticipated to be human carcinogens“ [119].

Of particular interest are epidemiological investigations, which showed that polymorphisms of enzymes which are involved in the activation and detoxification of HAs, affect the human risks for colon cancer. For example individuals, which were fast acetylators and had increased CYP1A2 levels, were found to have higher rates of colon cancer when they consumed high amounts of barbecued meat [120]. Furthermore, it is known from epidemiological studies, that the gastric cancer risks are high in countries with elevated red and processed meat consumption [121] and it was suspected, that HAs play an important role and account for the prevalence of this form of cancer in industrialized countries. As a consequence strong efforts were made to identify protective dietary compounds [122].

1.2.3.5. Protective effects of dietary constituents against HAs

Several nutritional constituents, which are known to inactivate HAs, caused protective effects against colon cancer in human studies [123]. These findings are an indirect indication on the relevance of “cooked food mutagens” on human cancer risk. So far hundreds in vitro and animal studies have been published which concern the protective effects of foods and certain
constituents against HAs [123]. Dietary fibers, pigments like chlorophylls and lactic acid bacteria inactivate HAs directly and reduce the uptake in the intestines, diterpenoids, which are present in coffee, inhibit the activation of CYP1A2 (for review see Schwab et al. [122]). One of the most important detoxification pathways is the conjugation with glucuronic acid. The protective effects against the formation of preneoplastic lesions in the large intestine by the consumption of cruciferous vegetables could be mechanistically explained. It is also notable that a reduction of exposure to HAs can be achieved via the mode of meat preparation, i.e. by the reduction of the cooking temperature [124] and/or use of marinades [125-126].

1.3. Detection of DNA-damage in human intervention trials by dietary factors

The design of the present study was based on previous investigations which were aimed to identify DNA and cancer protective properties in the human diet; therefore, the design and the endpoints which were used in such interventions are briefly described.

1.3.1. Design of nutritional studies

In principle it is possible to monitor the impact of consumption of specific foods and/or their constituents in studies which are based on the comparison of different groups of individuals (parallel design). Alternatively, intervention trials can be conducted, which have the advantage that inter-individual variations can be strongly reduced. Also these latter studies may have a parallel design; in the case of placebo controlled studies, which are considered to have a higher predictive value; it is also possible to use a cross-over design, in which one part of the study group consumes the test compounds/food first, whereas the other group consumes the placebo before the intervention with the test food (see figure 14). This strategy has the advantage that seasonal or long term effects, which can affect the outcome of SCGE-studies, can be avoided [127]. In order to reduce confounding factors caused by dietary components “run-in” phases can be included in intervention trials. “Wash-out” periods are also sometimes part of the intervention schemes to prove that the factor for which the study accounts for is indeed the active principle.
As described in detail in the materials and methods section, the main study was a placebo controlled trial which had a cross-over design, which included a “run-in” as well as a “wash-out” phase. The number of participants (n=22) was selected on the basis of results obtained in earlier trials in which the same parameters were monitored, (i.e. on the basis of calculations of the statistical power). The duration of the intervention phase was two weeks as it is known that this period is sufficiently high to detect the induction of drug metabolizing and antioxidant enzymes in human studies [127].

The follow-up trial with pure XAN had a parallel design and a lower number of participants was included. It was conducted to prove that XAN is the active principle of the drink which was investigated in the preceding larger study and it was assumed on the basis of the findings obtained in the main study, that 10 participants are a sufficiently high number to detect an effect.

1.3.2. Use of single cell gel electrophoresis assays (SCGE assays/Comet assays) in studies with dietary factors

A number of methods have been developed which can be used in human biomonitoring studies to detect factors which cause induction or prevention of DNA damage (for review see [128]). At present, one of the most widely used approaches are SCGE assays, which have the advantage that the cells do not require cell division to conduct measurements and that the studies are cost and time effective [127, 129]. The second most widely used approach are micronucleus-assays (MN-assays) with peripheral blood cells, but the intervention periods, which are required for these assays are substantially longer and it is necessary to cultivate the cells in presence of cytochalasin B under sterile conditions [130]. Other approaches such as DNA-adduct measurements and determination of altered DNA bases have been used in a number of trials as well, but they enable only the detection of the effects of specific compounds [131].

The alkaline version of the SCGE technique was developed in the late 1980’s by Tice et al. [132] and enables the detection of single and double strand breaks of apurinic sites [133]. The assay is based on the determination of DNA migration in an electric field and is increasingly used in genetic toxicology. The first results of a human nutrition study were published in the 1990ies by Duthie and her coworkers [134]. Substantial improvement of the predictive value of the SCGE experiments was achieved by Andrew Collins who developed protocols which allow the detection of oxidatively damaged bases by use of restriction
enzymes [127], changes of the sensitivity of the cells to ROS-induced DNA-damage [135] and alterations of the DNA repair capacity [136]. All these modifications have been successfully used in human intervention trials [127].

In the present investigation a new protocol was developed which enables to predict if intake of putative chemoprotective components (e.g. of XAN in our study) causes protection against genotoxic dietary carcinogens. Such attempts have already been made by Hölzl et al. [127] some years ago, but these attempts were based on the treatment of lymphocytes (before and after intervention) with promutagenic carcinogens in absence of metabolic activation mix and the concentrations which were used in that study were unphysiologically high. The only study which has been published with a similar approach so far is an intervention trial by Steinkellner et al. [137] who showed that consumption of coffee leads to protection against BPDE-induced DNA damage. In this study B(a)P was not activated with S9-mix, but the cells were treated with 0.4 μM BPDE, the main metabolite of the carcinogen. In order to reflect the situation in the human body in an optimal way, human derived liver S9-mix was used in the present study, which has the advantage that the substrate specificities of the different enzymes that catalyse the activation of the different compounds (in our case B(a)P, IQ and NDMA) are representative for humans (which is not the case with rat or mouse derived liver homogenates).

### 1.3.3. Investigation of alterations of the glutathione (GSH) levels in plasma

Glutathione (GSH) is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine [138]. GSH has several functions in the body. On one hand it is required for the detoxification of ROS [138] (this reaction is catalyzed by a specific antioxidative enzyme namely glutathion reductase, GPx). On the other hand, it is also a substrate for glutathione-S-transferase, which detoxifies a broad variety of macromolecules (see below). Reduced GSH levels are indicative for a disturbed redox-status in humans. An imbalance of GSH is observed in a wide range of pathologies, including cancer, neurodegenerative disorders, cystic fibrosis, human immunodeficiency virus (HIV) infections and aging [139]. Induction of the tripeptide levels was seen with beneficial food-constituents such as vitamin C, silymarin, α-lipoic acid, glutamine, methionine, and undenatured whey protein [140-143].
1.3.4. Investigation of alterations of the activities of glutathione-S-transferases (GSTs)

Glutathione-S-transferases comprise a number of isoenzymes and represent one of the most important xenobiotic drug metabolizing enzyme systems in mammals [144]. In humans, several isoenzymes have been identified which differ in regard to the tissue distribution and substrate specificities [145]. It is known, that dietary factors such as flavonoids, phenolic acids, allyl sulfides and breakdown products of glucosinolates induce GSTs in rodents and/or humans [146-150].

In the present study we included measurements of the overall GST-activity with the 1-chloro-2,4-dinitrobenzene (CDNB)-method; furthermore, the contents of the isoenzymes GST-\(\pi\) and GST-\(\alpha\) in plasma were determined with commercially available kits. The measurements were conducted for two reasons: (1) It is known from *in vitro* experiments with mammalian cells that the prenylated flavonoid XAN activates Nrf2 and induces GSTs [151-152]. (2) Furthermore, it is known that GSTs play a key role in the detoxification of PAHs including B(a)P [153] and also in the inactivation of electrophilic metabolites of IQ and other HAs (see schemes 9 and 13) [154].

The measurement of GST with the classical CDNB method provides information about the overall activity of several isoenzymes except GST-\(\theta\) [145]. GST-\(\alpha\) is mainly found in the liver and small intestine [155]. This class of isoenzymes is known to be induced in humans by certain types of cruciferous vegetables [156-157].

Furthermore, the plasma levels of GST-\(\pi\) were determined. It is the most widely distributed isoenzyme among the GSTs and present in many organs, predominantly in the epithelia cells of the urinary, digestive and respiratory systems. The specific occurrence pattern may reflect the role of the isoenzyme in the excretion of dietary carcinogens [158]. It is the most abundant form in many tissues, but not in the liver [159].
2. Material und Methods

2.1. Chemicals

Table 4 provides information about the chemicals, media and kits used in the present work.

