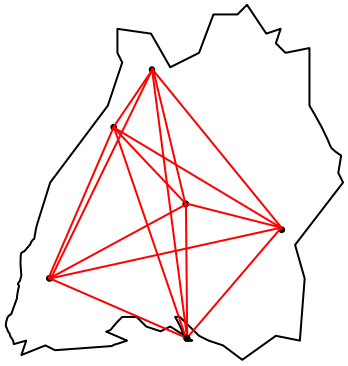
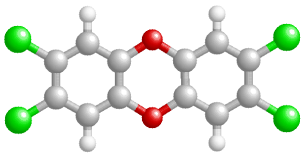
BW Toxnet Meeting

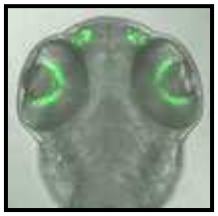
Tübingen 2008

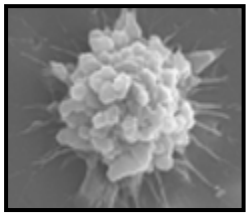


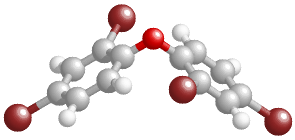
Baden-Württemberg-Toxicologists'-Meeting

July, 23rd 2008, University of Tübingen









Content

General Information

The conference takes place on Wednesday, July the 23rd 2008 from 10.15 to approx. 17.00

Conference Venue: Institute for Pharmacology and Toxicology, University of Tübingen, Lothar-Meyer-Building, Wilhelmstr. 56, 72074 Tübingen.

Public Transport: Busses 1 or 7 from Tübingen Main Station (Hauptbahnhof), direction Lustnau or Busses 2 or 6, direction WHO; Bus stop: Lothar-Meyer-Bau.

Parking: Parking is available opposite of the Lothar-Meyer-Bau in Wilhelmstr. or directly at the conference venue (please drive through Wächterstr.).

Please visit www.uni-tuebingen.de/toxikologie for detailed information on how to get to Lothar-Meyer-Bau. For further information please do not hesitate to contact us!

Organisers: Philip Marx-Stoelting, philip.marx-stoelting@uni-tuebingen.de, Tel.: 07071/2978285
Prof. Dr. Michael Schwarz, michael.schwarz@uni-tuebingen.de, Tel.: 07071/2977398

Program

10.15 – 10.30 Opening Remarks

10.30 – 12.45 Presentations (10min Presentation + 5min Discussion)

10.30 – 10.45 Presentation Albert Braeuning
10.45 – 11.00 Presentation Malgorzata Debiak
11.00 – 11.15 Presentation Julia Dempe
11.15 – 11.30 Presentation Melanie Esselen

} Session I Chair: Prof. Dr. H. Barth

11.30 – 11.45 Break

11.45 – 12.00 Presentation Jörg Fahrer
12.00 – 12.15 Presentation Joachim Orth
12.15 – 12.30 Presentation Florian Schmidt
12.30 – 12.45 Presentation Julia Strathmann

} Session II Chair: Prof. Dr. M. Metzler

12.45 – 13.30 Lunch Break

13.30 – 15.00 Postersession

15.00 – 15.15 Plenary session, Presentation of Working Groups

15.15 – 16.30 Working Groups (with coffee)

AG I: PhD-Students and Postdocs (Room 404, Philip Marx-Stölting)

AG II: Teaching staff (Room 329, Michael Schwarz)

16.30 – 17.00 Closing Session

Abstracts Presentations

BASAL AND INDUCIBLE CYTOCHROME P450 EXPRESSION IN β-CATENIN KNOCKOUT MICE

Albert Braeuning and Michael Schwarz

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Background: β-Catenin has been shown to play an important role in gene regulation in mouse liver, particularly of genes preferentially expressed in perivenous hepatocytes. Enzymes related to the metabolism of xenobiotics constitute one of the most important groups of perivenous genes. Basal expression of some cytochrome P450 isoforms has been demonstrated to be influenced by β-catenin signaling.

Objectives: The study was aimed to analyze the role of β-catenin on basal and inducible expression of drug-metabolizing enzymes *in vivo*.

Methods: Mice with liver-specific knockout of *Ctnnb1* (encoding β-catenin) were treated with different inducers of drug-metabolizing enzymes at 8 weeks of age. Expression analysis was performed by immunohistochemistry, Western blotting and real-time RT-PCR.

Results: Differential effects were observed with regard to the basal expression of drug-metabolizing enzymes in *Ctnnb1* knockout mice: Non-induced expression of most enzymes from phase I and II of drug metabolism was lower in the knockout mice when compared to wild-type controls, with only very few exceptions. Nuclear receptors AhR and CAR were also down-regulated.
Induction of the AhR by 3-MC and of CAR-dependent transcription by TCPOBOP and PB revealed an important role of β-catenin in the inducibility of drug-metabolizing enzymes *in vivo* showing a general stronger response in the wild-type group. Antioxidant-mediated enzyme induction was also inhibited in the *Ctnnb1* knockout mice. No significant differences between the two genotypes were seen for PXR- and PPAR-dependent effects.

Conclusions: The results demonstrate particular relevance of β-catenin signaling for basal as well as inducible expression of drug metabolizing enzymes.

NEW HIGH-THROUGHPUT METHOD FOR ASSESSING DNA INTERSTRAND CROSSLINKS

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Background : Nitrogen mustard derivatives, platinum compounds and mitomycins are common drugs applied in cancer therapy. Among the critical DNA lesions induced by these compounds are DNA interstrand crosslinks (ICL). While such lesions are highly mutagenic and pro-apoptotic, they are induced only to a minor extent, rarely exceeding 10% of total DNA damage. Thus, assessment of ICL formation and of the cellular repair capacity should be useful for optimization of the chemotherapy administered to patients. Monitoring has so far only been possible by using time-consuming specialized methods such as the comet assay. We have adapted a robust, automated high-throughput method, recently developed in our lab for the detection of DNA strand breaks, for the assessment of ICL.

Methods: The Fluorescence-Detected Alkaline DNA Unwinding assay (FADU) was originally described by Binboim & Javcak as a manually-operated assay. The assay is based on the strict relationship between the extent of alkali-induced DNA unwinding and number of DNA strand breaks. The amount of double stranded DNA following alkaline treatment is easily measured with intercalating fluorophore such as SybrGreen and is inversely proportional to initial amount of DNA strand breaks. For quantification of DNA cross-links, cells are additionally exposed to high dose of ionizing radiation. The number of cross-links formed is quantified as a decrease in DNA unwinding compare with irradiated controls.

Results: Our fully automated version appeared to be faster than currently used methods without having an impact on sensitivity of detection of DNA damage. It operates in a 96 well format allowing the synchronous analysis of multiple samples. Further scaling up might be easily performed. The procedure is completed within 4 h of time including sample preparation. So far, we have been able to detect DNA interstrand cross-links induced in human PBMC by pulse (1 h) treatment with 5 µg/ml mitomycin C or 2.5 µg/ml melphalan.

