Forschungszentrum Karlsruhe
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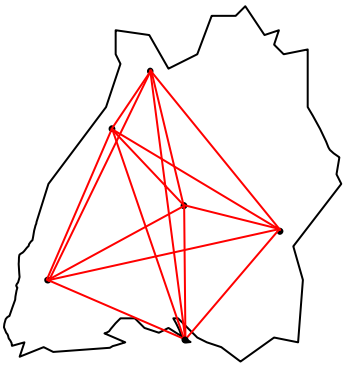
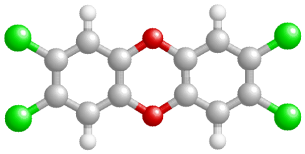
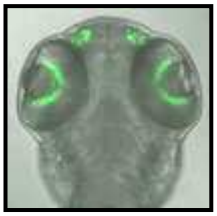
Karlsruhe Institute of Technology

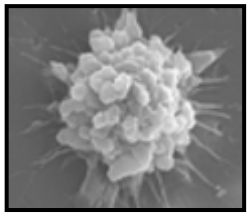
BW ToxNet 2007

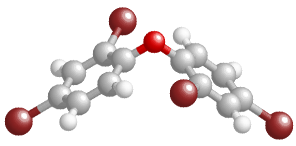


Baden Württemberger Toxikologen Treffen
10 Juli 2007, Forschungszentrum Karlsruhe









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General Information

Meeting venue:

July 10, 2007; 10:00 a.m.
Forschungszentrum Karlsruhe
Fortbildungszentrum für Technik und Umwelt
Hermann-von-Helmholtz-Platz 1
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BW-ToxNet meeting 2007

Date: July 10th, 2007
Venue: FTU, FZK, Karlsruhe

Organisation: Carsten Weiss, Uwe Strähle, Sabine Ntemir, Eva-Maria Kelter / ITG, FZK

10:00 h	Welcome	
10:05 - 10:20	Lixin Yang (FZK)	Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo
10:20 - 10:35	Markus Wahl (FZK)	Interactions of polybrominated diphenyl ethers with AhR signalling
10:35 - 10:50	Albert Bräuning (Uni Tübingen)	Regulation of drug-metabolizing enzymes by β-catenin-dependent signal transduction
10:50 - 11:05	Jochen vom Brocke (DKFZ)	Investigations on the mutation signature of 3-Nitrobenzanthrone in the human p53 sequence
11:05 - 11:20	Coffee Break	
11:20 - 11:35	Markus Fehr (Uni Karlsruhe)	Mechanisms of the genotoxic effect of *Alternaria* toxins
11:35 - 11:50	Joachim Orth (Uni Freiburg)	Activation of G-protein chimeras of various heterotrimeric G protein famililies by *Pasteurella multocida* toxin
11:50 - 12:05	Karin Heine (Uni Ulm)	ADP-ribosylation of actin by *Clostridium botulinum* C2 toxin results in apoptotic cell death of mammalian cells
12:05 - 12:20	Jörg Fahrer (Uni Konstanz)	High-affinity complex formation of poly(ADP-ribose) with specific binding proteins as a function of poly(ADP-ribose) chain length
12:20 - 12:35	Susanne Fritsch (FZK)	Incinerator fly ash provokes alterations of redox equilibrium and liberation of arachidonic acid in macrophages
12:35 - 13:35	Lunch	
13.35 - 15.15	Poster Session	
15.15 - 16.00	General Discussion	

Abstracts of the invited presentations

Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo.

Lixin Yang[1], Jules R. Kemadjou[1], Christian Zinsmeister[1], Matthias Bauer[1], Ferenc Müller[1], Michael Pankratz[1], Jens Jäkel[2,3] and Uwe Strähle[1]

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Abstract

Background: Organisms are in constant exchange with their environment. Chemicals trigger specific gene expression programs that protect or cause harmful toxic effects threatening the survival of the organism. In particular, early life stages are highly sensitive to toxic effects. Zebrafish embryos have a high but little explored potential as model for molecular developmental toxicology and toxicogenomics.
Methods and Results: We exposed zebrafish embryos to a range of 11 toxins and measured the changes in gene expression profiles by hybridizing cDNA to an oligonucleotide microarray. We obtained specific expression profiles and could predict the identity of the toxin from the expression profiles with high probability. Changes in gene expression were detected at toxin concentrations that did not cause morphological effects. The toxicogenomic profiles were highly stage-specific suggesting different responses at different life stages. Finally, we detected tissue specific-gene responses underscoring the sensitivity of the assay system.
Conclusion: Our results demonstrate that the genome of the zebrafish embryo can respond to toxin exposure in a highly sensitive and specific manner. Our work provides proof-of-principle for the use of the zebrafish embryo as a toxicogenomic model and highlights its potential for systematic, large-scale analysis of the effects of chemicals on the developing vertebrate embryo.

Interactions of polybrominated diphenyl (PBDE) ethers with AhR signalling

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Abstract

Background: Polybrominated diphenyl ethers (PBDE) are used as brominated flame retardants and accumulate in the environment. Toxic consequences of PBDE exposure are disturbances of the developing nervous system, impact on the endocrine system and tumor induction in rodents. The underlying toxic molecular mechanisms are poorly understood.
Objectives: Induction of the aryl hydrocarbon receptor (AhR) signalling pathway by PBDEs has been controversially discussed. AhR activation was mostly measured by induction of the AhR target gene *cyp1A1*. We focused on further downstream consequences of AhR activation including alterations in global gene expression, nuclear transition, proliferation, cell cycle distribution and developmental toxicity.
Methods: Affymetrix-Microarray analysis was performed in 5L rat hepatoma cells. Validation was carried out using real-time PCR in 5L and in BP8 cells, an AhR-deficient 5L subclone. Western blot analysis was done for some of the detected target genes. Inhibition of proliferation by AhR was measured using BrdU incorporation and FACS assays. Zebrafish embryos were exposed to PBDE samples and investigated for global changes in gene expression and developmental effects.
Results: A low grade purified PBDE sample contained traces of tetrabrominated dibenzofuran (TBDF) and activated AhR signaling in 5L cells, whereas the highly purified PBDE had no agonistic properties. These findings were corroborated in other cell lines and in zebrafish embryos. In addition, highly purified PBDEs appeared to inhibit AhR signalling.
Conclusions: PBDEs do not exert toxicity through activation of the AhR. Furthermore, PBDEs act on other xenosensors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) likely contributing to alterations in metabolism. Identification of novel target genes in rat hepatoma cells and zebrafish embryos provide potential mediators of PBDE toxicity.

Keywords: flame retardants, diphenyl ethers, toxicity, BDE 47, aryl hydrocarbon receptor, tetrabrominated dibenzofuran

Regulation of drug-metabolizing enzymes by β-catenin-dependent signal transduction

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Abstract

Background: Cytochrome P450 (Cyp) isoenzymes constitute the most important group of xenobiotic-metabolizing enzymes in the liver, exhibiting inducible expression regulated by different exo- and endogenous compounds via a set of ligand-activated transcription factors. However, the mechanisms that regulate basal Cyp expression in liver are poorly understood. Recently, overexpression of several Cyp isoforms as well as of nuclear receptors mediating Cyp induction, e.g. aryl hydrocarbon receptor (AhR) and constitutive androstane receptor, in mouse liver tumors with mutations in the *Ctnnb1* (encoding β-catenin) proto-oncogene has been reported (Stahl et al., Hepatology 42, 353-61, 2005).
Objective: To study Cyp induction by β-catenin.
Methods: Primary mouse hepatocytes were treated with inducers of the Wnt/β-catenin signaling pathway, leading to an upregulation of several Cyp mRNAs. To test whether the induction of Cyp1a by β-catenin was caused by an increase in mRNA levels of the AhR, a known target gene of β-catenin signaling, luciferase reporter vectors for human Cyp1a1 were constructed containing either the wild-type promoter sequence or mutated versions lacking the transcription factor binding sites for AhR/Arnt or β-catenin/TCF.
Results: Activation of the β-catenin pathway elevated the inducibility of Cyp1a mRNA by 2,3,7,8-tetrachloro-dibenzo-(p)-dioxin (TCDD). Depletion of β-catenin by siRNA reduced basal Cyp1a expression as well as attenuated induction of Cyp1a by TCDD. Luciferase activity was equally inducible in the wild-type and the AhR binding site-deficient vectors by stimulation of β-catenin signaling. Moreover, both basal and maximum TCDD-inducible luciferase activity was decreased by mutating the β-catenin/TCF binding site in the promoter. In BP8+ rat hepatoma cells with constitutive, non β-catenin-inducible expression of the AhR, activation of β-catenin by LiCl treatment led to an induction of Cyp1a mRNA comparable to that in the AhR wild-type parental cell line 5L.
Conclusions: In conclusion, the data suggest a direct participation of β-catenin in transcriptional regulation of basal and inducible expression of Cyp1a.