Table 4: Information about chemicals, abbreviations, their application, suppliers and CAS-numbers

<table>
<thead>
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<th>Abbreviation</th>
<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
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<td>Density gradient medium</td>
<td>Sigma(^1)</td>
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<td>RPMI</td>
<td>Cell culture medium</td>
<td>Sigma(^1)</td>
<td></td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td>Biofreeze</td>
<td>Freezing medium for lymphocytes</td>
<td>Biochrom(^2)</td>
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<td>Low melting point agarose</td>
<td>LMP agarose</td>
<td>Fixation of cells on slide</td>
<td>Invitrogen Life Technologies(^3)</td>
<td>9012-36-6</td>
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<tr>
<td>Normal melting point agarose</td>
<td>NMP agarose</td>
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<td>BSA</td>
<td>Buffer</td>
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<td>Di-methylsulfoxide</td>
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<th>Use</th>
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<td>Determination of vitality</td>
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**Chemical carcinogens**

<table>
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<td>Benzo(a)pyrene</td>
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<td>Chemical agent</td>
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<td>2-amino-3-methyl-3H-imidazo(4,5f)quinoline</td>
<td>IQ</td>
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<td>Test substance</td>
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**Human S9 mix**

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<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
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<td>Magnesium chloride</td>
<td>MgCl₂ x 6H₂O</td>
<td>Cofactor for S9-mix</td>
<td>PAA Laboratories GmbH&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>Glucose 6-phosphate</td>
<td>G-6-P</td>
<td>Cofactor for S9-mix</td>
<td>PAA Laboratories GmbH&lt;sup&gt;3&lt;/sup&gt;</td>
<td>56-73-5</td>
</tr>
<tr>
<td>Nicotinic adenine dinucleotide phosphate</td>
<td>NADP</td>
<td>Cofactor for S9-mix</td>
<td>PAA Laboratories GmbH&lt;sup&gt;3&lt;/sup&gt;</td>
<td>53-59-8</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>NaH₂PO₄ x H₂O</td>
<td>Cofactor for S9-mix</td>
<td>PAA Laboratories GmbH&lt;sup&gt;3&lt;/sup&gt;</td>
<td>89140-32-9</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>Na₂HPO₄</td>
<td>Cofactor for S9-mix</td>
<td>PAA Laboratories GmbH&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7758-79-4</td>
</tr>
</tbody>
</table>

**GSH–measurements**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfosalicylic acid</td>
<td>Reagent</td>
<td>Sigma&lt;sup&gt;1&lt;/sup&gt;</td>
<td>304851-84-1</td>
</tr>
<tr>
<td>Ellman’s reagent</td>
<td>Reagent</td>
<td>Sigma&lt;sup&gt;1&lt;/sup&gt;</td>
<td>68-78-3</td>
</tr>
<tr>
<td>Gluthathione (reduced)</td>
<td>GSH</td>
<td>Reagent</td>
<td>Sigma&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Bradford assay**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradford reagent</td>
<td>Dye</td>
<td>Bio-Rad Laboratories&lt;sup&gt;8&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**CDNB assay**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>CDNB</td>
<td>Sigma&lt;sup&gt;1&lt;/sup&gt;</td>
<td>97-00-7</td>
</tr>
</tbody>
</table>

**GST-α/GST-π determination**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Use</th>
<th>Supplier</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha GST ELISA</td>
<td>Kit</td>
<td>Argutus Medical&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Pi GST ELISA</td>
<td>Kit</td>
<td>Argutus Medical&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Sigma-Aldrich, Steinheim, Germany; <sup>2</sup> Steinheim, Germany; Biochrom AG, Berlin, Germany; <sup>3</sup> Invitrogen Life Technologies Ltd, Paisley, Scotland; <sup>4</sup> Merck, Darmstadt, Germany; <sup>5</sup> Nard Institute, Tokyo, Japan; <sup>6</sup> Trinova Biochem. GmbH, Gießen, Germany; <sup>7</sup> PAA Laboratories GmbH, Pasching, Austria; <sup>8</sup> Bio-Rad Laboratories GmbH, Vienna, Austria; <sup>9</sup> Argutus Medical Ltd., Dublin, Ireland
2.2. Solutions

2.2.1. Solutions for SCGE assays/Comet assays

Dulbecco’s PBS (pH = 7.4)
8.0 g NaCl, 1.15 g Na₂HPO₄ x H₂O, 0.2 g KH₂PO₄ and 0.2 g KCl were dissolved in 1000 ml distilled water and adjusted to pH 7.4 with NaOH.

Lysis solution:
2.5 M NaCl (146.1 g), 100 mM EDTA (37.2 g) und 10 mM Tris (1.2 g) were dissolved in 1000.0 ml distilled water. The pH was adjusted to 10 with NaOH. The solution was stored at 4° C. Triton X-100 (1%) and DMSO (10%) were added immediately before usage.

Electrophoresis buffer:
Stock solutions for NaOH and EDTA were prepared separately. For the 10 N NaOH solution 400.0 g of NaOH were dissolved in 1000 ml distilled water. For the 200 mM EDTA solution 14.89 g of EDTA were dissolved in 200.0 ml distilled water. Both stock solutions were stored at room temperature.
Before use the NaOH-stock solution (84.0 ml) was mixed with 14.0 ml of the EDTA-stock solution and ice cold distilled water to a total volume of 2700 ml. The final pH was 13.0.

Neutralisation buffer
0.4 M Tris (48.5 g) was dissolved in 1000 ml distilled water. The pH was adjusted to 7.5 with concentrated hydrochloric acid (HCl).
2.2.2. Solutions for GSH measurements

**Tris/EDTA buffer:**

0.5 M Tris (6.06 g) and 0.02 M EDTA (0.74 g) were dissolved in 100 ml distilled water. The pH was adjusted to 9.0 with concentrated hydrochloric acid (HCl).

**Sodium phosphate/EDTA buffer:**

Stock solutions for Na$_2$HPO$_4$/EDTA and NaH$_2$PO$_4$/EDTA were prepared separately. For the Na$_2$HPO$_4$/EDTA solution 1.78 g Na$_2$HPO and 0.74 g EDTA were dissolved in 100 ml distilled water (pH = 7.2). For the NaH$_2$PO$_4$/EDTA solution 1.4 g and 0.74 g were dissolved in 100.0 ml distilled water respectively (pH = 4.4). Before use both solutions were mixed and adjusted to a pH of 7.

**5% Sulfosalicylic acid**

5 g of sulfosalicylic acid was dissolved in 100 ml distilled water.

**Ellman’s reagent**

19.8 mg 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) was dissolved in 5 ml sodium phosphate/EDTA buffer.

**Glutathione (GSH)-solution**

A stock solution was prepared by dissolving 15.35 mg of GSH in 1.0 ml of sulfosalicylic acid. The stock solution was diluted 1:50 before use.

2.2.3. Solutions prepared for enzyme measurements

**Sodium phosphate buffer**

4.450 g Na$_2$HPO$_4$ and 3.450 g NaH$_2$PO were dissolved in 500 ml distilled water. The pH was adjusted to 6.8.
Glutathione (GSH)-solution
30.7 mg of GSH were dissolved in 1.0 ml of distilled water resulting in a concentration of 100 mM.

2,4-Dinitrochlorobenzene (CDNB)-solution
20.3 mg of CDNB were dissolved in 1 ml of ethanol.

2.3. Human derived liver S9 homogenate

The liver homogenate which was used for the experimental series was purchased from Trinova Biochem GmbH, Gießen, Germany.

2.3.1. Tissue origin

According to the information provided by the manufacturer, the tissues which were used for the preparation of the homogenate came from livers of organ donors, which were rejected for transplantations due to anatomical irregularities, histological findings, donor age, time in transit or the health status of the potential recipient. The supplier guaranteed that the tissue donors were free of infectious viruses such as HIV and hepatitis. The characteristics of the donors are listed in table 5.

Table 5: Characteristics of the donors (information provided by Trinova Biochem GmbH, Gießen, Germany)

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Age</th>
<th>Sex</th>
<th>Cause of death</th>
<th>Cold ischemia</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>M</td>
<td>Cerebral vascular aneurysm</td>
<td>22 h</td>
<td>Hypertension, alcohol, tobacco</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>M</td>
<td>Cerebral vascular aneurysm</td>
<td>14 h</td>
<td>Hypertension, tobacco</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>M</td>
<td>Intracranial hemorrhage</td>
<td>11.5 h</td>
<td>Alcohol</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>M</td>
<td>Head trauma</td>
<td>11 h</td>
<td>Tobacco, alcohol</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>M</td>
<td>Head trauma</td>
<td>17 h</td>
<td>Tobacco, alcohol, marijuana</td>
</tr>
</tbody>
</table>

1) M-male
2.3.2. Biochemical characteristics of the liver homogenate

The supplier provided information concerning the overall cytochrome P450 and protein contents of the homogenate as well as information on the activities of the cytochrome P450 subfamilies 1A1, 1A2 and 2A6 (see table 6). The vials with the pooled liver homogenate were stored at -80° C until use.

Table 6: Biochemical characteristics of the pooled liver homogenate (information provided by Trinova Biochem GmbH)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Content/specific activity</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>29.0 mg/ml</td>
<td>Determined by use of Bio-Rad reagents. (Bio-Rad Laboratories, Hercules, USA)</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>2.3 nm/ml</td>
<td>Assayed with dithionite difference spectra according to [160]</td>
</tr>
<tr>
<td>EROD activity (CYP1A1, CYP 1A2)</td>
<td>S.a. 8.8</td>
<td>Assayed using a modification of [161]; specific activities are expressed as picomoles hydroxresorufin formed/min/ mg S9 protein.</td>
</tr>
<tr>
<td>MROD activity (CYP1A2)</td>
<td>S.a. 16.4</td>
<td></td>
</tr>
<tr>
<td>BROD activity (CYP2A6)</td>
<td>S.a. 4.5</td>
<td></td>
</tr>
</tbody>
</table>

1) S.a.-specific activity

2.3.3. Preparation of S9 mix

Tubes with the liver homogenates were defrosted at room temperature and placed in a polystyrene box filled with crushed ice. Subsequently, cofactor solutions were added. The composition of the individual solutions is described in table 7. S9-mix was prepared freshly prior to each experimental series; all steps of the procedure were carried out at 0 - 4° C using cold, sterile solutions and glassware [162].

Table 7: Composition of S9-mix (10%)  

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (ml)</th>
<th>Molar concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver S9</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>MgCl2-KCl salts</td>
<td>0.20</td>
<td>1.65</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>NADP</td>
<td>0.40</td>
<td>0.10</td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.4</td>
<td>5.00</td>
<td>0.20</td>
</tr>
<tr>
<td>Sterile distilled H2O</td>
<td>3.35</td>
<td></td>
</tr>
</tbody>
</table>

1) The S9-mix was prepared according to Maron and Ames [163].

- 29 -
2.4. Composition of the drink and of the capsules used in the intervention trials

Table 8 shows a list of the ingredients of the XAN-drink which was used in the human intervention study. The composition of the capsules which were used in the follow-up trial is described in the same table. The composition of the placebo-drink was similar to the XAN-drink, but devoid of the flavonoid. Maleic acid was used instead of citric acid as it is known that it depicts DNA-protective properties. The pH of the drink was 3.8. The walls of capsules consisted of agarose, which was used instead of gelatine as several participants were Muslims.

