Division Toxicology and Cancer Risk Factors (C0200)

Head: Prof. Dr. Helmut Bartsch

Scientists:
Dr. Barbara Bertram
Dr. Heike Dally
Dr. Norbert Frank
Dr. Clarissa Gerhäuser
Dr. Reinhold Klein
Dr. Claudia Mayer
Dr. Jagadeesan Nair
Dr. Urmila Nair
Prof. Dr. Hans Osswald
Dr. Robert W. Owen
Dr. Odilia Popanda
Dr. Angela Risch
Dr. Margarita Rojas
Dr. Hans-Rudolf Scherf
Dr. Peter Schmezer
Dr. Bertold Spiegelhalder

Visiting scientists:
Dr. Roger Godschalk

Postgraduates:
Amira Gamal Eldeen
Beate Breitschopf
Reinhard Ebbeler
Alexander Frank
Kai Gassner
Elke Heiss
Jörg Hümmerich
Inge Schönfeld
Patrick Schweizer
Isabel Streck
Changping Xie

Undergraduates:
Vivianne Raedts
Andreas Vogt

Technical assistants:
Daniela Bodemer
Ursula Bollow
Christel Ditrich
Reinhard Gliniorz
Roswitha Haubner
Birgit Jäger
Karin Klimo
Jutta Knauft
Regina Merkel
Isabell Neumann
Ulrike von Seydlitz-Kurzbach
Peter Waas
Gerd Würtele
Otto Zelezny

Secretary:
Susanna Fuladdjusch

The overall goals of the Division involve: (i) identification of exogenous and endogenous cancer risk factors and elucidation of their mechanisms of action, (ii) characterization of cancer-preventive agents and proof of their efficacy in preclinical and clinical studies, (iii) development, based on advances in mechanistic knowledge, of new ultrasensitive detection methods for DNA-damage and biomarkers for cancer susceptibility that are useful for molecular epidemiology studies on cancer etiology and prevention and (iv) initiation and participation in such studies by contribution to methodology and design. This approach is aimed to provide data that are required to implement efficient cancer preventive measures either by elimination of risk factors or by interruption of disease development (chemoprevention).

While there is diversity in the Division's research activities, many studies fall under a common denominator, i.e. biomarker development and application (Chart 1). This follows a tier approach, before the markers are explored in large-scale epidemiological studies. Naturally, many of our investigations have reached only the first phases of this development, but recently larger-scale molecular epidemiological studies have been conducted [1].

Chart 1 *Rationale for developing and validating biomarkers or intermediate endpoints relevant to human carcinogenesis for use in molecular epidemiological or clinical settings [2]*

Epidemiological observations on risk and protective factors in human cancer

⇓

Mechanistic studies in experimental systems to establish biomarkers/intermediate endpoints as part of the causal chain

⇓

Development of (non-invasive) methods for exposure/risk markers

⇓

Validation in animal/human pilot studies

⇓

Exploration of markers in large-scale epidemiological investigations

⇓

Prime objectives in the area of biomarker development and human applications involve (i) to identify new sources of carcinogen exposure [3], especially those arising from endogenous sources [4, 5], (ii) to quantify carcinogen DNA-damage in populations and measure the effect of genetic predisposition [6,7], (iii) to identify high-risk groups [8, 9, 10, 11] and finally (iv) to verify the efficacy of preventive measures, e.g. through intervention with chemopreventive agents [1,12].

Since 1996, more intense activities on secondary cancer prevention by chemopreventive (antidysplastic) agents

have been commenced in the Division [13, 14, 15, 16]. As chemopreventive agents are structurally heterogeneous and mechanistically diverse, ongoing work aims at the identification and evaluation of new promising agents of natural origin [17,18,19,20] or synthetic analogues as lead compounds for the development of effective agents, the elucidation of their mechanism of action and ultimately proof of their preventive efficacy in high-risk groups with dysplastic lesions or in the general population.

Accessible dysplasias as predictive biomarkers during several month follow-up of antidysplastic treatment have been used to demonstrate the feasibility and benefit of cancer chemoprevention in small groups of high-risk subjects. These included a collaborative clinical intervention study using sulindac (an NSAID) in familial adenomatous polyposis (FAP) patients, providing limited evidence of cancer-preventive activity in mainly colectomized FAP patients [21]. A subsequent study showed an increased level of promutagenic etheno-DNA-adducts in colonic polyps of FAP patients [22]. Another study achieved partial reversion of oral dysplasias with an antioxidant combination in patients with leukoplakia or after surgery for primary carcinoma of the oral cavity [23]. A pan-European calcium fiber placebo-controlled intervention study has been completed in patients with sporadic colorectal adenomas [1].

*The characterization of new chemopreventive agents with anti-inflammatory/antioxidant properties, but low long-term toxicity call for a more intense interdisciplinary research network in secondary cancer prevention to include (i) conduct of clinical trials, taking into account accessible dysplasias for repeated direct dysplasia control following treatment with officinal and new antidysplastic drugs, (ii) development and validation of cancer-predictive biomarkers for less accessible dysplasias, (iii) subsequent development of new antidysplastic agents with higher preventive efficacy for a broader spectrum of different dysplasias. The creation of two new divisions of '**Chemoprevention**' (planned) and '**Clinical Epidemiology**' (since 1999) should provide the necessary reinforcement of this research program.*

To intensify the collaboration between laboratory scientists, clinicians and epidemiologists and to promote epidemiological studies on human cancer causes and prevention, multidisciplinary symposia were organized in 1999-2000:

- *Symposium "Mutagen Sensitivity, DNA Repair Capacity and Predisposition (AEK-Symposium), Heidelberg, March, 1999;*
- *Third Taiwanese-German Workshop on Cancer Causes and Prevention: Mechanisms, Preclinical and Clinical Studies, Heidelberg, July, 2000;*
- *International Workshop on Biomarkers in Cancer Chemoprevention, Heidelberg, February, 2000 (organized together with A.B. Miller; DKFZ and IARC, Lyon, France) [24]*

Publications (* = external co-author)

[1] Bonithon-Kopp, C.*.; Kronborg, O.*.; Giacosa, A.*.; Räth, U.*.; Faivre, J.*. [Experts:-Milan, C.*.; Fenger, C.*.; Piard, F.*.; Belghiti C.*.; Owen, R. W.; Pignatelli, M.*. Calcium and fibre supplementation in the prevention of colorectal adenoma recurrence: a placebo-controlled intervention trial from the European Cancer Prevention Organisation (ECP). Lancet 356 (2000) 1300-1306.

[2] Bartsch, H. Studies on biomarkers in cancer etiology and prevention: a summary and challenge of interdisciplinary research. Mutation Research 462(2-3) (2000) 255-279.

[3] Klein, R.G.; Schmezer, P.; Amelung, F..; Schroeder, H.-G.*.; Woeste, W.*.; Wolf, J.*. Carcinogenicity assays of wood dust and wood activities in rats exposed by long-term inhalation. Int Arch Occup Environ Health 74 (2001) 109-118.

[4] Bartsch, H.; Nair, J. New DNA-based biomarkers for oxidative stress and cancer chemoprevention studies. European Journal of Cancer 36 (2000) 1229-1234.

[5] Nair, J.; Barbin, A.*.; Velic, I.*.; Bartsch, H. Etheno DNA-base adducts from endogenous reactive species. Mutation Research 424 (1999) 59-69.

[6] Bartsch, H. Exocyclic adducts as new risk markers for DNA damage in man. In: B. Singer, H. Bartsch (eds.) Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis. IARC Sci. Publ. No. 150. (1999) IARC, Lyon, pp 1-16.

[7] Rojas, M.; Cascorbi, I.*.; Alexandrov, K.*.; Kriek, E.*.; Auburtin, G.*.; Mayer, L.*.; Kopp-Schneider, A.*.; Roots, I.*.; Bartsch, H. Modulation of benzo[a]pyrene diolepoxide-DNA adduct levels in human white blood cells by *CYP1A1*, *GSTM1* and *GSTT1* polymorphism. Carcinogenesis 21 (2000) 35-41.

[8] Bartsch, H.; Rojas, M.; Nair, U.; Nair, J.; Alexandrov, K.*. Genetic cancer susceptibility and DNA adducts: studies in smokers, tobacco chewers and coke oven workers. Cancer Detection and Prevention 23 (1999) 445-453.

[9] Schmezer, P.; Rupprecht, T.*.; Tisch, M.*.; Maier, H.*.; Bartsch, H. Laryngeal mucosa of head and neck cancer patients shows increased DNA damage as detected by single cell microgel electrophoresis. Toxicology 144 (2000) 149-154.

[10] Schmezer, P.; Rajaee-Behbahani, N.; Risch, A.; Thiel, S.*.; Rittgen, W.*.; Drings, P.*.; Dienemann, H.*.; Kayser, K.W.*.; Schulz, V.*.; Bartsch, H. Rapid screening assay for mutagen sensitivity and DNA repair capacity in human peripheral blood lymphocytes. Mutagenesis 16(1) (2001) 25-30.

[11] Wikman, H.; Risch, A.; Klimek, F.*.; Schmezer, P.; Spiegelhalder, B.; Dienemann, H.*.; Kayser, K.*.; Schulz, V.*.; Drings, P.*.; Bartsch, H. *hOGG1* polymorphism and loss of heterozygosity (LOH): Significance for lung cancer susceptibility in a caucasian population. Int J Cancer 88 (2000) 932-937.

[12] Nair, U.; Bartsch, H. Metabolic polymorphisms as susceptibility markers for lung and oral cavity cancer. In: Biomarkers in Cancer Chemoprevention. Miller, A.B. et al. (eds.), IARC Scientific Publications N° 154 (2001) (IARC, Lyon, France) 271-290.

[13] Freese, R.*.; Basu, S.*.; Hietanen, E.*.; Nair, J.; Nakachi, K.*.; Bartsch, H.; Mutanen, M.*.; Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production or hemostatic factors during a high-linoleic acid diet in healthy females. European Journal of Nutrition 38 (1999) 149-157.

[14] Gerhäuser, C.; Heiss, E.; Herhaus, C.*.; Klimo, K. Potential Chemopreventive Mechanisms of Chalcones. Chapter 4.4 Dietary Anticarcinogens and Antimutagens. Chemical and Biological Aspects, Ian Johnson and Roger Fenwick: RCS, Cambridge, UK (2000) 189-192.

[15] Owen, R.W.; Giacosa, A.*.; Hull, W.E.*.; Haubner, R., Spiegelhalder, B., Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. European Journal of Cancer 36 (2000) 1235-1247.

[16] Jung, M.*.; Brosch, G.*.; Kolle, D.*.; Scherf, H.; Gerhäuser, C.; Loidl, P.*. Amide analogues of trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. Journal of Medical Chemistry 42 (1999) 4669-4679.

[17] Song, L.L.*.; Kosmeder, J.W.*.; Lee, S.K.*.; Gerhäuser, C.; Lantvit, D.*.; Moon, R.C.*.; Moriarty, R.M.*.; Pezzuto, J.M.*. Cancer chemopreventive activity mediated by 4'-bromoflavone, a potent inducer of phase II detoxification enzymes. Cancer Research 59 (1999) 578-585.

[18] Owen, R.W.; Mier, W.*.; Giacosa, A.*.; Hull, W.E..; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. Food and Chemical Toxicology 38 (2000) 647-659.

[19] Owen, R.W.; Mier, W.*.; Giacosa, A.*.; Hull, W.E.*.; Spiegelhalder, B.; Bartsch H. Identification of lignans as major components in the phenolic fraction of olive oil. Clinical Chemistry 46 (2000) 976-988.

[20] Owen, R.W.; Giacosa, A.*.; Hull, W.E..; Haubner, R.; Würtele, G.; Spiegelhalder B.; Bartsch, H. Olive-oil consumption and health: the possible role of antioxidants. The Lancet Oncology Vol. 1 (2000) 107-112.

[21] Winde, G.*.; Schmid, K.W.*.; Brandt, B.*.; Mueller, O.*.; Osswald, H. Clinical and genomic influence of sulindac on rectal mucosa in familial adenomatous polyposis. Diseases of the Colon and Rectum 40 (1997) 1156-1168.

[22] Schmid, K.; Nair, J.; Winde, G.*.; Velic, I.*.; Bartsch, H. Increased levels of promutagenic etheno-DNA adducts in colonic polyps of FAP patients. International Journal of Cancer 87 (2000) 1-4.

[23] Barth, T.J.*.; Zöller, J.*.; Kuebler, A.*.; Born, I.A.*.; Osswald, H. Redifferentiation of oral dysplastic mucosa by the application of the antioxidants beta-carotene, alpha-tocopherol and vitamin C. International Journal for Vitamin and Nutrition Research 67 (1997) 368-376.

[24] Miller, A.B.; Bartsch, H.; Bofetta, P.*.; Dragsted, L.O.*.; Vainio, H.*. Biomarker in Cancer Chemoprevention. IARC Sci. Publ. No. 154 (2001) IARC, Lyon, France pp 1-294.

Chemoprevention (C0202)

C. Gerhäuser

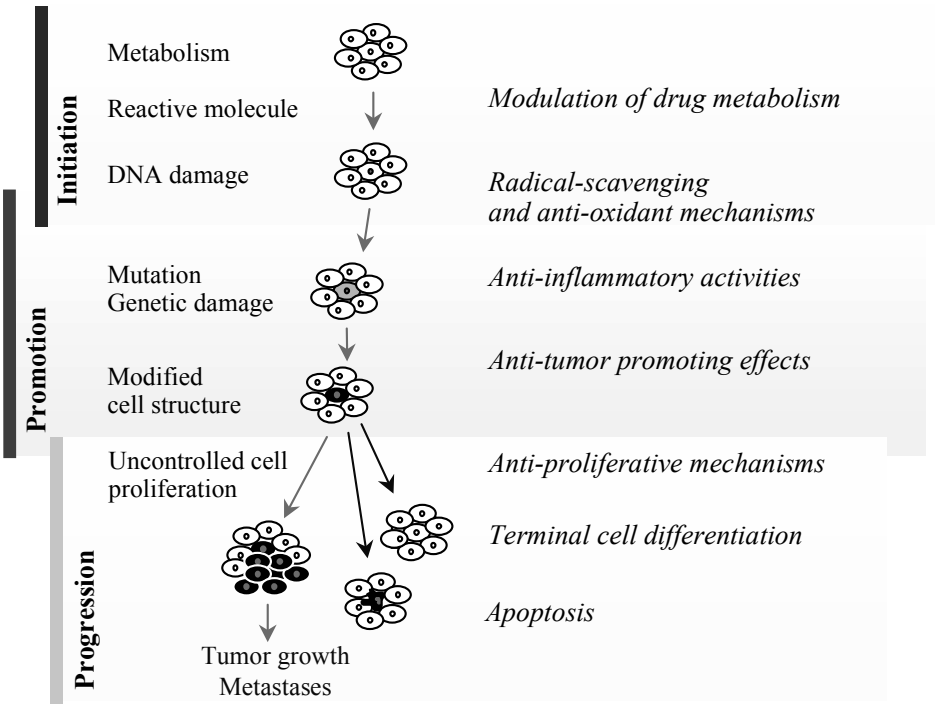
I. Identification and evaluation of novel potential cancer chemopreventive agents

Knowledge of molecular mechanisms is of importance for safe application of known, but also for further development of novel potential cancer preventive agents. The development of cancer is a multi-stage process which is generally divided into initiation, promotion and progression phases, as depicted in Scheme 1. Carcinogenesis can be regarded as an accumulation of genetic or biochemical cell damage which offers a variety of targets for chemopreventive agents to prevent or inhibit the slow progression from early genetic lesions to tumor development.

In the initiation phase, a carcinogen, either directly or after metabolic activation to a reactive molecule, interacts with intracellular macromolecules (DNA, proteins). This may cause DNA damage, which, if not repaired, can result in mutations and genetic damage. These mutations eventually lead to an altered expression of oncogenes and tumor suppressor genes or, e.g. continuous activation of protein kinases during the promotion phase, and finally result in modified cell structure, uncontrolled cell proliferation, tumor growth and metastases. This cascade of events offers a variety of targets for chemopreventive intervention at every stage. As indicated in Scheme 1, well established molecular mechanisms of chemoprevention include modulation of drug metabolism, anti-oxidant, radical-scavenging, anti-inflammatory, anti-tumor promoting and anti-proliferative activities as well as induction of terminal cell differentiation and apoptosis.