Keywords: Cytochrome P 450, Regulation, ß-Catenin, Liver

Investigations on the mutation signature of 3-Nitrobenzanthrone in the human p53 sequence

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Heinz H. Schmeiser
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Abstract

To test hypotheses on the origins of p53 mutations in human tumors, novel strategies are needed for generating mutation spectra experimentally. Recently, an assay employing Hupki (Human p53 knock-in) mouse embryonic fibroblasts (HUFs) has been developed. Utilizing this assay, we examined p53 mutations induced by 3-Nitrobenzanthrone (3-NBA), a carcinogen and environmental pollutant. Several immortalized cultures (cell lines) from more than 100 HUF primary cultures exposed at passage 1 for two to ten days to 2.0 and 7.5µM 3-NBA harbored mutations in the human DNA binding domain sequence of the Hupki p53 tumor suppressor gene or its splice sites. DNA was isolated, amplified and sequenced via the dye-terminator method to reveal manifest point mutations in the coding region of exons 4 – 9. Preliminary results indicate mostly A to G transitions and G to T transversions, some of which are at novel sites not found mutated in our previous experiments testing other mutagens (benzo(a)pyrene, aristolochic acid I, and UV light) nor in cell lines from untreated (control) cultures.
Apart from point mutations in the coding region (e.g. p53), a tumor suppressor can be inactivated through hypermethylation of the promoter region, which silences the gene (e.g. RASSF1A). This often coincides with genome-wide DNA-hypomethylation. Thus, a second focus is on the DNA-methylation status of the HUF cell-lines. To measure the degree of methylation, DNA was hydrolyzed to 3'-nucleotides, derivatized with a fluorescent dye and analyzed through micellary electrokinetic capillary chromatography with laser-induced fluorescence detection (CE-LIF). The degree of DNA-methylation in HUF cell lines that were mutant in p53 was significantly lower (3.8%) than that of primary cultures (4.0%, $p<0.001$), while that of cell lines wild-type in p53 was even lower (3.6%, $p<0.001$).

Keywords: p53 mutations, Hupki, 3-NBA, DNA-methylation

Mechanisms of the genotoxic effect of *Alternaria* toxins

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Abstract

Contamination of food and feed with toxin-producing fungi represent a major risk for diseases in humans and animals. Exposure to *Alternaria spp.*, especially *Alternaria alternata* has been associated with enhanced incidence of oesophageal cancer. Extracts of *A. alternata* have been described as genotoxic and mutagenic *in vitro*. However, the underlying mechanism of action has not been elucidated so far.
We investigated the genotoxic effect of the *Alternaria* toxins alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT) with emphasis on potential mechanisms involved. AOH and AME significantly increased the rate of DNA strand breaks in human colon carcinoma cells at concentrations ≥ 1 µM and 25 µM, respectively, measured as DNA strand breaks by single cell gel electrophoresis (comet assay). In contrast, ALT did not affect DNA integrity up to 100 µM. The rate of DNA strand breaks induced by AOH and AME were not modulated by formamidopyrimidine-DNA-glycosylase (fpg), thus excluding enhanced oxidative DNA damage.
AOH effectively competed with the minor groove binding ligand Hoechst 33258 with an EC_{50}-value of 8 ± 1 µM indicating substantial affinity to the minor groove of the DNA. A number of DNA minor groove binding ligands affect mammalian topoisomerases I and II. Therefore, we investigated the impact of *Alternaria* toxins on the different classes of topoisomerases. AOH was found to inhibit the catalytic activity of topoisomerase I at concentrations ≥ 50 µM. In contrast AME, bearing a methoxy group at position 9, did not affect the catalytic activity of topoisomerase I up to 100 µM. The catalytic activity of topoisomerase IIα and IIβ was significantly suppressed by AOH at ≥ 25 µM and ≥ 150 µM, respectively. AME did not affect the activity of topoisomerase IIβ up to 200 µM, but was found to be equipotent to AOH with respect to the inhibition of topoisomerase IIα. Thus, topoisomerase IIα was identified as the most sensitive target so far for both, AOH and AME. In accordance with the results in the comet assay, ALT did not affect the activity of topoisomerase I and II.
Furthermore, we investigated the mode of interaction of AOH with the potential target enzymes. AOH was found to stabilise the catalytically generated DNA-topoisomerase intermediate of topoisomerase I and II, thus acting as a so called topoisomerase poison. The stabilisation of the DNA-topoisomerase IIα intermediate was observed in the concentration range leading to enhanced DNA strand breaks in the comet assay.
In summary, AOH and AME were identified as potent inhibitors of topoisomerase IIα, which might at least contribute to the DNA strand breaking properties of these mycotoxins.

Keywords: *Alternaria*, alternariol, alternariol monomethyl ether, topoisomerase

ACTIVATION OF G-PROTEIN CHIMERAS OF VARIOUS HETEROTRIMERIC G PROTEIN FAMILIES BY *PASTEURELLA MULTOCIDA* TOXIN

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Abstract

The mitogenic *Pasteurella multocida* toxin (PMT) is the causative agent of atrophic rhinitis in pigs. PMT is known to activate signaling pathways in cells, which are linked to heterotrimeric G proteins. The toxin is one of the strongest activators of $G\alpha_q$-dependent phospholipase Cβ to induce inositoltrisphosphate production, Ca^{2+} mobilization and formation of diacylglycerol. The toxin also activates the small GTPase RhoA, resulting in formation of stress fibers and focal adhesions. The activation of RhoA depends on $G\alpha_q$ and $G\alpha_{12/13}$. In addition, PMT induces MAP kinase and STAT activation leading to alteration of gene expression.
Using chimeric α-subunits, consisting of parts from PMT-responsive and non-responsive G proteins, we were able to stimulate different effector pathways by PMT. In addition this approach led to the elucidation of the spectrum of PMT-activated G proteins.
Subsequently, several chimeras of $G\alpha_q$ and $G\alpha_{11}$, which is not a substrate of PMT, were constructed and introduced into $G\alpha_{q/11}$-deficient MEF. These studies resulted in the detection of a specific region at the N-terminus of $G\alpha_q$, which was necessary to allow stimulation of PLCβ by PMT.
Activation of $G\alpha_{13}$ by PMT could be studied by reconstitution of $G\alpha_{13}$ in $G\alpha_{12/13}$-deficient MEF. Additionally, chimeras of non-responsive $G\alpha_{11}$, and $G\alpha_{13}$ were activated by PMT and stimulated PLCβ as measured by inositoltrisphosphate accumulation in $G\alpha_{q/11}$-deficient cells. The similar region of $G\alpha_{13}$ and $G\alpha_q$ was important to transfer PMT-induced activation to $G\alpha_{11}$.
Finally, various chimeras of $G\alpha_q$ and $G\alpha_i/G\alpha_s$ were tested for response towards PMT. Chimeras were obtained, which after transfection and expression in HEK cells, mediated PMT effects. Activation of these chimeras by PMT caused alteration of adenylylcyclase activity as measured by cAMP accumulation.
The data indicate that a specific region in G proteins is responsible for activation by PMT and that PMT activates a much broader spectrum of G proteins than believed before.