Conclusions: The futures as sensitivity, high throughput, fast and easy handling and low costs make the automated FADU assay an attractive new candidate method for monitoring of DNA damage induced bifunctional DNA alkylating agents.

CELLULAR DISTRIBUTION AND EFFECTS OF CURCUMIN IN HUMAN CANCER CELL LINES

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Background: Curcumin is an unstable constituent of the Asian plant *Curcuma longa* and causes cell death in human cell lines by interfering with mitosis and leading to fragmented nuclei and disrupted microtubules. This process is named mitotic catastrophe.

Objectives: In order to elucidate the role of curcumin and its metabolites in this effect, cell lines with different metabolic activities (Ishikawa, HepG2 and HT29) were used to investigate the cellular distribution and the effects on DNA and mitosis.

Methods: Cell fractionation was used to separate cell lysate into cytosol and endoplasmatic reticulum, and fractions were analyzed to determine the amount of curcumin inside the cells. Moreover, cell culture medium was analyzed for curcumin and its metabolites.
To investigate the cellular effects, the cell cycle distribution was measured using fluorescence-activated cell sorting (FACS) and mitotic catastrophe was determined with the micronucleus assay.

Results: Despite their different metabolism of curcumin, all three cell systems responded with arrest in G2/M phase and mitotic catastrophe. No effects were observed when degradation products or metabolites were used. Fractionation of cells showed that concentrations of curcumin were higher in the endoplasmatic reticulum and cytosol than in the incubation medium by a factor of up to 150 and 8, respectively. No metabolites could be found in the cell fractions.

Conclusions: These results show that the parent curcumin molecule is responsible for mitotic catastrophe. The reductive metabolites and curcumin glucuronide did not exhibit any effects.

Keywords: Curcumin, Hexahydrocurcumin, Ishikawa, HepG2, HT29, Metabolism, Mitotic catastrophe

ANTHOCYANINS INTERFERE WITH THE DNA-DAMAGING PROPERTIES OF TOPOISOMERASE POISONS IN HUMAN COLON CARCINOMA CELLS

Melanie Esselen, Jessica Fritz, Tobias Flick and Doris Marko

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Background: Anthocyanins are naturally occurring coloured plant constituents, widely spread in fruits and vegetables of the daily diet. The none-glycosylated anthocyanidins bearing vicinal hydroxy groups at the B-ring, delphinidin and cyanidin, were found to potently suppress the catalytic activity of human topoisomerases I and II[1]. However, in contrast to many plant polyphenols *e.g*. quercetin and genistein, the anthocyanidins act as catalytic inhibitors, preventing the formation of the transient DNA-topoisomerase intermediate, the so called cleavable complex. However, little is known so far about the impact of anthocyanin-enriched food or food supplements on DNA-integrity *in vitro*.

Objective of the study was to elucidate whether an anthocyanin-rich extract and the aglycone delphinidin affect the DNA-damaging properties of the standard chemotherapeutics doxorubicin and etoposide.

Methods: Single cell gel electrophoresis (comet assay) was used to detect the impact of anthocyanins on the DNA-strand breaking effects of doxorubicin and camptothecin. Topoisomerase II induces transient double strand breaks in the DNA-backbone by forming a covalent DNA-topoisomerase intermediate. In the presence of topoisomerase II poisons, the intermediate is stabilised, thus prohibiting the release of the DNA strand. The so called ICE bioassay was performed to detect the level of topoisomerase covalently linked to DNA in cellular system. This assay is antibody-based and therefore specific for the respective topoisomerase isoforms.

Results: In the comet assay a concentration-dependent decrease of the strand breaking effects of doxorubicin by pre- and co-incubation with delphinidin or an anthocyanin-rich extract was observed. However, the inhibitory properties of delphinidin exhibited an apparent maximum at 10 µM. Furthermore, pre- and co-incubation with the test compounds suppressed significantly the level of the doxorubicin induced DNA-topoisomerase-intermediate, supporting the results in the comet assay. These data raises the question whether enhanced intake of anthocyanins for example by respective food supplements might counteract the effectiveness of therapeutic topoisomerase poisons.

Conclusion: In summary, anthocyanins represent a class of potent catalytic topoisomerase inhibitors, preventing the formation of the DNA-topoisomerase-intermediate. Their inhibitory properties are not limited of a cell-free system, but are also of relevance within intact cells. These results demand further investigations whether anthocyanin-enriched food or food supplements might interfere *in vivo* with standard chemotherapeutics.

The study funded by the grant MA 1659/6-1/2 of the Deutsche Forschungsgemeinschaft.

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

CLOSTRIDIUM BOTULINUM C2 TOXIN AS A NOVEL VEHICLE TO TRANSPORT BIOTINYLATED MOLECULES INTO MAMMALIAN CELLS: CLONING, EXPRESSION AND CHARACTERIZATION OF THE CELL-PERMEABLE CARRIER C2IN-STREPTAVIDIN

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The binary ADP-ribosylating toxin C2 from *C.botulinum* consists of the enzyme component C2I and the translocation component C2II, which is activated by proteolytic cleavage to C2IIa. The N-terminal part of C2I (C2IN) mediates the interaction of C2I and C2IIa, which is indispensable for the cellular uptake of the toxin into the host cell cytosol. Streptavidin, a tetrameric protein isolated from *streptomyces avidinii*, displays an exceptionally high affinity for biotin and is used in various applications in life-sciences and also drug delivery. Mutant streptavidins have recently been engineered, which show a decreased biotin-binding affinity and therefore allow for a reversible biotin-binding.

Objective of our project was to generate a fusion protein, based on the N-terminal part of C2I and a mutant dimeric streptavidin with a decreased affinity to biotin. This fusion protein should then be used as a novel, non-toxic cell-permeable carrier for biotinylated molecules in conjunction with C2IIa.

C2IN-streptavidin was cloned and overexpressed as a GST-tagged fusion protein in *E.coli* and purified to homogeneity yielding 400 mg of pure protein/1000 ml suspension culture. Further biochemical analysis using gelfiltration showed that the produced C2IN-streptavidin protein is capable of forming dimers, a prerequisite for efficient biotin-binding. In addition, the binding properties of the fusion protein were studied by means of an overlay blot technique and a gelshift assay, revealing a concentration-dependent binding of a biotinylated protein as well as a biotin-labeled oligonucleotide. Cellular uptake of the fusion construct was monitored by immunoblot analysis of Vero cells pre-treated with C2IN-streptavidin and C2IIa. Intracellular localization was confirmed by immunofluorescence and confocal laser scanning studies, providing clear evidence for successful cellular uptake. Moreover, a local accumulation of internalized C2IN-streptavidin was observed, most likely representing early endosomes. Further studies demonstrating the uptake of a biotinylated cargo are currently ongoing.

Taken together, we successfully expressed and characterized the carrier protein C2IN-streptavidin, which will provide a novel tool for the specific delivery and uptake of exogenous biotinlyated molecules into the cytosol of living mammalian cells.

