Pharmacology

Abstracts

Pharmacology 2006;78:144–160
DOI: 10.1159/000096485

Published online: October 19, 2006

12th Symposium of the Austrian Pharmacological Society (APHAR)

Joint Meeting with the Austrian Society of Toxicology (ASTOX)
Vienna, November 23–25, 2006

A1

TRPC3 Contributes to VEGF-Induced Ca^{2+} Signalling in Somatic Endothelial Progenitor Cells

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Recent evidence suggests that human adipose tissues host a significant fraction of somatic endothelial progenitor cells (EPCs). In culture, these cells generate colony-forming units (CFUs) that respond to vascular endothelial growth factor (VEGF) with further proliferation and are able to organize in tube-like structures. We observed abundant expression of TRPC3 channel protein in freshly isolated EPCs as well as in early EPC culture (24 h) by flow-cytometry and immuno-histochemical imaging. The population of TRPC3-expressing cells derived from the fresh adipose tissue preparation overlapped with the fraction expressing the selective stem-cell surface marker CD133. After 24 h in culture, $TRPC3^+$/$CD133^+$ CFUs were identified, which showed a heterogenous distribution of $TRPC3^+$ cells within the cell clusters, with TRPC3 expressing cells preferentially located in the outer rim of the colonies. Fura-2 Ca^{2+}-imaging experiments of these cell colonies revealed that VEGF-induced Ca^{2+} entry was significantly larger in TRPC3-expressing 'outer rim' cells as compared to cells in the centre of the clusters. Thus, TRPC3 is suggested as an element of the VEGF signalling pathway in EPCs, contributing to stem cell proliferation and may be essential for vasculogenesis. TRPC3 may be considered as a possible target that allows for selective interference with vasculogenesis and angiogenesis.

A2

Deletion of Y_2 Receptors by Local Infusion of Adeno-Associated Virus (AAV) Vectors Expressing Cre-Recombinase in $Y_2^{lox/lox}$ Mice and Subsequent Behavioural Testing

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Neuropeptide Y (NPY) is abundant in the central and peripheral nervous systems. It acts through Y_1, Y_2, Y_4 and Y_5 receptors and is involved in a variety of brain functions, including regulation of appetite and anxiety. When applied locally into the amygdala, NPY exerts an anxiolytic action, presumably mediated by Y_1 receptors. In contrast, stimulation of Y_2 receptors causes anxiety. Depletion of Y_2 receptors induces an anxiolytic phenotype, possibly by abolishing the release-inhibiting action of presynaptic Y_2 receptors. We now established site-specific deletions of Y_2 receptors in conditional $Y_2^{lox/lox}$ mice by local injection of an AAV-Cre-recombinase vector into the hippocampus, septum or amygdala. As controls, an AAV-GFP vector was injected in $Y_2^{lox/lox}$ mice at the same sites. Expression of Cre and GFP was demonstrated by in situ hybridization and immunohistochemistry. Deletion of Y_2 receptors and Y_2 mRNA was visualized by receptor autoradiography and in situ hybridization, respectively. It was neuron-specific and restricted to the injection sites. After bilateral injection of AAV-Cre vector into the amygdala, mice showed a tendency to-

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wards an anxiolytic phenotype in the light-dark test for anxiety. No anxiolytic effect was detected in mice after intra-hippocampal or intra-septal injections of AAV vectors. The experiments indicate that the anxiolytic effect of Y_2 receptor deletion may be generated in the amygdala.

A3

Deferoxamine Administration following Hypoxia-Ischemia in the Rat Brain: Neurotransmitter Concentrations and Histological Outcome

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Brain damage after hypoxia-ischemia is partly attributed to oxidative stress and is also related to the excitotoxicity. The aim of this study was to evaluate the effects of deferoxamine (Dfo), an antioxidant agent, on the release of glutamate, aspartate, glutamine and GABA and also its neuroprotective effect on the rat hippocampus after perinatal hypoxia-ischemia. Two groups of 7-day old rats underwent hypoxic-ischemic injury in the left cerebral hemisphere by left common carotid artery ligation followed by a 1-hour exposure to 8% oxygen. Immediately after hypoxia the control group (n = 10) received saline i.p. and the second group (n = 20) received Dfo mesylate (100 mg/kg). Seven days after the insult, an HPLC technique combined with fluorimetric detection was used for the amino acids determination in tissue homogenates, and histological analysis to determine cell death. In the control group, the most significant neural injury was identified in CA1 and to a lesser extend in dentate gyrus (d.g.), CA3 and CA4. The concentrations of glutamate, aspartate and GABA were significantly increased, whereas the glutamine levels were decreased. Dfo reduced the damaged neurons of the CA1 and CA3 regions statistically significantly. No effect was observed in CA4 and the d.g. Dfo didn't effect significantly the concentrations of glutamate, aspartate, glutamine and GABA. In conclusion, Dfo decreased the histological damage of the hippocampus without altering the neurotransmitter concentrations.