Table 8: Composition of the XAN-drink/XAN-capsules

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content/liter</th>
<th>Ingredients</th>
<th>Content/capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAN</td>
<td>12 mg</td>
<td>XAN</td>
<td>12 mg</td>
</tr>
<tr>
<td>Roasted malt extract</td>
<td>1 g</td>
<td>Agarose</td>
<td>100 mg</td>
</tr>
<tr>
<td>Maleic acid</td>
<td>42 mg</td>
<td>Lactose</td>
<td>422 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>40 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1000 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During the intervention phases the participants consumed one liter of the drink or one capsule daily (i.e. 12 mg XAN/day)

2.5. Design of the human intervention trials

2.5.1. Main human intervention study

22 participants (11 males/11 females) were recruited for the first study; all of them were healthy non-smokers and none of them consumed pharmaceuticals and/or food supplements. The demographic data are summarized in table 9.

Table 9: Demographic data of the participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Women (n=11)</th>
<th>Men (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>27.4 ± 5.3</td>
<td>25.0 ± 2.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165 ± 9.3</td>
<td>181 ± 7.8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>66.1 ± 10.0</td>
<td>80.8 ± 11.5</td>
</tr>
<tr>
<td>BMI</td>
<td>24.3 ± 3.6</td>
<td>24.7 ± 2.9</td>
</tr>
</tbody>
</table>

1) means ± SD
The design of the study is shown schematically in figure 14. Four days before the first sampling, the participants were asked to follow the food restriction guidelines which were developed to reduce inter-individual fluctuations and to control the uptake of flavonoids other than XAN. The participants were asked to avoid the consumption of nutritional supplements. Furthermore, the participants were asked to avoid extreme physical exercises prior and during the intervention phase, as it is known that it can cause oxidative damage [164-166]. The restriction-guidelines are summarized in table 10.

The participants consumed one liter of the study-drink between 9 am and 11 am over a period of 14 days. The study had a cross-over design and was placebo controlled. Also the placebo was consumed over a period of 14 days. Blood samples were taken from each participant after a “run-in” phase of 4 days before the start of the intervention and after 7 days and 14 days of consecutive consumption. Demographic data of the participants, their nutritional habits, as well as pulse and blood pressure were recorded.

Figure 14: Design of the study
Table 10: Restriction-guidelines

<table>
<thead>
<tr>
<th>Beverages/food/other</th>
<th>Not allowed</th>
<th>Allowed with restriction</th>
<th>Alternatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beverages</strong></td>
<td>energy drinks, coke</td>
<td>coffee (1 cup/day), black tea (1 cup/day), fruit juice (200 ml/day); wine (125 ml/day) or beer (500 ml/day)</td>
<td>mineral water, milk</td>
</tr>
<tr>
<td></td>
<td>liquor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Guinness and stout beer</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grain products</strong></td>
<td>cereals</td>
<td>whole grain bread (1 slice/day)</td>
<td>white bread, rice, pasta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td>cabbage, cabbage turnip, red cabbage, cauliflower, garden radish, Brussels sprouts</td>
<td>bell pepper (100 g/day), carrots (100 g/day), tomatoes (100 g/day), corn (100 g/day)</td>
<td>zucchini, potatoes, beans, lentils, peas, salad, cucumber, mushrooms</td>
</tr>
<tr>
<td><strong>Fruits</strong></td>
<td>kiwi, mango, papaya, sea, strawberries, rose hips, blueberries</td>
<td>lemon (1 piece/day), nectarine (1 piece/day), grapefruit (1 piece/day), apricot (1 piece/day), orange (1 piece/day)</td>
<td>apples, bananas, peas, water melons, peaches</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>vitamin and food supplements, aspirin, permanent medication</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5.2. Second human intervention trial

The second study comprised ten participants (5 males/5 females) and was conducted with encapsulated pure XAN, which was consumed daily between 9 am and 11 am over a period of two weeks. All participants were healthy non-smokers. Demographic data are listed in table 11. The experimental design was identical to the first human intervention study, but the study was not placebo-controlled and samples were collected at two time points (before the intervention and after 14 days of consumption). DNA migration induced by B(a)P and IQ was measured in lymphocytes before and after consumption of the capsules.

Table 11: Demographic data of the participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Women (n=11)</th>
<th>Men (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>31.6 ± 9.9</td>
<td>26.0 ± 2.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168 ± 5.9</td>
<td>185 ± 9.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.0 ± 7.7</td>
<td>90.3 ± 20.1</td>
</tr>
<tr>
<td>BMI</td>
<td>22.9 ± 1.9</td>
<td>26.4 ± 5.3</td>
</tr>
</tbody>
</table>

1) means ± SD
2.6. Sampling of lymphocytes and plasma

The blood of each participant was collected in six heparinized tubes (Vacutainer Systems, Becton Dickinson, Erembodegern, Belgium) (approximately 60 ml). The tubes were stored at 4° C prior to centrifugation (Sigma, Laboratory Centrifuges 4K15; 650 g, 10 min) at 4° C. Plasma (bright phase on top) was removed prior to freezing for additional analyses and stored at –80° C. The rest of the sample was diluted with 4 ml RPMI per heparin-tube and gently mixed with a pipette. After that the contents of two tubes were mixed with 15 ml Histopaque in an Accuspin-tube (Accuspin 50 ml, Sigma-Aldrich, Steinheim, Germany) and centrifuged (800 g, 15 min, 16° C). This procedure led to a sequential separation of the blood cells. The lymphocytes accumulated in a white ring between the RPMI (top phase) and Histopaque which contained erythrocytes (bottom phase). The lymphocytes were transferred with pipettes to 50 ml flasks and mixed with 12 ml RPMI. The suspensions were centrifuged (332 g, 10 min, 16° C), the supernatants removed and the pellets solved in 12 ml RPMI and centrifuged again (332 g, 10 min, 16° C). Subsequently, the supernatant was decanted and the pellet dissolved in 600 µl PBS. 100 µl of the suspension were mixed with 900 µl of freezing medium (Biofreeze) and transferred to a total of six cryotubes (2 ml, Greiner Bio-One, Frickenhausen, Germany). The tubes were stored in polystyrene boxes at -80° C over night and put to liquid nitrogen tanks the next day. Figure 15 depicts schematically the isolation of the lymphocytes.
2.6.1. Storage of the lymphocytes

Serum-free freezing medium (Biofreeze, Biochrom AG, Berlin, Germany) was used to store the lymphocytes in liquid nitrogen. This special medium does not contain DMSO, which is toxic to cells. After isolation of the lymphocytes, 100 μl of each cell suspension (approximately 10-25 billion cells) and 900 μl of Biofreeze were mixed gently and pipetted into a cryotube (Sigma-Aldrich, Steinheim, Germany). The tubes were wrapped in cotton wool and placed in a polystyrene box, which was stored in a -80° C freezer; subsequently, the tubes were transferred into liquid nitrogen after 24 hours.
2.6.2. Defrosting of lymphocytes

The lymphocytes were thawed in a water bath (37°C) and centrifuged (200 g, 5 min, at 4°C). Subsequently, the pellet was dissolved in PBS and washed once under exact the same conditions.

2.6.3. Determination of cell number and vitality

A Neubauer chamber was used to determine the vitality and the number of the cells with the trypan blue exclusion technique [167]. 20 µl of the cell suspension were mixed with 20 µl of the dye. After 60 seconds, 10 µl of the suspension were pipetted into a Neubauer counting chamber (LO-Laboroptik Ltd, Lancing, UK). The ratio between viable cells (unstained) and dead cells (blue) was determined by use of a light microscope (Nikon–TMS, Nikon GmbH, Düsseldorf, Germany) at 100-fold magnification.

2.7. Single cell gel electrophoresis assay (SCGE assay, Comet assay)

The protocol which was used in the SCGE experiments is shown schematically in figure 16. The experiments were conducted under standard conditions according to the general guidelines published by Andrew Collins et al. [168]. For each experimental point, three cultures were prepared and 50 cells were analysed.

After treatment of the cells with the chemical mutagens (chapter 2.7.1.), the vitality was determined (chapter 2.6.3.). Subsequently the cells were lysed and after electrophoresis (chapter 2.7.2.) DNA migration was determined (chapter 2.7.3.).
2.7.1. Treatment of the cells with chemical mutagens

The lymphocytes were incubated simultaneously with one of the mutagens and the human derived liver S9 activation mix in an Eppendorf tube at 37° C under constant shaking (300 rpm) for exactly 60 minutes. Subsequently, the tubes were immediately centrifuged (200 g, 5 min, at 4° C) and washed with 400 μl of PBS. This step was repeated once more before the vitality of the cells was determined in aliquots with the trypan blue method [167, 169].
2.7.2. Lysis and electrophoresis

Approximately $1 \times 10^5$ cells were mixed with 0.5% low melting point (LMP) agarose and pipetted onto a glass slide, which had been covered with 1.5% normal melting point (NMP) agarose. Subsequently, a cover slip was dropped onto the mix to ensure even distribution of the cells. To solidify the agarose, the slides were placed on a cooled metal plate. After solidification of the LMP agarose, the cover slips were removed and the slides were immersed in freshly prepared ice cold lysis solution for at least 60 minutes. Subsequently the slides were placed in a horizontal electrophoresis chamber (CBS Scientific Co., Del Mar, USA), which is filled with electrophoresis buffer for 30 minutes for unwinding of the DNA, thereafter that electrophoresis with 300 mA/25 V was conducted for another 30 minutes. Subsequently, the slides were washed twice with cooled neutralization buffer and once with H$_2$O before they were dried at room temperature.

2.7.3. Evaluation of slides

Before the evaluation, the gels were stained with 40 μl ethidium bromide (2.0 μg/ml). A fluorescence microscope (Nikon 027012, mercury lamp Osram HB10101AF, green filter 520 nm, objective Nikon Plan Fluor 10/0.3 magnification objective) was used for scoring. A camera (JVC TK-C1360 1/2-in. CCD) transferred the microscopic picture to a PC. The DNA-migration was examined using the semi-automated scoring program Comet Assay IV (Perceptive Instruments, Bury St. Edmunds, UK). The parameter which was recorded was the percentage of DNA in tail (tail intensity).