INFLUENCE OF Gβγ ON *PASTEURELLA MULTOCIDA* TOXIN-INDUCED ACTIVATION OF HETEROTRIMERIC G PROTEINS

J. Orth[1], I. Preuß[1], B. Kurig[2], B. Nürnberg[2] and K. Aktories[1]

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Albert-Ludwigs-Universität Freiburg, Albertstr. 25, D-79104 Freiburg
[2]Institut für Biochemie and Molekulare Biologie II, Universität Düsseldorf

Background: The intracellularly acting *Pasteurella multocida* toxin (PMT) affects several signal transduction pathways by stimulating heterotrimeric G proteins. PMT has been shown to activate the small GTPase Rho, the MAP kinase ERK and STAT proteins via the stimulation of two G protein families, $G\alpha_q$ and $G\alpha_{12/13}$. PMT action also results in an increase in inositol phosphates, which is due to the stimulation of PLCβ. Recent studies indicate that PMT distinguishes between the highly related $G\alpha_q$ and $G\alpha_{11}$. IP_3 accumulation induced by PMT depends on the activation of $G\alpha_q$.

Objectives: Recently, it was shown that Gβγ subunits are essential for the signal transduction of G_q. The influence of PMT on the composition of the G protein complex is still unknown. Additionally, it was studied whether PMT induces βγ-dependent signaling.

Methods and Results: To address the issue whether PMT stimulates G_q signaling via the monomeric α subunit or the heterotrimeric complex, the Gβγ subunits were sequestered by the C-terminus of β adrenergic receptor kinase (βARK-CT). The effect of sequestering Gβγ subunits was measured by a serum response element-dependent luciferase assay. Moreover, the mutants $G\alpha_q^{I25A,E26A}$ (incapable of binding βγ subunit) and $G\alpha_q^{G208A}$ (incapable of uncoupling βγ subunit) were used to determine if Gβγ subunits are necessary as positive effectors for PMT-induced activation. The two mutants were expressed in $G\alpha_{q/11}$-deficient mouse embryonic fibroblasts and PMT-induced stimulation was measured by inositol phosphate accumulation assay.
Additionally, we could show that not only Gα but also Gβγ subunits are activated by PMT. Therefore, we studied HEK293 cells co-expressing PtdIns-3,4,5-P_3- (PIP_3) sensitive pleckstrin homology (PH) domains fused to GFP together with phosphoinositide 3-kinase (PI3K) γ. Gβγ activates PI3Kγ leading to formation of PIP_3 as indicated by the recruitment of PIP_3-binding PH domain-containing proteins to the plasma membrane. In the presence of PI3Kγ, PMT, but not the inactive PMT-mutant, enhanced the redistribution of PH domains to the plasma membrane indicating the activation of Gβγ by PMT.

Conclusions: The data indicate that Gβγ subunits are essential for Gα-dependent signal transduction by *Pasteurella multocida* toxin. Additionally, activation of Gβγ signalling by PMT is shown.

HISTOPATHOLOGICAL ALTERATIONS IN THE THYROID SYSTEM OF ZEBRAFISH (*DANIO RERIO*) AFTER EXPOSURE TO PROPYLTHIOURACIL

Florian Schmidt, Thomas Braunbeck

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Background: In the past, several approaches to detect adverse effects of pollutants on the thyroid system of vertebrates have been developed with special emphasis on the South African claw frog, *Xenopus laevis*. Although, as permanent aquatic organisms, fish are highly affected by these so-called endocrine disruptors, studies into alterations of the thyroid system of fish under the influence of pseudothyroid acting substances are scarce.

Objectives and Methods: Therefore, effects of the reference compound propylthiouracil (PTU) on growth and development as well as on histopathology of potential target organs were analyzed in a modified early life stage test with the zebrafish (*Danio rerio*). Results were compared to corresponding tests conducted with *Xenopus laevis* in order to identify class- and species-specific differences.

Results: In zebrafish, PTU induced clear-cut dose-dependent hyperplasia and hypertrophy of thyroidal tissue concomitant with an increase in surrounding blood vessels. As a consequence, in both the highest (50 mg/L) and second highest (25 mg/L) concentration, macroscopically detectable goitre formation was observed, mainly consisting of small thyroid follicles and surrounding blood vessels. PTU also induced a concentration-dependent decrease of body weight, whereas body length was not affected. At the highest concentration, the size of the gills was diminished due to the enormous extent of thyroidal tissues. Hence, the disruption of the endocrine system may well have affected the fitness of the fish.

Conclusions: Such distinct histopathological effects may in future contribute to an easier identification and a more in-depth interpretation of changes induced by endocrine disrupting chemicals.

XANTHOHUMOL FROM HOPS IS ANTI-ESTROGENIC AND INHIBITS MAMMARY CARCINOGENESIS *IN VIVO*

Julia Strathmann, Karin Klimo, Renate Steinle, Norbert Frank, Clarissa Gerhauser

German Cancer Research Centre, Heidelberg, Germany

Background: Xanthohumol (XN), a prenylated chalcone from hops (*Humulus lupulus* L.) exerts a broad spectrum of chemopreventive actions. We have shown that XN inhibits tumor inititation, promotion and malignant progression by anti-oxidative effects, by modulation of xenobiotic metabolism, by anti-estrogenic and anti-angiongenic mechanisms, by inhibition of pro-inflammatory enzymes and by anti-proliferative mechanisms including the induction of apoptosis *in vitro*.

Objective: In continuation of these investigations, the aim of the present study was to analyze anti-/estrogenic properties of XN in the prepubertal rat uterotrophy assay and to determine mammary cancer preventive efficacy in the dimethylbenzanthracene (DMBA) rat model.

Methods: Assessing the prepubertal rat uterotrophy assay, juvenile rats (6 per group) were administered 17α-ethinylestradiol (EE) [1 µg/kg body weight (bw)] s.c. on three consecutive days and hysterectomized on day four.
To measure mammary cancer preventive efficacy of XN, female SD rats (20 per group, 6 weeks of age) were treated with 0, 10 and 100 mg XN/kg bw (XN-10, XN-100, dissolved in 0.75% EtOH and 1.5% Tween in drinking water) for 16 weeks. After an initial pretreatment for 9 days all animals were gavaged with a single dose of DMBA (dissolved in corn oil at 100 mg/kg bw).