II. Development and Establishment of bioassay systems

Given the great structural variety of phytochemicals, ongoing projects in our group are aimed at the identification



Scheme 1:
Cellular carcinogenesis and mechanisms relevant for cancer prevention

and evaluation of new promising agents of natural origin or synthetic analogs as lead compounds for the development of effective chemopreventive agents and the elucidation of their mechanism of action. Since isolation of active chemopreventive agents from plants based on activity-guided fractionation using *in vivo* animal models is not feasible due to time and cost factors, we have set up a battery of cell- and enzyme-based *in vitro* marker systems relevant for inhibition of carcinogenesis *in vivo.[2]*

Assay systems established in our laboratory include:

1. Modulation of drug metabolism

a. Inhibition of Phase 1 Cyp1A activity. β-Naphthoflavone-induced rat hepatoma cell preparations are used as a source of Cyp1A enzyme activity. Time-dependent dealkylation of 3-cyano-7-ethoxycoumarin (CEC) to 3-cyano-7-hydroxycoumarin is determined fluorimetrically in 96-well plates.

b. Induction of Phase 1 Cyp1A activity. Using CEC as a substrate, we have developed an extremely sensitive assay to measure induction of Cyp1A activity in intact Hepa1c1c7 (Hepa1) mouse hepatoma cells cultured in 96-well plates. This method is about 10-fold more sensitive than the commonly used EROD assay.

c. Induction of Phase 2 NAD(P)H:quinone oxidoreductase (QR). Induction of NAD(P)H:quinone oxidoreductase (QR) as a model Phase 2 enzyme is measured colorimetrically in cultured Hepa 1c1c7 cells as described previously.

d. Induction of total glutathione levels. Glutathione is considered as the most important intracellular antioxidant. In addition, it is necessary for GST-mediated conjugation reactions. Induction of total glutathione levels can be measured photometrically in hepatoma cell culture.

2. Radical scavenging effects and anti-oxidant mechanisms

a. Radical-scavenging effects. Radical scavenging potential is determined photometrically by reaction with 1,1-diphenyl-2 picrylhydrazyl (DPPH) free radicals in a microplate format.

b. Inhibition of TPA-induced superoxide radical formation in HL60 human promyelocytic leukemia cells differentiated to granulocytes is detected by photometric determination of cytochrome c reduction.

c. Scavenging of superoxide anion radicals generated non-enzymatically (PMS/NBT) or enzymatically in the xanthine/xanthine oxidase system.

3. Anti-inflammatory activities

a. Inhibition of Cox-1 and Cox-2 activity.

b. Inhibition of LPS-mediated iNOS induction in murine macrophages

4. Anti-tumor promoting effects

Inhibition of TPA-induced ODC induction in murine 308 cells

5. Anti-proliferative mechanisms

a. Inhibition of human DNA polymerase α is evaluated by measuring incorporation of radiolabeled thymidine tri phosphate into activated DNA.

b. Induction of cell differentiation in cultured HL-60 cells. Using a human promyelocytic leukemia cell line HL-60, the potential of plant extracts, natural components and synthetic derivatives to induce cellular differentiation to morphological and functional mature granulocytes and monocytes/macrophages is determined.

6. Mouse mammary organ culture (MMOC)

A drawback of *in vitro* investigations is the identification of false positive leads, i.e. compounds which show activity *in vitro*, but fail to inhibit carcinogenesis *in vivo.* Therefore, we have established an organ culture model using mouse mammary glands (MMOC) as a link between short-term *in vitro* and long-term *in vivo* carcinogenesis models. This system combines the advantages of an *in vitro* system (feasibility and handling, compound requirements, duration of the experimental procedure) with the complex cellular, metabolic and developmental conditions present in an entire organ. It is not only useful in identifying compounds and extracts with potential efficacy *in vivo*, but also allows investigation of mechanistic aspects of chemopreventive activity in a complex, but defined system. Further development of active compounds will include long-term *in vivo* carcinogenesis studies in animal models.

Taken together, these models allow fast (within days), sensitive and cost effective identification of promising lead compounds and plant extracts and have been utilized for activity-guided isolation of active principles. To date, a total of more than 1600 samples (plant constituents and synthetic analogs, extracts from various biological sources including medicinal plants, dietary components, mosses, marine bacteria, and fungi, and subfractions thereof) have been tested for biological activities in the bioassay systems described above (except MMOC). Based on these data, promising compounds and series of optimized structures were selected for detailed analyses and mechanistic investigations.

III. Description of selected projects

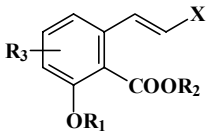
1. Modulation of drug metabolism

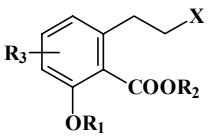
Bibenzyl derivatives of lunularic acid as novel lead compounds in chemoprevention

in cooperation with H. Becker and Th. Eicher, Universität des Saarlandes, Saarbrücken.

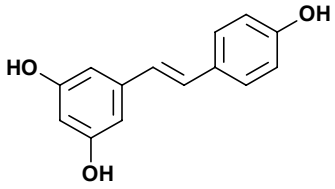
Liverworts *(Hepaticae)*, a category of mosses, are a unique source of bibenzyl derivatives of lunularic acid. These compounds display structural similarities with resveratrol found in grapes and red wine.

Screening in our series of assay systems indicative of inhibition of carcinogenesis *in vivo* led to the identification of bibenzyls as potent modulators of phase 1 and phase 2 metabolizing enzymes. CD values for the induction of quinone reductase (QR) activity in Hepa 1c1c7 cell culture were in the range of 0.03 to 51 µM depending on the substitution of the ring systems. These compounds did not induce QR activity in cultured BP^{r}c1 cells, indicating an aryl hydrocarbon (*Ah*) receptor-mediated bifunctional mechanism of induction, although the compounds have no similarity to known ligands of the A*h* receptor. In transient





X = Aryl, Heteroaryl, R_1, R_2 = H, CH_3, C_2H_5, R3 = OH, Br, AcO



Resveratrol

transfection experiments with QR-chloramphenicol acetyltransferase plasmid constructs, induction was confirmed to involve activation of the xenobiotic responsive element (XRE). Consequently, we could further demonstrate dose-dependent induction of Cyp1A activity in cultured Hepa 1c1c7 cells. Interestingly, selected compounds were also identified as potent inhibitors of Cyp1A activity with IC_{50} values in the nanomolar range. Ei-252 as a model compound demonstrated competitive inhibition with respect to the substrate 3-cyano-7-ethoxy-coumarin, determined by Lineweaver-Burk-, Dixon- and Cornish-Bowden plots of the results of kinetic experiments. This compound was further found to inhibit DMBA-induced preneoplastic lesion formation in mouse mammary glands in organ culture (MMOC). Based on these results, bibenzyls will be further investigated as readily available promising new cancer chemopreventive agents. [8,18]

2. Radical scavenging effects and anti-oxidant mechanisms

2a. Antioxidant and radical-scavenging potential of phenolic constituents of beer

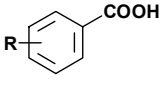
In cooperation with Axel Alt[1], Hans Becker[1], Horst Chmiel[2], [1]Universität des Saarlandes, Saarbrücken; [2]Gesellschaft für Umweltkompatible Prozesstechnik, Saarbrücken.

Beer is an important source of dietary antioxidant polyphenols (up to 1 g polyphenols/l). Moderate beer consumption is known to reduce the risk of coronary heart disease. In addition, beer has been reported to inhibit mutagenesis and DNA adduct formation induced by several carcinogens. Nevertheless, a detailed investigation of the antioxidant potential of beer constituents using physiologically relevant reactive oxygen species (ROS) as a basis for the possible application of beer and beer-related beverages in the prevention of cancer has not been performed so far. Consequently, we have initiated a study to evaluate composition and biological activities of beer (B) and a residue (R) rich in polyphenols removed from beer during the stabilization process. Extracts and a series of more than fifty purified compounds were tested for their potential to scavenge reactive 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. In addition, the capacity to scavenge superoxide anion radicals generated chemically or enzymatically was detected by reduction of nitroblue tetrazolium. Inhibition of TPA-induced superoxide anion radical formation in differentiated human promyelocytic leukemia cells was analyzed by cytochrome c reduction. Peroxyl and hydroxyl radical-scavenging ability was measured in a modified oxygen radical absorbance capacity (ORAC) assay. The tested polyphenolic constituents of beer and residue R mainly belong to five structural classes: benzoic and cinnamic acid derivatives (B+R), acetophenones (R), flavonoids (B+R) and catechins (B).

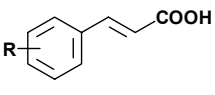
Catechins and flavonoids displayed the highest potential to scavenge DPPH radicals. In addition, all cinnamic acid derivatives with 4-OH- and additional 3-OH- or 3-OCH_3-substitution were potent DPPH radical scavengers. Protocatechuic, gallic and syringic acid were active benzoic acid derivatives. These initial results prompted us to investigate antioxidant potential using a series of ROS. Only flavonoids and catechins were able to scavenge chemically generated superoxide anion radicals at concentrations < 100µM. Activities in the cellular system were generally lower for all compounds tested. Importantly, in the ORAC assay, which was modified and validated in a microplate format, all compounds tested displayed high potential to scavenge hydroxyl radicals, whereas the capacity to scavenge peroxyl radicals was generally lower. In conclusion, this is the first investigation of the antioxidant potential of purified beer constituents as well as of polyphenols removed from beer during processing. Overall, the tested compounds displayed high antioxidant activity, especially versus the most reactive hydroxyl radicals involved in lipid peroxidation processes. [13]

2b. Ellagic acid induces antioxidant mechanisms in cultured human hepatocellular carcinoma cells HUH-7

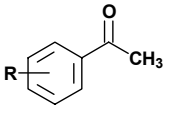
Dietary factors play an important role in modulating the development of certain types of human cancers. Chemopreventive agents are found in all foods, especially fruits and vegetables. Ellagic acid is a dietary polyphenol present in fruits and nuts including raspberries, strawberries and walnuts. It possesses both anti-mutagenic and anti-carcinogenic activities, thus, anti-carcinogenic effects



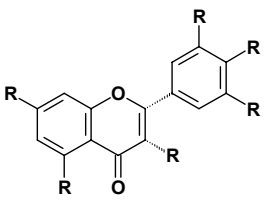
Benzoic acid derivatives



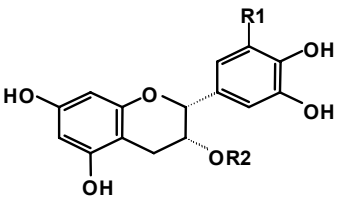
Cinnamic acid derivatives



Acetophenones



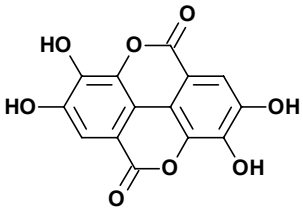
Flavonoids
(R = H, OH, OCH_3)



Catechins
(R_1= H, OH; R_2=H, gallic acid)

were demonstrated in various rodent chemoprevention models. Ellagic acid is known to modulate the activation of carcinogens by inhibition of phase 1 cytochrome P450 enzymes and by induction of phase 2 enzymes which are involved in carcinogen detoxification.

The present study aimed to investigate whether ellagic acid affects antioxidant mechanisms that may contribute to its anti-carcinogenic activity.



Ellagic acid

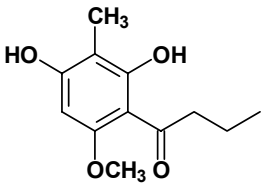
To achieve this goal we studied the effect of ellagic acid treatment on the different antioxidant mechanisms in human hepatocellular carcinoma cells (HUH-7). Ellagic acid at low concentrations was found to increase total antioxidant capacity of HUH-7 cells against ROO• and OH• radicals in the ORAC assay. Ellagic acid significantly increased total intracellular thiol levels and moderately increased the GSH/GSSG ratio. This increase in total thiols was not only due to GSH levels but mainly due to thiol-containing proteins, which might include cysteine-rich proteins like metallothionein (MT) or enzymes like catalase and thioredoxin reductase. Consequently, ellagic acid significantly enhanced metallothionein I mRNA expression in competitive RT-PCR analyses and non-significantly increased the total amount of metallothionein protein, determined by ELISA analyses. Ellagic acid enhanced the activity of antioxidant enzymes (catalase and thioredoxin reductase), especially at low concentrations, and significantly inhibited induced lipid peroxidation in HUH-7 cells. This may be due to the high radical-scavenging activity of ellagic acid. Taken together, our studies support the role of ellagic acid as a cancer chemopreventive agent acting by multiple antioxidant mechanisms. Further studies will focus on thioredoxin and on factors involved in regulation of MT mRNA expression after ellagic acid treatment. [14]

3. Anti-inflammatory activities

3a. Inhibition of cyclooxygenase 1 (Cox-1) activity by *Dryopteris* phlorophenone derivatives

In cooperation with C.J. Widen[1], G.J. Kapadia[2], [1]Maunulan Aptekki, Helsinki, Finland; [2]Dept. of Pharm. Sci., School of Pharmacy, Howard University, Washington, D.C., USA.

Cyclooxygenase 1 (Cox-1) is a key enzyme in metabolism of arachidonic acid and production of prostaglandins (PGs) as hormone-like mediators of inflammation. Elevated levels of PGs have been associated with carcinogenesis and tumor promotion. As a part of our program for identification and mechanistic investigation of potential cancer chemopreventive agents, we have tested a series of about fifty phlorophenone derivatives for *in vitro* inhibition of cyclooxygenase 1 (Cox-1) activity. Microsomes of sheep seminal vesicle were used as a source of Cox-1, and oxygen consumption during PG formation was monitored. The compounds tested comprised of monomeric acylphloroglucinols and di-, tri- and tetrameric phlorophenone derivatives. These agents have previously been tested for inhibition of EBV-early antigen activation by phorbol esters (Kapadia *et al*., Cancer Let. 105, 161-165, 1996).

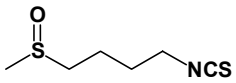


Isoaspidinol

The monomeric compound isoaspidinol was identified as a potent inhibitor of Cox-1 activity with a halfmaximal inhibitory concentration (IC_{50}) of 2.3 µM. Interestingly, replacement of the 4-hydroxy group by a 4-methoxy-group significantly reduced the inhibitory potential (12% inhibition at a test concentration of 100 µM). The isomeric compounds o-desaspidinol B and desaspidinol B, which lack the methyl group at position 3, were slightly less active than isoaspidinol with IC_{50} values of about 10 µM. Substitution of the butyryl- by a propionyl-side chain (desaspidinol P) further reduced the inhibitory activity (IC_{50} value: 17.3 µM). Of the dimeric compounds tested, desaspidin and flavaspidic acid BB were moderately active (IC_{50} values: 15.7 and 21.1 µM, respectively). Based on these data, the anti-inflammatory and chemopreventive activity of selected acylphloroglucinols will be further investigated. [19]

3b. Mechanistic investigation of sulforaphane-mediated cancer chemopreventive potential

Sulforaphane is an aliphatic isothiocyanate found as a glucosinolate-precursor in cruciferous vegetables like broccoli. Its chemopreventive activity, shown by inhibition of chemically-induced rat mammary carcinogenesis, has mainly been attributed to the modulation of carcinogen metabolism.



Sulforaphane

As an additional mechanism of action, we have recently demonstrated that sulforaphane potently inhibits LPS-mediated nitric oxide (NO) production in Raw 264.7 macrophages with a halfmaximal inhibitory concentration (IC_{50}) of 0.7 µM. Excessive production of NO during infection and chronic inflammation is considered as a causative factor of cellular injury and cancer e.g. via nitrosative deamination and lipid peroxidation. Sulforaphane did not directly interact with NO, nor inhibited the enzymatic activity of inducible nitric oxide synthase iNOS). Rather, western blot analyses revealed a dose- and time-dependent decrease of LPS-stimulated iNOS as well as Cox-2 protein expression [1].