Keywords: heterotrimeric G protein, bacterial protein toxin, *Pasteurella multocida* toxin

ADP-ribosylation of actin by *Clostridium botulinum* C2 toxin results in apoptotic cell death of mammalian cells

Karin Heine and Holger Barth

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Abstract

The cytoskeleton represents one of the cutting sites of eucaryotic cells. Hence it is not surprising that it is a favoured target of toxins secreted by various pathogenic bacteria. The binary *Clostridium botulinum* C2 toxin directly attacks the actin cytoskeleton of eukaryotic target cells by mono-ADP-ribosylation of G-actin at Arg-177, leading to a complete depolymerization of actin filaments within about 3 h. For the C2 toxin the molecular mode of action and the immediate cytopathic effects are well characterized. However, the final fate of treated cells is not completely understood.
Here we demonstrate that the cytopathic effect of C2 toxin was non-transient and toxin-treated cells stayed round. Once the total amount of actin was ADP-ribosylated, no unmodified actin was detected in such cells, even after 48 h of incubation. Here we figured out that the finale fate of C2 toxin-treated HeLa cells is apoptotic cell death. The ADP-ribosylation of the total amount of cellular actin itself did not correlate simultaneously with the occurrence of apoptotic cells. However, within a lag of about 20 h, apoptosis was observed in about 20 - 50% of the cells using the Annexin/ propidium iodide staining. Apoptosis was also confirmed within this time range applying the Nicoletti method to detect fragmented DNA. For further confirmation we used immunoblot analysis to detect PARP-1 cleavage occurring in presence of activated caspases-3/-7. We saw that the DNA repair enzyme PARP-1 got obviously cleaved 15 h after toxin treatment as well as we could inhibit its caspase dependent cleavage by using a general caspase inhibitor (z-VAD-fmk) for up to 48 h.
The “delayed” cell death induced by C2 toxin was in contrast to the “fast” apoptotic effects observed with *Clostridium difficile* TcdB that became evident after about 3 - 5 h of incubation with the toxin. The large cytotoxins TcdA and TcdB from *Clostridium difficile* mono-glucosylate Rho-GTPases. Rho proteins are master regulators of the actin cytoskeleton, therefore the treatment of cells with TcdB results in its destruction. We state that in the same time-frame, as the Rho-GTPases are entirely glucosylated, apoptotic cells first show up.
Other Rho-inactivating toxins, such as C3 transferase from *Clostridium limosum* or the variant TcdB-1470, caused cell rounding but did not induce any cell death within the same time-frame as mentioned above. This striking distinct behaviour compared to C2 toxin-treated cells clearly demonstrates that the destruction of the actin cytoskeleton accompanied by cell rounding was not sufficient to induce cell death.
Further experiments will be needed to elucidate the link between ADP-ribosylation of actin by bacterial toxins and the induction of apoptotic cell death in mammalian cells in more detail.

Keywords: Binary bacterial toxins, *Clostridium botulinum* C2 toxin, apoptosis, ADP-ribosylation, actin

High-affinity complex formation of poly(ADP-ribose) with specific binding proteins as a function of poly(ADP-ribose) chain length

J. Fahrer[1], R. Kranaster[2], M. Altmeyer[1], A. Marx[2] and A. Bürkle[1]

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Abstract

Background:
Poly(ADP-ribosyl)ation is one of the very early cellular responses to genotoxic insults and is catalyzed by the family of poly(ADP-ribose) polymerases (PARPs). Using NAD^+ as substrate, PARPs synthesize the biopolymer poly(ADP-ribose) (PAR) comprising up to 200 ADP-ribose moieties. PAR was demonstrated to specifically interact with a plethora of proteins implicated in DNA damage checkpoint and repair.
Objectives:
Aim of our study was to characterize the noncovalent interaction between PAR and specific binding partners such as p53 in terms of selectivity and affinity.
Methods:
PARP-1, the xeroderma pigmentosum-A [XPA] protein and p53 were overexpressed in Sf9 insect cells using the baculovirus system and purified to homogeneity. PAR was synthesized *in vitro* and end-labeled using the carbonyl-reactive linker biocytin hydrazide. Following anion exchange HPLC fractionation, the fractions collected were monitored for chain length on modified sequencing gels. Interaction of separated PAR chains and recombinant proteins were analyzed by electrophoretic mobility shift assay [EMSA]. Moreover, real-time surface plasmon resonance (SPR) was used to assess binding kinetics.
Results:
PAR was successfully conjugated with a biotin moiety as revealed by neutravidin ELISA and semidry blots followed by streptavidin-POD detection. HPLC separated polymers were analyzed on modified sequencing gels revealing isolated PAR chains from 5 – 65 ADP-ribose units. EMSA interaction studies of separated PAR chains with p53 and XPA displayed differential binding properties in a chain length-dependent manner. p53 underwent complex formation with long ADP-ribose chains (55-mer) starting at low nM concentrations ($K_{D\text{-}p53}$= $1.31x10^{-7}$M)]. Interestingly, we observed that p53 induces the formation of at least three specific complexes with long PAR. p53 was also able to bind short PAR chains (15mer, $K_{D\text{-}p53}$= $2.46x10^{-7}$M), yet forming only one defined complex. By contrast, XPA did not specifically interact with short polymer, but produced a complex with long ADP-ribose chains (55mer) ($K_{D\text{-}XPA}$= $3.21x10^{-7}$M). SPR analysis corroborated the EMSA results showing no interaction of XPA with immobilized short PAR chains (14mer), but strong binding to long PAR chains (63mer). Moreover, p53 binding was observed for both short and long PAR chains and supported the finding that three different p53-PAR complexes are formed with long ADP-ribose chains.
Discussion:
Specific end-labeling of PAR chains provided a novel tool to study the interaction of PAR and proteins in solution as well as in solid phase. We demonstrated for the first time that PAR-protein interactions exhibit extraordinary high affinities in the nM range and displayed an as yet unknown selectivity. These findings emphasize the physiological importance of PAR and provide a clue of how PARPs might orchestrate cellular processes such as DNA repair by controlled PAR synthesis of varying complexity.

Keywords: Poly(ADP-ribose) – affinity studies – DNA repair – tumor suppressor p53

Incinerator fly ash provokes alterations of redox equilibrium and liberation of arachidonic acid in macrophages

Susanne Fritsch, Harald F. Krug, C. Weiss and Silvia Diabaté

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Abstract

Background:
Numerous epidemiological studies associated exposure to elevated environmental fine particulate matter with mortality rates as well as adverse health effects, in particular in susceptible individuals or humans with pre-existing pulmonary or cardiovascular diseases. However, underlying mechanisms relevant for toxicity are not completely understood. Especially the relation of oxidative stress and inflammatory responses, including lipid signalling, need to be elucidated in more detail.
Objectives:
The aim of the present study was to examine the effect of incinerator fly ash (IFA) as a model of environmental particulate matter on the formation of reactive oxygen species (ROS) and their ability to induce oxidative stress in RAW264.7 macrophages. Furthermore, the liberation of arachidonic acid (AA) and its metabolites was investigated.
Methods:
Free AA and its metabolites were detected by labelling with [^{14}C]-AA acid prior to the incubation with particles, subsequent lipid extraction and separation by thin layer chromatography; Western Blot analysis; Detection of lipid mediators by enzyme immunoassays. ROS were assessed by DCF fluorescence, and cellular content of glutathione and glutathione disulfide was quantified according to the enzymatic cycling method.
Results:
The interaction of IFA with macrophages increases free AA, COX-2 protein as well as prostaglandin E_2 levels. AA liberation depends on an increase of the intracellular calcium concentration. Additionally, AA mobilisation was blocked selectively by an ERK1/2 pathway-specific inhibitor, while inhibition of p38 MAPK (mitogen activated protein kinase) had no effect. Furthermore, IFA induces oxidative stress, indicated by the formation of ROS, changes in intracellular glutathione content and induction of the antioxidative protein hemeoxygenase-1. Interestingly, ERK1/2 phosphorylation and mobilisation of AA are linked to the generation of ROS, since pre-treatment with N-acetyl-cysteine (NAC) blocked both the ERK1/2 activation and the level of free AA induced by IFA.
Conclusions:
IFA induced ROS seems to be the primary event, which triggers ERK activation and subsequent AA mobilisation in macrophages.
Keywords: arachidonic acid; incinerator fly ash; macrophages; oxidative stress; particulate matter; reactive oxygen species

Poster abstracts

1.) Mutations and DNA adducts induced by *N*-hydroxy-3-aminobenzanthrone in the human p53 gene using the Hupki mouse model

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Heinz H. Schmeiser
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Abstract

To test hypotheses on the origins of p53 mutations in human tumors, novel strategies are needed to generate mutation spectra experimentally. Recently, an assay employing Hupki (Human p53 knock-in) mouse embryonic fibroblasts (HUFs) has been developed.
Utilizing this assay, we examined p53 mutations induced by *N*-hydroxy-3-aminobenzanthrone (*N*-OH-3-ABA) an active metabolite of 3-nitrobenzanthrone, a carcinogen and environmental pollutant. HUFs were exposed at passage 1 to 2 µM *N*-OH-3-ABA for 6 days. DNA was isolated, amplified and sequenced via the dye-terminator method to reveal manifest point mutations in the coding region of exons 4 – 9. To measure the degree of DNA adducts DNA was isolated, hydrolyzed to 3'-nucleotides and analysed with the ^{32}P-postlabeling method. Cell viability was determined in *N*-OH-3-ABA treated HUFs by a cell couting device (CASY Cell Counter TTC, Schärfe Systems, Reutlingen).
Cell viability was 70% at 2 µM *N*-OH-3-ABA after 5 days exposure. *N*-OH-3-ABA formed 3-NBA specific DNA adducts at a level of 1 adduct/ 10^7 normal nucleotides. All 36 primary HUF cultures were immortalised after treatment. Mutation spectra in the human p53 gene in exons 4-9 will be presented.