2.8. GSH measurements of plasma samples

The measurements were conducted in collaboration with W. Huber (Medical University of Vienna, Institute of Cancer Research) and his coworker R. Bohacek. 100 μl of the plasma samples were mixed with 400 μl 5% sulfosalicylic acid and 1.0 ml of Tris buffer. The mixture was pipetted into a cuvette; subsequently, the first measurement was conducted. After addition of 50 μl ice cold Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB), the second measurement was made. Per sample 5 photometric determinations were conducted.
The method is based on the use of Ellman's reagent, which can be used to measure low-molecular mass thiols such as GSH in biological samples, for example in blood [170].

Thiols react with Ellman’s reagent, dissociating the disulfide bond which leads to the formation of 2-nitro-5-thiobenzoate (NTB’). The latter compound ionizes to the NTB$^{2-}$ dianion in water under alkaline and neutral conditions. NTB$^{2-}$ is yellow and can be quantified spectrophotometrically. The rapid chemical reaction is depicted in figure 17.

Figure 17: Reaction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) with a thiol (R-SH) (adapted from [171]).

This reaction is stoichiometric; one mole of thiol releases one mole of NTB. The formation of the reaction product was determined with a spectrophotometer (Hitachi U-2001, Japan) by measuring the absorbance of light at 412 nm.

2.9. **Determination of the protein concentrations**

The protein contents in plasma were determined spectrophotometrically according to Bradford [172]. The measurements were conducted in collaboration with W. Huber (Medical University of Vienna, Institute of Cancer Research) and his coworker R. Bohacek. The plasma samples were diluted 1:1000 with PBS (pH = 7.4). 10 μl of this rarefaction were mixed with 790 μl distilled water and 200 μl Bradford reagent. The samples were incubated for 5 minutes at room temperature and measured spectrophotometrically with a Beckman DU 600 (Beckman Coulter, Brea, USA). Per sample 5 measurements were conducted in parallel.

The assay is based on the absorbance shift from 465 to 595 nm of the dye Coomassie Brilliant Blue G-250 when bound to proteins. Under acidic conditions the dye is red, but when binding to proteins occurs it changes its color to blue.
2.10. Measurement of overall GST activity in plasma

In 1974 Habig et al. [173] developed a protocol for the determination of GST activities in human plasma and saliva. The method is based on the binding of glutathione to the substrate 1-chloro-2,4-dinitrobenzene (CDNB). As a reaction product, 2,4-dinitrophenyl-glutathione is formed, it absorbs light at a wavelength of 340 nm [173]. Figure 18 shows the scheme of the reaction.

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{NO}_2 \\
\text{Cl} & \quad \quad \text{GSH} & \quad \quad \text{GST}^\# & \quad \quad \text{O}_2\text{N} & \quad \text{NO}_2 \\
\end{align*}
\]

1-chloro-2,4-dinitrobenzene \quad 2,4-dinitrophenyl-glutathione

Figure 18: Reaction of GSH with CDNB (adapted from [173])

The extinction coefficient of 2,4-dinitrophenyl-glutathione is 9.6. The spectrophotometric analysis of enzyme activity is based on product formation and is a composite result of the activity of each isoenzyme present in plasma [173].

980 μl natriumphosphate buffer, 10 μl GSH solution, 10 μl CDNB and 10 μl sample were mixed directly in the cuvette and the reaction time was 180 seconds and the temperature of the reaction mix was 37°C. The wavelength was adjusted to 340 nm. Per individual sample two replicates were measured. The blank was determined using distilled water instead of sample and was also determined in duplicate.

2.11.a Measurement of GST-α in plasma samples

The GST-α content in plasma was determined with a quantitative immunoassay (Alpha GST ELISA, Argutus Medical Ltd., Dublin, Ireland). The measurement is based on the sequential addition of sample, enzyme conjugate and substrate to microassay wells coated with anti-GST-α IgG. The colour intensity is proportional to the amount of GST-α present in the sample. Wash buffer (TBST) and calibrators were prepared according to the instructions of the manufacturer. Immediately before the start of the assay the samples were diluted 1/5 by
adding 50 μl sample to 200 μl sample diluents. Microassay wells were placed in the assay plate and after that calibrators (0, 2.5, 5, 10, 20, 40, and 80 μg/l), positive controls and diluted samples were added to the microassay plates in duplicate (100 μl/well). Subsequently, the plate was covered and incubated at room temperature for 60 min and shaken gently (350 rpm). Thereafter the cover was removed and each well was washed 4 times with washing buffer (250 μl/well). After the washing steps the plate was firmly tapped against a pile of paper towels. In the next step 100 μl conjugate were added to each well. Again the microassay plates were incubated at room temperature for 30 minutes and shaken gently (350 rpm). After that the plate was washed 4 times as described above. In the next step 100 μl of substrate were added using a multichannel pipette and incubated at room temperature in the dark for exactly 15 minutes with no shaking. 100 μl of stop solution is added to each well using again a multichannel pipette. After that the plate was immediately read at 450 nm and 630 nm as reference using the Tecan Infinite 200 plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.11.b Measurement of GST-π in plasma samples

The GST-π content in plasma was determined with a quantitative immunoassay (Pi GST ELISA, Argutus Medical Ltd., Dublin, Ireland). The test procedure is similar to that of the GST-α measurements, but a different antibody was used (anti-GST-π IgG).

2.12. Statistical analysis

2.12.1. Comet results

The mean tail intensities (% DNA in tail) from 50 cells per slide (3 slides per person per time point) were calculated and used for statistical analysis with GraphPad Prism 5.02 (Graph Pad Software, San Diego, USA). As suggested in international recommendations for SCGE assays, the Kruskal Wallis-test without assumption of Gaussian distribution was applied, followed by post hoc Dunn’s comparison [174]. P-values ≤ 0.05 were considered as statistically significant.
2.12.2. Results of GSH measurements and enzyme measurements

Measurements of the overall (CDNB) activity and the concentrations of the individual isoenzymes were conducted in duplicate; whereas the GSH and protein contents in plasma were analyzed in five individual measurements per sample. Statistical significances were analyzed with the Student’s t-test. For all comparisons, p-values $\leq 0.05$ were considered as statistically significant. The statistical calculations were conducted via the program GraphPad Prism 5.02 (Graph Pad Software, San Diego, USA).
3. Results

The results section of the thesis consists of three main chapters. The first describes the findings of preliminary experiments which were conducted in order to determine the optimal concentrations of the three genotoxic food related carcinogens. The second chapter concerns the results of the first intervention study with a XAN-containing refreshment drink and contains data of single cell gel electrophoresis assays and results of enzyme measurements, whereas the third part describes the second human intervention study which was performed with encapsulated XAN.

3.1. Results of the preliminary experiments

Prior to the human intervention studies, preliminary experiments with different concentrations of the genotoxic carcinogens were conducted in order to establish dose-response relationships with the three substances. The experiments were conducted as described in detail in chapter 2.7. (page 35).

It can be seen in figures 19, 20 and 21 that all three compounds caused induction of DNA damage in presence and absence of metabolic activation mix. In the case of NDMA, a significant effect was seen in absence of S9-mix with concentrations ≥ 200 μM, whereas much more pronounced DNA-migration was observed in presence of the enzyme mix. A ten-fold lower concentration (20 μM) induced a significant increase of the tail intensity (figure 19-C) under these conditions. As shown in figures 19-B and 19-D, no signs of acute toxicity were seen in both experimental series.
Figure 19: Effects of different concentrations of NDMA in peripheral human lymphocytes. Cells from three healthy individuals (1 male, 2 females) were used. Per experimental point three cultures were treated, three slides were prepared and 150 cells were scored. Lymphocytes were exposed to different concentrations of NDMA in absence or in presence of human derived liver S9-mix for 60 minutes. The vitality of the cells was determined with the trypan blue exclusion technique (figure 19-B and figure 19-D). DNA migration was measured in SCGE assays which were performed under standard conditions (20 min unwinding, 20 min electrophoresis) (figure 19-A and figure 19-C). Bars represent means with standard deviations. c - solvent control (PBS); p-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.

Also in experiments with B(a)P induction of DNA damage was detected without S9. However, the effects were again more pronounced in presence of exogenous activation mix. With 2.5 μM the percentage of DNA in tail was three-fold higher as that seen in the controls (figure 20-C). As shown in figures 20-B and 20-D the vitality of the cells was under all experimental conditions ≥ 80%.
Figure 20: Effects of different concentrations of B(a)P in peripheral human lymphocytes. The experiments were conducted as described in the legend to figure 19. c-solvent control (DMSO); p-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.

Also with the third substance (IQ), induction of comet formation was detected in absence of activation mix. In both experimental series, identical concentrations were tested, but addition of the liver enzyme homogenate caused an approximately 2-fold increase of DNA-migration at the lowest dose (1 mM), which was statistically significant (figure 21-C). As shown in figure 21-D the vitality of the cells was not affected after exposure of the cells to the heterocyclic aromatic amine.
Figure 21: Effects of different concentrations of IQ in peripheral human lymphocytes. The experiments were conducted as described in the legend of figure 19. c-solvent control (DMSO); p-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.

3.2. Results of the human intervention trial with the XAN-drink

On the basis of the results obtained in the dose-response experiments, a concentration of 30 μM NDMA was used in the main study to investigate the impact of consumption of the XAN-drink on the extent of DNA-migration. The doses of B(a)P and IQ were 5.0 μM and 1.0 mM, respectively.

3.2.1. Results from the single cell gel electrophoresis assays with the chemical carcinogens

It can be seen in figure 22-B that the nitrosamine induced comet formation and the extent of NDMA-induced DNA damage declined marginally (-3.7%) after 14 days of consecutive
consumption of the drink. This effect failed to reach statistical significance. The findings which were obtained with the placebo are shown in figure 22-A. Also in this case no significant changes were detected.