Results: In the prepubertal rat uterotrophy assay, treatment with EE alone significantly increased uterine wet weights from 22.6 ± 2.44 mg to 79.0 ± 13.6 mg (average ± standard deviation, $p<0.001$ by ANOVA). Co-treatment with XN (100 mg/kg bw) i.p. reduced uterine weights by 24.8% to 68.3 ± 11.5 mg, whereas s.c. administration of XN at the same dose had no effect (80.8 ± 11.5 mg). XN treatment without EE stimulation did not influence uterine weights. Quantitative analyses of mRNA expression of estrogen-dependent target genes in the uteri confirmed anti-estrogenic potential of XN. Expression of C3 complement, IGF-1 and GAPDH was 111-, 1.4-, and 1.8-fold induced by EE application. Interestingly, XN treatment by both routes of application significantly inhibited EE-induced expression of all three genes (C3 complement i.p. 30.4%, s.c. 40.6%, $p<0.05$; IGF-1 i.p. 59.2%, s.c. 33.9%, $p<0.05$; GAPDH i.p. 62.3%, s.c. 57.5%, $p<0.05$).
In the DMBA-induced rat mammary carcinogenesis model, tumor incidence was not significantly influenced by either XN treatment (DMBA 71%, XN-10 47%, XN-100 64%). However, XN treatment at the high dose significantly reduced tumor multiplicity (1.1 ± 0.3 *vs*. DMBA 2.2 ± 1.7 and XN-10 3.0 ± 2.2, $p<0.05$) and tumor burden (0.75 ± 1.3% *vs*. DMBA 2.5 ± 4.1%, $p=0.07$). Also, treatment with XN at 100 mg/kg bw resulted in a significantly higher bodyweight ($p<0.05$) at the end of the study, compared to XN-10 and DMBA treatment groups, suggesting that the animals' general health condition was positively affected by XN.

Conclusion: Overall these data demonstrate mammary cancer preventive efficacy of XN. Multiple mechanisms, including modulation of DMBA metabolism, anti-estrogenic and anti-proliferative activity may account for these effects and are under current investigation.

Abstracts Poster

GENE-TEQ: STANDARDIZATION OF RESULTS IN DNA IMPAIRMENT FOR THE COMET ASSAY

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Within ecotoxicology to date, there is no comparable and standardized method to assess genotoxic effects of monosubstances and environmental samples, respectively. The aim of the present study was to search for a genotoxic substance which can be used to develop a comparable index to quantify genotoxic effects caused by environmental samples containing a complex mixture of different pollutants. Hence, the production of DNA breaks by direct and indirect genotoxic substances and carcinogenics was examined in a rainbow trout liver cell culture (RTL-W1) using the comet assay. The direct genotoxic substances tested were Methanesulfonic acid methyl ester (MMS), N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) and Methylnitrosourea (MNU), and the indirect genotoxic substances tested were Cyclophosphamide (CPP), Dimethylnitrosamine (DMNA) and 4-Nitroquinolineoxide (4-NQO). First of all, each substance was tested in the neutral red assay to avoid any acute cytotoxicity in the used concentration range. In the next step profiles of the dose response relationship for all substances were investigated using the comet assay. Based on these results MNNG seems to be an adequate chemical for a comparable quantification of genotoxic effects caused by environmental samples. Furthermore, it is important to investigate whether such standardization also is possible when using other cell lines, since it is well documented that different organisms show other sensitivities for genotoxic substances. At the moment, this work is being conducted for a hamster lung cell line (V79).

INVESTIGATIONS ABOUT THE STRUCTURE OF THE CHORION OF EGGS FROM *DANIO RERIO*

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The fish acute toxicity test is still a mandatory test used in chemical toxicity assessment. 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, the fish embryo toxicity test has been developed to replace the acute fish toxicity test. The fish embryo test with *Danio rerio* is obligatory for whole effluent testing in Germany (DIN 38415-T6) since 1st of January 2005. 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 required to improve the correlation between the acute fish test and the alternative fish embryo tests. There is evidence, that some of the discrepancies probably originate from a possible protecting effect of the chorion which surrounds the fish embryo during the whole test period.
In the first part of the present study the morphological structure of the chorion of eggs from *Danio rerio* at different stages was analyzed using light, transmission electron (TEM) and scanning electron microscopy (SEM). The aim of the second part of this investigation was to assess if the structure of the chorion could be modified by the non-ionic tenside Triton X-100. This agent is usually used in biochemistry to release membrane proteins in their native form. The Triton X-100 concentrations used in this study were below acute embryotoxicity.
First results showed that the surface structure of the chorion is just slightly modified until 60 h after fertilization, while the inner structure is not changing. However, the chorion of an unfertilized oocyte has a different structure than those of a fertilized egg. The unfertilized oocyte shows pore channels with microvilli, whereas in the fertilized eggs those channels are closed from the outer side of the chorion. However, from the inner layer the channel is still visible. Nevertheless, the surface structure of the chorion has been modified after the treatment with Triton X-100 in two different concentrations (1 µg/L and 3 µg/L). These effects are similar to those produced by aging.
In further investigations the influence on the permeability of the chorion will be determined for another tenside, perfluorooctane sulfonate (PFOS). PFOS is persistent in the environment and has been detected in a wide range of organisms including aquatic vertebrates. PFOS has been suggested to increase cell membrane permeability, e.g. by affecting membrane fluidity. Thus, the toxicity of other pollutants could potentially increase due to an enhanced chemical uptake. The toxicity potentiation behaviour of PFOS in chemical mixtures will be studied in fish on both short- and long-term basis. Furthermore, the adverse effects of PFOS in subsequent generations will be determined by a two-generation approach.

COMPARATIVE CHARACTERIZATION OF HUMAN AND MURINE PARP-1

Sabine Lehmann, Aswin Mangerich, Sascha Beneke, and Alexander Bürkle

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Background: The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) consists of three main functional domains: (i) an N-terminal DNA binding domain (DBD), (ii) an intermediate automodification domain (AD) and (iii) a C-terminal catalytic domain (CD). Upon DNA damage PARP-1 binds to DNA strand breaks via its DBD and becomes highly catalytically active resulting in the production of poly(ADP-ribose). This biopolymer attaches to many nuclear proteins including PARP-1 itself via its AD, thereby triggering the activation of the cellular DNA repair machinery. In general, maintenance of genomic stability is assumed to antagonise the organismal ageing process. In line with this, we demonstrated previously that the maximum poly(ADP-ribosyl)ation capacity in mononuclear blood cells positively correlates with mammalian longevity. Thus, short-lived species such as rats showed the lowest, and long-lived species such as humans the highest poly(ADP-ribosyl)ation capacity. Subsequent *in vitro* analyses with purified human and rat PARP-1, revealed that the human enzyme exhibits an at least 2-fold higher poly(ADP-ribosyl)ation capacity compared to its rat orthologue. This leads to the conclusion that the species-dependent differences in maximum cellular poly(ADP-ribosyl)ation capacities might be caused at least in part by evolutionary divergence in PARP-1 protein sequences.

Objective: The present study is investigating if this situation holds also true when comparing human (h) PARP-1 with its murine (m) orthologue. A higher poly(ADP-ribosyl)ation capacity of hPARP-1 compared to mPARP-1 is to be expected, considering the evolutionary close relationship between mouse and rat. Furthermore, this study should investigate which domain(s) of the PARP-1 enzyme might be responsible for possible species-specific differences in poly(ADP-ribosyl)ation capacities. Therefore, human and murine as well as chimeric PARP-1 enzymes are to be expressed recombinantly and subsequently characterized on a biochemical level.