Keywords: p53 mutations, Hupki, *N*-OH-3-ABA, 3-NBA, DNA-adducts

2.) Title: Uptake Mechanisms and Toxicological Aspects of Synthetic Nanoparticles in Human Cells

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Abstract

Background: Synthetic nanoparticles show a broad range of usage in science, technology and medicine. Hitherto for most of the new nanomaterials little is known about cellular uptake mechanisms and biological effects, such as genotoxicity. Therefore, information about safety and potential hazard of nanoparticles is needed. The entry of particles into living organisms presupposes their uptake mainly by epithelial cells or phagocytes. This process is strongly dependent on the size and the surface characteristics of the nanoparticles. In any case, nanoparticles have various possibilities to find their way across cell membranes. Once internalized, they may affect the integrity of the cell in different ways. The induction of oxidative stress plays a major role and is suggested to be able to lead e.g. to DNA damage via oxidation of bases.
Objectives: Investigation of cellular uptake, acute toxicity and genotoxicity of synthetic nanoparticles in human cells
Methods: In our *in vitro* studies, we investigated the uptake and toxicological aspects of synthetic nanoparticles (silica, vanadium oxide and titanium dioxide) in the human lung epithelial cell line A549. Acute toxicity was determined using the MTT cell viability assay. The comet assay was used to assess possible genotoxicity caused by DNA strand breaks.
Results: For silica and titanium dioxide nanoparticles, no acute toxic effect could be measured. SiO_2 particles were taken up into the cells in a dose and time-dependent manner. These particles were localized in the cytoplasm, partly in agglomerates. In contrast to SiO_2 and TiO_2, vanadium oxide particles exhibited strong toxicity. Nanosized particles displayed a higher toxicity than the corresponding bulk material. Comet assay experiments revealed an increased DNA damage after 36 h in cells treated with nanoscaled V_2O_3, but not with nanoscaled V_2O_5.
Conclusions: Exposure of human cells to synthetic nanoparticles may result in uptake of the particles and induction of biological effects (acute toxicity, genotoxicity,…). Our results indicate that these processes may depend on the size and surface characteristics of the nanoparticles.

Keywords: synthetic nanoparticles, cellular uptake, acute toxicity, genotoxicity

3.) MECHANISMS AND CONSEQUENCES OF GENOTOXIN INDUCED ACTIVATION OF STRESS-ACTIVATED-PROTEIN-KINASES IN MAMMALIAN CELLS

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Abstract

Background: The polycyclic aromatic hydrocarbon and environmental pollutant benzo[a]pyrene (B[a]P), a constituent and contaminant of cigarette smoke, automobile exhaust, industrial waste and even food products, is carcinogenic to rodents and humans. B[a]P binds to the intracellular aryl hydrocarbon receptor thereby inducing its own metabolism by cytochrome P450s leading to the formation of a highly reactive electrophilic compound, the ultimal carcinogen B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE).
Objectives: Our studies address the mechanisms by which BPDE leads to activation of stress-activated-protein kinases (SAPKs) and aim at the identification of the primary target(s) for BPDE induced signaling.
Methods: Western blot, survival assays, RNAi, Comet assay
Results: Within minutes of exposure we find rapid and strong phosphorylation of both SAPK family members JNKs and p38s in murine and human cells. The upstream kinases MKK3/6 and SEK1 are activated after BPDE exposure. By the use of specific inhibitors of signaling mediators upstream of JNKs and p38 we could demonstrate a role of c-src like kinases in SAPK-activation by BPDE. By depleting individual family members of c-src like kinases by the use of siRNAs, c-lyn kinase could be identified contributing to SAPK activation by BPDE**.** Survival assays with p38 wt and $p38^{-/-}$ murine fibroblasts showed an impaired cell growth after BPDE treatment in p38 deficient cells.
Conclusions: BPDE induced phosphorylation of p38 and JNK depends on activation of src-like kinases based on inhibitor and RNAi knock-down studies. Activation of p38 is necessary for the cellular survival after BPDE treatment.

Keywords: BPDE, JNK, p38, PP-1, siRNA, src-like kinases

4.) Autocatalytic processing of the *Escherichia coli* Cytotoxic Necrotizing Factor 1 (CNF1)

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Abstract

The Cytotoxic Necrotizing Factor 1 (CNF1) is produced by pathogenic *Escherichia coli* strains, causing alteration of the host cell actin cytoskeleton and thereby promoting bacterial invasion of blood-brain barrier endothelial cells. Neonatal meningitis and urinary tract infections are the consequence of this invasion. CNF1 belongs to a family of bacterial toxins that target small GTPases like Rho, Rac and Cdc42 by deamidating the GTPase at position E63/61. Other highly related toxins of this family are CNF2, also produced from *E. coli* and CNFy from *Yersinia pseudotuberculosis* strains. All CNFs are 115 kDa single chain AB toxins with an N-terminal receptor binding and a C-terminal enzymatic domain. Both domains are connected by a putative translocation domain with two highly hydrophobic helices involved in membrane translocation. It is generally accepted, that CNF1 enters the host cell through receptor mediated endocytosis. After binding of the toxin to its so far unknown receptor –it is discussed that laminin is involved- the toxin is endocytosed in a clathrin independent manner. Acidification in the late endosomes leads to a conformational change of the toxin, promoting an insertion of the translocation domain into the membrane. It was speculated that the toxin enters the cytosol as a holotoxin. Using a monoclonal antibody with a recognition site in the catalytic domain of CNF1, we detect a 55-60 kDa fragment in the cytosolic fraction of intoxicated cells. Here we show that the processing of CNF1 is autocatalytic and dependent on an intrinsic serine protease activity.

Keywords: CNF1, CNFy, bacterial toxin, small GTPases, *Escherichia coli* toxin, *Yersinia pseudotuberculosis* toxin

5.) Alteration of steroidogenesis in H295R cells after exposure to sediments of the Danube river

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Abstract

Despite an improvement of water quality, an obvious decline in fish populations in the upper Danube river has been observed since the early 90th century. Over the past three years a series of scientific studies has been conducted to research this issue. Within the scope of these studies, an increased ecotoxicological effectiveness of sediments of different locations along the Danube river within both acute and mechanism-specific bioassays has been detected.
While there has been increasing awareness of endocrine active chemicals to interact with the reproduction of wild fish populations, to date there have been no efforts to investigate the potential role of particle-bound endocrine disrupting substances in the upper Danube river. To assess the non-receptor mediated endocrine disrupting potential of sediment samples of the Danube river, two novel bioassays using the human H295R cell line have been applied. The effects on the synthesis of steroid hormones and the expression of genes of the major steroidogenic enzymes were investigated using quantitative ELISA and RT-PCR techniques, respectively. The most significant change in gene expression was a 10-fold up-regulation of the CYP11B2 gene. Furthermore, an induction of the 3βHSD2 and CYP19 genes was observed. In addition, the sediment extracts of three locations caused an induction of the analysed steroid hormones.
The results of this study indicate an endocrine disrupting potential of sediment samples collected at certain locations along the Danube river. It is still unclear, however, what the relevance of these findings for the observed decline of fish populations is.

Key words: Fish decline, H295R, steroidogenesis, endocrine disruption

6.) Teratogenetic and genotoxic evaluation of several perfluorinated chemicals (PFCs)

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Abstract

Perfluorinated chemicals (PFCs) are fully fluorinated organic compounds which have been manufactured for decades and are widespread in industrial and commercial products. The current knowledge regarding the potential effects of PFCs towards biological systems is highly insufficient. This lack of investigations is alarming since PFCs have been reported to be widely distributed on a global basis with detectable concentrations in both humans and wildlife. What further emphasizes the need for additional knowledge is the recent documentation that perfluorooctane sulfonate (PFOS) can enhance the toxicity of other compounds by increasing the permeability of cell membranes. In this study the cytotoxic, genotoxic and embryotoxic potential of PFOS and three fluorotelomer alcohols (FTOHs) were investigated using cell bioassays (V79) and the zebrafish (*Danio rerio*) embryo assay. In addition, PFOS was combined with the positive control of each testsystem in order to identify any enhanced toxicity caused by interactive effects. Of the PFCs tested only PFOS showed cytotoxic potential towards V79 cells. PFOS combined with 3,4-dichlorophenol did not induce an increased cytotoxicity. In the micronucleus test, PFOS did not cause an induction of micronucleated V79 cells, but increased the genotoxic potential of the positive control
3,4-cyclophosphamide with metabolic activation. Only PFOS and perfluoro-1-octanol caused lethal toxicity towards zebrafish embryos (EC_{50} = 96.6 mg/L (93.9-99.2) and 291.5 mg/L (262-321) respectively) whereas all PFCs tested induced sublethal effects within the investigated concentration range. PFOS combined with 3,4-dichloroanilin caused a twofold increased mortality rate in zebrafish embryos, indicative of an interactive effect.