To further elucidate the mechanism of sulforaphane-mediated iNOS induction, RT-PCR analyses confirmed that sulforaphane regulates iNOS expression at the transcriptional level. A pivotal transcription factor in LPS-mediated iNOS induction is NF-κB. In unstimulated macrophages, it is located in the cytosol as a complex with IκB (inhibitor of

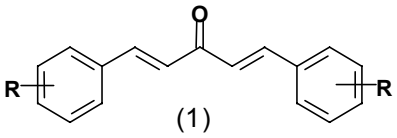
NF-κB), which is phosphorylated and degraded upon LPS-stimulation, thus releasing NF-κB and allowing nuclear translocation and initiation of transcription. In electrophoretic mobility shift assay (EMSA) experiments sulforaphane was shown to impair DNA-binding of NF-κB without affecting the DNA-binding of AP-1. Using western blotting and immunofluorescence detection of I-κB and NF-κB subunits p65 and p50, respectively, we could demonstrate that sulforaphane does neither interfere with induced degradation of IκB nor with nuclear translocation of NF-κB. Interestingly, treatment of Raw macrophages with sulforaphane caused a rapid decrease in intracellular GSH levels, which might influence the redox-sensitive activation, translocation and transactivation of NF-κB. Taken together, our data indicate that sulforaphane mediates its chemopreventive effects additionally by anti-inflammatory mechanisms. [11]

4. Anti-tumor promoting effects
Inhibition of phorbol ester-induced ornithine decarboxylase acticity by distyrylketones and curcuminoids

In cooperation with S. Jones, F. Woldu, V. Balasubramanian, G.J. Kapadia, Dept. of Pharm. Sci., School of Pharmacy, Howard University, Washington, D.C., USA.

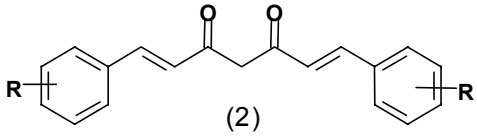
Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines and is highly inducible by growth-promoting stimuli. ODC activity and the resulting polyamines are essential for cellular proliferation, but long-term elevated levels have been associated with tumor promotion. Therefore, it can be assumed that agents able to inhibit tumor promoter-induced ODC activity and to regulate polyamine synthesis may be viewed as good candidates for cancer chemotherapy and chemoprevention.

As a part of our program for identification and mechanistic investigation of potential cancer chemopreventive agents, we have tested a series of fifty non-polar distyrylketones and curcuminoids for *in vitro* inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ODC activity in cultured mouse 308 cells.



(1)

Distyrylketones



(2)

Curcuminoids

Distyrylketones (1) were identified as potent inhibitors of ODC induction. The unsubstituted compound displayed a halfmaximal inhibitory concentration (IC_{50}) of 2.2 µM. Structure activity relationship analyses revealed that methoxy-substitutions at both aromatic rings modulated the inhibitory potential and resulted in compounds with IC_{50} values in the range of 0.2 to 21 µM (3,4,5 > 3,4 > 2,4,6 > 3,5 >> 4 > 2,3 >2,4). Chlorine-substitution at positions 2 or 3 and methyl-substitution at position 2 were tolerated (IC_{50} values: 2.0 -2.3 µM), whereas methyl-substitution at position 3 reduced the inhibitory potential (IC_{50} value: 9.7 µM).

Interestingly, most curcuminoids (2) analyzed were inactive at a test concentration of 10 µM. Curcumin as a reference compound was found to inhibit ODC induction with an IC_{50} value of 24 µM. Only one analog with 2,4,5-trimethoxy-substitution was more active than curcumin (IC_{50} value: 7.4 µM). Based on these data, inhibition of tumor promotion by distyrylketones will be further investigated.

5. Anti-proliferative mechanisms
5a. Inhibition of DNA polymerase a by potential cancer chemopreventive agents

In cooperation with H. Becker[1], G.J. Kapadia[2], H.P. Nasheuer[3], [1]University of Saarbrücken, Saarbrücken; [2]Dept. of Pharm. Sci., School of Pharmacy, Howard University, Washington, D.C., USA; [3]Inst. of Mol. Biotechnology, Jena.

Accumulation of genetic damage during carcinogenesis can result in uncontrolled cell proliferation due to e.g. continuous activation of oncoproteins. DNA polymerase α is the only eukaryotic DNA polymerase that can initiate DNA synthesis *de novo*. Furthermore, it seems to be involved in mechanisms that control cell cycle S-phase entry, but no specific role in DNA repair has been assigned to this enzyme. These characteristics render it an attractive target for anti-proliferative strategies which might contribute to chemopreventive activity in the promotion or progression phase of carcinogenesis.

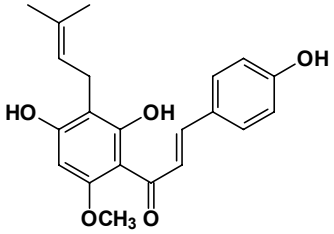
Consequently, we have established an *in vitro* bioassay system to identify and mechanistically investigate novel polymerase inhibitors using a recombinant human DNA polymerase α-primase complex (DNA pol/prim). As an example, (–)-epigallocatechin gallate from green tea was identified as a potent inhibitor with a halfmaximal inhibitory concentration (IC_{50}) of 1.4 ± 0.2 µM. Inhibition was non-competitive with respect to the radio-labeled nucleotide substrate (TTP) and the DNA template. (–)-Epicatechin gallate was slightly less active (IC_{50}: 7.9 µM ± 1.5 µM), whereas (+)-epicatechin and (–)-epigallocatechin were inactive (IC_{50} values >500 µM).

Furthermore, bartramiaflavone, a biflavonoid isolated from mosses (*Bartramia sp.)* was discovered as a novel potent inhibitor of DNA pol/prim with an IC_{50} value of 0.6 ± 0.4 µM. In addition, a series of differentially substituted 1,4-naphthoquinones was investigated. Juglone (5-hydroxy-1,4-naphthoquinone) and 5,8-dihydroxy-1,4-naphthoquinones (e.g. shikonin, arnebin-1) completely inhibited TTP incorporation into newly synthesized DNA at a test concentration of 500 µM. These results emphasized the importance of hydroxy-substitution at positions 5 and/or 8 for potent inhibition. Based on these data, structural requirements for inhibition of DNA polymerase a have been deduced.

5b. Xanthohumol from hop (*Humulus lupulus*) as a novel potential cancer chemopreventive agent

In cooperation with Alt, A. and Becker, H., Universität des Saarlandes, Saarbrücken.

The identification, evaluation, mechanistic investigation and utilization of dietary components, natural products or their synthetic analogs as potential cancer chemopreventive agents in the form of functional foods or nutraceuticals has become an important issue in current health- and cancer-related research. To this end, xanthohumol (XN), a prenylated chalcone from hop (*Humulus lupulus*) and a series of natural and semi-synthetic analogs and related hop constituent were tested in a broad-spectrum of *in vitro* bioassays.



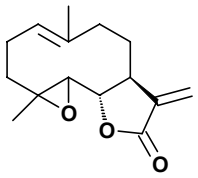
Xanthohumol

Of all hop constituents and analogs tested, XN was identified as the most promising agent with multiple hitherto unknown activities indicative of cancer preventive potential. XN has been reported previously to modulate carcinogen metabolism and to act by cytotoxic/-static mechanisms; however, a conclusive characterization of its chemopreventive potential was missing. XN was able to scavenge a variety of physiological relevant radicals including peroxyl, hydroxyl, and superoxide anion radicals more effectively than the known antioxidant Trolox. Anti-initiating mechanisms by modulation of enzymes involved in carcinogen metabolism and detoxification were confirmed. For the first time, XN was characterized as an effective anti-inflammatory agent. It was found to inhibit both the constitutive form of cyclooxygenase Cox-1 and, more importantly, the inducible Cox-2 which is linked to carcinogenesis. In cultured Raw 264.7 murine macrophages, XN was shown to decrease lipopolysaccharide-mediated inducible nitric oxide synthase (iNOS) induction. Another novel aspect of chemopreventive potential of XN can be seen in its multiple anti-proliferative mechanisms. XN was found to inhibit human DNA polymerase α, the only eukaryotic polymerase that can initiate DNA synthesis *de novo*. This effect might be responsible for the previously described inhibition of cell growth. Using alkaline phosphatase induction in the Ishikawa cell line, XN was identified as an anti-estrogen without possessing estrogenic potential. Additionally, XN was found to induce terminal cell differentiation in cultured HL-60 cells. Differentiation markers and a decrease in cellular proliferation were detected at a concentration range of 0.8- 6.25 µM. Most importantly, XN at nanomolar concentrations prevented carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture (MMOC), providing a first direct proof for its chemopreventive potential. Investigations on bio-availability, efficacy and safety in animal models are ongoing. Together, our data provide promising evidence for novel preventive applications of XN and hop products. [12]

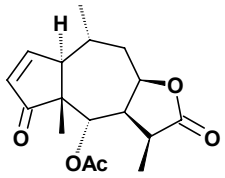
5c. Induction of HL-60 cell differentiation by sesquiterpene lactones

In cooperation with C.A. Klaas[1], I. Merfort[1], V. Castro[2], [1]Institute of Pharmaceutical Biology, Albert-Ludwigs-University, Freiburg; [2]Escuela de Quimica, Universidad de Costa Rica, San Jose, Costa Rica.

Cancer can be regarded as an imbalance between cell proliferation and cell differentiation, i.e. cell maturation and development to a defined cell type. Consequently, induction of cell differentiation to a normal, not cancerous phenotype is regarded as a valid mechanism of cancer chemoprevention and chemotherapy. Naturally occurring sesquiterpene lactones (SLs) have been shown previously to possess anti-inflammatory and anti-tumoral potential. Anti-inflammatory activity was linked to the inactivation of transcription factor NF-κB by alkylation of its p65 subunit. To investigating a possible correlation between NF-κB inhibition and induction of cell differentiation, we utilized the human promyelocytic leukemia cell line HL-60. Cellular differentiation to morphological and functional mature granulocytes, monocytes or macrophages was determined by monitoring cellular properties, i.e. reduction of nitroblue tetrazolium (NBT) after TPA-challenge, appearance of nonspecific (NSE)/specific acid esterase (SE) and a decrease in cellular proliferation.



Parthenolid



Dihydrohelenalin acetate

For SLs possessing a reactive α-methylene-γ-lactone moiety, e.g. Parthenolid, we observed good correlation between inhibition of NF-κB and of cell proliferation (r^2=0.96), but no correlation with induction of cell differentiation. Rather, weak NF-κB inhibitors, including α-methylene-butyrolactone used as a reference compound and SLs without the α-methylene-γ-lactone moiety, e.g. dihydrohelenalin acetate, were identified as potent differentiation inducers by NSE straining, indicating cell maturation along the monocytic lineage. Taken together, we could demonstrate that induction of HL-60 cell differentiation by SLs is independent of NF-κB inhibition. Further mechanistic studies will be performed to reveal other potential targets and to allow structure-activity relationship analyses.

5d. Potential mechanisms of induction of cell differentiation by histone deacetylase inhibitors

In cooperation with M. Jung, University of Münster.

Histone deacetylase (HDAC) inhibitors have been shown to be able to induce differentiation in various cell types *in vitro*. The present work investigate the induction of differentiation in various cancer cell lines treated with HDAC inhibitors and focus on studying the molecular basis thereof. Two major types of HDAC inhibitors were included in our studies, sodium butyrate (SB) and trichostatin A (TSA). SB

is particularly interesting because it has been found that butyrate is a metabolite of dietary fiber which is produced by anaerobic microorganisms in the large bowel. Cell differentiation of colon cancer cell lines was investigated by induction of brush border glycoprotein alkaline phosphatase (ALP) activity. Histone acetylation was studied by AUT gel electrophoresis. Protein expression of p21 and pRB was detected by western blotting techniques using specific antibodies. ALP activity in colon cancer cell lines LIM 1215 and HCT 116 was elevated more than 10-fold by treatment with SB. AUT-PAGE analysis confirmed hyperacetylation of histone H4 as early as 6 h after SB-treatment. This indicated a potential association of HDAC-inhibitory activity with the capability of inducing cell differentiation. Interestingly, however, TSA effectively induced hyperacetylation in these cell lines but failed to induce ALP activity as a marker of differentiation. SB- and TSA-treatment of LIM1215 and HCT 116 cells led to a significant induction of p21, a cell cycle progression inhibitor, from 6h to 24h post-treatment, after which levels began to decline. This effect seemed to be p53-independent, as induction of differentiation and p21 expression could also be observed in SB-treated p53-null HL-60 cells as well as in a p53-knockout derivative of the HCT 116 cell line. Further studies are intended to reveal a direct link between inhibition of histone deacetylation and differentiation induction, and the role of p21 and p53 in this process. [5,16]

External funding: Wissenschaftsförderung der Deutschen Brauereiwirtschaft e.V. , 1.11.1999 – 21.12.2001,
Dr. C. Gerhäuser/Prof. H. Becker (Uni Saarbrücken)

Publications and published meeting abstracts
(* = external co-author)

[1] Gerhäuser, C., Heiss, E., Herhaus, C. Sulforaphane inhibits inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (Cox-2) expression in murine macrophages. *Proc. Am. Assoc. Cancer Res*. **40**, 359 (1999).

[2] Gerhäuser, C. *In vitro* mechanism-based screens are of value. *Toxicol. Lett*. Suppl. 1/99, W5-1, 19, 1999.

[3] Heiss, E, Herhaus, C., Klimo, K., Gerhäuser, C. Inducible nitric oxide synthase (iNOS) expression is a target for the anti-inflammatory action of chalcones. *J. Cancer Res. Clin. Oncol.* **125** (Suppl.), S29 (1999).

[4] Herhaus, C. and Gerhäuser, C. Modulation of drug-metabolizing enzymes by non-polar chalcones. *J. Cancer Res. Clin. Oncol.* **125** (Suppl.), S38 (1999).

[5] Jung, M.*, Brosch, G.*, Kölle, D.*, Scherf, H., Gerhäuser, C., Loidl, P.*, Amide Analogues of Trichostatin A as Inhibitors of Histone Deacetylase and Inducers of Terminal Cell Differentiation. *J. Med. Chem.* **42**, 4669-4679 (1999).

[6] Lee S.K.*, Luyengi L.*, Gerhäuser C., Mar W.*, Lee K.*, Mehta R.G.*, Kinghorn A.D.*, Pezzuto J.M.* Inhibitory effect of munetone, an isoflavonoid, on 12-O-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase activity. *Cancer Lett*. **136**, 59-65 (1999).

[7] Song, L.L.*, Kosmeder, J.W.*, Lee, S.K.*, Gerhäuser, C., Lantvit, D.*, Moon, R.C.*, Moriarty, R.M.*, and Pezzuto, J.M.* Cancer chemopreventive activity mediated by 4'-Bromoflavone, a potent inducer of Phase 2 detoxification enzymes. *Cancer Res*. **59**, 578-85 (1999).

[8] Gerhäuser, C., Heiss, E., Klimo, K., Neumann, I., Becker, H.*, Eicher, Th.*, Bartsch, H. Bibenzyl derivatives as novel lead compounds in chemoprevention. *Proc. Am. Assoc. Cancer Res*. 41, 412 (2000).

[9] Ha, T.*, Gerhäuser, C., Zhang, W.*, Ho-Chong-Line, N.*, and Fourasté, I.* New Lanostanoids from Ganoderma Lucidum (Polyporaceae) that induce NAD(P)H:quinone oxidoreductase in cultured Hepa1c1c7 murine hepatoma cells. *Planta Medica* 66: 681-684 (2000).

[10] Gerhäuser, C., Heiss, E., Herhaus, C., Klimo, K., Potential Chemopreventive Mechanisms of Chalcones. Chapter 4.4 in: *Dietary Anticarcinogens and Antimutagens. Chemical and Biological Aspects.* Ian Johnson and Roger Fenwick: RCS, Cambridge, UK (2000), pp. 189-192.

[11] Gerhäuser, C., Alt, A., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., Scherf, H., Frank, N., Bartsch, H., Becker, H., Xanthohumol from hop *(Humulus lupulus)* as a novel potential cancer chemopreventive agent. *Proc. Am. Assoc. Cancer Res*. **42,** 18, 2001.

[12] Gerhäuser, C., Alt, A., Gamal-Eldeen, A., Neumann, I., Frank, N., Cmiel, H., Bartsch, H., and Becker, H. Antioxidant and radical-scavenging potential of phenolic constituents of beer. *Proc.Am. Assoc. Cancer Res.* **42**, 19, 2001.

[13] Gamal-Eldeen, A., Gerhäuser, C., Frank, N., Bartsch, H. Ellagic acid induces antioxidant mechanisms in cultured human hepatocellular carcinoma cells HUH-7. *J. Cancer Res. Clin. Oncol.* **127** (Suppl. 1), S.34, 2001.