A4

Evidence for Trapping of a Charged Phenylalkylamine in $Ca_V1.2$

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Voltage-gated calcium channels are in a closed conformation at rest and temporary open when the membrane is depolarised. Here we study the access of permanently charged phenylalkylamine ((–)qD888) to its binding pocket in the channel pore. We exploit mutations of a conserved isoleucine in position I781 by threonine/proline in segment IIS6 of $Ca_V1.2$ that have previously been shown to shift channel activation and inactivation in parallel by 30–40 mV in the hyperpolarising direction [Hohaus et al: J Biol Chem 2005;280:38471–38477]. Shifted channel gating enabled us to evoke currents with identical kinetics at different potentials. We show here that the development of channel block by (–)qD888 (applied to the intracellular site) is not affected by voltage. Recovery from block at rest was accelerated at more hyperpolarised voltages. Our findings support the conclusion that $Ca_V1.2$ must be opening widely up to enable free access of the charged (–)D888 molecule to its binding site whereas drug dissociation from the closed channel conformation is restricted by bulky channel gates. Functional data indicating a location of a trapped (–)D888 molecule close to the central pore region are supported by a homology model illustrating that the closed $Ca_V1.2$ accommodates a large cation such as (–)qD888.

A5

STAT5 – A Novel Target for the Treatment of Abelson-Induced Leukemia?

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Imatinib has changed the therapy and life of patients suffering from myeloid and lymphoid leukaemia caused by Abelson oncogenes. Unfortunately, some of the patients become resistant during therapy, novel approaches are therefore required to complement imatinib treatment. The transcription factor STAT5 is constitutively activated in Abelson-induced malignancies and has been proposed as key regulator for disease development. We have recently shown that cells that lack the transcription factor STAT5 are entirely resistant to transformation with Abelson oncogenes. The current study aims at investigating whether STAT5 is necessary for disease progression and therefore qualifies as therapeutic target. We relied on using transgenic mouse models for B lymphoid leukaemia that allow the deletion of STAT5 after onset of disease and on cells derived from fetal livers of $STAT5^{(-/-)}$ mice (embryonic day 14). This approach allows for investigating the mechanisms how STAT5 contributes to transformation and expansion of malignant clones: deletion of STAT5 in cultured leukemic cells resulted in an arrest in the G_1 phase of the cell cycle. Conversely, expression of STAT5 by retroviral gene transfer rendered the cells more resistant against the pro-apoptotic and growth inhibitory effects of imatinib treatment. Finally, we complemented STAT5 deficient primary cells by retroviral rescue using different mutants of STAT5. This strategy allowed us to define the structural requirements of STAT5 for leukaemia initiation. Our data suggest that targeting STAT5 is a useful therapeutic strategy. A combinatorial approach using imatinib and STAT5 inhibition might be beneficial to lower the risk for the development of drug resistance upon imatinib treatment.

A6

The Effect of ARNO/Cytohesin-2 (an Exchange Factor for Arf6) on A_{2A} Adenosine Receptors in Rat Pheochromocytoma (PC12) Cells

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The A_{2A} receptor is one of the G protein-coupled receptors, which desensitizes upon prolonged agonist stimulation. In order to understand the biological function of its unusually long C-terminus, we screened a human library for proteins capable of binding to the last 120 amino acids of the A_{2A} receptor. We identified ARNO/cytohesin-2 as a binding partner. In this study we investigated the impact of ARNO on A_{2A} receptor signaling in PC12 cells. These cells express endogenously the A_{2A} receptor. We established cell lines with inducible expression of ARNO or its catalytic inactive mutant E156K. Neither ARNO nor the mutant had an effect on receptor expression, signaling via adenylyl cyclase activation or long-term de- and re-sensitization kinetics. In order to investigate effects of ARNO on A_{2A} receptor short-term de- and re-sensitization we employed a FRET-based cAMP signaling assay. Cells were transfected with plasmids coding for the regulatory and the catalytic subunit of PKA fused to CFP and YFP, respectively. Accumulation of cAMP results in the dissociation of the PKA subunits, which can be measured in single cells as a loss of FRET. The presence of dominant negative ARNO accelerated the recovery of A_{2A} receptor and led to a pronounced signaling response when cells were re-challenged with the agonist. Our results indicate that the interaction with ARNO/cytohesin-2 stabilizes short-term desensitization of the A_{2A} receptor to prevent a sensory overload in PC12 cells.