**Figure 22: Effects of XAN consumption on NDMA induced DNA migration in human peripheral lymphocytes.** Figure 22-B shows the results which were obtained with a XAN containing drink; figure 22-A describes the findings which were obtained with a placebo (roast malt drink without XAN). 22 healthy participants (11 males, 11 females) consumed 1.0 liter of a XAN-containing drink or a placebo over a period of 14 days. Blood samples were collected at several time points, namely immediately before the start of the intervention (0 d), 7 days (7 d) and 14 days (14 d) after consecutive consumption. The lymphocytes were isolated as described in chapter 2.6. The experiments were conducted as described in the legend of figure 19 with a dose of 30 μM NDMA. Per person and time point 3 slides were prepared and 150 cells were scored. SCGE-measurements were conducted under standard conditions (30 min unwinding, 30 min electrophoresis). Figures 22-C and 22-D depict the vitality of the cells, which were measured with the trypan blue exclusion technique. Bars with error bars represent means with standard deviations. Labels on the y-axis indicate the percentage of DNA in tail or vitality in percent, labels on the x-axis the time points of sampling. c- solvent controls (PBS); p-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.
The results which were obtained under identical experimental conditions with B(a)P and IQ are summarized in figures 23 and 24. It can be seen that B(a)P induced a significant increase of comet formation which was reduced after the consumption of the XAN drink. The tail intensities declined after 7 days by 15.5% and after 14 days by 19.2% (in both cases $p \leq 0.05$).

**Figure 23: Effects of XAN consumption on B(a)P induced DNA migration in human peripheral lymphocytes.** The B(a)P concentration which was used in all experiments was 5 μM. The experiments were conducted as described as in the legend of figure 19; $p$-values $\leq 0.05$ were considered as statistically significant and are marked with asterisks.
IQ induced comet formation in the lymphocytes also in the intervention study with the XAN-containing drink. The reduction seen after 7 days of continuous intake of the drink was 12.8% and 14.7% after 14 days of the intervention period, respectively. Both effects were statistically significant ($p \leq 0.05$). It can be also seen in figures 23-C, and 23-D that the vitality of the cells was between 80 and 100% in all experiments.

Figure 24: Effects of consumption of a XAN-drink on IQ induced DNA migration in human peripheral lymphocytes. The IQ concentration which was used in all experiments was 1.0 mM. The experiments were conducted as described as in the legend of figure 19; $p$-values $\leq 0.05$ were considered as statistically significant and are marked with asterisks.
3.2.2. Determination of the glutathione (GSH) levels in plasma

The results are summarized in Figure 25. At the end of the intervention trial the concentrations of the tripeptide were 17.9 % lower than at the start of the study, but the effect did not reach significance.

![GSH contents in plasma before and after intervention with the XAN containing drink.](image)

**Figure 25: GSH contents in plasma before and after intervention with the XAN containing drink.** The levels of the tripeptide were determined spectrophotometrically with Ellman’s reagent as a substrate according to the protocol of Boyne [171] (see chapter 2.8.). Bars represent means with standard deviations of GSH contents measured in duplicate per sample (n = 22). The x-axis represents the time points of sampling. P-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.

3.2.3. Impact of the consumption of XAN on the overall glutathione-S-transferase (GST) levels in plasma

The results of the enzyme measurements are depicted in figure 26. It can be seen that consumption of none of the two drinks had a significant impact on the overall (CDNB) activity of GST.
Figure 26: GST activity in plasma before and after intervention with a XAN-drink. The levels of the enzyme were determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene as a substrate according to the protocol of Habig [173] (see chapter 2.10.). The protein concentrations were determined spectrophotometrically according to Bradford [172] (see chapter 2.9.). Bars represent means with standard deviations of GST activity measured in duplicate per sample (n = 22). Labels on the y-axis represent activity in $\mu$mol/min/µg protein; labels on the x-axis represent the time point of sampling. P-values $\leq 0.05$ were considered as statistically significant and are marked with asterisks.

3.2.4. Impact of the intervention on the activities of GST-\(\pi\) and GST-\(\alpha\) in plasma

The alterations of the isoenzymes before and after intake of the drinks are depicted in figures 27 and 28. The levels of GST-\(\pi\) decreased slightly after the consumption of the XAN-drink and of the placebo; however, these effects failed to reach significance. In figure 28-B it can be seen that the activity of the isoenzyme GST-\(\alpha\) was clearly increased (by 58.4 %) after the intervention, while no such effect was observed in participants who consumed the drink without XAN (figure 28-A).
Figure 27: GST-π contents in plasma before and after consumption of a XAN-drink. The levels of the enzyme were determined with the quantitative immunoassay Pi GST ELISA (see chapter 2.11b). Bars represent means with standard deviations of GST-π contents measured in duplicate per sample (n =22). Labels on the y-axis represent GST-π contents in μg/l, labels on the x-axis represent the sampling times.

Figure 28: GST-α contents in plasma before and after intervention with a XAN-drink. The levels of the enzyme were determined with the quantitative immunoassay Alpha GST ELISA (see chapter 2.11a). The measurements were conducted in a similar way as described in the legend of figure 27. P-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.
3.3. Results of the follow-up study with encapsulated XAN

The findings from a trial in which the participants consumed an identical amount (12 mg/person/day) of the flavonoid as that contained in the refreshment drink in encapsulated form over a period of two weeks are summarized in figures 29 and 30. It can be seen that a similar pattern of changes of the extent of chemically induced DNA-damage was observed. B(a)P- and IQ-induced comet formation were significantly reduced by 14.6% and 23.6%, respectively.

Figure 29: Effects of consumption of pure XAN on B(a)P induced DNA migration in human peripheral lymphocytes. 10 healthy participants (5 males, 5 females) consumed a XAN containing capsule (12 mg/person/day) over a period of 14 days. Blood samples were taken at several time points, namely immediately before the start of the intervention and after 14 days of consecutive consumption. The lymphocytes were isolated as described in chapter 2.6. The experiments were conducted as described in the legend of figure 19 with a dose of 5.0 μM B(a)P. Per person and time point 3 slides were prepared and 150 cells were scored. SCGE-measurements were conducted under standard conditions (30 min unwinding, 30 min electrophoresis), the vitality of the cells was detected with the trypan blue assay. Bars represent means with standard deviations. The bars indicate the percentage of DNA in tail or vitality in percent, labels on the x-axis the time points of sampling. c–solvent control (DMSO); P-values ≤ 0.05 were considered as statistically significant and are marked with asterisks.
Figure 30: Effects of consumption of pure XAN on IQ induced DNA migration in human peripheral lymphocytes. The IQ concentration which was used in all experiments was 1.0 mM. The experiments were conducted as described in the legend to figure 19. P-values $\leq 0.05$ were considered as statistically significant and are marked with asterisks.
4. Discussion

The present study yielded three major results, namely (1) the development of a modified SCGE-protocol which can be used to detect protective effects of dietary constituents in human intervention trials, (2) evidence for the fact that XAN, a prenylated hop flavonoid, protects humans against induction of DNA-damage by two representatives of important groups of chemical carcinogens which are contained in the human diet and (3) it was found that the protective effects are probably due to induction of GST-α, which catalyses the detoxification of a broad variety of DNA-reactive electrophilic metabolites of chemical carcinogens.

4.1. The development of the protocol

The basic principle of the modified SCGE-protocol (see figure 16) is the combination of human derived liver S9 with SCGE-experiments in which peripheral lymphocytes were collected before and after consumption of a putative protective compound. Already in the 1960s it was clear that most indicator cells which are used in \textit{in vitro} genotoxicity assays are devoid of drug metabolizing enzymes which catalyze the activation of important groups of carcinogens such as PAHs, HAs, nitrosamines, certain mycotoxins and many more. Therefore H. Malling developed in 1971 a protocol for the so-called “S9-mix”, a liver derived enzyme homogenate which was prepared from “activated” rodent livers in order to ensure optimal activity of the enzyme [175]. NADPH and several cofactors were added to the fraction which was isolated after homogenization of hepatic tissue by centrifugation at 9000 g [175]. This metabolic activation mix is added to bacterial indicator cells [163], but also to stable mammalian cell lines which are devoid of phase I enzymes in \textit{in vitro} tests [176]. Routine tests are performed in most cases with liver homogenates from rats which are usually pretreated with enzyme inducers such as Aroclor 1254 or with combinations of phenobarbital and 3-methylcholanthrene [177]. In some studies, liver enzyme preparations from other species and/or other organs were used [178-179]. In the present study, we used human derived S9 preparation which has the advantage that the enzymes reflect a substrate specificity of the different enzymes in man. It is known that distinctive differences exist in regard to the activity and specificity of cytochromes P450 in humans [180-181].
Human derived liver homogenates were used earlier in a number of model studies with different genotoxic carcinogens, but due to their limited availability they were not employed in routine testing [182]. Some of these studies were conducted to elucidate species differences and in most trials bacterial indicator cells were used. Also the model compounds which were included in the present study have been tested with human derived liver mixtures. IQ was investigated in several series [183-184] and it was shown in Salmonella/microsome assays that use of pooled human S9-mix leads to a lower number of induced revertants as compared to the mutants yield which were obtained with rat S9 [181, 185]. Also B(a)P was tested and a more potent effect was seen with rat derived liver homogenates [181, 185]. In the case of NDMA, however, it was found that the human derived liver mix is approximately 8-fold more potent in terms of mutagenic activity as S9-mix from rats [181, 185].

Due to lack of co-factors, phase II enzymes are not represented in the S9-mix [186]; however, some of them are active in peripheral lymphocytes, which were used in the present study as indicator cells. Apart from GSTs which are the most important detoxification system in mammals and humans, also a number of other enzymes including those which are involved in the detoxification of ROS (haem oxygenase (HO), superoxide dismutase (SOD), glutathione peroxidase (GPx)) are found in these cells [187-189].