[14] Heiss, E., Klimo, K., Neumann, I., Gerhäuser, C., Anti

-proliferative mechanisms of Xanthohumol from hop *(Humulus Lupulus)* in *in vitro* breast cancer chemoprevention models. *J. Cancer Res. Clin. Oncol.* **127** (Suppl. 1), S.47, 2001.

[15] Gerhäuser, C., Scherf, H.R., Klaas, C.R., Castro, V., Merfort, I., Induction of HL-60 cell differentiation sesquiteroene lactones. *J. Cancer Res. Clin. Oncol.* **127** (Suppl.1), S.46, 2001.

[16] Xie, C.P., Scherf, H.R., Neumann, I., and Gerhäuser, C., Potential mechanisms of inductio of cell differentiation by histone deacetylase inhibitors *J. Cancer Res. Clin. Oncol.* **127** (Suppl. 1), S. 46, 2001.

Patents

[17] No. 100 15 525.1. Synthetische Derivate von Lunularsäure, Arzneimittel enthaltend diese Verbindungen, Verfahren zur Herstellung der Lunularsäurederivate sowie deren Verwendung. Erf. Gerhäuser, Eicher*, Pick*. Patentanmeldung eingereicht beim Deutschen Patentamt am 30.3.2000

[18] Erfindungsmeldung vom 28.3.2000. Acylphloroglucinols and phlorophenones as anti-inflammatory, metabolism-modulatory, anti-proliferative, anti-oxidant, and potential cancer chemopreventive agents. Erf. Gerhäuser, Kapadia*, Widen*, Penttilä*.

Keywords: plant constituents, , NADP(H):quinone oxidoreductase, nuclear factor κB, , MMOC (mouse mammary organ culture).

Intervention Studies and Characterization of Cancer Protective Food Components (C0207)

B. Spiegelhalder in collaboration with R.W. Owen

1.1. Intervention studies and colorectal cancer

R.W. Owen, G. Würtele, B. Spiegelhalder, H. Bartsch, J. Wahrendorf[1], B. Hofstad*, M. Vatn*, C. Bonithon-Kopp** and J. Faivre**

[1]Division of Epidemiology, DKFZ; *Rikshospitalet, The National Hospital, Medical Department A, Pilestredet 32, 0027 Oslo, Norway; **ECP Colon Cancer Working Group, Registre des Tumeurs Digestives de la Côte-d'Or, Faculté de Medicine, 7 Boulevard Jeanne d'Arc, 21033, Dijon, France.

A high content of calcium (Owen, 1998 Recent Results in Cancer Res. Vol.146, 195-213) and/or fibre (Hill *et al*, 1997 *Eur J Cancer Prev* 6, 512-514) in the diet is regarded to be protective against colorectal cancer. The major mechanisms of action are thought to be chelation (cal-

cium) and dilution (fibre) of potentially co-carcinogenic intestinal lipids. To test this hypothesis a number of intervention studies in adenoma patients have been embarked upon. In a pilot placebo-controlled calcium intervention trial (35 patients) intervention had no greater effect than placebo on reduction of intestinal cell proliferation (Weisgerber *et al* 1996 *Gut*, 38, 396-402). In another slightly larger study (110 patients) intervention with calcium and antioxidants had a small but insignificant effect on the repression of growth of adenomas left *in-situ* but a significant effect on the formation of new adenomas (Hofstad *et al*, 1998 *Digestion* 59, 148-156). Publication of the data pertaining to these studies is now complete.

Finally in collaboration (Faivre *et al*, 1997 *Eur J Cancer Prev* 6, 132-138) with the European Agency for Cancer Prevention (ECP) a pan-European (involving 10 countries) long-term (3 year) calcium/fibre (fybogel) placebo-controlled intervention study in patients (665) with sporadic adenoma has recently been completed. At the DKFZ (during 1997 and 1998) intestinal lipid and mineral content of the stools was completed in 1003 Inclusion and Intervention stool specimens.

The relation between diet, cell proliferation, adenoma recurrence, intestinal lipids and minerals, blood DNA and antioxidant status and the effect of intervention on these parameters is being evaluated and manuscripts prepared for publication.

The clinical data [2] shows that overall, calcium intervention had a small but non-significant preventive effect on the recurrence of adenomas and this attained statistical significance in those patients whose habitual intake of calcium was very low. On the other hand intervention with fibre had a significant enhancing effect on the recurrence of adenomas. This effect was particularly enhanced in Northern European men. Furthermore intervention with fibre had a clear dose dependent interactive effect with habitual dietary calcium intake.

The hypothesis that bile acids are involved in the adenoma-carcinoma sequence was not upheld by any of the above studies but the data from the ECP study is currently undergoing exhaustive statistical analyses.

1.2. Phenolic and lipid components of seasoning oils and their precursors in relation to cancer of the colorectum and breast

R.W. Owen, R. Haubner, B. Spiegelhalder, H. Bartsch, W.E. Hull[1], A. Giacosa* and P. Srivatanakul**

[1]Central Spectroscopy, DKFZ; *Istituto Nazionale per la ricerca sul cancro, Istituto scientifico per lo studio e la cura del tumori, Genova, Italy; **National Cancer Institute, Bangkok, Thailand.

1.2.1. Olive drupes and olive oil

The Mediterranean diet is associated with decreased incidence of a range of diseases, especially cancer of the colorectum and breast. A major reason for this is the high consumption of olive oil which contains over 70% of its li

pids as the monounsaturated long chain fatty acid oleic acid (n-9) and in addition a range of phenolic substances. Because high intakes of polyunsaturated long chain fatty acids (n-6) have recently been implicated in the aetiology of breast cancer (Nair *et al*, 1997 *Cancer Epidemiol. Biomarkers Prev*. 6, 597-601) and to better determine the mechanisms by which olive oil is superior to other oils in its health protecting properties the phenolic (antioxidant) and lipid components was evaluated in a range of olive and seed oils (n = 30) currently on the Italian market.

Extraction, GLC and HPLC protocols have been developed to maximise detection and separation of the lipid and phenolic components. A range of major antioxidants have been identified by mass spectrometry and nuclear magnetic resonance spectroscopy including squalene, terpenoids, simple phenols, secoiridoids, lignans and flavonoids. The detection and characterization of lignans and flavonoids is a novel phenomenon in olive oil.

These studies have been extended to an evaluation of the phenolic antioxidant content of olive drupes. Both black and green olives contain very high concentrations (10-20 times higher than in extravirgin olive oil) of these substances and therefore may represent an even greater contribution to the health promoting properties of the Mediterranean diet.[1]

All of the individual phenolic compounds extracted and isolated from olive oils have potent antioxidant properties and compare favorably with the classic antioxidant, vitamin E. These data may have a bearing not only on chemopreventive strategies but also on future epidemiological studies which are recommended to take into account not only the types and grades of oils but also the intake of olive drupes.

These studies are also being extended to olive oils and olive drupes from a range of different countries within the Mediterranean basin and to a range of different products derived from olives currently on the Italian market.

1.2.2. Palm, Sesame, Soy oils in comparison to other seasoning oils in Thailand

Along with the diet of the Mediterranean basin of Europe, food consumption within countries of Eastern civilizations are also likely to provide a rich source of antioxidant substances which are not present in typical Western diets. A good example of this is Thailand which has an extremely low incidence of colon and breast cancer. To test this hypothesis a collaborative study has been established between the Division of Toxicology and the National Cancer Institute, Bangkok. A range (n = 20) of seasoning oils currently on the Thai market were studied by the same analytical techniques applied in the olive oil studies. Crude palm oil was found to contain substantial amounts of antioxidant phenolic acids such as 3,4-dihydroxy benzoic acid, vanillic acid, *p*-coumaric acid. In contrast refined palm oils (n = 6) were devoid of these phenolic acids but two were found to contain considerable amounts of what appears to be a synthetic antioxidant. In comparison to butylated hydroxyanisole and butylated hydroxytoluene (BHT) it was found to contain two hydroxyl moeities on the phenol ring. This was confirmed by both GC/MS and NMR and has been assigned the structure butylated dihydroxyanisole

(BDHA). Considerable variation in the concentration of this substance in refined palm oils was evident and raises the question, is it a synthetic addition or a natural product? Probably the natural antioxidants in crude palm oil are destroyed in the refining process and it is possible that BDHA is formed artificially during the refining process from BHT which may be added to preserve the keeping quality of palm oil. Studies are in progress to elucidate how industrial refining of palm oil is conducted in Thailand so that the presence of this new substance can be rationalized. Of the other oils studied, sesame oil was found to be particularly rich in two non-polar antioxidant phenolics i.e. sesamin and sesamolin. Levels of up to 2g/kg were detected. Precursor sesame seeds however contained a more complex profile and contained in addition the mono-, di- and tri-glucosides of sesaminol and pinoresinol glucoside along with free sesaminol, sesamolinol, pinoresinol and samin. Also the presence of substantial quantities of lariciresinol, iso-lariciresinol, matairesinol, syringaresinol and vanillic acid have been detected for the first time. In contrast soy along with sunflower, corn and peanut oils are devoid of antioxidant sustances belonging to these classes. The structures of the compounds described in this study were identified by ESI-MS, GC/MS and NMR. Work is in progress to isolate sufficient of these antioxidants for a compreshensive screen of their anticancer potential in collaboration with Dr Clarissa Gerhäuser and Dr Peter Schmezer.

1.2.3. Linseeds and linseed oil in relation to faecal mammalian lignans

Mammalian lignans are formed in the large intestine by microbial transformation of dietary precursors and they are considered to be protective against breast cancer especially because of their similar structure to Tamoxifen. The lignans formed are termed enterodiol (ENND) and eneterolactone (ENNL) and the major precursor is deemed to be secoisolariciresinol diglucoside (SDG) which is a major component of a complex phenolic polymer (CPP) in linseeds. Linseed oil in contrast to e.g. olive and sesame oils contains very low levels of SDG because the CPP is insoluble in oil matrices. Although SDG has been isolated and identified as a major component of the CPP we have conducted further investigations into the nature of the polymer. After extraction and methanolysis of the polymer we have demonstrated that SDG is bound within an array of phenolic acids such as *p*-coumaric, ferulic and caffeic via glucose intermediate linkages. Therefore high intakes of linseeds in the diet will not only afford a rich source of substrate for the formation of mammalian lignans in the large intestine where they can exert local protective effects against colon cancer and via absorption, at other sites, but also provides considerable amounts of antioxidant phenolic acids after digestion of the polymer in the large bowel which should enhance the protective effects of the lignans.

The exact nature of all the polymeric fractions are currently being subjected to a range of spectroscopic methods so that their definitive structures can be delineated and work is in progress to provide sufficient quantities for comprehensive screening of their chemopreventive potential.

1.3. Faecal phenols

R. W. Owen, B. Spiegelhalder and H. Bartsch

Many of the compounds isolated from various seasoning oils and their precursors are glycosides and therefore a tool used along with spectroscopic techniques to determine their structures is deglycosylation. Commercial enzymes are very ineffective at deglycosylating phenolic glycosides i.e. under prescribed conditions only 50% effectiveness after one weeks incubation. Therefore we adopted a different approach. The faecal matrix is rich in a variety of glycosidases and deglycosylation can be completed in hours in phosphate buffer with very small amounts of faecal matrix. In studies with SDG for example, complete conversion via the monoglucoside to secoisolariciresinol is evident after 3 h. It was noted however in these experiments that phenolic antioxidants not derived from SDG were evident in the HPLC and GC/MS chromatograms. These were identified as metabolites of phenolic antioxidants present in olive drupes and olive oil and therefore were ‘contaminants´ in the faecal matrix. Pilot studies with the faecal matrix alone confirmed this. Surprisingly phenolic antioxidants within the faecal matrix are resistant to extraction by organic solvents but under the conditions of the deglycosylation experiments are released into the aqueous medium which then allows extraction with these fluids.

A study was therefore conducted with faecal samples drawn from the ECP calcium/fibre intervention study and the phenolic antioxidants were identified by both GC/MS and ESI-LC/MS. This allowed their quantitation using single ion monitoring techniques and over thirty phenolic antioxidant compounds can be detected in the faecal matrix. The new methodology has been applied to as yet samples from three countries in the ECP study i.e. Denmark, Germany and Italy representing a north-south gradient. In this pilot study a remarkable correlation between the content of the phenolic antioxidant substances in the faecal matrix and the incidence of both breast and colon cancer has been demonstrated.

1.4. Fermentation studies

R. W. Owen, B. Spiegelhalder and H. Bartsch.

In previous work published in the 1980´s a limited pathway of mammalian lignan formation from linseed meal was proposed but the exact mechanism was not elucidated. In our studies on linseeds (1.2.3) gram quantities of SDG were isolated and purified and therefore we endeavored to shed further light on the nature of this pathway. To achieve this, anaerobic fermentation experiments were conducted. Briefly, Brain Heart Infusion broth supplemented with reducing agents and the substrate SDG (0.5 mg/ml) were inoculated with faecal matrix (1%) and incubated at 37°C for 72 h in an anaerobic chamber. The fermentation broths were extracted on extrelute and during silicic acid column chromatography were separated into a series of fractions. After identification of the metabolites in the fractions by GC/MS and ESI-LC/MS they were isolated and purified by semi-preparative HPLC and their structures confirmed by

NMR. This has allowed us to formulate a complete pathway of SDG transformation to the mammalian lignans by the faecal microflora. Now, sufficient amounts of the mammalin lignans can be isolated and purified for comprehensive screening of their anticancer potential.

Furthermore in our studies on faecal phenols (1.5) it was noted that although many individuals excreted metabolites of olives and olive oil antioxidant precursors, the lignans (1.2.1) were not detected as part of the phenolic fraction. Therefore studies on the fate of lignans derived from olive oil, sesame oil and sesame seeds were studied in a similar manner to SDG. The data shows that lignans derived from olive oil (acetoxypinoresinol, pinoresinol), sesame oil (sesamin, sesamolin) and sesame seeds (sesamolinol, lariciresinol, isolariciresinol, matairesinol) are also metabolized by the faecal microflora to the mammalian lignans ENND and ENNL. This may explain the discrepancy that has been observed between the intake of SDG (which until now was considered to be the only precursor of these metabolites) and mammalian lignan formation in the large bowel. Also a number of novel intermediate lignans have been detected and isolated and studies are in progress to elucidate their definitive structures by GC/MS, ESI-LC/MS and NMR.

1.5. Antioxidant content of red wines

R.W. Owen, G. Würtele, B. Spiegelhalder and H. Bartsch.

The antioxidant profile and content of red wines is well researched and a number of different classes have been identified. However studies with human volunteers who were instructed not to consume olive derived products for two weeks continued to excrete readily detectable levels of tyrosol and hydroxytyrosol in the faecal matrix. It was considered unlikely that these antioxidants were constituents of either meat, vegetables or fruit which the volunteers were allowed to consume. Rather a more likely source was beverages such as tea or wine. Therefore a comprehensive analysis of the phenolic antioxidant content of tea (green and black) and red wine was conducted. The profiles in tea were identical to those already published but red wine is found to contain substantial amounts of hydroxytyrosol and tyrosol along with several other compounds which have not been previously described as components of red wine. The major classes of antioxidants were first separated by column chromatography into a series of fractions from which their nature was identified by HPLC, GC/MS and ESI-LC/MS. This has enabled a number of various LC/MSD programs to be devised which allows the antioxidant profiles of wines to be analyzed by direct injection (1-20 µL) of the wine (a total of only 200 µL is required to obtain a complete profile) into the LC/MSD without any prior work-up using single ion monitoring techniques. At present over fifty antioxidant substances can be detected and identified in red wines by this method and due to the ease of analysis will allow rapid comparisons of the antioxidant content of wines worldwide in our ongoing chemoprevention studies.

1.6. Assessment of the role of reactive oxygen species in the aetiology and promotion of cancer

R.W. Owen, G. Würtele, B. Spiegelhalder and H. Bartsch.

HPLC methods have been developed (Owen *et al*, 1996,Gut 38,591-597) for the reliable determination of phytic acid (PA) and reactive oxygen species (ROS) in human faeces. Stool PA content shows a strong correlation with the excretion of minerals such as iron, calcium and magnesium but exhibits no relation with cell proliferation. HPLC (Owen *et al*, 1996, Eur. Cancer Prev. 5,233-240) allows the simultaneous monitoring of the hypoxanthine/xanthine oxidase system and ROS generation, enables the kinetics of the process to be evaluated in detail and has been successfully applied to the monitoring of ROS production by the faecal matrix.