A7

A Site of Interaction between the Selectivity Filter and the Inner Vestibule of the Voltage-Gated Na^+ Channel

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Voltage-gated Na^+ channels consist of 4 domains each of which is composed of 6 transmembrane α helices (S1–S6). The four S6 segments assemble around the central axis of the pore, thereby forming the inner part of the conduction pathway. The extracellular loops connecting S5 and S6 segments (P-loops) fold back into the membrane to form the outer vestibule and the selectivity filter. Thus, the extracellular part of the pore is created by the P-loops whereas the intracellular portion is made up by the S6 segments. This raises the question whether P-loops and S6 segments do functionally interact with each other. Molecular modelling suggests that in the $rNa_V1.4$ channel (rat skeletal isoform) the amino acid K1237 at the base of the P-loop of domain III is in close proximity to the residue I1575 of the adjacent S6 segment of domain IV. To test for a functional interaction between these residues we created both single and double mutations at these sites. We tested the impact of these mutations on the distribution between fast and slow inactivated states elicited by a 1 s depolarization to –20 mV. Both in wild-type channels and in the single mutants K1237E (P-loop) and I1575A (S6 segment) ~20% of channels recovered from fast inactivation, i.e. with a time constant of 6 ms. In contrast, 87 ± 3% of channels carrying both mutations (K1237E/I1575A) recovered from fast inactivation ($p < 0.01$ vs. single mutations). These data suggest that P-loops and S6 segments can form functional interactions at sites 1237 and 1575 during inactivation gating.

Grant support: FWF P 17509-B11.

A8

Universal C-Terminal Modulation of Voltage and Ca^{2+}-Dependent Gating in L-Type Ca^{2+} Channels

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Tonic neurotransmitter release at retinal synapses is mediated by Ca^{2+} influx through slowly inactivating $Ca_V1.4$ L-type voltage-gated Ca^{2+} channels (LTCCs) lacking Ca^{2+}-dependent inactivation (CDI). $Ca_V1.4$ α1 subunit mutations cause human congenital stationary night blindness type-2 (CSNB2). Here we report the functional properties of K1591X (KX), a truncation mutant lacking the C-terminus after the IQ-motif in HEK-293 cells (+β3+α2δ1). Half-maximal activation voltage ($V_{0.5act}$) for KX was significantly shifted to more negative potentials (shift: I_{Ba}: –8.6 mV; I_{Ca}: –13.2 mV) shown in whole-cell patch-clamp recordings. In contrast to WT, KX showed robust calmodulin (CaM)-dependent CDI. Removal of the last 55 or 122 (C_{122}) C-terminal amino acid residues restored CaM-dependent CDI and shifted $V_{0.5act}$ of the corresponding truncation mutant to more negative potentials (shift I_{Ba}: –9.7; I_{Ca}: –9.5 mV). Gating changes were reversed by co-expression of C_{122}. Fluorescence resonance energy transfer experiments in living cells revealed binding of C_{122} to C-terminal motifs mediating CDI in other Ca^{2+} channels. The absence of this modulatory mechanism in the CSNB2 mutant KX underlines its importance for normal retinal function in humans. Similar Ca^{2+} and voltage-dependent properties were observed in a C-terminally truncated $Ca_V1.3$ splice variant expressed in neurons suggesting a universal role of the C-terminus for LTCC gating modulation.

Support: FWF (P-17159) and University of Innsbruck.

A9

Duramycin Effects on hERG Potassium and Voltage-Gated Sodium Currents: A QT-Prolongation Risk?