7.) Dioxin like activity of sediments from the Danube River using three different cell lines and chemical analysis

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Abstract

This investigation is a consequence of a distinct fish decline in the upper Danube River since the beginning of the 1990s. In contrast to the decline of fish population, former studies have repeatedly documented that the water quality along the Danube River is clearly improved. However, the conclusion of a first investigation in 2002 – using in vitro-biotest systems – was that a high ecotoxicological hazard potential is associated with local sediments.
In comparison to the highly polluted rivers Rhine and Neckar in Germany, the present study documented that sediment samples from the upper Danube River showed very high AhR-mediated activity in the used biotests (GPC.2D, DR-CALUX and EROD assay). The combination of fractionation techniques and different in-vitro tests revealed that the main induction could not be explained by priority pollutants, even though the concentrations of priority PAHs were very high. In conclusion, this investigation shows that the high induction rates are mainly mediated by non-priority PAHs. Due to the effects of PAHs towards fish and the well known connection between dioxin-like activity and carcinogenicity, the link between contamination and the fish decline cannot be ruled out. Hence, there is an urgent need to broaden the toxicological and environmental knowledge regarding to non-priority compounds, as a basis for risk assessment.

8.) A microarray approach to investigate complex pollution of river sediments with zebra fish embryos

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Abstract

For the ecotoxicological assessment of complex environmental samples, a vast majority of potentially toxic pollutants needs to be considered. It is possible to simultaneously determine the total hazard of all substances using bioassay approaches; however, toxic effects can frequently not be attributed to chemically analysed priority pollutants.
There is a controversial debate if application of microarrays will be able to contribute to the assessment of hazard potential and to the identification of pollutants in complex samples. Whereas recently published studies document the general suitability of microarray techniques for the characterization of chemicals, little is know about assessing of complex environmental samples, especially sediment.
Hence, a microarray labelled with 10000 genes, developed and applied at the Karlsruhe Research Center for the investigation of chemicals was used to clarify whether gene expression profiles generally suit for sediment extracts. Accordingly, the present study aimed at developing zebrafish-based microarrays for (1) the assessment of biological effectiveness of sediment samples (biomarker approach), (2) the elucidation of toxicity-related mechanisms (metabolism) and (3) the identification of pollutant (classes) responsible for the toxic effects.
As a results, the changes in gene expression provided sensitive biomarkers of exposure to reveal the acute stress situation of embryos exposed to sediment (extracts). Many of the regulated genes, however, showed an unspecific or unknown function. Gene expression analysis could clearly differentiate between exposure to extract, sediment or controls and can possibly be used to determine classes of toxicants that contribute to effects if compared to expression patterns of single substances. However, it is hardly possible to identify single substances via specific expression patterns. Furthermore, for the extract experiments it was not possible to distinguish between the sample sites only based on expression pattern even though fold-changes correlated with biological effects.
This may be attributed to different test concentrations (LOEC) used and that no dose-response relationships were investigated.

Keywords: Microarray, zebrafish embryos, sediment, expression analysis

9.) Development of a flow-through system for the fish embryo toxicity assay

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Abstract

The fish acute toxicity test is still a mandatory component in chemical toxicity testing. Within the framework of the new European Chemicals Policy (REACH), animal testing should – whenever possible- be reduced or even replaced by alternative methods (3Rs). To achieve this objective, the fish embryo toxicity test has been developed to replace the acute fish toxicity test and is mandatory for effluent testing in Germany using a DIN guideline. Based on this protocol, a proposal for a new guideline on fish embryo toxicity for chemicals testing has been submitted to the OECD by the German Federal Environment Agency. However, further optimization is necessary to improve the correlation between the fish and the fish embryo tests.

For this purpose, a flow-through system for the fish embryo toxicity test is developed within the project introduced here, it is shown that it is technically feasible to modify commercially available polystyrene 24-well-plates to enable a 48h fish embryo test under continuous flow of test substance solutions. Within this project, substances with significantly diverging results in the conventional fish test compared to the static fish embryo test have been identified. First results using such test substances in our flow through system will be discussed. Moreover, the project will attempt at elucidating the reasons for these discrepancies, and further modifications to the protocol will be implemented.

Keywords: REACH, OECD, fish embryo toxicity test, flow-through system

10.) Expanded possibilities: Optimization and new applications for Membrane Dialysis Extraction (MDE)

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Abstract

Background: Membrane Dialysis Extraction (MDE) is a recently developed technique for organic soil and sediment extraction as worst case scenario. The procedure minimizes the risk of degradation of thermally labile substances and excludes organic macromolecules due to size restriction. Nevertheless, extraction efficiency had been shown to be comparable to conventional exhaustive Soxhlet extraction.
Objectives: MDE currently is being optimized in terms of time and material consumption, and further possible applications are investigated.
Methods and Results: Pre-extraction of the membranes was optimized by combining distillation and Soxhlet extraction into a multiflux cold extraction system (Preextraction Soxhlet Cold Technology, PRESCOT). This facility allows membrane cleaning within 24 h while reducing solvent consumption to less than a third compared to the commonly deployed hexane bath over 1 week.
Based on promising results from preliminary experiments, MDE was performed with native sediments. In order to investigate the extraction efficiency of MDE using native sediments, comparative testing of extracts of native and freeze-dried samples was carried out using bioassays and chemical analysis. Results for the Neutral Red retention assay using RTL-W1 cells and the Fish egg test with Danio rerio showed similar effects of both extracts, indicating comparable extraction stringency.
Conclusions: The extraction of native sediments with the initiation of the process on site avoids possible alteration of the samples by transportation, storage and drying processes. Thus, resulting extracts will provide the most realistic insight into the toxic potential of sediment samples.
With the PRESCOT facility, membrane cleaning becomes significantly less time and solvent consuming which reduces expenses and increases the procedure's efficiency.

Keywords: Membrane dialysis extraction, sediment toxicity, native sediment samples, biotests

11.) Impact of apple polyphenols on key elements of the Wnt-pathway

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Abstract

Background: Glycogen synthase kinase-3β (GSK3β) is one of the key elements of the Wnt pathway, involved in the regulation of β-catenin homeostasis. The inhibition of GSK3β kinase activity might lead to the onset of β-catenin/TCF/LEF-mediated gene transcription, representing a potentially mitogenic stimulus. Recent studies showed that apple polyphenols possess several biological properties which might be of interest with respect to the prevention especially of colorectal cancer.
Objective of the study was to elucidate whether apple polyphenols affect key elements of the Wnt-pathway, which might limit their usefulness in the prevention of colon carcinogenesis.
Methods: GSK3β kinase assay; Western Blot analysis with antibodies against GSK3β kinase, phosphorylated β-catenin and β-catenin.
Results: The effect of a polyphenol-rich apple juice extract (AE02) on immunoprecipitated GSK3β from HT29 cells. AE02 was found to effectively inhibit isolated GSK3β as well as intracellular GSK3β kinase activity in HT29 human colon carcinoma cells. The inhibition of enzyme activity occurs at polyphenol concentrations corresponding to the concentration of these compounds in the original apple juice, which represented a consumer-relevant apple juice blend. In accordance with the inhibition of GSK3β kinase activity by AE02, treatment of HT29 cells resulted in a significant decrease of phosphorylated β-catenin. However, unexpectedly, also the total intracellular β-catenin level was found to be diminished, indicating that the interference of the apple constituents with GSK3β was not associated with a stabilization of β-catenin in HT29 cells.
Conclusion: The results let assume that at consumer-relevant concentrations apple polyphenols do not mediate growth stimulating effects in HT29 cells via the Wnt-pathway.