Recent developments (Owen *et al*, 1997 I Cancer Clin Oncol 12 (Suppl. 1) 2, 1998 Eur I Cancer Prev (Suppl. 2), S.41-54) utilizing a refined HPLC method has shown clearly that the capacity of faeces to generate ROS is not related to the bacteria present. Rather ROS generation is supported by an as yet unidentified soluble factor within the faecal matrix. Studies are in progress to identify this soluble factor and a refined assay system has been re-applied to the faecal samples studied thus far and to additional patient and population groups to finally establish whether or not PA, iron and ROS generation alone or in combination within the large intestine have any bearing on the aetiology of colorectal cancer. This data is currently being evaluated.

1.7. Reactive oxygen species (ROS) and gastric cancer

R.W. Owen, G. Würtele, B. Spiegelhalder, H. Bartsch, H. Bauer[1] and J. Rudi*
[1]Division of Molecular Toxicology, DKFZ; *Department of Gastroenterology, Krehl Klinik, Heidelberg.

Infection with Helicobacter pylori is assumed to be a risk factor for the development of gastric carcinomas, but the molecular mechanism for this is still unknown. Therefore the upregulation of reactive oxygen species (ROS) in gastric juice of human gastritis patients and methylation/demethylation of the p53 gene in the human gastric carcinoma cell line AGS by *H. pylori* were studied. Gastric juice of 31 patients (with and without gastritis) were analyzed for their potential to generate ROS as measured by hydroxyl radical attack on the aromatic probe salicylic acid. The products (dihydroxy benzoic acids) were analyzed by high performance liquid chromatography. Gastric juice samples of twenty (64.5%) patients with chronic *H. pylori* gastritis generated elevated concentrations of ROS, compared to only 8 (21.6%) of gastritis patients without *H. pylori* infection.

The methylation status of wild type p53 gene in the gastric tumor cell line AGS (ATCC:CRL-1739) was also evaluated with respect to *H. pylori* infection (type:60190,vac A genotype,cag A-positive). In incubation experiments with *H. pylori* the 5-methyldC of CpG regions of p53 was analyzed by the MspI/HpaII restriction enzyme method using 14

sense/antisense primer pairs at CCGG restriction sites of p53 intron1 – intron7, exon1 exon5, exon6, exon7, exon8.

Methylated cytosines at CpG sites could be detected in intron1, exon5, exon7, exon8. After the incubation of cells either with *H. pylori* bacteria or the supernatant of culture media from *H. pylori* a higher degree of methylation in the CpG islands of p53 could not be detected. Demethylation was only shown in intron1. Stomach infections with *H. pylori* appear to be associated with the potential for a higher rate of ROS formation by the gastric juice. This may represent one step in the mechanism of cancer formation in the stomach. There was no upregulation of p53 methylation, but the endogenous methylation grade at cytosines is being investigated further.

2. Under Planing Projects

2.1. Chemopreventive potential of phenolic antioxidants isolated from seasoning oils and their precursors.

R.W. Owen, A. Risch, C. Gerhäuser, P. Schmezer, W.E. Hull, B. Spiegelhalder and H. Bartsch.

In our ongoing cancer chemopreventive studies we have particular interest in the health benefits of the Mediterranean diet and therefore during the last 18 months we have developed new extraction and HPLC protocols (Owen *et al*, 2000[4-7]) to isolate and identify the important antioxidant phenolic substances within olive and other seasoning oils (and their precursors). To date a range of major antioxidants have been identified by mass spectrometry and nuclear magnetic resonance spectroscopy including simple phenols, secoiridoids, lignans and flavonoids. All of the individual compounds have potent antioxidant properties as judged by the scavenging of reactive oxygen species in the hypoxanthine/xanthine oxidase HPLC assay described by Owen *et al*. (1996)Eur I Cancer Prev 5, 233-240. In addition the secoiridoids and lignans also display potent inhibition of the enzyme xanthine oxidase which has recently become established as having a major influence on the carcinogenic process in animal models.

These new data have ramifications for the chemoprotective effect of the Mediterranean diet because olive oil especially is a major component and justifies a defined research program to isolate the active principles in bulk so that their antineoplastic properties can be comprehensively screened in a range of *in vitro* and animal model systems prior to conducting intervention studies in humans.

Therefore the aim of this project is to isolate in bulk the known antioxidant phenolic compounds from olive oil either by extraction from the oil or by chemical synthesis and down-stream processing on a semi-industrial scale.

To assess the potency of these antioxidants in a variety of *in-vitro* tests e.g.
a) Quinone reductase.
b) Comet assay.
c) Mouse mammary organ culture.

To conduct animal experiments utilizing the various rat models for breast, colon and lung cancer to assess the anti-carcinogenic potencies of the individual phenolic antioxidants.

To develop Phase 1, 2 and 3 study protocols in humans.

To undertake clinical intervention studies with the most promising antineoplastic phenolic substances based on the data generated by the Phase 1-3 studies. Pre-cancerous patient groups (breast, colon, lung) with a range of defined polymorphic profiles will be subjected to randomized intervention protocols with the chemopreventive agents.

2.2. Unsaturated fatty acids and oestrogen metabolites: lipid peroxidation and oxidative stress as risk modifiers of breast cancer, a case-control study based on biomarkers

K.H. Adzerson*, F Beldmann*, G Bastert*, J. Nair, R.W. Owen and H. Bartsch
*Frauenklinik der Universität Heidelberg, Abteilung für Allgemeine Frauenheilkunde und Geburtshilfe.

Epidemiological and experimental data show correlations between an elevated risk for breast cancer and the dietary intake of long-chain fatty acids (LCFA). While consumption of fat per se is not strongly associated, evaluation of intake of individual fatty acids show that diets high in polyunsaturated long-chain fatty acids (PUFA) of the n-6 series is deleterious while high intake of PUFA of the n-3 series and monounsaturated long-chain fatty acids (MUFA) may be protective. It is imperative that the associations between fat intake in terms of the relative proportions of the specific n-3, n-6 and, n-9 LCFA is clearly elucidated so that future chemopreventive strategies can be devised for high risk groups.

To this end a collaborative study between the Department of Gynecology and Obstetrics, University of Heidelberg Women's Clinic and the Division of Toxicology and Cancer Risk factors is currently being designed involving 240 cases and controls. The intake of the various LCFA classes will be evaluated by dietary questionnaire and related to a variety of end-points such as fatty acid composition and profiles in breast adipose tissue, exocyclic DNA adducts in breast epithelial tissue and white blood cells and serum antioxidant status (carotenoids, ascorbic acid, tocopherols and polyphenols).

2.3. Effects of moderate versus low impact rehabilitative exercise programs on oxidative DNA damage and repair in patients with treated colorectal cancer

H. Allgayer*, R.W. Owen, J. Nair, and H. Bartsch
*Oncology Department, Rehabilitation Clinic Ob der Tauber, der LVA Württemburg, Bad Mergentheim.

Increased oxidative DNA products currently are believed to be involved in early stages of tumor initiation and promotion such as breast and colon cancer. Based on this knowledge the so called biomarker concept has been put forward assuming that measuring DNA damage products derived from interactions with reactive oxygen and/or lipid peroxidation such as 8-oxo-dG, 8-oxo dAdenine and/or etheno-DNA base adducts can be used as quantifiable and modifiable risk indicators. Dietary intervention including low fat, supplementation with vitamins C and E, or short chain fatty acids, and, the intake of certain plant ingredients in healthy volunteers, tumor patients and ani-

mals has been found to be associated with decreased levels of DNA damage products paralleled in most instances also by a diminished cancer risk and/or relapse rates.

Life style aspects are also increasingly coming into the focus of interest as higher levels of physical exercise are found to be associated with decreased cancer risk especially breast and colorectal cancer and, in concordance with the biomarker concept, with decreased levels of DNA damage products in volunteers and animals.

A more detailed knowledge of short and long term effects of different levels of physical activity on DNA damage and tumor relapse is presently available for cancer patients, but would be of particular importance in further clinical management of these patients. In addition detailed knowledge of exercise effects on DNA repair mechanisms also are of clinical relevance, because strengthening such repair may further contribute to decreased DNA damage and cancer (relapse) risk. As colorectal cancer is the second most frequent malignancy in the Western world this project will focus on patients with these tumors.

The aims are to compare short and long term effects of moderate versus low intensity rehabilitative exercise programs in patients with treated (surgery, chemotherapy, radiation) colorectal cancer on the levels of DNA damage products and repair activity (etheno-DNA adducts in blood cells, urine and stool samples) and relapse states, using a controlled and randomized trial.

Publications (* = external co-authors)

[1] Owen, R.W., Giacosa*, A., Hull, W.E.*, Haubner, R., Würtele, G., Spiegelhalder, B. and Bartsch, H. (2000). Olive oil consumption and health: the possible role of antioxidants. *Lancet Oncology* 1, 107-112.

[2] Bonithon-Kopp*, C., Kronborg*, O., Giacosa*, A., Räth*, U. and Faivre*, J. (2000). [Experts:-Milan*, C., Fenger*, C., Piard*, F., Belghiti*, C., Owen, R. W., and Pignatelli*, M. Calcium and fibre supplementation in the prevention of colorectal adenoma recurrence: a placebo-controlled intervention trial from the European Cancer Prevention Organisation (ECP). *Lancet* 356, 1300-1306.

[3] Haines*, A., Hill*, M.J., Thompson*, M.H., Owen, R.W., Williams*, R.E.O., Meade, T.W. and Wilkes, H. (2000). A prospective study of faecal bile acids and colorectal cancer. *Eur J Cancer Prev* 9, 317-323.

[4] Owen, R.W., Giacosa*, A., Hull*, W.E., Haubner, R., Spiegelhalder, B. and Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur J Cancer* 36, 1235-1247.

[5] Owen, R.W., Mier*, W., Hull*, W.E., Giacosa*, A., Spiegelhalder, B. and Bartsch, H. (2000). Identification of lignans as major components in the phenolic fraction of olive oil. *Clin Chem* 46, 976-988.

[6] Owen, R.W., Mier*, W., Giacosa*, A., Hull*, W.E., Spiegelhalder, B. and Bartsch, H. (2000). Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chem Toxicol* 38, 647-659.

[7] Owen, R. W., Spiegelhalder, B. and Bartsch H (2000). Generation of reactive oxygen species by the faecal matrix. *Gut* 46, 225-232.

[8] Bartsch, H., Nair, J. and Owen, R. W. (1999). Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis* 20, 2209-2218.

[9] Fadden*, K., Hill*, M.J., Latymer*, E., Low*, G. and Owen, R.W. (1999). Steroid metabolism along the gastrointestinal tract of the pig. *Eur J. Cancer Prev* 8, 35-40.

[10] Owen R.W. (2001). Biomarkers in colorectal cancer. In: Biomarkers in cancer chemoprevention (A.B. Miller, H. Bartsch, L. Dragsted, H. Vainio). IARC Scientific Publications No 154, 101-112.

[11] Owen R.W. (2000). The role of nutritional factors: colon cancer. In: *Carcinogenic and anticarcinogenic factors in food* (G. Eisenbrand, A.D. Dayan, P.S. Elias, W. Grunow, J. Schlatter Eds), DFG, Wiley-VCH, 43-75.

Genetic Toxicology and DNA Repair (C0203)

P. Schmezer

In vitro genotoxicity is a property of most human and many rodent carcinogens, but often a (tissue-)specific metabolism has to occur to form the ultimate DNA reactive species. We have, therefore, focused our work on the use of metabolically active mammalian cells (human and rodent) freshly isolated from various organs/tissues to study the genotoxic activity of compounds. This goal can effectively be reached only when assays are used which allow the detection of genotoxicity on the single cell level. In this case cells isolated from small human biopsies can be analyzed. The microgel electrophoresis technique (alkaline comet assay) is an appropriate method for this purpose. We have optimized this technique to study genotoxicity as well as specific types of DNA lesions such as oxidative damage. In addition, we use the microgel electrophoresis technique to study DNA repair. Our research activities included investigations on airborne genotoxic/carcinogenic compounds [3,12], mainly in the respiratory tract. To identify possible genotoxic carcinogens, mutagenicity was studied in transgenic rodent mutation assays (BigBlue®, Muta®Mouse) and by *hprt* T-cell cloning [1,6].

More recently, we have focused our work on the identification of high risk individuals in human population studies: In co-operation with epidemiological and clinical partners, an optimized alkaline comet assay is used to monitor cellular mutagen sensitivity (induced by chemicals or radiation) and DNA repair capacity in peripheral blood lymphocytes. Additionally, PCR techniques are applied to identify individuals carrying specific repair enzyme polymorphisms. Furthermore, gene expression of DNA repair enzymes is studied using different methods: DNA array technology, multiplex RT-PCR, and multiprobe-RNase-protection-assay. The identification of high risk individuals carrying increased mutagen or radiation sensitivity, reduced DNA repair capacity, or specific defects in DNA repair enzymes has substantial preventive implications: These individuals could be targeted for intensive cancer screening, and they could be enrolled into chemopreventive trials. Finally, a new activity consists in the search and evaluation of compounds capable to induce cellular repair mechanisms.

1. Genotoxic effects in human epithelial cells of the upper aerodigestive tract

P. Schmezer, T. Rupprecht, U. Harréus, R. Gliniorz, C. Mayer

In cooperation with: H. Maier, M. Tisch, Bundeswehrkrankenhaus, Ulm

In numerous epidemiological studies, environmental and occupational compounds such as chromate, benzo[a]-pyrene, and N-nitroso-diethanolamine have been shown to be of potential carcinogenic risk on human epithelial cells of the upper aerodigestive tract. We used the alkaline microgel electrophoresis assay to directly demonstrate the genotoxicity of such compounds in different mucosal cells isolated from human biopsies of the upper aerodigestive tract: nose, paranasal sinuses, mouth, pharynx, larynx, and tonsils [2]. The cells were freshly isolated from the tissue samples by enzymatic digestion, and 0.5 – 1x10^6 cells per donor were obtained with a viability of 80-100%. After *in vitro* incubation with the potential carcinogens, the cells were subjected to the microgel electrophoresis assay, and the results were evaluated regarding the personal history of each donor including exposure to tobacco smoke, alcohol, and occupational compounds. Chromate induced strong genotoxic damage in the nasal and paranasal sinus epithelia as well as in mucosa cells of the larynx. N-nitroso-diethanolamine caused significant damage in oral epithelial cells and showed also DNA damaging effects in cells isolated from pharynx and larynx tissue. Benzo[a]-pyrene induced only a low level of DNA strand breakage in mucosal cells of mouth, pharynx and larynx. Significant individual differences were apparent for tissue samples of different donors. The genotoxic effect induced in cells of donors with chronic alcohol consumption was significantly higher than in cells of patients without chronic alcohol abuse. Our results demonstrate that the microgel electrophoresis assay is a useful tool to investigate genotoxicity in human epithelial cells of different parts of the upper aerodigestive tract. The assay is able to detect individual differences in sensitivity as well as possible target sites for the genotoxic activity of potential carcinogens.

Major risk factors for cancer of the oral cavity, pharynx and larynx are smoking and excess alcohol consumption. Since long-term survival rates of head and neck cancer patients have not substantially increased, new preventive strategies including the use of cancer chemopreventive agents have to be developed. We used our standardized alkaline microgel electrophoresis assay as a sensitive and rapid tool to detect DNA damage on a single cell level: In a pilot study, macroscopically normal laryngeal mucosa biopsies obtained by surgery from head and neck cancer patients (n=29) and from hospital controls (n=22) were analyzed [5]. As compared to controls, cells from head and neck cancer patients showed a significantly elevated DNA damage without any further genotoxic treatment ($p<0.01$). We conclude that this increased background DNA damage in laryngeal epithelia could result from genetic alterations caused by smoking and alcohol leading in accord with the field cancerisation hypothesis to a gradual decrease of genomic stability and malignancy. The microgel electrophoresis assay is suitable to be further explored as a rapid screening method in larger clinical studies i) to identify high-risk subjects carrying cells with decreased genomic stability, and ii) to verify the efficacy of chemopreventive regimens to prevent or slow down the development of head and neck cancer in high-risk persons.