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Drug-induced prolongation of the QT interval, e.g. based on hERG potassium current inhibition or impairment of sodium current inactivation, has become a major safety concern during drug development. Duramycin (DM), a peptide antibiotic, is in clinical development for the treatment of cystic fibrosis. It becomes deposited in cellular membranes where it binds to phosphatidylethanolamine. DM may thereby change biophysical membrane properties and perturb ion channel function. Thus, its application possibly carries the risk to elicit a QT-prolongation. Here, we tested the effects of DM on hERG potassium and voltage-gated sodium currents of various mammalian cell types in whole cell patch clamp studies. We found that DM bath concentrations between 1 nM and 0.1 μM did not generate any effects on these currents. Concentrations ≥0.3 μM, however, reduced the amplitudes of hERG potassium currents. Moreover, sodium current amplitudes were reduced, and fast inactivation kinetics was slowed in the presence of DM in a concentration-dependent manner. The reported effects of DM on ion channel function may be generated by disruption of biophysical membrane properties rather than by specific interaction with ion channel proteins. Our data suggest that, under therapeutic conditions (i.e. administration via inhalation), DM is unlikely to elicit a QT-prolongation, because the observed plasma concentrations are far below those required to affect hERG potassium and voltage-gated sodium currents.

A10

Brain Lesions in Aortic Denervated vs. Sham-Operated Rats

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Introduction: Rapid control of blood pressure is a fundamental function of autonomic nervous system, involving both medullary and supramedullary centres and various neurotransmitters, including glutamate and GABA. Aim of the present study was the evaluation of potential brain damage after aortic denervation in rats. **Methods:** Male Wistar rats underwent aortic denervation (ADN) or sham operation under urethane anesthesia. During that period and for 2 h after completion of the procedure the animals' blood pressure was continuously recorded via iliolumbal artery catheterization. Afterwards, rats were sacrificed; the brain was sectioned in specific levels corresponding to CA1, CA3, cingulate cortex and cerebellar Purkinje cells and examined for dead neurons under light microscope. **Results:** Aortic denervation didn't cause acute alterations in systolic blood pressure. Quantification of the density of dead neurons revealed significantly greater percentage of dead neurons in CA1, CA3 and cingulate cortex and lower percentage in cerebellar Purkinje cells in ADN rats compared with Sham operated rats. **Conclusions:** In conclusion, aortic denervation induces acute neuronal damage in specific brain regions.

A11

Visualizing the Membrane Mobility of Corticotropin-Releasing Factor Type 2 Receptors (CRF2-R) by Fluorescence Microscopy

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Corticotropin-releasing factor is a neuropeptide that controls the production of glucocorticoids within the hypothalamic-pituitary-adrenal axis. Increasing evidence suggests an involvement of CRF-dependent cellular effects for addiction and drug dependence. To understand these effects, we conducted a study to elucidate the basic properties of membrane mobility and oligomeric state of CRF2-Rs. We used three techniques: fluorescence resonance energy transfer, fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. We found that CRF2-Rs oligomerize at the cellular membrane, consistent with findings in other GPCRs. These oligomers were also detected at the plasma membrane of stably transfected HEK cells by FCS; we described receptor complexes with two diffusion times, namely 2 ± 0.7 ms and 30 ± 5 ms. High concentration of the physiological ligand CRF induced a change within the distribution of the complexes (from 62 ± 4% of the slower complexes under basal conditions to 38 ± 7% at 100 nM CRF, and 38 ± 5% of the faster complexes under basal conditions to 62 ± 8% at 100 nM CRF). Changes in the diffusion properties of the receptors were detected only by agonist-binding and not by the antagonist alpha-helical-CRF[9–41] in a saturating concentration. Therefore, we concluded that the reorganization of CRF2-R complexes at the plasma membrane, upon activation, may play a key role for its function, for its downstream signaling to different G-proteins and/or for desensitization of CRF2-Rs.

A12

A Conserved Glutamate in the Transmembrane Domain 2 Governs the Conformational Switch in the Transport Cycle of Serotonin Transporter

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The alternate access model describes transmembrane substrate translocation across the lipid bilayer by transporters of neurotransmitter/sodium symporter (NSS) family. The model postulates the existence of outward and inward facing conforma-

tions of a transporter, that interchange to allow substrate translocation. However, how the inward-outward conformational switch is triggered is unclear. To address this issue we focused on Glu136 of the serotonin transporter (SERT). This residue is conserved in transmembrane domain 2 of NSS transporters and has been shown to be crucial for their function, oligomerization and trafficking. Three substitutions were introduced, resulting in SERT-E136D, SERT-E136Q, and SERT-E136A, which were all correctly inserted into the plasma membrane. SERT-E136Q and SERT-E136A failed to support substrate influx into cells, whereas SERT-E136D did so at a reduced rate. Binding assays using 2β-[^{3}H]carbomethoxy-3β-(4-iodophenyl)tropane together with substrate efflux experiments performed with SERT and the mutants, supported the conjecture that the mutant transporters preferentially adopted the inward facing conformation: the conformational equilibrium of SERT was shifted progressively (E136D > E136Q > E136A) to the inward-facing conformation. Our results indicate that Glu136 takes part in a hydrogen bonding network crucial for the transporter conformational switch. This conclusion is fully supported by a structure of a homologous bacterial leucine transporter.