Keywords: apple juice extract, phloretin, phloridzin, quercetin, glycogen synthase kinase-3β, β-catenin, HT29

12.) ROLE OF THE ESTROGEN RECEPTOR IN THE DIETHYLSTILBESTROL-INDUCED DISRUPTION OF THE EXPRESSION OF WNT5A AND WNT7A IN HUMAN ENDOMETRIAL (ISHIKAWA) CELLS

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Abstract

Genes of the WNT family are involved in signal transduction cascades important for development and carcinogenesis. WNT5A is supposed to be an upstream regulator of WNT7A expression in a signaling pathway that transmits information among stromal and epithelial cells of the endometrium. Disruption of this pathway is associated with morphological abnormalities of the female reproductive tract, and mice treated *neonatally* or *in utero* with the trans-placental acting carcinogen diethylstilbestrol (DES) show the same abnormalities, whereas estrogen receptor (ER)alpha knockout mice are insensitive towards DES.
The aim of the present study was to determine the influence of the synthetic estrogen DES on the mRNA expression of WNT5A and WNT7A in a human endometrial adenocarcinoma cell line (Ishikawa cells) by reverse transcription/competitive PCR. The role of the ER was examined by (i) the determination of the mRNA expression and the enzyme activity of the alkaline phosphatase (ALP), which is regulated by the ER in Ishikawa cells, (ii) the use of the ER antagonist ICI 182,780 (ICI), and (iii) silencing of the ERalpha by RNA interference using siRNA.
After treatment of Ishikawa cells with DES at concentrations that stimulated the expression of the ALP for 6-48 h, a significant decrease in the mRNA levels of WNT7A was observed reaching maximum reduction after 48 h. DES-induced reduction of WNT7A mRNA levels was prevented by the ER antagonist ICI as well as by knock down of ERalpha by RNA interference. After treatment with DES for 48 h and longer, mRNA levels of WNT5A correlated with those of WNT7A; however, there were pronounced differences after treatment periods of 24 h and less. In conclusion, when challenged with DES, the Ishikawa cell line reacts similar to the endometrial epithelium of mice exposed to DES *in utero* or *neonatally*, thus providing a powerful tool for the investigation of endocrine disrupting of the female reproductive tract.

13.) PHYTOESTROGENS MODULATE THE EXPRESSION OF 17BETA-ESTRADIOL-METABOLIZING ENZYMES IN CULTURED MCF-7 CELLS

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Abstract

The activation of 17beta-estradiol (E2) to 2-hydroxyestradiol (2-HO-E2) and the more genotoxic 4-hydroxyestradiol (4-HO-E2) and oxidation to the respective quinones constitutes a risk factor in hormonal carcinogenesis. 2-HO-E2 is formed by cytochrome P450 (CYP)1A1, and 4-HO-E2 is formed by CYP1B1. Both are detoxified by catechol-O-methyltransferase (COMT), whereas their quinones are inactivated by NADPH-quinone-oxidoreductase (QR). Since the soy isoflavones genistein (GEN) und daidzein (DAI) are widely consumed due to their putative protective function in breast carcinogenesis, we examined the influence of E2, GEN und DAI on CYP1A1/1B1, COMT, and QR expression in MCF-7 cells by reverse transcription/competitive PCR. CYP1A1 and COMT enzyme activity were determined using ethoxyresorufin and quercetin as substrates. Furthermore, estrogen receptor (ER)-regulated cell proliferation was determined by E-screen. E2, GEN, and DAI inhibited the expression of CYP1A1, COMT, and QR. The maximum effect (reduction by about 60%) was obtained with 100 pM E2, 1 microM GEN, and 10 microM DAI which also induced the most pronounced cell proliferation in the E-screen. In contrast, expression of CYP1B1 was only slightly affected. CYP1A1 and COMT mRNA levels correlated with enzyme activities. The ER antagonist ICI182,780 blocked the effects of E2 and the isoflavones. Thus, GEN and DAI at estrogen-active concentrations stimulate the formation of the more genotoxic metabolites of E2 and inhibit the detoxification of catechol and quinone estrogens in estrogen-responsive tumor cells.

14.) Alternaria toxins: DNA strand-breaking activity in mammalian cells *in vitro*

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Abstract

Fungi of the genus *Alternaria* infest numerous food items but also grow on other materials, e.g. soil, wall papers and textiles. *Alternaria alternata* is the most abundant of more than forty *Alternaria* species and produces several toxins including alternariol (AOH) and alternariol methyl ether (AME). These *Alternaria* toxins are frequently detected in moldy wheat and other grains, in pecans, in various fruits, e.g. tomatoes, olives, melons, apples and raspberries, and in processed fruit products such as apple and tomato juice. Consumption of food contaminated with *Alternaria* toxins has been associated with an increased incidence of esophageal cancer, and there are several reports on the mutagenicity and genotoxicity of AOH and AME. In most assays, AOH appears to exhibit a more pronounced genotoxicity than AME.

In the present study, the DNA strand-breaking activity of the two Alternaria mycotoxins has been compared in three cell lines with different metabolic capabilities, i.e. Chinese hamster V79 and human HepG2 and HT-29 from liver and intestine, respectively. DNA single strand breaks induced by the test compounds were determined by using the technique of alkaline unwinding.

AOH und AME were found to induce strand breaks in a concentration-dependent manner in all three cell lines. The incidence was about equal for AOH and AME in V79 cells which do not metabolize the mycotoxins, and exceeded the incidence observed in HepG2 and HT-29 cells. The human cells were not capable of oxidative metabolism but exhibited UDP-glucuronosyltransferase (UGT) activity. AOH was more extensively glucuronidated than AME in HepG2 cells, resulting in a higher incidence of strand breaks upon treatment of these cells with AME. HT-29 cells were much more efficient than HepG2 cells in glucuronidating AOH and AME. For example, 24 h after incubating HepG2 cells to AOH or AME, about 50% of the dose was still present as unconjugated compound, but the rate of strand breaks was similar to a 1 h incubation. In contrast, AOH and AME were completely glucuronidated in HT-29 cells after 24 h, and strand breaks were no longer detectable at this time point. Our results suggest that glucuronidation of AOH and AME suppresses the genotoxic activity of these mycotoxins. In support of this assumption is the observation that curcumin, a known inhibitor of UGT activity, increased the incidence of AOH- and AME-induced strand breaks in HT-29 cells.

This study was supported by the State of Baden-Württemberg (Research Program “Mycotoxins” as part of the Research Initiative “Food and Health”).

15.) Methylation of catechol estrogens in human cancer cells

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Abstract

The endogenous steroid hormone 17ß-estradiol (E2) plays a major role in the etiology of breast and endometrial cancer. For the tissue and plasma levels of E2, its phase I and phase II metabolism are very important. Major phase I metabolites are estrone (E1) and the four catechol estrogens 2-HO-E2/-E1 and 4-HO-E2/-E1. These catechols undergo metabolic redox-cycling to generate reactive oxygen species and semiquinone/quinone intermediates which may damage DNA. 4-HO-E2 is a carcinogen, whereas the methylation product of 2-HO-E2, i.e. 2-MeO-E2, is believed to protect against cancer. Methylation and glucuronidation are part of phase II metabolism, but only glucuronidation leads to the complete inactivation and excretion of E2 and its metabolites.

The aim of the present study was to elucidate the methylation of the catechol estro-gens in HepG2 liver cancer cells, which show active UDP-glucuronosyltransferases, and to compare these results with earlier studies in MCF-7 breast cancer cells. HepG2 cells were incubated with each of the four catechol estrogens, the media was extracted with ethyl acetate and the metabolites were analyzed by HPLC. Whereas 2-HO-E2/-E1 gave rise to two methylation products each, only one product was detected with 4-HO-E2 and 4-HO-E1. The formation of the products was observed for 24 h. Moreover, coincubations of the different catechol estrogens were conducted in order to determine the mutual influence on their methylation.
The results of this study are consistent with those obtained in MCF-7 cells, indicating that 2-HO-E2/-E1 but not the carcinogenic 4-HO-E2/-E1 are good substrates of COMT. Therefore, 2-HO-E2/-E1 are first methylated and their methylation products then eliminated by glucuronidation. For 4-HO-E2/-E1, glucuronidation appears to be more important for inactivation than methylation.