Results and Methods: Two different expression systems are currently being tested regarding their suitability for PARP-1 (113 kD) protein expression: firstly, protein overexpression in *E. coli*, and secondly, protein expression in baculovirus-transfected *Sf*9 insect cells. For both systems, we successfully generated suitable DNA constructs that carry either the human (two different polymorphic variants) or murine full-length PARP-1 cDNAs as well as chimeric cDNAs thereof. Chimeric cDNAs consist of the human DBD and the murine AD/CD, and vice versa. All sequences to be expressed contain an N-terminal hexahistidine tag to enable enzyme purification. Detailed expression analyses in *E. coli* revealed only low expression levels of the full-length PARP-1 protein, possibly due to toxic effects of PARP-1 overexpression or due to size and nature of the heterologous protein. Consequently, we are currently focusing on the establishment of PARP-1 protein expression in the baculovirus/*Sf*9 insect cell system.

Outlook and Conclusions: In future experiments, the different variants of recombinant PARP-1 will be analysed with respect to their poly(ADP-ribosyl)ation capacity and their interaction behaviour. Therefore, this study should give valuable insight into species-specific biochemical properties of PARP-1 which should form the basis for the interpretation of phenotypic analyses of transgenic mouse models with heterologous expression of hPARP-1. Furthermore, potentially higher poly(ADP-ribosyl)ation capacity of hPARP-1 compared to its murine orthologue would pave the way for the generation of hypermorphic hPARP-1 knock-in mice. It will be interesting to assess the lifespan and aging rate of such hPARP-1-expressing mice.

HOST CELL PEPTIDYL PROLYL CIS/TRANS ISOMERASES ARE REQUIRED FOR TRANSLOCATION OF THE *CLOSTRIDIUM BOTULINUM* C2 TOXIN INTO THE CYTOSOL OF MAMMALIAN CELLS

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The binary C2 toxin of *Clostridium botulinum* consists of the C2IIa component, responsible for binding and translocation of the toxin into the host cell cytosol and the separate enzyme component C2I, which mono-ADP-ribosylates G-actin of eukaryotic cells. Following receptor-mediated endocytosis, C2IIa facilitates the translocation of C2I from the lumen of acidified early endosomes into the cytosol. For this purpose, C2IIa forms pores into endosomal membranes, through which C2I translocates into the cytosol. As previously showed by our group, C2I requires unfolding to pass the C2IIa pore and has to be refolded in the cytosol to fully display its enzymatic activity. This step is dependent on the activity of the host cell chaperone Hsp90.

In the current study, we used the specific pharmacological inhibitors ciclosporine A and FK506 (Tacrolimus) to investigate whether the activity of host cell peptidyl-prolyl cis/trans isomerases (PPIases) are also involved in the cellular uptake of the C2 toxin. Ciclosporine A (inhibitor of cyclophilins) and FK506 (inhibitor of FK-binding proteins) delayed significantly the C2 toxin effects on HeLa and Vero cells. Thereto, we observed the toxin-induced morphological changes of the cells. Cells, which have been treated with C2 toxin in the presence of one of the inhibitors, also showed less ADP-ribosylated actin in the cytosol than those without inhibitor. We were able to exclude an influence of the inhibitors on the ADP-ribosyl-transferase activity of C2I as well as on the early steps of toxin internalization.

Under experimental conditions, the direct translocation of cell-bound C2 toxin across the cytoplasmic membrane into the cytosol was blocked in the presence of either ciclosporine A or FK506, suggesting that PPIase activity is also required for translocation of C2I across endosomal membranes. Using biotin-labelled C2I as bait, we were able to co-precipitate cyclophilin A and Hsp90 from C2 toxin treated HeLa cells, implying binding of C2I to cyclophilin A and Hsp90 in the cytosol of intact cells.
Taken together, our results proof the direct interaction of C2I with cyclophilin A and Hsp90. To our knowledge, this is the first report describing the involvement of peptidyl-prolyl cis/trans isomerases in the uptake of a bacterial toxin into the cytosol of eukaryotic cells.

CLOSTRIDIUM BOTULINUM C2 TOXIN INDUCES DELAYED CASPASE-DEPENDENT APOPTOSIS IN MAMMALIAN CELLS

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The actin cytoskeleton represents a crucial target for a variety of intracellular acting protein toxins, which are produced by pathogenic bacteria. The binary C2 toxin from *Clostridium botulinum* is composed of the transport component C2IIa and the separate enzyme component C2I, which mono-ADP-ribosylates G-actin inn the host cell cytosol thus leading to depolymerization of actin filaments and breakdown of the actin cytoskeleton. Cell rounding results within 3 hours of incubation with C2 toxin, yet there is no immediate cell death occuring.

Here we investigated the long-term responses of mammalian cell lines (HeLa, Vero) following C2 toxin treatment. Observed cells stayed round for up to 48 h even though the toxin was removed from the medium after its internalization into the cells. The entire actin was ADP-ribosylated within 3 to 7 h; however, ADP-ribosylation was no signal for enhanced cellular degradation of modified G-actin. Moreover, there was no obvious degradation of the C2I protein in the host cell cytosol, thus the C2I ADP-ribosyltransferase remained active for at least 48 h. After ~ 15 h there was first evidence for apoptotic cell death. Restructuring of the cytoplasmic membrane as well as DNA-fragmentation were observed in C2 toxin-treated cells, i.e. representing two hallmarks of apoptosis. Furthermore, the activation of executioner caspase-3 and the cleavage of Poly-ADP-ribosyltransferase-1 (PARP-1), as a known caspase-3 substrate, were observed via immunoblot analysis. Employment of specific caspase-8 and caspase-9 inhibitors revealed the involvement of these initiator caspases in C2 toxin-induced cell death, too.

From our data, we conclude that the long-lived nature of C2I in the host cell cytosol is crucial for the non-reversible cytotoxic effect of C2 toxin, resulting in delayed apoptosis of the tested mammalian cell types.

LIPOSOME-MEDIATED UPTAKE OF CATECHOLS INTO CHINESE HAMSTER V79 LUNG FIBROBLASTS

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Background: Catechols are unstable, rather polar and chemically reactive substances. Numerous reactions of catechols are described in the literature, e.g. redox cycling leading to semiquinone/quinone intermediates and reactive oxygen species, or complex formation with metal ions. Moreover, the polarity of catechols impedes their diffusion through the lipid bilayer of the cells. Taken together, these factors account for the low concentrations of catechols in cells usually observed after direct incubation.

Objectives: We examined the stability of quercetin, a model catechol, in cell culture medium and produced liposomes containing quercetin. Liposomes are spherical vesicles composed of a bilayer membrane and are often used for drug delivery. The aim of the present study was (i) to increase the concentration of quercetin in V79 cells by packing it into liposomes, and (ii) to thereby increase the number of quercetin-induced DNA strand breaks in these cells.