A13

Xanthines Foster Up-Regulation of Export-Deficient Variants of the A_1 Adenosine Receptor

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Cell-permeable xanthine molecules act as pharmacochaperones on the A_1 adenosine receptor. Using A_1 receptor variants with wild-type sequence or with sequence mutations in the receptor carboxyl terminus we have assessed the mechanism by which the pharmacochaperones up-regulate the receptor. Both the high- and low-affinity antagonist ligands, 1,3-dipropyl-8-cyclopentylxanthine and isobutyl-methylxanthine, caused an increase in receptor density; agonists, by contrast, had no effect. Treatment with pharmacochaperones increased the surface expression of export-deficient receptors to a level similar to that of wild-type. We believe that the pharmacochaperone acts to release the receptor from being arrested by components of the endoplasmic reticulum (ER) quality control. (i) Although protein export depends on proper folding we have no evidence that chaperone ligands acted as folding scaffolds, neither when the receptor was expressed in bacterial nor in mammalian cells. (ii) Upon receptor overexpression an antagonist radioligand failed to bind to receptors retained in the ER even though the receptors tested were perfectly functional once located to the plasma membrane. (iii) Only if ER export was blocked did the addition of the pharmacochaperone result in specific radioligand binding, hence properly folded receptor in ER membranes. This implies that the export-deficient mutants are functional receptors but slow in export – probably due to tight interaction with ER-associated heat shock proteins.

A14

Retinoic Acid Induces Sensitization of Cyclic AMP Formation in a Neuroblastoma Cell Line

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Differentiation of SH-SY5Y-neuroblastoma cells to a neuron-like phenotype also leads to sensitization of cellular cAMP formation. Over a 4-day time course, receptor- and forskolin-mediated stimulation of cAMP formation increased by ~50-fold. In isolated cell membranes we found a similar but much smaller increase in the catalytic activity of adenylyl cyclase. Although sensitization was initiated by the treatment of cells with all-trans retinoic acid (RA) we did not find transcriptional regulation of adenylyl cyclase isoforms (or of other components controlling cellular cAMP levels). Nevertheless, sensitization was diminished by inhibitors of nuclear, i.e. transcriptional activity. In our working hypothesis, RA is the cause of sensitization but eventually supersensitivity of adenylyl cyclase is due to an indirect, maybe autocrine effect. This is based on the following observations. (i) Supersensitivity of cAMP production occurred upon a delay of ~30 h after withdrawal of RA. The addition of conditioned cell medium, however, shortened the delay. (ii) PP-1, an inhibitor of src-tyrosine kinases, reduced sensitization but only if it had been added before cAMP formation was enhanced; the inhibition by PP-1 was not reversible. (iii) Epidermal growth factor (EGF) is a candidate autocrine factor because its addition to RA-treated cells further enhanced supersensitivity. This raises the issue if RA affects cAMP signalling pathways in nerve cells.

A15

Cdk6 – for the Better or Worse in Tumor Development?

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Dysregulation of AP-1 family members is a frequent event in human cancer. A loss of the AP-1 family member JunB was observed in human myeloid and B lymphoid leukaemia, although the consequences of JunB loss has remained unclear. We have recently shown that loss of JunB accelerates leukaemia formation using transgenic mouse models. JunB deficient leukaemic cells proliferate faster and display a strong expression of the cell cycle kinase Cdk6. These findings are mirrored by experiments performed in mice deficient for c-Jun, an antagonist of JunB. C-Jun-deficient tumor cells induce leukaemia with a significant delay, are impaired in their proliferative capacity and do not express Cdk6. We therefore propose that Cdk6 is a key modulator of leukaemia development. Our concept is supported by observations in patients suffering from B lymphoid malignancies, where chromosomal translocations involving cdk6 have been described. We have shown that mice deficient for cdk6 develop leukaemia with a prolonged latency and reduced signs of disease. In contrast, forced expression of Cdk6 by retroviral gene transfer blocks leu-

kaemia development. We designed experiments that aim at clarifying this apparent contradiction and define the molecular context that turns Cdk6 from a tumor suppressor to a tumor promoter. Small molecular weight inhibitors against cyclin-dependent kinases have been developed and are currently undergoing clinical trials. Hence, it is of obvious relevance to explore how the presence or absence of catalytically active cdk6 effects the onset and evolution of leukaemic cells.