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16.) Accumulation of curcumin in membranous structures of human Ishikawa endometrial cancer cells

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Abstract

Curcuminoids are constituents of the rhizomes of the Asian plant *Curcuma longa,* which are commonly called turmeric. The yellow pigment from turmeric is used as a spice and consists mostly of curcumin with lower amounts of mono-demethoxy-curcumin and bis-demethoxy-curcumin. Curcuminoids have been reported to exhibit anti-oxidative, anti-inflammatory and anti-carcinogenic properties.
The aim of the present study was to characterize the accumulation of curcumin in membraneous structures of human Ishikawa cells, a cell line derived from a human endometrial carcinoma. Cells were incubated with cell culture media in the presence or absence of fetal calf serum (FCS) for various time points (0-30 h), and incubation media was collected and cells were harvested. After ultracentrifugation, cells were divided into three fractions: cell debris, microsomes and cytosol. The incubation media were extracted with ethyl acetate and the cell fractions with Folch reagent, and analyzed by HPLC.
Upon incubation, curcumin accumulated in the cells in a time-dependent manner, showing that it passed the plasma membrane very rapidly. The accumulation was even faster in the absence of FCS, implying that curcumin is able to bind strongly to FCS.
The major portion of curcumin was found in cell debris, a smaller part in microsomes and little in cytosolic fractions. In cytoplasm, a large quantity of metabolites (hexa-hydro- and octa-hydro-curcumin) was detected instead of curcumin. In cell culture media, curcumin decreased whereas metabolites increased. In comparison with curcumin, plasma membrane permeability of hexa-hydro-curcumin was reduced.
In summary, this study has shown that curcumin penetrates human Ishikawa cell plasma membranes very rapidly and binds to the endoplasmic reticulum. Metabolites of curcumiin were found in the cytosolic fraction. In contrast, after incubation with hexa-hydro-curcumin, only small amounts were found in the cells.

17.) Lack of anti-oxidative effectiveness of delphinidin in cell culture

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Abstract

Background: Several epidemiological studies indicate that flavonoids in fruits and vegetables might contribute to a reduction of the risk to develop life-style related diseases, such as cancer or artherosclerosis. Anthocyanins, one group of flavonoids, are a widespread source of naturally occuring food colorants. The glycosides of delphinidin are among the most frequent anthocyanins in fruits and vegetables. During the past decade, the intake of anthocyanins was implicated with antioxidative activity. In numerous studies, the scavenging of reactive oxygen species by anthocyanins and especially their respective aglycones has been reported in different cell-free systems.
Objectives: In the present study we addressed the question whether the antioxidative properties of delphinidin are also of relevance within intact cells. In previous studies we showed that in the comet assay delphinidin induces DNA strand breaks in HT29 cells after 1 h of incubation in concentrations ≥ 50 µM [1].
Methods: With a modified comet assay, using the bacterial repair enzyme formamidopyrimidine glycosylase (fpg) we determined the proportion of oxidative-mediated DNA modifications. In order to determine probable antioxidative properties of delphinidin within HT29 cells, a two step incubation protocol was applied. HT29 cells were pretreated with delphinidin under serum-free conditions for one hour and then serially incubated with either menadion (10 µM) or t-butyl hydroperoxide (tBHP, 50 µM) to induce oxidative stress.
Results: Incubation of HT29 cells with delphinidin for one hour resulted in a significant increase of fpg-sensitive sites in a concentration of 100 µM, indicative for prooxidative effects. In contrast to results in cell-free systems, delphinidin failed to suppress the oxidative damage by both, menadion as well as t-butylhydroperoxide. Moreover, the serial application of 50 µM delphinidin followed by a respective oxidative stressor (menadion or tBHP) resulted in substantially enhanced oxidative DNA damage, corresponding to the sum of the damage implicated by incubation with the single compounds. When delphinidin and menadion were applied in parallel even an overadditive increase of oxidative DNA damage was observed.
Conclusions: In summary, delphinidin failed to mediate antioxidative effects in HT29 cells, but even induced oxidative DNA damage itself in concentrations ≥ 50 µM. These results raise the question towards the usefulness of anthocyanins as potential antioxidants in vivo.

[1] Habermeyer, M.; Fritz, J.; Barthelmes, H.U.; Christensen, M.O.; Larsen, M.K.; Boege, F. and Marko, D. (2005): Chem. Res. Tox., 18(9):1395-1404

Keywords: anthocyanins, antioxidant, comet assay, delphinidin, DNA damage, DNA strand breaks, HT29 cells

18.) Suppression of autophosphorylation of the EGFR by different ellagitannins

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Abstract

Background: Ellagitannins are hydrolyzable tannins which belong to the class of plant polyphenols. They have been shown to mediate antiproliferative, apoptotic and antioxidant effects, but little is known about the underlying mechanisms.
Objectives: In the present study we investigated the effects of ellagitannins on cell growth in the human colon carcinoma cell line HT29. The protein tyrosine kinase activity of the isolated epidermal growth factor receptor (EGFR) preparation was effectively blocked by ellagitannins at nanomolar concentrations. To address the question whether the interference with the activity of the isolated EGFR also plays a role within intact cells, effects on the phosphorylation status of the EGFR, as a measure for its activity, were determined in the human vulva carcinoma cell line A431, in which the EGFR is overexpressed. Inhibition of the upstream located EGFR is expected to suppress the activation of the subsequent mitogen-activated protein kinase (MAPK) cascade, leading to the inhibition of cell growth.
Methods: The effects on cell growth were determined using the sulforhodamine B assay. Inhibition of the isolated EGFR was performed using a protein tyrosine kinase assay. Modulation of the autophosphorylation of the EGFR in intact cells was determined by Western Blot analysis.
Results: Our results demonstrated that the inhibition of the EGFR activity is not limited to the cell-free system, but is also observed in intact cells at micromolar concentrations.
Conclusions: Ellagitannins appear to possess bioactive properties, which affect cellular signaling cascades regulating cell growth.

Keywords: EGFR, ellagitannins

19.) Zinc supplementation affects the cellular poly(ADP-ribosyl)ation capacity

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Abstract

Background: Poly(ADP-ribosyl)ation is a reversible posttranslational modification of nuclear proteins and represents an early cellular response to DNA damage generated by endogenous and exogenous damaging agents in mammalian cells. The synthesis of poly(ADP-ribose) chains covalently attached to target proteins is catalysed by poly(ADP-ribose)polymerases (PARPs), using NAD^+ as substrate. Poly(ADP-ribosyl)ation is involved in several cellular processes including DNA repair and maintenance of genomic stability. Poly(ADP-ribosyl)ation is mostly catalyzed by PARP-1, an abundant nuclear enzyme that binds via its zinc finger motifs to DNA with single or double strand breaks. Zinc binding has shown to be essential for PARP-1 activation. A decrease in cellular poly(ADP-ribosyl)ation has been implicated in the ageing process. Its decreased function might be related to age-related zinc deficiency.

Objectives: Our aim was to determine cellular poly(ADP-ribosyl)ation capacity as function of age and zinc status in humans.

Methods: Healthy old subjects from various European countries were recruited for the ZINCAGE project and supplemented orally with 10mg zinc per day for 7 weeks. Blood was taken before and after zinc supplementation and the plasma zinc level was determined with a Thermo XII Series ICP-MS. We assessed cellular poly(ADP-ribosyl)ation capacity of peripheral blood mononuclear cells (PBMC) by using a recently established flow cytometry-based assay (Kunzmann et al., Immun Ageing 2006;3:8) as function of age and nutritional zinc status.

Results: Our results reveal a positive correlation between cellular poly(ADP-ribosyl)ation capacity and zinc status in human PBMC ($p<0.05$). We could also confirm a decrease of PARP-1 activity with donor age, highlighting the role of PARP in the ageing process.

Conclusions: The results demonstrate that zinc supplementation in elderly people can increase the cellular poly(ADP-ribosyl)ation capacity of their PBMC. We speculate that this may help maintain integrity and stability of the genome more efficiently and so contribute to an extension of healthspan.

Keywords: PARP-1, Poly(ADP-ribosyl)ation capacity, zinc supplementation, aging

20.) Quantification of DNA strand breaks by An automated version of the fluorescence-detected alkaline DNA-unwinding (FADU) assay

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Abstract

Background: *Quantification of DNA strand breaks and repair in living cells is an important endpoint in the assessment of genotoxicity.*

Objectives: Our aim was to improve the available methodology beyond the current state-of-the-art.

Method/Results: We established an automated version of the Fluorescence-detected Alkaline DNA Unwinding (FADU) assay based on the use of a commercial laboratory robot. The very high precision and reproducibility of our new assay makes it possible to measure the effect of very low concentrations of genotoxic agents after 5 minutes incubation and to assess the repair capacity of the cells at 5 minute intervals. After preparation of cells the assay time is only 2 hours.

Conclusions: The new method can be used not only in experimental research but also for screening and characterization of drugs that induce DNA strand breaks. Furthermore, it is possible to detect endogenous DNA strand breaks. Thus the assay is useful to investigate differences between groups of individuals, e.g. smokers/non smokers, young/old, before chemotherapy/after chemotherapy.