As shown in figures 19-21 (pages 43-45) we found clear induction of comet formation in the lymphocytes with the different model compounds in the presence of human derived S9-mix. According to our knowledge, no SCGE-experiments have been conducted with this model, but it was shown that B(a)P and BPDE cause significant induction of chromosomal aberrations and MN in human lymphocytes after activation with liver S9 from rats and humans as well [190-192]. Also with IQ and NDMA induction of MN and of chromosomal damage was reported from experiments with peripheral human lymphocytes after activation with exogenous liver homogenate [193-195].

It is notable that we found in the preliminary experiments also an increase of DNA migration in absence of activation mix with all three model compounds. This phenomenon can be explained by the fact that other metabolic activation pathways may lead to activation and/or spontaneous formation of electrophilic metabolites and may cause these effects. In this context it is notable that Nersesyan et al. [196] reported earlier on induction of DNA-migration by IQ, B(a)P and several other carcinogens in human lymphocytes without S9. However, since the concentrations of the test compounds which were required to cause measureable effects were substantially higher and do not reflect the situation in humans, we decided to use the liver homogenate incubation protocol.
4.2. DNA-protective effects of XAN in humans

The following chapters describe the protective effects of the XAN drink and of the pure compound which were observed in the intervention trials. As mentioned in the introduction section, indication for DNA protective effects against different genotoxic carcinogens was observed in earlier in vitro studies with XAN and also in in vivo studies with rats. The results of these earlier investigations are listed in table 12.

Table 12: Impact of XAN on DNA-stability and on the activities of drug metabolizing enzymes: results of earlier experiments

<table>
<thead>
<tr>
<th>Experimental model/indicator cells</th>
<th>Results/remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella/microsome gene mutation test</td>
<td>↓ revertants induced by MeIQx and PhIP</td>
<td>[197]</td>
</tr>
<tr>
<td>SCGE-assay with human hepatoma HepG2 cells</td>
<td>↓ DNA-strand breaks induced by MeIQx and PhIP</td>
<td>[197]</td>
</tr>
<tr>
<td>CBMN-assay with human hepatoma HepG2 cells</td>
<td>↔ frequencies of MNi, NPBs, NBs and NDI while coexposure of the cells with XAN and PhIP</td>
<td>[197]</td>
</tr>
<tr>
<td>QRT-PCR gene expression analysis in human hepatoma HepG2 cells</td>
<td>↑ gene expression of CYP1A1, CYP1A2 and UGT1A ↓ gene expression of GSTA1 and SULT1A1</td>
<td>[197]</td>
</tr>
<tr>
<td>CBMN-assay with human hepatoma HepG2 cells</td>
<td>↓ formation of DNA-strand breaks induced by IQ and B(a)P</td>
<td>[198]</td>
</tr>
<tr>
<td>DPPH-assay with human hepatoma HepG2 cells</td>
<td>↓ damage caused by t-BOOH in cells pretreated with XAN</td>
<td>[198]</td>
</tr>
<tr>
<td>Spectrophotometric methods in rat hepatoma H4IIE cells</td>
<td>↓ activity of CYP1A</td>
<td>[6]</td>
</tr>
<tr>
<td>Spectrophotometric measurements in murine hepatoma Hepa 1c1c7 cells</td>
<td>↑ activity of QR</td>
<td>[6]</td>
</tr>
<tr>
<td>Spectrophotometric measurements in murine hepatoma Hepa 1c1c7 cells</td>
<td>↑ activity of QR</td>
<td>[199]</td>
</tr>
<tr>
<td>Spectrophotometric measurements in murine hepatoma Hepa 1c1c7 cells</td>
<td>↓ single strand DNA-breaks induced by menadione</td>
<td>[199]</td>
</tr>
<tr>
<td>Salmonella/microsome gene mutation test</td>
<td>↓ revertants induced by IQ</td>
<td>[200]</td>
</tr>
</tbody>
</table>
Table 12: continued

<table>
<thead>
<tr>
<th>Experimental model/indicator cells</th>
<th>Results/remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCGE-assay with human hepatoma HepG2 cells</td>
<td>↓ DNA-strand breaks induced by IQ</td>
<td>[200]</td>
</tr>
<tr>
<td>SCGE-assay with liver and colon cells from F344 rats</td>
<td>↓ DNA-strand breaks induced by IQ in liver and colon cells</td>
<td>[14]</td>
</tr>
<tr>
<td>GST-P⁺ foci in livers of F344 rats</td>
<td>↓ numbers and area of GST-P⁺ foci</td>
<td>[14]</td>
</tr>
</tbody>
</table>

1) QR: quinone reductase, MeIQx: 2-mono-3,4-dimethylimidazo(4,5-f)quinoline; PhIP: 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, MN: micronucleus, NPB: nucleoplasmic bridges; NB: nuclear bud; NDI: nuclear division index; CYP: cytochrome; UGT: uridine 5'-diphospho-glucuronosyl-transferase; SULT: sulfotransferase; t-BOOH: tert-butyl hydroperoxide; GST-P⁺ foci: glutathione S-transferase placental form (GST-P)-positive foci

4.2.1. Protection of XAN against B(a)P-induced DNA-migration

The results which were obtained in the intervention trial with the XAN drink and with the pure flavonoid are shown in figure 23 (page 47) and figure 29 (page 52). The findings are indicative for substantial protective effects. These observations confirm the results which were published by M. Filipic and her coworkers [198], who showed that XAN protects human hepatoma cells against B(a)P-induced comet formation.

4.2.2. Inhibition of IQ-induced DNA-damage by XAN

XAN was also protective against induction of IQ-induced DNA migration. It is notable that earlier in vitro studies with bacterial and mammalian indicator cells showed protective effects, which were also seen in experiments with rats in which induction of DNA migration was reduced in inner organs (i.e. in the liver and colon) when the animals were pretreated with XAN [14]. As mentioned above, these DNA-protective effects were paralleled by inhibition of formation of preneoplastic foci in both organs.

4.2.3. Lack of a protective effect against NDMA-induced DNA-damage

As described in the results section (page 45 and 46), no significant protection against NDMA-induced DNA damage was seen in the present study. According to our knowledge no results
from earlier investigations are available in which potential protective effects against nitrosamines were investigated with the prenylated flavonoid.

4.2.4. Molecular mechanism of protection

Intense efforts to identify dietary compounds which protect against DNA damage and cancer induced by nitrosamines, PAHs and HAs have been made in the past. The results of these investigations are described in a number of review articles. A comprehensive description of anti-mutagenicity and anti-carcinogenicity studies with HAs can be found in the articles of Schwab et al. [23] and Dashwood et al. [201], protective studies against nitrosamines, B(a)P and other PAHs are summarized in the paper by Weisburger [202].

A broad variety of mechanisms which led to the prevention of DNA-damage and to a reduction of tumor formation has been identified (for review see [203]). In the case of B(a)P the most relevant detoxification pathway is conjugation with glutathione, which is catalyzed by glutathione-S-transferase. Therefore, induction of this enzyme plays a major role in the protection against this compound and other PAHs. In regard to inactivation of electrophilic metabolites of IQ, UGT plays a dominant role [204]. However, as mentioned above it was also reported that GST may inactivate electrophilic metabolites.

As described in the results section clear evidence for induction of GST-α after consumption of XAN in the plasma of the participants was found. This observation provides a plausible explanation for the prevention of B(a)P- and IQ-induced DNA-damage. It is notable that induction of GST-α was also seen in a number of earlier experiments with dietary constituents, for example with Brassica and Allium vegetables in human studies [156-157, 188, 205]. In this context it is notable that these dietary constituents were also found to be protective against DNA damage caused by PAHs and HAs [206-209].

4.3. Comparison of the protective potency of XAN with other chemopreventive dietary constituents

The dose of XAN (12 mg/person/day) which was used in the present study was chosen on the basis of the amount which was tested in an in vivo study with rats by Ferk et al. [14]. This
amount is substantially lower as the doses of other compounds which were required to cause protective effects against PAHs and HAs.

In tables 13 and 14 some results of in vivo studies are listed in which protective effects against B(a)P were investigated. The human equivalent doses of the different plant derived anti-mutagens/anti-carcinogens are shown in the left columns and were calculated according to Shannon Reagan-Shaw et al. [210]. It can be seen that the human equivalent doses (HED) of the substances which are required to cause significant protective effects (reduction of DNA-damage and/or tumor rates) were at least 6-fold higher than the XAN-dose which was found to be effective in the present study. It is notable that, apart from these in vivo studies with laboratory rodents, also results of a few human trials were published in which protection against BPDE-induced DNA damage was monitored in lymphocytes in intervention trials. The findings of these studies are listed in table 13. All interventions were conducted with complex foods, therefore it is not possible to compare the results with the present study.