A16

The Signaling Mute hCMV Chemokine Receptor US28R129A Prevents Melanoma Growth

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Introduction: Virally encoded homologues of human chemokine receptors have evolved as powerful mediators of cell fate. The expression of some, e.g. ORF74, has been linked to tumorigenesis, where the high constitutive activity of the receptor has been linked to cell proliferation. In addition, chemokine secretion by cancer cells leads to tumor progression. US28, a human cytomegalovirus (HCMV) encoded receptor, is known to scavenge chemokines from the cellular environment. Hence, we investigated if de novo expression of US28 and a mutated version of US28 (R129A) devoid of its constitutive signaling capacity can influence tumor growth and proliferation in melanoma cell lines. **Methods:** We transduced two human melanoma cell lines, SBcl2 and 451Lu, with a lentiviral vector encoding US28 (wt) or the signaling mute US28R129A (R129A) and subsequently inoculated SCID mice with these cells. **Results:** US28R129A exhibits profound anti-proliferative activity in melanoma cells. Moreover, tumor growth of low- and high-tumorigenic melanoma cells transduced with US28R129A was impaired in SCID mice. **Conclusions:** These effects may be due to (i) chemokine scavenging, since US28 is known to act as a chemokine ‘sink’ and thus might remove inflammatory chemokines from tumor surrounding tissue or (ii) the lack of constitutive signaling in US28R129A, therefore impairing downstream proliferative pathways like p38 MAP kinase, CREB- or NFκB-transcription. These findings suggest that signaling mute virally encoded chemokine receptors may represent a novel approach to interfere with tumor-promoting effects of chemokines in solid tumors.

A17

Role of the Prostaglandin D_2 Receptors, CRTH2 and DP, in Eosinophil Chemotaxis and Mobilization from Bone Marrow

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Prostaglandin (PG) D_2 is a major mast cell product and acts via two receptors, the DP receptor and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). While CRTH2 has been shown to mediate the chemotaxis of eosinophils, basophils and Th2-type T lymphocytes, the role of the DP receptor in these effects has remained unclear. Here we report that PGD_2 induced the rapid release of eosinophils from the bone marrow of the in situ perfused hind limb of the guinea-pig and that this effect was inhibited by either the DP receptor antagonist BWA868c or the CRTH2 receptor antagonists TM30089. The CRTH2-selective compound 13,14-dihydro-15-keto-PGD_2 likewise caused eosinophil release from the bone marrow, and this was inhibited by TM30089 but not BWA868c, which demonstrates the selectivity of the DP antagonist. To a lesser extent BWA868c also inhibited the in vitro chemotactic response to PGD_2 of human peripheral blood eosinophils, which was completely abolished by TM30089. The chemotactic responsiveness of eosinophils to eotaxin was markedly enhanced in the presence of PGD_2, and this effect was reversed by TM30089 but not BWA868c. The DP-selective agonist BW245c did not mimic the effects of PGD_2 on eosinophil release from the bone marrow or chemotaxis, which suggests the involvement of a novel subtype of the DP receptor. In conclusion, our data demonstrate that DP receptors co-mediate the PGD_2-induced mobilization of eosinophils from the bone marrow and chemotaxis. DP receptor antagonists can block these responses and might hence be useful for the treatment of allergic disease.

K1

No Immunomodulatory Effect of Fosfomycin in Experimental Endotoxemia in Humans

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Introduction: Fosfomycin is an antibiotic used for therapy of sepsis and severe soft tissue infections, exerting modulatory effects on proinflammatory cytokines. It was shown that survival rates of septic mice were increased from 30 to 80%, only due to the immunomodulatory effects of the antimicrobially inactive enantiomer of the drug. The present study was performed to evaluate the effects of fosfomycin on selected cytokines in an experimental endotoxemia model in humans. **Methods:** Twelve volun-

teers received an intravenous bolus of 2 ng/kg lipopolysaccharide (LPS) on two study days. In a cross-over design, they received either 8 g of fosfomycin or placebo intravenously two hours after the injection of LPS. Plasma protein levels of TNF-α, IL-1β and IL-6 were determined by ELISA, and in leucocytes on mRNA level by RT-PCR. The expression of the cell surface markers CD11b, CD54, and CD130 on monocytes and neutrophils was investigated by flow cytometry. **Results:** The concentration-versus-time profiles of TNF-α, IL-1β, and IL-6 on protein and on mRNA level were almost identical on both study days with either fosfomycin or placebo. The same was also observed for cell surface markers CD11b, CD54, and CD130. **Conclusion:** The present data suggest that fosfomycin administered at therapeutic doses is not able to exert notable effects on cytokines in humans during endotoxemia.