2. Quantitative assessment of poly(ADP-ribosyl)ation

P. Schmezer, N. Rajaee-Behbahani, C. Mayer, O. Zelezny, R. Gliniorz, P. Waas, U. Bollow, B. Bertram

In cooperation with: A. Bürkle, University of Newcastle, UK; H. Becher, Department of Tropical Hygiene and Public Health, University of Heidelberg; H. Ramroth, Division of Clinical Epidemiology, DKFZ; A. Dietz, Department of Otolaryngology, Head and Neck Surgery, University of Heidelberg

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that is catalytically activated by DNA strand interruptions. It catalyses the covalent modification of proteins with ADP-ribose polymers, using NAD^+ as precursor. We have studied the DNA damage-induced formation of poly(ADP-ribose) in intact human peripheral blood lymphocytes by *in situ* immuno-fluorescence detection. The response of lymphocytes to bleomycin, which is known to induce DNA single and double strand breaks, was investigated with regard to polymer formation. For this purpose, a quantitative approach was developed to assess more accurately the immunostaining of polymer formation by computerized image analysis [7]. As an application of this new method, we have determined the polymer formation following bleomycin treatment in quiescent human peripheral blood lymphocytes versus mitogen activated cells. Quiescent human lymphocytes showed a similar basal immunostaining for the polymer compared to phytohemagglutinin-activated cells, expressed as relative mean pixel intensity (1.3±0.8 and 2.2±0.9, respectively; $p<0.3$). After bleomycin treatment, there was a clear-cut enhancement of polymer immunostaining, with phytohemagglutinin-activated cells showing significantly higher pixel intensity values than non-activated cells (9.2±1.4 and 4.2±1.0, respectively; $p<0.005$). As expected, in the presence of the ADP-ribosylation inhibitor 3-aminobenzamide (3-AB), the pixel intensity value of polymer immunostaining was decreased in both quiescent and phytohemagglutinin-activated lymphocytes to 1.2±0.7 and 1.5±0.9, respectively. Our findings reveal (i) that mitogen-stimulated, intact lymphocytes show enhanced polymer formation following bleomycin treatment, and (ii) that our new quantitative immuno-fluorescence assay coupled with computerized image analysis is reliable and sensitive enough to detect changes in polymer formation rate.

This newly developed assay has been further applied in a clinical case-control study. As it is known that defects in DNA repair pathways have been associated with increased risks for cancer in humans, we investigated whether acquired or inherited changes in the activity of PARP alter the risk for laryngeal cancer. In a case-control study on genetic, lifestyle and occupational risk factors for laryngeal cancer, the PARP activity was assessed as DNA

damage-induced poly(ADP-ribose) formation in human peripheral blood lymphocytes by quantitative immunofluorescence analysis. Polymer formation was determined as the cellular response to bleomycin, in lymphocytes from 69 laryngeal cancer patients and 125 healthy population controls. The frequency of bleomycin-induced polymer formation, measured as mean pixel intensity, was significantly lower in cases (74.6; standard error [SE] = 3.7) than in controls (94.5; SE = 3.5) and was not influenced by smoking, age or sex. There was no significant difference between cases (59.1; SE = 5.2) and controls (50.5; SE = 3.7) in basal polymer formation (in cells not treated with bleomycin). When the lowest tertile of polymer formation was used as the reference, the odds ratio (OR) for the highest tertile of bleomycin-induced polymer formation was 0.26 (95% confidence interval [CI], 0.08-0.53, p = 0.01). Peripheral blood lymphocytes from laryngeal cancer patients thus showed significantly less bleomycin-induced poly(ADP-ribose) formation. Our results suggest that a reduced capacity of somatic cells to synthesize poly(ADP-ribose) might be associated with an increased risk for laryngeal cancer. The underlying mechanism remains to be investigated.

Concerning the search and evaluation of natural compounds capable of inducing cellular repair mechanisms we started experiments with (-)-epigallocatechin gallate (EGCG), the main constituent of green tea. Tea does not belong to the many botanicals with mutagenic and carcinogenic potential [13], but shows remarkable preventive effects against the development of cancer and cardiovascular disease. EGCG turned out to be an effective inducer of poly(ADP-ribose) in Nalm6 cells, a pre-B-leukemia cell line, and in human lymphocytes. As soon as 10 min after the addition of EGCG to the cells, poly(ADP-ribose) was strongly enhanced in Nalm6 and in lymphocytes, persisting for 60 min in the leukemia cells and >4h in the lymphocytes. Preliminary results on a concomitant effect of EGCG on the DNA-level using the microgel electrophoresis assay revealed a slight DNA-damage.

3. DNA repair capacity and mutagen sensitivity as risk markers for non-small cell lung cancer

P. Schmezer, C. Mayer, N. Rajaee-Behbahani, A. Risch, R. Gliniorz, O. Zelezny, P. Waas, O. Popanda

In cooperation with: W. Rittgen, Biostatistics Unit, DKFZ; P. Drings, H. Dienemann, K.W. Kayser, V. Schulz, Thoraxklinik Heidelberg- Rohrbach; A. Bach, M.C. von Brevern, BASF-Lynx, Heidelberg

Individual susceptibility to carcinogens is an important determinant of disease risk. It is influenced by host factors such as the ability to repair DNA lesions. We have developed a microgel electrophoresis assay for use in molecular epidemiological studies in order to identify subjects who are at high risk [10]. The assay was validated in a case-control study on non-small cell lung cancer [11]. Peripheral blood lymphocytes were collected from 160 cancer patients and 180 control patients without cancer and from the same hospital, and stored at –80°C. After thawing, the phytohaemagglutinin-stimulated cells were treated with bleomycin at 20µg/ml for 30 min and the extent of DNA damage and DNA repair capacity were determined: Bleomycin sensitivity was significantly higher in lung cancer patients than in tumor-free hospital controls ($p<0.0001$). DNA repair capacity, after 15 min repair time, in lymphocytes of non-small cell lung cancer patients and controls was 67% and 79.3%, respectively ($p<0.0004$). There was no correlation, in either patient or control group, between the bleomycin sensitivity and DNA repair capacity with age or gender. The median values of DNA repair capacity and sensitivity in controls were used as cut-off points for calculating odds ratios (OR). After adjustment for age, gender and smoking status, the cases vs. controls had reduced DNA repair capacity (OR = 2.1; 95% confidence limit: 1.1-4) and increased bleomycin sensitivity (OR = 4; 95% confidence limit: 2.2-7.4). Both endpoints were independent risk factors for smoking-related cancer. Repeated analysis of peripheral lymphocytes from the same individual demonstrated good reproducibility of the assay. Cryopreservation of the lymphocytes for more than 12 months did not significantly affect their sensitivity. These results show that our standardized microgel electrophoresis assay is suitable for determining individual sensitivity to mutagens and DNA repair capacity: It is sensitive and faster than cytogenetic assays, and can be applied to native and cryopreserved peripheral blood lymphocytes. Validation of this assay in large prospective studies for the identification of subjects at high cancer risk is now warranted.

For characterization and identification of DNA repair genes which might be responsible for the observed impaired repair, we developed a cDNA array for expression analysis of human DNA repair genes: PCR fragments were amplified from IMAGE cDNA clones of more than 70 genes, known to be directly or indirectly involved in DNA repair. Following verification by sequence analysis, the fragments were spotted on arrays. The arrays are now used to produce transcriptional profiles from lung cancer patients with normal and impaired DNA repair. First results showed a good reproducibility of this technique: a less than 2-fold variation was observed in expression levels for most of the genes of the same patient in repeated analyses. On the other hand, transcriptional profiles of two lung cancer patients differed considerably: More than 50% of the signals showed a difference of > 2 - 7-fold. A pilot study including expression profiles of ca 30 non-small cell lung cancer patients with high and low DNA repair capacity is currently being performed.

4. Mutation analysis of DNA polymerases in tumor cells

O. Popanda, P. Waas

In cooperation with: H.W. Thielmann, T. Flohr, J. Dai (DKFZ), E. Hagmüller, Kreiskrankenhaus Bad Friedrichshall

Results are presented in the research report of the division “Interactions of carcinogens with biological macromolecules” (head: Prof. Dr. Dr. H. W. Thielmann) in detail.

According to the mutator hypothesis proposed by L. A. Loeb, the multistep process of tumor development

might be caused by mutations arising in genes that play a key role in maintaining genomic integrity; among them would be those which are involved in DNA replication and repair. For example, mutations in genes coding for DNA polymerases might impair their catalytic functions, particularly their copying fidelity. In consequence, a large number of mutations would accumulate in replicated genes, contributing to the stepwise development of cancer.

Several aspects of the mutator hypothesis were investigated using i) malignant Novikoff hepatoma as a model system [4] and ii) sporadic human colon cancers [8].

i) Catalytic properties of DNA polymerases α and δ from highly malignant Novikoff hepatoma cells were compared with those of the enzymes from normal rat liver. Several parameters were found to be abnormal, among them were: sensitivity towards various inhibitors and cytostatic drugs. In addition, the sedimentation velocities of the DNA polymerase α-primase complexes were altered. The deviating catalytic properties were correlated with mutations found in the genes for DNA polymerase δ and in the 70 kDA subunit of DNA polymerase α suggesting that mutant polymerase genes can encode a mutator polymerase.

ii) Surgical specimens derived from patients with sporadic human colon cancer (clinical cooperation partner: Prof. Dr. E. Hagmüller, Kreiskrankenhaus Bad Friedrichshall) were scrutinized for mutations in genes coding for DNA polymerases α and δ and for the replication factors A and C. Six human colon cancer cell lines (with or without a defect in mismatch repair) were also examined. In 6 out of 19 tumors and in the cell lines, mutations in the DNA polymerase δ gene were detected. Some of them were positioned in or near domains essential for enzymatic functions and are, therefore, likely to alter the enzyme's catalytic activity. As the mutations found in tumors were also detected in the normal tissue, we suggest that these mutations represent DNA polymorphisms. Whether these polymorphisms can contribute to the development of human tumors has to be investigated further.

The analysis of 10 tumors and the cell lines revealed only silent mutations in the genes coding for DNA polymerase α and replication factor C α indicating that cells containing mutations in these genes may not be viable. In replication factor A, a further polymorphism was detected. In cooperation with clinical and epidemiological partners, studies on polymorphisms in DNA repair genes including DNA polymerases will be performed [9].

Publications (* = external co-author)

[1] Schmezer,P. Eckert,C. Induction of mutations in transgenic animal models: BigBlue® and Muta®Mouse, in: The Use of Short- and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation, DB McGregor, JM Rice and S Venitt (eds.), *IARC Scientific Publication 146 (1999) 367-394*

[2] Harréus,U. Schmezer,P. Kuchenmeister,F. Maier,H. *Genotoxic effects in human epithelial cells of the upper aerodigestive tract. *Laryngo-Rhino-Otol. 78 (1999) 176-181*

[3] Klein,R.G. Schmezer,P. Hermann,R. Waas,P. Spiegelhalder,B. Bartsch,H. Strong nasal carcinogenicity and genotoxicity of 1-nitroso-4-methylpiperazine after low dose inhalation in rats. *Carcinogenesis 20 (1999) 1629-1631*

[4] Popanda,O. Flohr,T. Fox,G. Thielmann,H.W. A mutation detected in DNA polymerase delta cDNA from Novikoff hepatoma cells correlates with abnormal catalytic properties of the enzyme. *J Cancer Res Clin Oncol. 125 (1999) 598-608*

[5] Schmezer,P. Rupprecht,T. Tisch*,M. Maier*,H. Bartsch,H. Laryngeal mucosa of head and neck cancer patients shows increased DNA damage as detected by single cell microgel electrophoresis. *Toxicology 144 (2000) 149-154*

[6] Mayer,C. Schmezer,P. Freese*,R. Mutanen*,M. Hietanen*,E. Obe*,G. Basu*,S. Bartsch,H. Lipid peroxidation status, somatic mutations and micronuclei in peripheral lymphocytes: a case observation on a possible interrelationship. *Cancer Letters 152 (2000) 169-173*

[7] Rajaee-Behbahani,N. Schmezer,P. Bürkle*,A. Bartsch,H. Quantitative assessment of bleomycin-induced poly(ADP-ribosyl)ation in human lymphocytes by immunofluorescence and image analysis. *J Immunol Methods 244 (2000) 145-51*

[8] Popanda,O. Zheng,C. Magdeburg*,J.R. Buttner*,J. Flohr,T. Hagmüller*,E. Thielmann,H.W. Mutation analysis of replicative genes encoding the large subunits of DNA polymerase alpha and replication factors A and C in human sporadic colorectal cancers. *Int J Cancer 86 (2000) 318-24*

[9] Wikman,H. Risch,A. Klimek,F. Schmezer,P. Spiegelhalder,B. Dienemann*,H. Kayser*,K. Schulz*,V. Drings*,P. Bartsch,H. *hOGG1* polymorphism and loss of heterozygosity (LOH): significance for lung cancer susceptibility in a caucasian population. *Int J Cancer 88 (2000) 932-7*

[10] Schmezer,P. Rajaee-Behbahani,N. Risch,A. Thiel,S. Rittgen,W. Drings*,P. Dienemann*,H. Kayser*,K.W. Schulz*,V. Bartsch*,H. Rapid screening assay for mutagen sensitivity and DNA repair capacity in human peripheral blood lymphocytes. *Mutagenesis 16 (2001) 25-30*

[11] Rajaee-Behbahani,N. Schmezer,P. Risch,A. Rittgen*,W. Kayser*,K.W. Dienemann*,H. Schulz*,V. Drings*,P. Thiel,S. Bartsch,H. Altered DNA repair capacity and bleomycin sensitivity as risk markers for non-small cell lung cancer. *Int J Cancer 95 (2001) 86-91*

[12] Klein, R.G. Schmezer, P. Amelung, F. Schroeder*, H.-G. Woeste*, W. Wolf*, J. Carcinogenicity assays of wood dust and wood additives in rats exposed by long-term inhalation. Int Arch Occup Environ Health 74 (2001) 109-118

[13] Bertram, B. Hemm*, I. Tang*, W. Mutagenic and carcinogenic constituents of medicinal herbs used in Europe and in the USA. *Die Pharmazie 56 (2001) 99-120*

Biomarkers (C0206)

J. Nair

Cooperations: A. B. Miller et al., G. Fürstenberger DKFZ; K.H. Adzersen, G. Bastert, Frauenklinik Heidelberg; P. Galle, University Hospital, Mainz; G. Winde, Klinikum Kreis Herford, Herford; E.W. Vogel, Leiden University, Leiden, The Netherlands; Jacques Laval, Institute Gustave Roussy, Villejuif, France; A. Barbin, IARC, Lyon, France; B. Tudek, Instytut Biochemii i Biofizyki, Warsaw, Poland; R.H. Elder, Paterson Institute for Cancer Research, Manchester, UK; F.J. van Schooten, University Maastricht, the Netherlands; R. Srám, Technical Academy of Science, Prague; Czech Republic; K.H. Beger, University Ulm; P. Dollara, University of Florence, Italy.

The group's main programs involve further extension and application of the expertise gained on the development and validation of methods for analyzing DNA-adducts as biomarkers, for understanding the mechanism of their for-

mation from exogenous and endogenous reactive species and to apply these markers in human biomonitoring and chemoprevention trials. The formation of adducts from a carcinogen or the proximate metabolite of carcinogen (exogenous or endogenous) is one of the earliest damage to the genome in the cells. If not repaired, the adduct formation in a surviving cell, will lead to a mutation upon cell division and the accumulated mutations that disrupt genomic integrity leads to cancer.

The studies concluded so far established the validity of measurement of etheno-DNA adducts to assess endogenous DNA damage due to oxidative stress and lipid peroxidation. This included assessment of DNA damage caused by chronic infection/inflammation and dietary factors such as intake of polyunsaturated fatty acids (ω-6 PUFA) and oxidative stress caused by metal storage diseases. The most common method for evaluating oxidative DNA damage is the measurement of oxidized DNA base 8-oxo-deoxyguanosine, however, uncertainties exist on the use of this biomarker due to artifact formation. The measurement of more stable secondary DNA damage such as etheno-DNA adducts proved to be more reliable to assess the DNA damage caused by increased oxidative stress and lipid peroxidation (LPO). The following investigations are accomplished in order to link increased etheno adduct formation as a result of enhanced lipid peroxidation due to inflammation and intake dietary ω-6 PUFA.