Table 13: Influence of dietary constituents on the genotoxic activity of B(a)P in animal experiments \(^1\)

<table>
<thead>
<tr>
<th>Test system/test compound/human equivalent doses</th>
<th>Dose/treatment</th>
<th>Results/remark</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal aberration (CA) and MN in bone marrow cells, comet assay in peripheral blood cells of Swiss albino mice Soy isoflavons (SI) HED: 1.6, 3.2 mg/kg b.w.</td>
<td>SI (20 or 40 mg/kg b.w.) feeding for 7 days B(a)P (125 mg/kg b.w.) 1x p.o., sacrifice after 30 h for CA or 28 h MN</td>
<td>CA ↓ 49.5% (20 mg/kg), ↓ 69.3% (40 mg/kg) MN ↓ 36.9% (20 mg/kg), ↓ 57.3% (40 mg/kg) DNA damage (F-value) ↓ 76.6% (20 mg/kg), ↓ 100% (40 mg/kg)</td>
<td>[211]</td>
</tr>
<tr>
<td>Bone marrow MN in NMRI mice Isoquercetin (isoQ) HED: 1.1 mg/kg b.w.</td>
<td>isoQ (13.9 mg/kg b.w.) i.p. B(a)P (150 mg/kg b.w.) 1x i.p., sacrifice after 48 h</td>
<td>MN ↓ 32.9%</td>
<td>[212]</td>
</tr>
<tr>
<td>Test system/test compound/human equivalent doses</td>
<td>Dose/treatment</td>
<td>Results/remark</td>
<td>Ref.</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
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<td>------</td>
</tr>
<tr>
<td>Chromosomal aberration (CA) and sister chromatid exchanges (SCE) assays in the bone marrow and MN assay in peripheral blood cells of Swiss albino mice</td>
<td>Cur (140 mg/kg b.w.) by gavage for 5 days B(a)P (40 mg/kg b.w.) 1x i.p.</td>
<td>CA ↓ 43% MN ↓ 48% SCE ↓ 46%</td>
<td>[213]</td>
</tr>
<tr>
<td>Curcumin (Cur) HED: 11.4 mg/kg b.w.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA and SCE assays in the bone marrow and MN assay in peripheral blood cells of Swiss albino mice</td>
<td>BTP (200 mg/kg b.w.) by gavage for 5 days B(a)P (40 mg/kg b.w.) 1x i.p.</td>
<td>MN ↓ 55% SCE ↓ 45%</td>
<td>[213]</td>
</tr>
<tr>
<td>Black tea polyphenols (BTP) HED: 16.2 mg/kg b.w.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCGE assay in ICR mice Quercetin (Q) HED: 40.5, 81.1, 162.2 mg/kg b.w.</td>
<td>Q (0.5, 1, 2 g/kg b.w.) feeding for 90 days B(a)P (100 mg/kg b.w.) 1x i.p.</td>
<td>Tail length in ♀ mice ↓ 33.9% (1 g/kg), ↓ 34.5% (2 g/kg) Tail length in ♂ mice ↓ 14.9% (0.5 g/kg), ↓ 23% (1 g/kg), ↓ 33.7% (2 g/kg) % DNA in tail in ♀ mice ↓ 12% (0.5 g/kg), ↓ 72% (1 g/kg), ↓ 76.3% (2 g/kg) % DNA in tail in ♂ mice ↓ 59.1% (0.5 g/kg), ↓ 60.6% (1 g/kg), ↓ 69.4% (2 g/kg)</td>
<td>[214]</td>
</tr>
<tr>
<td>SCGE assay in ICR mice Quercetin (Q) HED: 40.5, 81.1, 162.2 mg/kg b.w.</td>
<td>Q (0.5, 1, 2 g/kg b.w.) feeding for 90 days B(a)P (100 mg/kg b.w.) 1x i.p.</td>
<td>Tail length in ♀ mice ↓ 33.9% (1 g/kg), ↓ 34.5% (2 g/kg) Tail length in ♂ mice ↓ 14.9% (0.5 g/kg), ↓ 23% (1 g/kg), ↓ 33.7% (2 g/kg) % DNA in tail in ♀ mice ↓ 12% (0.5 g/kg), ↓ 72% (1 g/kg), ↓ 76.3% (2 g/kg) % DNA in tail in ♂ mice ↓ 59.1% (0.5 g/kg), ↓ 60.6% (1 g/kg), ↓ 69.4% (2 g/kg)</td>
<td>[214]</td>
</tr>
</tbody>
</table>
### Table 13: continued

<table>
<thead>
<tr>
<th>Test system/ test compound/human equivalent doses</th>
<th>Dose/treatment</th>
<th>Results/remark</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduction of tumor rates and volume</strong></td>
<td></td>
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<tr>
<td>Lung tumor rates in Swiss albino mice</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hesperidin (H)</td>
<td>H (25 mg/kg b.w.) p.o. 1 day before B(a)P treatment for 16 weeks (group 1) or 8 weeks after B(a)P treatment for 8 weeks (group 2)</td>
<td>Tumor rates in group 1 ↓ 50% tumor rates in group 2 ↓ 36.3%</td>
<td>[215]</td>
</tr>
<tr>
<td>HED: 2.0 mg/kg b.w.</td>
<td>B(a)P (50 mg/kg b.w.) 2x/week p.o. over 4 weeks</td>
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<tr>
<td>Lung tumor rates and tumor volumes in A/J mice</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Polyphenon E (mix of green tea catechins)</td>
<td>Polyphenon E (20, 40, 60, 80 mg/kg b.w.) feeding for 20 weeks starting one week after B(a)P treatment</td>
<td>Tumor rates ↓ 45.46% (80 mg/kg b.w.) Tumor volume ↓ 64.7% (20 mg/kg b.w.), ↓ 78.3% (40 mg/kg b.w.), ↓ 89.5% (60 mg/kg b.w.), ↓ 93.6% (80 mg/kg b.w.)</td>
<td>[216]</td>
</tr>
<tr>
<td>HED: 1.6, 3.2, 4.9, 6.5 mg/kg b.w.</td>
<td>B(a)P (100 mg/kg b.w.) 1x i.p.</td>
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<tr>
<td>Lung tumor rates and tumor volumes in A/J mice</td>
<td></td>
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</tr>
<tr>
<td>Deguelin (D)</td>
<td>D (5, 10 mg/kg b.w.) feeding for 22 weeks; B(a)P (100 mg/kg b.w.) 1x i.p.</td>
<td>Lung tumor rates ↓ 36% (5 mg/kg b.w.) ↓ 55.5% (10 mg/kg b.w.) Tumor volume ↓ (5 mg/kg b.w.), ↓ 77.8% (10 mg/kg b.w.)</td>
<td>[217]</td>
</tr>
<tr>
<td>HED: 0.4 mg/kg b.w.</td>
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</tbody>
</table>

1) B(a)P – benzo(a)pyrene; HED – human equivalent dose; MN – micronucleus; CA – chromosomal aberration; SCE – sister chromatid exchange; SCGE assay – single cell gel electrophoresis assay; i.p. – intraperitoneal; p.o. – oral administration; b.w. – body weight
Table 14: Influence of food and beverages on the genotoxic activity of B(a)P/BPDE in human intervention trials 1)  

<table>
<thead>
<tr>
<th>Test system/test foods</th>
<th>Dose/Treatment</th>
<th>Results/Remark</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human intervention study (n=8 ♀) DNA adduct measurements (modified 32P –postlabelling assay) with peripheral blood lymphocytes blueberry/apple juice (quercetin-rich)</td>
<td>participants consumed 1 l of blueberry/apple juice over 4 weeks ex vivo treatment of lymphocytes with 1µM B(a)P</td>
<td>↔ BPDE-DNA adducts both after 2 and 4 weeks</td>
<td>[218]</td>
</tr>
<tr>
<td>Human intervention study (n=168) DNA adduct measurements (modified 32P –postlabelling assay) with peripheral blood lymphocytes blueberry/apple juice (quercetin-rich)</td>
<td>participants consumed 1 l of blueberry/apple juice over 4 weeks ex vivo treatment of lymphocytes with 1 µM B(a)P</td>
<td>BPDE-DNA adducts ↑ 28.4%</td>
<td>[219]</td>
</tr>
<tr>
<td>Human intervention study (n=6) SCGE assay with whole blood Hot mustard</td>
<td>participants consumed 20 g of hot mustard (isothiocyanates) over 4 days, blood collected each 12 h whole blood exposed to 5µM BPDE</td>
<td>Tail moment ↓ 33.9% (24 h), ↓ 45% (48 h), ↓ 34% (72 h), ↓ 14.3% (6 d)</td>
<td>[220]</td>
</tr>
<tr>
<td>Human intervention study (n=6) SCGE assay with peripheral lymphocytes Unfiltered coffee</td>
<td>Participants consumed 1 l of unfiltered coffee over 5 days ex vivo treatment of lymphocytes with 0.4 mM BPDE</td>
<td>Tail length ↓ 45%</td>
<td>[137]</td>
</tr>
</tbody>
</table>

1) B(a)P–benzo(a)pyrene; BPDE-benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide; SCGE assay–single cell gel electrophoresis assay

The results which were obtained with IQ in some animal studies are shown in table 15. Again, evidences for protective effects were restricted to doses which were substantially higher as the amounts of XAN which were consumed by the participants in the present investigations.
Table 15: Influence of dietary constituents on the carcinogenic activity of IQ in laboratory rodents

<table>
<thead>
<tr>
<th>Test system/test compound/human equivalent doses</th>
<th>Dose/Treatment</th>
<th>Results/Remark</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduction of preneoplastic foci</strong></td>
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<tr>
<td>GST-P⁺ foci in livers of F344 rats</td>
<td></td>
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</tr>
<tr>
<td>Quercetin (Q)</td>
<td>Q (600 mg/kg b.w.) feeding for 1 week; IQ (100 mg/kg b.w.) by gavage 12 h after partial hepatectomy + phenobarbital + galactoseamine</td>
<td>↓ 23% number of GST-P⁺ foci, ↔ foci area</td>
<td>[16]</td>
</tr>
<tr>
<td>HED: 97.3 mg/kg b.w.</td>
<td></td>
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<tr>
<td>Ellagic acid (EA)</td>
<td>EA (600 mg/kg b.w.) feeding for 1 week; IQ (100 mg/kg b.w.) by gavage 12 h after partial hepatectomy + phenobarbital + galactoseamine</td>
<td>↔ number of GST-P⁺ foci, ↔ foci area</td>
<td>[16]</td>
</tr>
<tr>
<td>HED: 97.3 mg/kg b.w.</td>
<td></td>
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<tr>
<td>ACF in the colons of BALB/cA nude mice (n=10/group)</td>
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<tr>
<td>Epigallocatechin gallate (EGCG)</td>
<td>EGCG (5, 10, 20 mg/kg b.w.) feeding for 2 weeks; IQ (50 mg/kg b.w.) by gavage over 2 weeks; sacrifice after 6 weeks</td>
<td>number of ACF ↔ (5 and 10 mg/kg) ↓ 73% (20 mg/kg)</td>
<td>[15]</td>
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<tr>
<td>HED: 0.4, 0.8, 1.6 mg/kg b.w.</td>
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<tr>
<td>GST-P⁺ foci in livers of F344 rats</td>
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<td></td>
</tr>
<tr>
<td>Diallyl sulfide (DAS)</td>
<td>DAS (200 mg/kg b.w.) feeding for 8 weeks; IQ (100 mg/kg b.w.) 1x by gavage</td>
<td>↓ 29% number of GST-P⁺ foci</td>
<td>[16]</td>
</tr>
<tr>
<td>HED: 32.4 mg/kg b.w.</td>
<td></td>
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<tr>
<td>Vanillin (V)</td>
<td>V (600 mg/kg b.w.) feeding for 8 days; IQ (100 mg/kg b.w.) 1x by gavage</td>
<td>↓ 28% number of GST-P⁺ foci</td>
<td>[16]</td>
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<tr>
<td>HED: 97.3 mg/kg b.w.</td>
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<tr>
<td><strong>Reduction of DNA damage</strong></td>
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<tr>
<td>DNA adducts in livers of Sprague-Dawley rats</td>
<td>Chlorophyllin (150 mg/kg b.w.) by gavage; IQ (50 mg/kg b.w.) 1x by gavage</td>
<td>↓ ca. 50%</td>
<td>[221]</td>
</tr>
<tr>
<td>Chlorophyllin</td>
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<tr>
<td>HED: 24.3 mg/kg b.w.</td>
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</table>
Table 15: continued