K2

Intravenous Administration of Clonidine Reduces Intraocular Pressure and Alters Ocular Blood Flow

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Introduction: The aim of the present study was to evaluate the effect of intravenously administered clonidine on ocular blood flow in healthy volunteers. **Methods:** A randomized, double-masked, placebo controlled, two-way cross-over study was performed in 12 healthy young volunteers. Clonidine (0.2 μg/kg) or placebo was administered intravenously over 10 min. Ocular hemodynamics were measured using laser Doppler flowmetry, laser Doppler velocimetry, and a retinal vessel analyzer. **Results:** Clonidine significantly decreased mean arterial pressure (MAP) and intraocular pressure (IOP). Calculated ocular perfusion pressure decreased significantly by –8.7 ± 8.7% after infusion of clonidine ($p < 0.01$ versus placebo). Retinal arterial diameters increased by +4.4 ± 2.7% ($p = 0.012$ versus placebo). Red blood cell velocity decreased by –16 ± 14% ($p < 0.01$ versus placebo) after infusion of clonidine. Hence, calculated retinal blood flow decreased by –14 ± 12% ($p = 0.033$ versus placebo). Choroidal blood flow increased by +18 ± 19% ($p < 0.01$ versus placebo) and optic nerve head blood flow by +16 ± 23% ($p = 0.046$ versus placebo) 30 min after administration of clonidine but both returned to baseline thereafter. **Conclusions:** The short-time increase in choroidal and optic nerve head blood flow indicates a transient vasodilatory effect of clonidine due to an unknown mechanism. The decrease in retinal blood flow indicates clonidine-induced vasoconstriction in the retinal microvasculature.

K3

The Effects of Short Term Statin Pre-Treatment and Subsequent Low Dose Endotoxemia on Circulating Endothelial Progenitor Cells in Healthy Volunteers

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Background: Endothelial progenitor cells (EPCs) are a specific subtype of haematopoietic stem cells that contribute to the repair of injured endothelium. Statin treatment has been shown to increase EPC counts in patients with stable coronary artery disease. Levels of circulating EPCs have been investigated in different inflammatory diseases. However, so far no data exist whether short term statin pretreatment influences circulating EPCs in subsequent systemic inflammation. **Objective:** To explore the effect of statin pre-treatment on endothelial progenitor cells in human low grade inflammation. **Methods:** Randomized, double-blind, placebo-controlled three way cross-over trial in six healthy male volunteers with a washout-time of at least six weeks between treatment periods. Each volunteer received three treatment periods in random order consisting of 5 days of oral simvastatin (80 mg/day), rosuvastatin (40 mg/day) and placebo treatment. On day 5 of each study period, subjects received LPS (2 ng/kg i.v.). **Results:** Statin pre-treatment led to a significant increase in endothelial progenitor cells but could not suppress the endotoxemia induced EPC decrease over the observation period. **Conclusion:** Short term statin therapy significantly increases EPCs in healthy volunteers. However, this could not counteract the decrease of circulating EPCs after LPS infusion.

K4

Transcriptional Profiling of the Peripheral Blood Response after Statin Treatment and during Endotoxin-Induced Inflammation

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Introduction: In addition to their potent cholesterol-lowering action, statins exert pleiotropic immunomodulatory effects. While statins are known to exhibit anti-inflammatory effects by post-translationally attenuating monocyte effector pathways, little is known about effects on global leukocyte gene expression. We used GeneChips to study transcriptional changes in an in vivo model of acute inflammation and explore potential impact of statin treatment on gene expression in circulating blood cells. **Methods:** In this double-blinded, placebo-controlled, crossover study, 6 healthy, male volunteers were randomized over three periods to receive either simvastatin (80 mg/day), rosuvastatin (40 mg/day) or placebo for 4 days before challenged with lipopolysaccharide (LPS; 2 ng/kg). Blood samples, drawn before and after

statin treatment and 4 h after LPS administration, were fractionated and stabilized. RNA was isolated from captured leukocytes, subjected to cRNA synthesis, and then hybridized to genome arrays comprising 50,000 gene transcripts. **Results:** Analysis of expression data revealed that statins exert relatively little effect on leukocyte gene expression. However, LPS induced a vast transcriptional change of the leukocyte transcriptome, which seemed not to be significantly modulated by statins. **Conclusion:** This is the first in vivo study to examine genome-wide effects on the leukocyte transcriptome induced by statin treatment and the genetic program induced during endotoxemia.