1. Formation of etheno adducts 1,N^6-ethenodeoxyadenosine (εA) and N^3,4-ethenodeoxycytidine (εdC) were investigated in diseases, prone to develop into colon cancer such as familial adenomatous polyposis(FAP), ulcerative colitis (UC) and Crohn's disease (CD). εdA and εdC were elevated in both FAP and CD where as in UC εdC alone was elevated. The increased etheno-DNA adducts in FAP may resulting from increased arachidonic acid metabolism due to overexpression of phospholipase A2 and cyclooxygenase 2. In case of CD and UC the increased adduct formation may be due to increased oxidative stress/lipid peroxidation as a consequence of chronic inflammation and overproduction of nitric oxide .[3]
2. Formation of etheno adducts were found to be higher in DNA of pancreatic tissues obtained from chronic pancreatitis patients compared to normal pancreas.
3. Detection of 1,N^6-ethenodeoxyadenosine (εdA) in human urine by immunoaffinity-HPLC-fluorescence.
   In order to investigate the mechanism of formation and repair of these lipid peroxidation induced ε-base adducts in human, which appear to be a complex one due to the influence of dietary factors, hormonal metabolism, inflammatory processes etc., a non-invasive assay is desirable. For this purpose we have developed a specific and sensitive urinalysis assay for 1,N^6-ethenodeoxyadenosine (εdA) using immunoaffinity-HPLC-fluorescence. Using urinary εdA as biomarker it is now possible to study the DNA damage due to oxidative stress and lipid peroxidation in human caused by different etiology such as high dietary fatty acid and low antioxidant intake, inflammatory process etc. using this non-invasive and easily available biological samples [9].
4. An immunohistochemical method has been developed for the measurement of εdA at the cellular level and successfully applied in rat liver exposed to vinyl chloride and iron overload.[5]

Ongoing Activities and Future Plans:

1. Elucidation of role of etheno adducts in chronic inflammation /infection induced oxidative stress

The role of nitric oxide synthase on the formation of etheno DNA adducts is being further investigated in p53 knock out mice which were infected with *C. parvum* (in Collaboration with Dr. C. Harris and Dr. P. Hussain, NIH, Bethesda, USA).

2. Investigation of formation of etheno DNA adducts in mitochondrial DNA and comparison with the nuclear DNA

DNA damage in mitochondrial DNA has recently received enhanced attention as mitochondria play a major role in apopotosis. It is proposed to investigate the relative etheno-DNA adduct formation in mitochondrial and nuclear DNA in animal models (LEC rats) in response to oxidative stress.

3. Investigation of etheno-DNA adducts in brain and liver DNA of Oxyrats which attain premature aging due to increased oxidative stress

The result obtained in this study is expected to determine the DNA damage due to oxidative stress/lipid peroxidation in the brain DNA, (Supported by INTAS, in collaboration with Dr J. Laval. CNRS, Villejuif, France).

4. Investigation of the role of COX-2 and LOX on the formation of etheno DNA adducts.

Studies on DMBA-TPA mouse skin carcinoma revealed a close correlation of etheno DNA adducts and 8- and 12-, HETES; the products of LOX pathways of arachidonic acid metabolism (Nair et al 2000)[1]. Increased etheno DNA adducts were detected in the polyp epithelia of FAP patients (Schmid et al 2000)[3]. It is further planned to investigate the relative roles of COX-2 and LOX using cell lines expressing high levels of these enzymes (In collaboration with Dr. G. Fürstenberger, DKFZ Heidelberg)

5. Method development for the analysis of εdG

Inspite of existing evidence for the *in vitro* and *in vivo* formation of εdG, no sensitive methods are currently available for its analysis. It is proposed to develop and validate an ultrasensitive method for εdG based on immunoaffinity cleanup, ^{32}P-postlabelling TLC/HPLC methods similar to that developed for εdA and εdC. For this purpose, hybridoma cell lines that produce antibodies against εdG were kindly provided by Dr. S.S. Hecht (Cancer Center, University of Minnesota Hospital and Clinics, Minneapolis, USA). Antibody will be coupled with Protein A-Sepharose to prepare immunoaffinity columns which will be characterized for the specificity and retention capacity of the adducts. Reference compounds, [^{3}H]-εdG, 3'-and εdG monophosphate will be synthesized.

6. Investigations on the formation of ε-DNA adducts in humans in relation to fatty acid intake and hormonal metabolism

Although high incidence and mortality rates for breast cancer are reported across Europe, these differ according to geographical location within the EU (~120 and ~60/100,000 in Western-Northern and Southern European countries, respectively. The variation can be attributed to difference in the types of dietary fat intake. The most established risk factor for breast cancer is length of free estradiol exposure during the life time. It is been shown in the past that diet rich in ω-6 fatty acid induce high but variable levels of ε-DNA adducts in female volunteers. Studies described here propose to investigate the role of fatty acid intake and estradiol metabolism and the role of redox-cycling on the formation of ε-DNA adducts in pre-menopausal female subjects. Fatty acids are known to modulate the oxidative pathway of estradiol and 4-hydroxy estradiol, a rodent carcinogen, acts as a pro-oxidant that could initiate redox-cycling [6]. Selection of samples from humans: Buffy coat and serum samples from premenopausal adult women, who participated in the EPIC study from one EU country, are available for study. Additionally, breast adipose tissue, blood and urine samples will be collected from cancer patients. Control samples will be obtained from healthy women, either undergoing reduction mammoplasty, or presenting with an unclear mammogram and/or who undergo a diagnostic breast operation with a possible benign outcome. These samples will be used to establish a correlation between fatty acid intake, estradiol metabolites and etheno DNA adducts in European women. Analyses of ε-DNA adducts, fatty acid content and estradiol-metabolites: These samples will be used to establish correlation between fatty acid intake, estradiol metabolites and ε-DNA adducts in European women. ε-DNA adducts (εdA and εdC) in white blood cells and breast epithelial cells will be measured by immunoaffinity/^{32}P-postlabelling [9] and εdA in urine will be measured by immunoaffinity-HPLC-fluorescence detection [9]. Fatty acids will be analyzed in adipose tissue and erythrocytes by GC-MS. Estradiol and its metabolites 2- and 4- hydroxy estradiol and 2- and 4-methoxy estradiol will be analyzed by (GC-MS) (under EU-Contract n° QLK4-2000-00286).

7. The role of etheno-DNA adducts in the etiology of atherosclerosis

Cardiovascular diseases and cancer are both characterized by uncontrolled proliferation of cells, share common risk factors and autopsy studies demonstrated that atherosclerosis and cancer tend to occur in the same individuals. These observations led to the hypothesis that atherosclerosis may develop like a benign tumor according to an initiation-promotion mechanism. Indeed, there is evidence for mechanistic similarities between the carcinogenic and the atherogenic process, but the etiology of atherosclerosis still needs to be further elucidated. Oxidative stress and lipid peroxidation are known to play a key role in the onset and progression of atherosclerosis and cancer. Lipid peroxidation products can bind covalently to cellular DNA to form etheno-DNA adducts. These etheno-DNA adducts were found to be highly promutagenic lesions if not repaired by specific glycosylases, and are therefore promising candidates to test the hypothesis, whether DNA damage is involved in the pathogenesis of atherosclerosis. Enhanced oxidative stress has been found in apolipoprotein E knock-out mice (ApoE-KO mice), which develop severe spontaneous atherosclerosis. ApoE-KO mice offer an excellent model to investigate the role of oxidative stress and subsequent formation of etheno-DNA adducts in the etiology of vascular diseases. The role of etheno-DNA adducts, induced by lipid peroxidation, in the etiology of atherosclerosis will be further elucidated in this forthcoming project. For this, the ApoE knock-out animal model will be explored. In this project the German Cancer Research Center (Division of Toxicology and Cancer Risk Factors) will collaborate with Prof. Dr. M. Daemen (Dept. of Cardiology and Pathology) and Dr. F.J. Van Schooten (Dept Health Risk Analysis and Toxicology) at the Maastricht University in the Netherlands.

8. Development and validation of a new immuno-enriched ^{32}P-postlabelling assay for the detection of O^4-ethyl-dT.

Among about a dozen different alkylation products induced in genomic DNA by *N*-nitroso carcinogens, O4-ethyl-thymidine (O^4-etdT) can be considered as a major premutational lesion causing AT→GC transition mutations. Although O^4-etdT is initially formed at far lower amounts than other alkylation products in cellular DNA, it accumulates because of its inefficient repair and may thus play an important role in carcinogenesis. We established a sensitive and specific immuno-enriched 32P-HPLC assay with which sub-femtomolar levels of O^4-etdT can be detected. First, DNA is enzymatically digested to 2'-deoxynucleoside-3'-monophosphates, and O^4-etdT is subsequently immunoprecipitated by saturated ammonium sulfate using specific monoclonal antibodies (ER-01). The precipitate is redissolved and desalted using a centrifugation filter, which retains the O^4-etdT-antibody interaction product. After recovery by inversion of the filter, O^4-etdT is simultaneously 5'-radiolabeled with [γ–^{32}P]ATP and 3'-dephosphorylated by T4-polynucleotide kinase at pH 6.8 to yield a 5'-monophosphate-O^4-etdT. Separation of O^4-etdT from residual normal nucleotides and an internal standard (1,*N*^{6}-ethenodeoxyadenosine, εdA) can be performed by reversed-phase HPLC with online detection of radioactivity. The recovery of the adduct dependent predominantly on the antibody concentration and was found to be >80%. Using this method, O^4-etdT levels were determined in calf thymus DNA treated with *N*-ethyl-*N*-nitrosourea (ENU) at concentrations ranging from 0.01 to 100 mM, and a reproducible dose dependent formation of O^4-etdT was observed. Theoretically, this newly developed assay is sufficiently sensitive to detect O^4-etdT in human samples and is also applicable to other types of DNA adducts.

External Funding: Marie Curie Fellowship Program from EU; EU Contract MCFI-2000-01241. EU-Contract QOL-2000-4.2.1 Oxidative Stress and Chronic Diseases: Exocyclic DNA Adducts as Markers for Disrupted Genomic Integrity and Risk (awarded in March 2001).

Publications (* = external co-author)

[1] Nair J, Fürstenberger, G, Bürger F, Marks, F and Bartsch, H. Promutagenic Etheno-DNA Adducts in Multistage Mouse Skin Carcinogenesis: Correlation with Lipoxygenase-Catalyzed Arachidonic Acid Metabolism. Chem Res Toxicol. 2000 ;13:703-709.

[2] Bartsch H, Nair, J. New DNA-based biomarkers for oxidative stress and cancer chemoprevention studies. Eur J Cancer. 36:1229-1234,2000.

[3] Schmid K, Nair, J, Winde G, *Velic I, *Bartsch H. Increased levels of promutagenic etheno-DNA adducts in colonic polyps of FAP patients. Int J Cancer. 87, 1-4, 2000.

[4] Bartsch, H., *Phillips, D.H., Nair, J.,* Hewer, A., *Meyberg - Solomeyer, G., and *Grischke, E.M. Lack of evidence for tamoxifen- and toremifene-DNA adducts in lymphocytes of treated patients. . Carcinogenesis, 21: 845-847, 2000.

[5] Yang, Y., Nair, J., *Barbin, A., and Bartsch, H. Immunohistochemical detection of 1,N(6)-ethenodeoxyadenosine, a promutagenic DNA adduct, in liver of rats exposed to vinyl chloride or an iron overload. . Carcinogenesis, 21: 777-781, 2000.

[6] Bartsch, H., Nair, J., and Owen, R.W. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. . Carcinogenesis, 20: 2209-2218, 1999.

[7] Bartsch, H., Rojas, M., Nair, U., Nair, J., and *Alexandrov, K. Genetic cancer susceptibility and DNA adducts: studies in smokers, tobacco chewers, and coke oven workers. . Cancer Detect Prev, 23: 445-453, 1999.

[8] *Freese, R., *Basu, S., *Hietanen, E., Nair, J., *Nakachi, K., Bartsch, H., and *Mutanen, M. Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemostatic factors during a high-linoleic acid diet in healthy females. . Eur J Nutr. 38: 149-157, 1999.

[9] Nair, J. Lipid peroxidation-induced etheno-DNA adducts in humans. . IARC Sci Publ,No. 150, 55-61, 1999.

[10] Nair, J., *Barbin, A., *Velic, I., and Bartsch, H. Etheno DNA-base adducts from endogenous reactive species. . Mutat Res, 424: 59-69, 1999.

Genetic Polymorphisms as Cancer Risk Markers (C0207)

B. Spiegelhalder

Worldwide, lung cancer has a very high incidence for men, and its incidence is increasing among females. Lung cancer has a bad prognosis, as therapeutic measures have only limited success. While tobacco smoking is strongly associated with lung cancer risk, only 20% of all heavy smokers develop lung cancer. Many genetic and molecular biological studies point towards a polygenic heritable predisposition for lung tumors. Xenobiotic metabolism of environmental carcinogens and DNA-repair processes are two important ways in which individual susceptibility to environmental carcinogenesis can be affected [2]. Genetic polymorphisms in enzymes involved either in detoxification of procarcinogens, such as *CYP1A1*, CYP2A6, CYP2E1, *GSTM1*, *GSTT1*, *GSTP1*, *NAT1* or *NAT2* or in repair of DNA-damage incurred as a result of exposure, may affect lung cancer susceptibility. The combination of homozygous mutated *CYP1A1* and the *GSTM1**0/*0 genotypes, for instance, has been shown to lead to a stronger increase of anti-benzo(a)pyrene diol-epoxide DNA adduct levels than *CYP1A1* and *GSTM1* wild-type, in individuals with similar exposure levels [1], and in smokers an association has also been observed with the combined NAT and GST genotypes [8]. At-risk genotypes can therefore be used as susceptibility markers, with the aim of identifying high risk individuals. There are indications that interindividual differences as a result of genetic polymorphisms may be of particular importance to cancer risk at low dose exposures. Among a group of smokers with low cigarette consumption (low nicotine-cotinine levels in blood) those with the slow *NAT2* genotype had higher adduct levels than fast acetylators, while this difference was much less marked in heavier smokers (Vineis et al 1994 *Nature* 369: 154-156). Similar results were observed in smokers in Japan in connection with *CYP1A1* and *GSTM1* polymorphisms and lung cancer. The better characterization of the relevance of gene-environment interactions in the context of carcinogenesis is of great importance for preventive measures such as the setting of exposure threshold values, public health campaigns, and chemopreventive approaches.

1. The relevance of enzyme polymorphisms in lung cancer risk

A. Risch, H. Wikman, H. Dally, S. Thiel, K. Gassner, P. Schmezer, N. Rajaee-Behbahani, B. Spiegelhalder, H. Bartsch

In cooperation with Prof. Dr. med. Peter Drings, Prof. Dr. med. Hendrik Dienemann, Prof. Dr. Dr. med. Klaus Kayser, Prof. Dr. med. V. Schulz, Thoraxklinik, Heidelberg-Rohrbach and Dr. Lutz Edler, DKFZ.

In a hospital based case-control study the frequency of different genetic polymorphisms in xenobiotic metabolising enzymes and of differences in DNA-repair capacity is being investigated. Occupational and smoking history of lung cancer patients and cancer free hospital controls is recorded, and blood and tissue samples are collected. Samples are genotyped for different genetic polymorphisms by PCR/RFLP based assays. These include: *NAT1*, *NAT2*, *GSTM1*, *GSTT1*, *GSTM3*, *GSTP1*, *CYP1B1*, *CYP1A1*, *hOGG1* and *MPO*. Genotyping for further genetic polymorphisms of possible relevance is planned. A modified alkaline microgel electrophoresis assay [5] was used to determine mutagen sensitivity and DNA repair capacity after bleomycin-induced DNA damage and showed a significant positive correlation ($p<0.005$) between increased lung cancer risk and mutagen sensitivity and a negative correlation with repair capacity ($p< 0.005$) [7]. Over 1000 samples have been collected, and over 700 have been genotyped for N-acetyltransferases [6] and glutathione-S-transferases, where it could be shown that it is important to distinguish between the different histological subtypes of lung tumors, to evaluate the risk associated with different genotypes. Genotyping for *hOGG1* revealed no increased risk associated with any allele our Caucasian population, and studies on LOH in lung tumors at the hOGG1 site showed that this gene located at 3p21 frequently does display LOH, however, preferential loss of a certain allele was not observed [4].

This study is/was funded by the Deutsche Krebshilfe (H.D), the Verein zur Förderung der Krebsforschung (A.R.), the EU-Marie-Curie Fellowship Program (H.W.) and the Gottlieb Daimler und Karl Benz Stiftung (H.W.)