Methods: The amount of quercetin in cell culture medium and in V79 cells was detected by HPLC analysis with an UV/VIS detector. For the preparation of liposomes, a liposome kit containing L-α-phosphatidylcholin (63 µmol), stearylamine (18 µmol) and cholesterol (9 µmol) was used. The induced DNA strand breaks were measured using the alkaline unwinding technique.

Results: When V79 cells were incubated with free quercetin, a rapid decline of quercetin in the cell culture medium but low intracellular concentration was observed. In contrast, incubation with liposomes containing quercetin gave rise to a decreased degradation of quercetin in the culture medium and an increase of quercetin within the cells. Despite these results, quercetin packed in liposomes was not able to induce more DNA strand breaks than quercetin without liposomes.

Conclusion: These results show that packing a catechol into liposomes leads to a higher stability of the catechol and an increased passage through the lipid bilayer. However, the liposome-packed catechol appears to exert less genotoxic activity than the free catechol.

Keywords: Catechol, Liposome, Quercetin, DNA strand break, V79 cells, Alkaline unwinding

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GLUCURONIDATION OF ALTERNARIA MYCOTOXINS BY HUMAN MICROSOMES AND RECOMBINANT UDP-GLUCURONOSYLTRANSFERASES

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Background: Fungi of the *Alternaria* species grow in regions of moderate climate and infest numerous food and feed items, e.g. cereals, vegetables, and fruits. Major *Alternaria* toxins include alternariol (AOH), alternariol-9-methyl ether (AME), and altenuene (ALT).

Objectives: The aim of the present study was to investigate the glucuronidation of these polyphenolic mycotoxins *in vitro*.

Methods: AOH (which has three hydroxyl groups), AME (two hydroxyl groups) and ALT (three hydroxyl groups) were incubated with human hepatic and intestinal microsomes, or with supersomes, i.e. microsomes from Sf9 insect cells expressing the human UDP-glucuronosyltransferase (UGT) isoforms 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, or 2B15, in the presence of UDPGA and the incubations analyzed by HPLC with UV detection and LC-MS.

Results: With AOH and AME, rapid formation of two monoglucuronides was observed with each myotoxin. In contrast, the enzymatic activity for the glucuronidation of ALT was about 1000-fold lower than for AOH and AME, and only small amounts of one monoglucuronide of ALT were formed. The two AOH glucuronides were generated in a 1:1 ratio by hepatic but a 9:1 ratio by intestinal microsomes. With AME, microsomes from both tissues markedly (95-100%) preferred the formation of one of the two glucuronides. The exact chemical structures of the individual glucuronides, i.e. the position of the glucuronide moiety, is under investigation. Glucuronidation of AOH and AME was also studied with supersomes expressing various UGT isoforms. The activities of the various UGT isoforms were determined using typical substrates, e.g. 17ß-estradiol for UGT1A1 and 1A3, trifluoperazine for UGT1A4 and 7-hydroxy-4-trifluoromethylcoumarin for the other UGTs. All isoforms except UGT1A4 and 1A6 were able to glucuronidate both AOH and AME, and most isoforms had a somewhat higher activity for AME. UGT1A9 was the most active isoform, followed by 1A1, 1A8, 1A10 and 2B15. The UGT isoforms exhibited different preferences for the formation of the two AOH glucuronides, but all isoforms except UGT1A7 generated almost exclusively the same AME glucuronide as found with microsomes.

Conclusion: Our study has demonstrated that AOH and AME are good substrates for glucuronidation, whereas ALT is very poorly glucuronidated. The patterns of AOH glucuronides, but not of AME glucuronides, vary between tissues and appear to depend on the tissue-specific expression of UGT isoforms. These data are of relevance for the elimination of Alternaria mycotoxins in exposed humans.

Keywords: Alternariol, Alternariol methyl ether, Altenuene, Glucuronidation, Microsomes, UGT isoforms

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POLYBROMINATED DIPHENYL ETHERS AND ARYLHYDROCARBON RECEPTOR AGONISTS: DIFFERENT TOXICITY AND TARGET GENE EXPRESSION

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Background: Polybrominated diphenyl ethers (PBDEs) accumulate in the environment and in humans. PBDEs are developmental neurotoxicants, disturb the endocrine system and induce tumors in rodents. However, underlying mechanisms of PBDE toxicity are still insufficiently understood. Some reports demonstrated activation of the aryl hydrocarbon receptor (AhR) by PBDEs based on induction of its target gene *cyp1A1*. In contrast, also inhibition of AhR activation by PBDEs has been observed.

Objectives: In the present study, we used different PBDE congeners (BDE47, 99, 153 and 209) and analyzed their effects on AhR signaling. In addition to *cyp1A1* induction we monitored several known downstream consequences of AhR activation in various cell lines and zebrafish. Furthermore, we studied PBDE toxicity in zebrafish embryos to identify novel PBDE target genes.

Methods: AhR activity was studied in hepatoma cells by investigation of multiple AhR target genes (microarray and real time PCR analysis), nuclear translocation and inhibition of proliferation. Zebrafish embryos were examined for developmental toxicity and identification of novel target genes.

Results: PBDEs did not activate but rather inhibited AhR signaling. Moreover, BDE47 specifically induced malformations in zebrafish embryos. BDE47 induced toxicity and changes in gene expression were clearly different from those provoked by AhR agonists.

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

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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). BPDE can bind to nucleophilic macromolecules such as proteins and DNA. The formation of BPDE-DNA-adducts is able to cause mutations.

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, FACS, BrdU 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. In the same time period BPDE induced DNA damage (strand breaks) could be detected. The DNA damage recognition by ATM/ATR is not a prerequisite for BPDE induced SAPK activation. The cellular consequences of BPDE treatment were tested in survival assays with p38 wt and $p38^{-/-}$ murine fibroblasts. P38 deficient cells are more sensitive than wt cells but show no increased apoptosis rate.

Conclusions: The BPDE induced phosphorylation of p38 and JNK correlates with BPDE induced DNA damage. The DNA damage recognition proteins ATM/ATR are not involved in this process. The activation of p38 is necessary for the cellular survival after BPDE treatment.

Keywords: BPDE, JNK, p38, ATM/ATR

ACTIVATION OF G_i BY *PASTEURELLA MULTOCIDA* TOXIN

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Background: *Pasteurella multocida* toxin (PMT) is one of the strongest activators of G□$_q$-dependent phospholipase C (PLC) β to induce inositol trisphosphate 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 $G\alpha_{12/13}$. Interestingly, $G\alpha_{11}$, a member of the G_q family is not stimulated by PMT. In addition, PMT induces MAP kinase and STAT activation.

Objectives: Here we report that PMT is not specific for G_q or $G_{12/13}$ but activates $G\alpha_i$.