Keywords: Genotoxicity, DNA damage, DNA strand breaks, DNA repair, quantification, automation

21.) Role of Connexins in Phenobarbital-mediated tumour promotion

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Abstract

Background*:* Connexin (Cx) 26 and 32 are the major gap junction proteins in liver. Like other connexins, they play an important role in tumour promotion and progression. In previous studies we could show that Cx32 is essential for phenobarbital (PB)-mediated tumour promotion in mouse liver.
Objective: To investigate whether Cx26 plays a similar role in PB-mediated tumour promotion.
Methods: We injected Cx26-deficient mice and control mice with a single dose of N-nitrosodiethylamine (DEN 90µg/g) and subsequently kept one group on a diet containing 0,05% PB for 35 weeks, while a control group was kept on a PB-free diet. At the end of the experiment, the carcinogenic response in the liver of the animals was monitored.
Results: Mice from PB-treatment groups showed a strongly increased liver weight compared to mice treated with DEN alone, which was mostly due to a much higher tumour burden. Relative liver weights of PB-treated Cx26 knockout mice were slightly smaller than those of the respective wildtype mice. The tumour response in PB-treated mice of both strains was very similar, but the number of larger tumours was lower in PB-treated knockout mice. Cx26 knockout mice treated with DEN alone did not show an increase in tumour-prevalence when compared to Cx26 wildtype mice nor were there significant differences in terms of tumour-size between strains, which is in accordance with earlier findings.
Conclusions: Our present data show that elimination of Cx26 has only minor effects on chemically induced mouse hepatocarcinogenesis, in contrast to effects seen in Cx32 knockout mice, which demonstrate strongly increased susceptibility for DEN-induced hepatocarcinogenesis and a lack of response to PB-mediated tumor promotion. Since the major structural difference between Cx26 and Cx32 lies in a missing cytosolic C-terminal domain in Cx26, this may play an important role in PB-mediated signal transduction.

Keywords: Connexin, HCC, Liver, Phenobarbital

22.) GPR49 is a target of ß-catenin and NFkB signalling

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Abstract

Background: G-protein coupled receptors are among the most promising drug targets. GPR49, also called LGR 5 is a seven transmembrane domain G-Protein coupled receptor that was recently reported to be up-regulated in human hepatocellular and colon carcinoma bearing mutations in the ß-catenin gene. Both tumours are among the most frequent tumours worldwide and a major cause of death.
Objective: To investigate the expression of GPR49 after stimulation of ß-catenin and NFkB signalling.
Methods: Primary hepatocytes were kept on DMEMF12 medium and treated with LiCl, SB 216763, TNFa, SC514 and WNT3a. Total RNA was isolated from mouse liver tumours and primary hepatocytes by TRIZOL method. RNA was quantified by quantitative real time PCR using a Light Cycler (Roche).
Results: Our results show that GPR49 is up-regulated in mouse liver tumours, which harbour a mutation in the *Ctnnb* gene but also demonstrate its up-regulation in primary hepatocytes after treatment with GSK3ß inhibitors like LiCl or SB216763 and WNT3a conditioned cell culture medium. These results give further proof for a regulation of GPR49 by the canonical Wnt signalling pathway. In addition, we could show that GPR49 is down-regulated after stimulation of NFkB-dependent signalling, an effect that could be specifically blocked by SC514, an inhibitor of this signalling pathway.
Conclusion: These findings suggest that GPR49 is regulated by ß-catenin and is also a possible target of NFkB signalling.

Keywords: HCC, GPR49, ß-Catenin, NFkB

23.) A cell permeable fusion toxin as a tool to study the consequences of actin-ADP-ribosylation caused by the *Salmonella enterica* virulence factor SpvB in intact cells

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Abstract

The virulence factor SpvB is a crucial component for the intracellular growth and infection process of *Salmonella enterica*. The SpvB protein mediates the ADP-ribosylation of actin in infected cells and is assumed to be delivered directly from the engulfed bacteria into the host cell cytosol. Here we used the binary *Clostridium botulinum* C2 toxin as a transport system for the catalytic domain of SpvB (C/SpvB) into the host cell cytosol. A recombinant fusion toxin composed of the enzymatically inactive N-terminal domain of C2I and C/SpvB was cloned, expressed and characterized *in vitro* and in intact cells. When added together with C2II, the C2IN-C/SpvB fusion toxin was efficiently delivered into the host cell cytosol and ADP-ribosylated actin in various cell lines. The cellular uptake of the fusion toxin requires translocation from acidic endosomes into the cytosol and is facilitated by Hsp90.
No differences concerning the immediate cytopathic effects of the C2IN-C/SpvB fusion toxin and the C2 toxin were obvious as both toxins induced ADP-ribosylation of actin at arginine-177, depolymerization of actin filaments, resulting in cell rounding. However, the long-term host cell responses to C2IN-C/SpvB and C2 toxin were different. The cytopathic effect of C2-toxin was not reversible and C2 toxin-treated cells finally died. In contrast, cells, treated with C2IN-C/SpvB, recovered. Recovery of cells correlated with the degradation of C2IN-C/SpvB in the host cell cytosol and *de novo* synthesis of actin in intoxicated cells. Thus, the different stability of the toxins in the cytosol determines the final fate of intoxicated host cells.
In ongoing experiments, we investigate whether the findings obtained with the artificial C2IN-C/SpvB protein can be transferred to the full length SpvB and whether SpvB becomes degraded in the cytosol of *Salmonella*-infected cells.

Keywords: Bacterial toxins, *Salmonella enterica* SpvB, actin, ADP-ribosylation, *Clostridium botulinum* C2 toxin, protein transport, translocation, degradation

24.) Clostridium botulinum C2 toxin: a new scenario for the interaction of the binary toxin with eukaryotic cells

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Abstract

C2 toxin from *Clostridium botulinum* is the prototype of binary actin-ADP-ribosylating toxins. Binary toxins are unique AB-type toxins as they consist of two individual non-linked proteins, called components, which interact in a precisely coordinated manner to intoxicate eukaryotic target cells. C2 toxin is composed the enzyme component C2I (~50 kDa), an ADP-ribosyltransferase, and C2II (~80 kDa), which represents the binding/translocation component. To become biologically active, C2II is cleaved near its N-terminus. The resulting activated C2IIa monomers (~60 kDa) spontaneously assemble to mono-heptamers, which bind to carbohydrate receptors on the cell surface. C2IIa mediates the cellular uptake of C2I because C2I exclusively binds to cells via C2IIa heptamers. The C2IIa/C2I toxin complex is taken up into cells via receptor mediated endocytosis and C2I translocates from acidified endosomes into the cytosol. This step is facilitated by C2IIa pores in the endosomal membranes and by the host cell chaperone Hsp90. In the cytosol, C2I mono-ADP-ribosylates G-actin at position Arg-177, leading to depolymerization of F-actin and to a destruction of the cytoskeleton. While the uptake mechanism of C2I was studied in detail during the past years, the early steps of toxin binding and complex formation of C2IIa with C2I are not completely clear.
Here we demonstrate that C2IIa and C2I form stable toxin complexes *in vitro*, in the absence of the cellular receptor. Preformed C2 toxin complexes could be detected by gel electrophoresis methods when C2I was pre-incubated *in vitro* with C2IIa. We used radiolabeled C2I to demonstrate that most likely three molecules of C2I interact with one C2IIa heptamer. The toxin complexes, formed *in vitro*, were biologically active. Treatment of Vero and CaCo-2 cells with either the preformed C2I/C2IIa complex or with the combination of the single components showed that the complex enhanced the cytopathic effect, indicating that it harboured enhanced cytopathic activity. One reason for the enhanced cytopathic activity might be that binding of C2I to C2IIa facilitates the subsequent interaction of C2IIa heptamers with the cellular receptor. We performed immunoblot analysis to address this question. However, our results indicate that binding of C2I to C2IIa did not influence the binding of C2IIa to cells.
Based on our new findings, we propose an extended model for the formation of an active C2 toxin complex. Two scenarios leading to toxin complex formation are possible: 1. C2IIa binds to the cell receptor creating a docking platform for C2I. 2. A second pathway includes complex formation of C2IIa and C2I prior to interaction of the toxin with target cells. The second pathway might be more effective when low concentration of the toxin components are present in the medium, thus C2IIa “takes what it gets first”, either C2I or the receptor.

Keywords: Binary bacterial toxins, *Clostridium botulinum* C2 toxin, binding to cell surface, stoichiometry, complex formation