<table>
<thead>
<tr>
<th>Test system/test compound/human equivalent doses</th>
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<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA adducts in livers of F344 rats Indole-3-carbinol (I3C) HED: 9.7 mg/kg b.w.</td>
<td>I3C (60 mg/kg b.w.) feeding for 8 weeks; IQ (50 mg/kg b.w.) 1x p.o. injection, sacrifice 48 h later</td>
<td>↑ ca. 100% (after 8 h); ↓65% (after 24-28 h)</td>
<td>[222]</td>
</tr>
<tr>
<td>DNA adducts in livers and colons of F344 rats Conjugated linoleic acid (CLA) HED: 48.6 mg/kg b.w.</td>
<td>CLA (300 mg/kg b.w.) feeding for 4 weeks; IQ (100 mg/kg b.w.) by gavage every 2 days over 2 weeks</td>
<td>↓ 59% (colon), ↔(liver)</td>
<td>[223]</td>
</tr>
<tr>
<td>DNA adducts in livers of F433 rats β-carotene HED: 3.2 mg/kg b.w.</td>
<td>β-carotene (20 mg/kg b.w.) feeding for 8 days; IQ (100 mg/kg b.w.) 1x by gavage</td>
<td>↓ 50%</td>
<td>[224]</td>
</tr>
<tr>
<td>DNA adducts in livers of F433 rats α-tocopherol HED: 12.9 mg/kg b.w.</td>
<td>α-tocopherol (80 mg/kg b.w.) feeding for 8 days IQ (100 mg/kg b.w.) 1x by gavage</td>
<td>↓ 30%</td>
<td>[224]</td>
</tr>
</tbody>
</table>

1) IQ - 2-amino-3-methyl-3H-imidazo(4,5f)-quinoline; HED - human equivalent dose; i.p. – intraperitoneal, p.o. – oral administration; GST-P⁺ foci - glutathione S-transferase placental form (GST-P)-positive foci; ACF – aberrant crypt foci

Since no evidence for protective effects of XAN against NDMA-induced DNA-damage was found in the present study, the effects of other dietary compounds against nitrosamines are not discussed in detail; typical examples for compounds which inhibited DNA-migration and cancer are flavonoids such as quercetin and naringenin, green tea polyphenolics and isothiocyanates to name a few [225-227].
5. Conclusions

The results of the present study show that consumption of the prenylated flavonoid XAN leads to prevention of DNA damage caused by the food specific carcinogens B(a)P and IQ. Since it is assumed that these compounds induce cancer via induction of genomic instability, these findings can be taken as an indication that the flavonoid protects humans against cancer risks caused by exposure to these compounds, which are considered to play a role in the etiology of human cancer. The present findings are in agreement with results of earlier *in vitro* studies with human cell lines and also with *in vivo* experiments with rats, which indicated that the prevention of IQ-induced DNA damage in inner organs leads to the prevention of formation of preneoplastic foci. The results of enzyme measurements show that the protective effects are probably due to induction of the GST isoenzyme GST-α which is known to detoxify electrophilic metabolites of diverse compounds. As described in detail in the discussion section a number of other dietary constituents have been found protective towards B(a)P, IQ and other representatives of PAHs and HAs. However, the unique characteristic of XAN is that it was found to cause protective effects at dose levels which were at least 6-times lower as the doses which were required with other chemopreventive dietary constituents.

Taken together, our findings indicate that XAN is a highly promising cancer protective compound in humans.
References


74. IARC, Certain Polycyclic Aromatic Hydrocarbons and Heterocyclic Compounds. IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS. Vol. 3. 1973, Lyon, France: IARC.


Appendix

This certificate is in recognition of

C. Pichler

winner of a Mutagenesis/Oxford University Press Poster Award
at the European Environmental Mutagen Society meeting,
Warsaw, 2012

Editor-in-Chief, Mutagenesis

Figure 31: Mutagenesis/Oxford University Press Poster Award
1. Aim of the study

Xanthohumol (XN) is a prenylated flavonoid which is found in beer. Recent findings from in vitro studies indicate that it is a promising chemopreventive agent (for details see: Gerhauser C et al). Aim of the study was to find out if an alcohol-free drink containing XN protects humans against DNA-damage caused by radic.

2. Methods

- Healthy, non-smoking volunteers (n=22; 11 males/11 females) consumed a drink containing XN (12 mg/dL) over a period of 14 days. To reduce the impact of other factors, they were asked to restrict their diet (limited intake of certain fruits, vegetables, spices and beverages) and to avoid excessive physical activities. Blood samples were collected at different timepoints: (1) before the intervention, (2) after 7 days and (3) after 14 days (study design see Figure 2).

- Blood was collected by venipuncture at all timepoints, subsequently peripheral lymphocytes were isolated by density centrifugation.

- The impact of the flavonoid-intake on DNA-stability parameters was monitored with the alkaline single cell gel electrophoresis (SCGE) assay under standard conditions, after ROS (H_2)O_2 treatment and with lesion-specific enzymes (FFPG and EndoIII) as the method of Collins et al. 1997. Apart from that we monitored alterations of DNA-migration by "activated" human liver-S9 treated carcinogens benz(a)pyrene (BaP), 2-amino-3-methyl-4H-imidazo[4,5-F]quinoline (IQ), N-nitrosodimethyl-amine (NDMA).

- Furthermore, attempts were made to elucidate the molecular mechanisms of protection. The important detoxification enzyme GST-tα was monitored by use of the Bio tear HEPATR+Alpha, a quantitative enzyme immunoassay kit. The test procedure is based on the sequential addition of the samples, a enzyme-conjugate and a substrate to microwells coated with anti-GST-tα IgG. The color intensity is proportional to the amount of GST-tα present in the sample.

3. Results

- SCGE assay

While under standard conditions, which reflect endog. formation of single (SSBSs) and double strand breaks (DSBs), no changes of DNA-migration were observed at different timepoints. A significant reduction (43.7%) of the formation of oxidised purines was detected after the intervention (14 days) by use of the restriction enzyme formamidopyrimidine glycosylase (FFPG) (see Figure 3). After the treatment of the cells with ENDO III no differences in DNA migration could be seen at the various timepoints. Comet formation after ROS-exposure was significantly reduced (28.2%) (see Figure 3). Furthermore, we observed a significant decrease of BaP-, and IQ-induced DNA-migration in the lymphocytes (reduction 19.2% and 14.2% respectively). No significant alteration was seen with NDMA.

- Enzyme immunoassay

The activity of the ikezyme GST-tα was clearly increased by 58.4% after the intervention (see Figure 5). No such effect was detected when the participants consumed the drink without XN.

4. Conclusions

Overall, our results suggest that intake of XN protects human against oxidative and chemically induced DNA-instability.

Figure 3a: SCGE assays with peripheral lymphocytes after ROS-exposure. Bars indicate means ± SD. From each experimental endpoint 150 cells (3 sides) were evaluated. Stars indicate statistical significance (p<0.001).

Figure 3b: Results of SCGE assays with peripheral lymphocytes after ROS-exposure. Bars indicate means ± SD. From each experimental endpoint 150 cells (3 sides) were evaluated. Stars indicate statistical significance (p<0.001).

Figure 4: Results of SCGE assays with lymphocytes after chemical treatment with IQ (50μM). Bars indicate means ± SD. From each experimental endpoint 150 cells were evaluated. Stars indicate statistical significance (p<0.001).

Figure 5: Enzyme Immunoassay

The activity of the ikezyme GST-tα was clearly increased by 58.4% after the intervention (see Figure 5). No such effect was detected when the participants consumed the drink without XN.

Overall, our results suggest that intake of XN protects human against oxidative and chemically induced DNA-instability.

References:


Figure 32: Poster presented at 42nd EEMS-Meeting in Warsaw, Poland (16th – 20th September 2012)
Danksagung

Das Zustandekommen der vorliegenden Arbeit ist erst durch die Hilfe und Unterstützung vieler Personen möglich geworden. Aus diesem Grund möchte ich folgenden Personen meinen besonderen Dank aussprechen:

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Curriculum vitae

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Citizenship: Austria

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2004 - 2005 Army service in the Austrian Armed Forces  
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2011 - 2013 Diploma thesis in the working group of Prof. Dr. Knasmüller at the Institute of Cancer Research in Vienna

**Jobs**

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**Conference attendance**

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8.11.2012 Oral presentation at the conference “Lifestyle, Oxidative Stress and Diabetes Mellitus” in Modra, Slovakia

**Languages**

German - native language  
English - fluent