2. The relevance of enzyme polymorphisms to the risk of developing lung cancer at a young age

A. Risch, B. Spiegelhalder, H. Bartsch

In cooperation with Prof. Dr. Dr. H.-Erich Wichmann, Dr. Irene Brüske Hohlfeld, Dr. Michaela Kreuzer, GSF, München

55 young lung cancer patients (age < 45) were compared to a similarly sized group of older (aged 55-69) lung cancer patients matched for smoking exposure and another similarly sized control group of age matched population controls. All patients and controls were recruited as part of a larger epidemiological study. Individuals were genotyped for known polymorphisms in *NAT2*, *GSTM1*, *GSTT1* and *CYP1A1*. Statistical analysis did not reveal an association between any of the examined polymorphisms and risk of developing lung cancer at a young age.

3. Development of high-throughput genotyping methods, and identification of new alleles

A. Risch, B. Spiegelhalder, H. Bartsch

In order to identify the role that genetic polymorphisms may play in individual risk assessment, large case-control studies need to be conducted. PCR-RFLP methods are state of the art for the screening of large sample numbers for known genetic polymorphisms, however, as the number of polymorphisms to be investigated increases, even larger studies, and higher throughput genotyping are required. Methods employing fluorescence-based capillary PCR followed by melting curve analysis for the detection of mutations (Roche, LightCycler) have been developed for *NAT2* [6], *CYP1A1* and *CYP1B1*. Further such methods, as well as those for the identification of new previously unknown alleles are under development.

4. Exposure to cigarette smoke and genetic polymorphisms as risk markers for breast cancer in the general population

A. Risch, B. Spiegelhalder, H. Bartsch

In cooperation with Dr. Jenny Chang-Claude, Silke Kropp, DKFZ-Div. Clinical Epidemiology

Four possible susceptibility markers, N-acetyltransferases 1 and 2 (*NAT1* and *NAT2*), cytochrome P450 2A6 (CYP2A6) and glutathione-S-transferase T1 (*GSTT1*) may be relevant for breast cancer in identifying high risk subjects. *NAT1* and *NAT2* metabolise aromatic heterocyclic amines, which are found in cigarette smoke at relatively high concentrations. CYP2A6 metabolizes several nitrosamines found in tobacco smoke, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is believed to be one of the major human lung carcinogens and possibly causes tumors also at other sites. *GSTT1* is responsible for the detoxification of ethylene oxide formed from ethene in cigarette smoke as well as for glutathione-dependent activation of certain halomethanes also present in cigarette smoke. A significantly increased risk of breast cancer in women who actively smoke and are slow (*NAT2*) acetylators has been shown. There are also indications that enzyme polymorphism may have a particular impact on cancer risk at low-level carcinogen exposure. Given that one in twelve women in the Federal Republic of Germany will suffer from breast cancer in her lifetime and the prevalence of smoking in the population is high, the association of smoking with breast cancer is of both etiologic and public health importance.

The objectives of this study are to determine if genetic polymorphisms in *NAT1*, *NAT2*, CYP2A6, and *GSTT1* alter the ability of women to detoxify or activate tobacco-related carcinogens and thereby increase their susceptibility to breast cancer. Questionnaire data on active cigarette smoking and blood samples collected and stored for subsequent molecular genetic analysis were already available from a recently completed population-based case-control study of breast cancer diagnosed by the age of 50. Data on exposure to environmental tobacco smoke have now been collected for an overall assessment of active and passive smoke exposure of the study subjects. Genotyping for *NAT2*, *NAT1*, CYP2A6 and *GSTT1* has been carried out using PCR-RFLP and fluorescence based PCR methods on breast cancer patients and age-matched controls from the general population. Genotyping for *NAT1* is still under way. The concomitant analysis of smoking exposure data and genetic susceptibility markers will allow i) investigation of the extent of gene-environment interactions and ii) clarification of the association between smoking and breast cancer risk in genetically defined subgroups of the general population.

This study is funded by the Deutsche Krebshilfe.

5. Investigation of the potential role of *GSTM1, GSTT1* and *GSTP1* polymorphisms, as modulators of antioxidative capacity, in modifying telomere length and as possible risk factors for vascular dementia

A. Risch, H. Bartsch

In cooperation with T. von Zglinicki, University of Newcastle, UK, Dept. of Gerontology, Institute for the Health of the Elderly.

Progressive cerebrovascular atherosclerosis and consecutive stroke are among the most common causes of dementia. However, specific risk factors for vascular dementia are still not known. Human telomeres shorten with each cell division *in vitro* and with donor age *in vivo*. In human fibroblasts *in vitro*, the telomere shortening rate decreased with increasing antioxidative capacity. A good intra-individual correlation between the age-corrected telomere lengths in fibroblasts and peripheral blood mononuclear cells has been observed. In 186 individuals including 149 geriatric patients (age range, 55-98 yr), leukocyte telomeres in patients with probable or possible vascular dementia were significantly shorter than in three age-matched control groups, namely in cognitively competent patients suffering from cerebrovascular or cardiovascular disease alone, in patients with probable Alzheimer's dementia, and in apparently healthy control subjects. No correlation with either telomere length or diagnosis was found in a random subgroup of 75 patients to polymorphisms in the apolipoprotein E and glutathione-S-transferase *GSTM1, GSTT1* and *GSTP1* genes. Telomere length may be an independent predictor for the risk of vascular dementia [3].

Publications (* = external co-author)

[1] Rojas M, *Alexandrov K, *Cascorbi I, *Brockmöller J, *Likhachev A, *Pozharisski, K, *Bouvier G, *Auburtin G, *Mayer L, *Kopp-Schneider, A, *Roots I and Bartsch H (1998). High benzo[a]pyrene diol-epoxide DNA adduct levels in lung and blood cells from subjects with combined *CYP1A1* MspI/MspI-GST1*0/*0 genotypes. *Pharmacogenetics* 8: 109-118.

[2] Bartsch H., Nair U., Risch A., Rojas M., Wikman H., *Alexandrov K. (2000) Genetic Polymorphims of CYP Genes, Alone or in Combination, as a Risk Modifier of Tobacco-related Cancers. *Cancer Epidemiology, Biomarkers and Prevention* 9, 3-28.

[3] *von Zglinicki T., *Serra V., *Lorenz M., *Saretzki G.,*Lenzen-Großimlighaus R., *Geßner R., Risch A., *Steinhagen-Thiessen E. (2000) Short telomeres in patients with vascular dementia: an indicator of low antioxidative capacity and a possible risk factor? *Lab. Invest.* 80(11):1739-1747.

[4] Wikman H., Risch A., *Klimek F., Schmezer P., Spiegelhalder B., *Dienemann H., *Kayser K.,* Schulz V., *Drings P., Bartsch H. (2000) *hOGG1* Polymorphism and Loss of Heterozygosity (LOH): Significance for Lung Cancer Susceptibility in a Caucasian Population, *Int. J. Cancer* 88, 932-937.

[5] Schmezer P., Rajaee-Behbehani N., Risch A., Thiel S., Rittgen W., *Drings P., *Dienemann H., *Kayser K., Schulz V., and Bartsch H. (2001) Rapid screening assay for mutagen sensitivity and DNA repair capacity in human peripheral blood lymphocytes, *Mutagenesis* 16(1), 25-30.

[6] Wikman H., Thiel S., Jäger B., Schmezer P., Spiegelhalder B., Edler L., *Dienemann H., *Kayser K., Schulz V., *Drings P., Bartsch H., Risch A. (2001) Relevance of N-Acetyltransferases 1 and 2 (*NAT1*, *NAT2*) Genetic Polymorphisms in non-Small Cell Lung Cancer, *Pharmacogenetics* 11, 157-168.

[7] Rajaee-Behbahani N., Schmezer P., Risch A., *Rittgen W., *Kayser K., *Dienemann H., *Schulz V., *Drings P., Thiel S., Bartsch H. (2001) Altered DNA repair capacity and bleomycin sensitivity as risk markers for non-small cell lung cancer, *Int. J. Cancer*, 95, 86-91.

[8] Godschalk R.W.L., *Dallinga J.W., Wikman H., Risch A., *Kleinjans J.C.S., *Bartsch H.,* Van Schooten F.-J. (2001) Modulation of DNA and protein adducts in smokers by genetic polymorphisms in *GSTM1*, *GSTT1*, *NAT1* and *NAT2*. *Pharmacogenetics in press.*

Genotype Dependence of Carcinogen DNA Adduct Levels (C0207)

B. Spiegelhalder

1. Modulation of benzo(a)pyrene diolepoxide – DNA adduct levels in human white blood cells by *CYP1A1, GSTM1 and GSTT1* polymorphism

M. Rojas, K. Alexandrov

In cooperation with I. Cascorbi, I. Roots, Charité, Humbolt University, Berlin ; G. Auburtin, L. Mayer, Institut National de l'Environnement et des Risques, France

The modulation of benzo(a)pyrene diol-epoxide (BPDE) DNA adduct levels by polymorphisms in the *CYP1A1, GSTM1 and GSTT1* genes was assessed in leukocytes of Caucasian males. *BPDE-DNA* adducts were detected in 52 % samples with a 100-fold variation. All samples with the *GSTM1* active genotype showed non-detectable adducts and the subjects with *GSTM1*0/*0* had detectable adducts. Among the *GSTM1*- deficient individuals , the *CYP1A1*1/*1* genotype showed a lower adduct level compared with heterozygous mutated *CYP1A1 *1/*2* genotypes. When all variable were dichotomized, statistical evaluation showed that *CYP1A1* status, *PAH* exposure and smoking had significant effects on adduct levels which increased in order : *CYP1A1*1/*1 < CYP1A1(*1/*2 or *2A / *2A)*; environmental exposure < occupational exposure < ; nonsmokers < smokers. Higher levels of *BPDE-DNA* adducts in individuals with the combined *CYP1A1(*1/*2 or *2A/*2A)-GSTM1*0/*0* genotype suggest that these genotype combinations are at increased risk for contracting lung cancer when expose to PAH.
In a review we have summarized the results of case-control studies published since 1990 on the effects of genetic variants of *CYP1A1, 1A2, 1B1, 2A6, 2D6, 2E1, 2C0, 2C19, 17* and *19* alone or in combination with detoxifying enzymes as modifiers of the risk for tobacco-related cancers.

CYP polymorphisms were found to be moderate in terms of penetrance and relative risk, with odds ratios ranging from 2 to 10. Some CYP1A1/GSTM1 0/0 genotype combinations seem to predispose the lung ,esophagus, and oral cavity of smokers to an even higher risk for cancer or DNA damage , requiring, however, confirmation.

Publication (* = external co-author)

[1] Rojas, M., Cascorbi, I.*,Alexandrov, K., Kriek, E*., Auburtin, G*:, Mayer, L*.,Koop-Schneider, A., Roots, I*, Bartsch, H: . Modulation of benzo(a)pyrene diolepoxide DNA adduct levels in human white blood cells by *CYP1A1, GSTM1*0/*0 and GSTT1* polymorphisms, Carcinogenesis 21 (2000) 35-41.

[2] Bartsch, H., Nair, U., Risch, A., Rojas, M., Wikman, H. *Alexandrov, K. Genetic polymorphism of *CYP* genes , alone or in combination, as a risk modifier of tobacco-related cancers. Cancer epidemiology, Biomarkers and Prevention 3 (2000) 3-28

2. Benzo(a)pyrene diolepoxide-DNA adduct levels in human bronchial layer and lung parenchyma ; the role of *CYP1A1, GSTM1, MPO* genotypes and their expression

M. Rojas, K. Alexandrov

In cooperation with J.M. Vignaud, Laboratoire d'Anotomie pathologique, Centre Hospitalier Universitaire de Nancy, France; I. Cascorbi, Ernst Moritz Arndt University of Greifswald Medical Faculty, Greifswald

In this ongoing study we compared the formation of benzo(a)pyrene diolepoxide-DNA adduct levels in human bronchial epithelial cells and lung parenchyma from lung cancer patients. All samples showed the presence of this adduct. Higher formation of the adduct was found in DNA from bronchial layer in comparison to the lung parenchyma. Also high variation was observed between the different samples. These results are being correlated with the *CYP1A1*, *GSTM1* and *MPO* genotypes and their expression.

3. Myeloperoxidase –463A variant strongly reduces benzo[a]pyrene diol-epoxide DNA adducts in skin of coal tar treated patients.

M. Rojas, R. Godschalk, K. Alexandrov

In cooperation with : I. Cascorbi, Ernst Moritz Arndt University of Greifswald Medical Faculty, Judith Ostertag, University Hospital Maastricht (AzM). Department of Dermatology, The Netherlands

The skin of atopic dermatitis patients provides an excellent model to study the role of inflammation in benzo[a]pyrene (BaP) activation, since these individuals are often topically treated with ointments containing high concentrations of BaP. In this study [1], we determined by HPLC-fluorescence detection (Alexandrov et al., Cancer Res., 52 (1992), 6248-6253) the BaP-diolepoxide (BPDE)-DNA adduct levels in human skin after topical treatment with coal tar and their modulation by the -463G → A myeloperoxidase (MPO) polymorphism, which reduces *MPO* mRNA expression. BPDE-DNA adduct levels were 2.2 and 14.2 adducts per 10^8 nucleotides for *MPO*-463AA/AG and -463GG, respectively. The predominant BaP tetrol observed was tetrol I-1, which is derived after hydrolysis of the *anti*-BPDE-DNA adduct. The tetrol I-1/ II-2 ratio corresponding to the *anti*/ *syn* ratio was 6.7. The ^{32}P-postlabelling assay was also performed and thin layer chromatograms showed a major spot with the chromatographic location corresponding to BPDE-DNA. The mean values of the BPDE-DNA adduct spots were 3.8±2.4 per 10^8 nucleotides for *MPO*-463AA/AG (n=3) and 18.4±11.0 per 10^8 nucleotides for *MPO*-463GG (n=7), respectively (P=0.03). One individual with the homozygous mutant genotype (-463AA) even had a 13-fold lower adduct level (1.4 per 10^8) as compared to *MPO*-463GG subjects. In conclusion, these data showed for the first time (i) the *in vivo* formation of BPDE-DNA adducts in human skin treated with coal tar and (ii) that the *MPO*-463AA/AG genotype strongly reduced BPDE-DNA adduct levels in human skin.

Publication (* = external co-author)

[1] M. Rojas, R. Godschalk , K. *Alexandrov, I. *Cascorbi, E. *Kriek, J. *Ostertag, F.-J. *Van Schooten and H. Bartsch: Myeloperoxidase-463A variant reduces bento (a) pyrene diolepoxide DNA adducts in skin g coal far treated patients. Carcinogenesis (in press).

4. Efficacy of new chemopreventive cigarette filter to reduce benzo[a]pyrene-DNA adducts in MCF-7 and LH-60 cells

M. Rojas, K. Alexandrov, H.R. Scherf, I. Cascorbi*, I. Emami**

Co-operation Research Project: DKFZ, *Ernst-Moritz-Arndt-Universität Greifswald, **Biosyntec (France)

Cigarette smoking is the major risk factor for lung cancer. While it is crucial to prevent addiction to tobacco, and to enhance the efficacy of smoking cessation and reduction programs, these approaches have had limited impact. Chemoprevention is one approach to decreasing lung cancer risk in addicted smokers. Polycyclic aromatic hydrocarbons (PAH), examplified by benzo[a]pyrene (B[a]P), tobacco-specific nitrosamines (e.g. NNK) and free radicals / reactive oxygen species (ROS) are considered to be important carcinogens involved in lung cancer induction in smokers.

A new cigarette filter was developed by Biosyntec, France, which can trap ROS and thus can interfere with the metabolic activation of inhaled lung carcinogens. This filter containing a specific galenic formulation of a rosemary extract, can significantly reduce the free radicals concentration in cigarette smoke. We studied the efficacy of this new chemopreventive filter to block initiation of carcinogenesis by benzo[a]pyrene in MCF-7 and LH-60 cells. MCF-7 cells have high *CYP1A1* enzyme activity and little myeloperoxidase activity, while LH-60 cells have high myeloperoxidase activity and low level of *CYP1A1* enzyme activity. Cells treated with tobacco smoke solution obtained from cigarettes with the new filter showed a strong inhibition (40-50%)of B[a]P-DNA adducts formation in comparison to cells treated with tobacco smoke obtained with a conventional filter. These results lead to the assumption that it should be possible using this filter to reduce the capacity of tobacco smoke to form the critical tumorigenic adduct between B[a]P and DNA. This effect is now confirmed in *in vivo* experiments in smoking human volunteers