Methods and Results: We show that PMT decreases basal, isoproterenol and forskolin-stimulated cAMP accumulation in intact Swiss 3T3 cells, inhibits adenylyl cyclase activity in cell membrane preparations, and enhances the inhibition of cAMP accumulation caused by lysophosphatidic acid via EDG receptors. PMT-mediated inhibition of cAMP production is independent of toxin activation of $G\alpha_q$ and/or $G\alpha_{12/13}$ as shown by using mouse embryonic fibroblasts deficient in $G\alpha_{q/11}$ or $G\alpha_{12/13}$. Although the effects of PMT are not inhibited by PTx, PMT blocks PTx-catalyzed ADP-ribosylation of G_i. PMT also inhibits steady-state GTPase activity and GTP binding of G_i in Swiss 3T3 cell membranes stimulated by lysophosphatidic acid.

Conclusions: The data indicate that PMT is a novel activator of G_i, modulating its GTPase activity and converting it into a PTx-insensitive state.

EFFECTS OF ENGINEERED AND COMBUSTION-DERIVED NANOPARTICLES IN LUNG CELLS

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Background: Inhalation of engineered nanoparticles may induce similar responses in the lung as combustion derived nanoparticles, which have been shown by epidemiologic studies to cause adverse health effects. However, the mechanisms relevant for the biological effects of both types of nanoparticles are still insufficiently known.

Objectives: Here we compare the effects of engineered nanoparticles (TiO_2, Carbon Black) with those of fly ash, a complex mixture of different particle sizes including nano-sized particles and of different chemical components, and quartz and CdO as positive controls. As target cells we used the human lung epithelial cell lines BEAS-2B, Calu-3 and A549. Biological endpoints were viability, generation of reactive oxygen species (ROS) and expression of anti-oxidant enzymes on mRNA and protein level.

Methods: LDH assay, WST-1 assay, ROS detection, Western blot, qRT-PCR

Results: Fly ash induced the formation of intracellular ROS as measured by oxidation of the fluorescent dye H_2DCF and resulted in a significant loss of cell viability in BEAS-2B. In A549 and Calu-3, fly ash was not toxic. Nanoscaled TiO_2 and Carbon Black induced a moderate LDH leakage in A549, but not in BEAS-2B and Calu-3. However, only fly ash exposure resulted in an expression of the anti-oxidative enzymes heme oxygenase-1 (HO-1) and γ-glutamate-cysteine ligase (γGCL). Treatment with the antioxidant N-acetyl cysteine (NAC) prevented fly ash-induced ROS formation and induction of HO-1 mRNA and protein. Surprisingly, although nano-CB increases ROS levels it did not induce HO-1.

Conclusions: These data demonstrate that the generation of intracellular ROS by nanoparticle exposure may differentially regulate cellular responses such as expression of anti-oxidant enzymes. The reasons for these differences are not yet known. We suppose that in the case of fly ash a combination of different metals may trigger the observed induction of oxidative stress and anti-oxidative responses.

Keywords: Fly ash, TiO_2, Carbon Black, ROS, HO-1, γGCL, NAC

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GENOTOXIC POTENTIAL OF SYNTHETIC METAL OXIDE NANOPARTICLES

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Background: Synthetic nanoparticles show a broad range of usage in science, technology and medicine. Nanoparticles are produced on industrial scale and used as additives for drugs, cosmetics, and many other applications. However, for most of the new nanomaterials little is known about biological consequences and possible adverse effects. Nanoparticles containing transition metals are expected to be especially prone to modify DNA because of their high capability to produce reactive oxygen species. Vanadium oxides are used e.g. as pigments and catalysts; moreover, ultrafine particles containing vanadium oxide are released into the atmosphere by the combustion of vanadium-rich petroleum.

Objectives: Investigation of the generation of reactive oxygen species and genotoxicity of synthetic vanadium oxide nanoparticles in human lung cells

Methods: In our study, we used a human lung epithelial cell line (A549) and both bulk and nanoscaled V_2O_3 and V_2O_5. Production of reactive oxygen species was measured fluorimetrically. DNA damage was analyzed by comet assay and micronucleus test. Solubility of vanadium oxide species was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES).

Results: Biological effects on lung cells were varying for different vanadium oxide species. Only soluble vanadium oxides were found to be able to generate reactive oxygen species both in cells and in a cell-free system. An increased DNA damage was observed in cells treated with nanoscaled V_2O_3 and bulk V_2O_5 for 36 h and 48 h, but not with bulk V_2O_3 and nanoscaled V_2O_5. No induction of micronuclei was observed.

Conclusions: Bulk material and nanoparticles of the same chemical origin exert different effects on cells. Furthermore, vanadium oxide nanoparticles may possess a genotoxic potential. These findings emphasize the importance of thorough toxicity testing for nanomaterials in order to prevent threats to human health.

Keywords: synthetic nanoparticles, vanadium oxides, reactive oxygen species, genotoxicity

DNA DAMAGING PROPERTIES OF COMPLEX *ALTERNARIA ALTERNATA* EXTRACTS IN HT29 CELLS

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Background: Fungi of the genus *Alternaria* produce mycotoxins such as alternariol (AOH), alternariol monomethylether (AME), altenuene (ALT) and tenuazonic acid (TA) which have been described as cytotoxic, genotoxic and mutagenic *in vitro* and *in vivo*. Due to their ubiquitous occurrence and their potential to considerable toxin production even under relatively low temperature conditions, *Alternaria spp.* are associated with spoilage of refrigerated foods and stored feeds. As the most prominent *Alternaria* toxins show relatively low acute toxicity, rather little effort has been made to determine the toxic/genotoxic potential of the complex fungal extracts so far.

Objectives: The aim of our present study was to establish and optimise culture conditions for different strains of *Alternaria alternata* to obtain extracts with defined, reproducible mycotoxin profiles. The DNA damaging effects of these extracts were investigated in cultivated human tumor cell lines.

Methods: The following *Alternaria alternata* strains were used: DSM 62006, DSM 62010, DSM 12633 and DSM 1102. The fungi were cultivated on moist rice at 25℃ in the dark and rice samples extracted with acidified water/ ethyl acetate. Levels of AOH, AME, ALT and TA were determined via HPLC. Genotoxic effects were determined via single cell gel electrophoresis (comet-assay) using human colon carcinoma cells (HT29) after 1 h of incubation time. A modification was thereby implemented using formamidopyrimidine DNA glycosylase (fpg) from *E. coli* in order to detect possible oxidative damages to the DNA.

Results: Reproducible *Alternaria* extracts were obtained, with respect to the absolute amount as well as the relative composition of the quantified toxins. In the comet assay, the *A. alternata* DSM 12633 extract exhibited potent genotoxic properties. Compared to the corresponding concentrations of pure AOH, the *A. alternata* DSM 12633 extract showed a significantly enhanced rate of DNA strand breaks. Moreover, treatment with fpg resulted in a significant increase in DNA strand breaks, whereas the according concentrations of AOH didn't differ between fpg treated and untreated samples.

Conclusion: The *A. alternata* DSM 12633 extract possesses genotoxic properties which substantially exceeds the DNA damaging effects of the *Alternaria* toxins identified so far. The extract also leads to a considerable induction of fpg sensitive sites in the DNA (possibly 8-oxo-guanine, formamidopyrimidines or products of N^7-adduct formation) that cannot be observed with equivalent concentrations of AOH, AME or TA.

TOXICOLOGICAL EFFECTS OF METALLIC PLATINUM-NANOPARTICLES IN HUMAN COLON CARCINOMA CELLS

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Background: Most modern cars are equipped with a three-way catalytic converter which uses platinum (Pt) as redox catalyst. However, these catalytic converters emit platinum as Pt-nanoparticles. These nanostructured particles accumulate at traffic hot-spots and might enter the food chain as a contaminant on the surface of food plants.
In contrast to platinum-halogen compounds and platinum in its oxidised form (Pt^{2+}, Pt^{4+}) little is known so far about the toxicological relevance of nanostructured Pt-particles. Especially ultrafine particles with a large ratio between mass and surface are expected to show the most significant toxicological effects.

Objectives: Our studies on the toxicological relevance of Pt-nanoparticles were focussed on the modulation of the redox status and the maintenance of DNA integrity.
The aim of the present study was to answer the question whether metallic platinum particles are able to invade human colon carcinoma cells (HT29) and influence biological molecules like glutathione and DNA. Glutathione (GSH) in its reduced form plays a crucial role in the defence against a multitude of toxicological relevant species and is involved in the suppression of oxidative stress. Therefore, the total intracellular GSH level is one parameter in the assessment of the cellular redox status.
The study was carried out with special emphasis on the dependence of the toxicological effects on the mean size of the respective particles.

Methods: In this study HT29 cells were incubated with different preparations of platinum particles (Pt > 100 nm, Pt < 100nm, Pt < 30nm) for 3 h and 24 h. The cellular uptake of platinum particles in HT29 cells was analysed by electron microscopy. The potential influence of platinum nanoparticles on cellular GSH level was investigated by GSH assay according to Tietze. Furthermore, DNA strandbreaking potential was determined by single cell gel electrophoresis (comet assay).

Results: Pt-particles < 30 nm were found to suppress the intracellular GSH level in a concentration range of 0.1 - 10 ng/cm^2 after 3 h of incubation. By elongating the incubation time up to 24 h, effects of Pt-particles < 30 nm on the cellular GSH level were strongly enhanced. At a concentration of 1.0 ng/cm^2 more than 80 % of the intracellular GSH level was suppressed. Furthermore, substantial DNA damage was observed in the comet assay after 3 h of incubation with these Pt-particles (< 30 nm). After 24 h of incubation the rate of DNA damage was still enhanced. In contrast, Pt-Particles > 100 nm showed a significant but much lower increase of DNA strand breaks and did not exhibit GSH depleting properties in the tested concentration range.

Conclusion: In summary, we found that platinum nanoparticles are internalised by human colon carcinoma cells (HT29). Hence, they affect the cellular glutathione level and affect DNA integrity. The observed effects correlate with the particle size in an inverse manner such that the cellular effects are enhanced with a decrease in the particle size and an increase of the incubation time.

THE REPROGLO-ASSAY FOR *IN VITRO* REPRODUCTIVE TOXICITY TESTING

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Background: Developmental defects leading to severe malformations and dysfunctions can result from alterations in the differentiating embryo caused by exogenous embryotoxic compounds. During early embryonic development five highly conserved signaling pathways are very important for the regulation of differentiation: the Wnt-, TGF-β-, Notch-, Hedgehog- and Receptor-Tyrosine-Kinase-signaling pathway. For *in vitro* assessment of teratogenic effects, alterations of these signaling pathways could be measured using reporter constructs comprising pathway-specific DNA binding elements controlling a luciferase reporter gene. In the context of REACH, such *in vitro* methods for the assessment of reprotoxicity are needed more than ever.

Aim: To develop a reporter-based *in vitro*-test for reprotoxic effects

Materials and Methods: D3 murine embryonic stem cells were transfected with a reporter construct specific for the Wnt signaling pathway. Stable transfectants in their undifferentiated state were treated for 24h with potential embryotoxic chemicals. A combination of a resazurin-based cell viability assay and a luciferase activity assay was used to determine effects on the signaling pathway. For high throughput, the assay was carried out on 96-well plates. Retinoic acid and valproic acid as embryotoxic model compounds were tested in non-cytotoxic dose ranges of 0.03 to 0.3 mM (RA) and 0.01 to 1 mM (VA).

Results: Valproic acid showed an activation of the Wnt signaling pathway in the dose range from 0.01 to 1 mM, although there seems to be a threshold concentration of about 0.2 mM. Retinoic acid also showed an upregulation with a maximum at a concentration of 0.03 mM, followed by a downregulation of Wnt signaling up to 0.3 mM. Several other chemicals are at the moment in the phase of testing.

Discussion: The results demonstrate that the ReproGlo-assay can help to elucidate the complexity of signalling pathways during embryonic development and detect the influence of embryotoxic compounds. Efforts are made in our lab to establish a ReproGlo-assay also with a human stem cell line (human sacrococcygeal teratomas, hsct)

GPR49 IS A TARGET OF ß-CATENIN AND NFkB SIGNALLING

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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 NFκB 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). Cloning of a luciferase reporter vector containing a part of the mGPR49 promotor sequence by PCR and ligation with pGL3b (Promega). The reporter assay was performed as well on 55.1c cells, derived from a DEN induced mouse liver tumour bearing a deletion of exon 2 in the β-catenin gene, as on Cos7 cells that were cotransfected with pCIneo-S33Y, a plasmid coding for mutated and therefore constitutive active β-catenin. Additional treatment with LiCl and SB 216763 followed.

Results: Our results show that GPR49 mRNA levels are 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 findings are confirmed by reporter gene assays using the GPR49 promotor pGL3b reporter vector, which result in an increased luciferase signal in β-catenin mutated, pCIneo-S33Y cotransfected and LiCl treated cells.
Thus our studies indicate a regulation of GPR49 by the canonical Wnt signalling pathway.
In addition, we could show that GPR49 mRNA levels are down-regulated after stimulation of NFκB-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, NFκB

Information on Working Groups

Working groups are thought to enhance networking activities among Baden-Württemberg's toxicologists. They are planned to be held in two different sessions, one for Diploma and PhD-students and Postdocs and one for teaching staff, senior scientists and professors. Activities of the working groups are planned to be continued.

The first groups aims are to exchange experiences on methods and experimental procedures, facilitate exchange of junior researchers among institutes and enhance networking activities of young scientists from the region of Baden-Württemberg working in the area of toxicology. The session will take place in room 404 of the Lothar-Meyer-Building from 15.15 to 16.30 on the conference day.

The second groups aims are to enhance networking activities among teaching staff, to develop common curricula and to facilitate exchange of teachers with a certain expertise as much as to enhance options for common grant applications and strengthening common research activities. The session will take place in room 329 of the Lothar-Meyer-Building from 15.15 to 16.30 on the conference day.

List of Participants

Due to data privacy protection
the list of participants was deleted
in this on-line document.