K5

Exogenous Recombinant Human Cu,Zn Superoxide Dismutase Does Not Affect Vascular Dysfunction in Acute Inflammation

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Introduction: Endothelial dysfunction in inflammation is associated with increased oxygen radical formation. Transient endothelial dysfunction of resistance arteries induced by low dose *E. coli* endotoxin (LPS) is mitigated by antioxidants. Recombinant human Cu,Zn superoxide dismutase (rhSOD) scavenges O_2^- and might improve endothelial function after LPS. **Methods:** Pharmacokinetics and tolerability of i.v. rhSOD were confirmed in healthy humans (n = 18). The effect of 600 mg i.a. rhSOD on vasodilation to i.a. acetylcholine (ACh) and glyceroltrinitrate (GTN) was investigated by venous-occlusion forearm plethysmography in 20 healthy male volunteers (27 ± 4 years) 210 minutes after LPS (20 IU/kg, i.v.) in a placebo-controlled parallel group study. **Results:** LPS caused mild transient flu-like symptoms, and had no effect on SOD plasma concentrations. Forearm blood flow (FBF) was dose-dependently increased by ACh and GTN, which was blunted after LPS. rhSOD increased SOD plasma concentrations from 0.32 ± 0.34 to 42.44 ± 4.44 μg/ml, but had no impact on vascular reactivity, flu-like symptoms, or markers of oxidative stress. rhSOD alone did not affect vascular reactivity in control experiments (n = 3). **Conclusion:** Vascular dysfunction after LPS cannot be improved by high doses of rhSOD. Extracellular O_2^- is unlikely responsible for impaired reactivity to pharmacologic vasodilation. Different therapeutic targets might be required in severe systemic inflammation and vascular dysfunction.

K6

Effects of the Specific Interleukin-8 Inhibitor Reparixin on Endotoxin-Induced Inflammation

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Introduction: Reparixin antagonizes interleukin-8 (IL-8) on the level of signal transduction in vitro. We hypothesized that IL-8 mediates some of the reactions occurring during acute inflammation and specifically that IL-8 may be a mediator of endotoxin induced neutrophilia. **Methods:** The study was a randomized, double-blind, placebo-controlled, parallel group trial. Twenty healthy volunteers were randomized to receive either reparixin (12) or placebo (8) intravenously. One hour after the start of reparixin/placebo infusion a bolus of 2 ng/kg endotoxin was infused over 1–2 min. Blood samples were obtained over 24 h. **Results:** Reparixin, being metabolized to ibuprofen, suppressed serum thromboxane B_2 levels by 78% as compared to baseline and control at 8 h, respectively. LPS-induced neutrophilia was not significantly affected by reparixin in human volunteers. Consistently, reparixin did not alter the lymphocyte or monocyte counts and had no effect on LPS-induced systemic inflammation as measured by tumor necrosis factor alpha (TNF-α) or interleukin-6 (IL-6) release. Regulation of IL-8 receptors CXCR1 and CXCR2 and the degranulation marker CD11b showed the expected kinetics. Reparixin had no effect on thrombin formation as measured by prothrombin fragment (F_{1+2}). **Conclusions:** Our study showed that reparixin was safe but had no impact on endotoxin-induced inflammation. In contrast to previous studies with its metabolite ibuprofen, reparixin does not enhance inflammation in this model.

K7

Increased Temporal Lobe Efflux of R-[^{11}C]Verapamil in Patients with Pharmacoresistant Epilepsy

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Introduction: Regional overexpression of the multidrug transporter P-glycoprotein (P-gp) in epileptic brain tissue may lower target site concentrations of antiepileptic drugs and thus contribute to pharmacoresistance in epilepsy. **Methods:** We used the P-gp substrate R-[^{11}C]verapamil (VPM) and PET to test for differences in P-gp activity between epileptogenic and non-epileptogenic brain regions of 7 patients with drug-resistant unilateral temporal lobe epilepsy (TLE). We compared VPM kinetics in ho-

mologous brain volumes of interest (VOIs) located ipsilateral and contralateral to the seizure focus. Results: In several temporal lobe (TL) regions that are known to be involved in seizure generation and propagation ipsilateral influx rate constants k_1 and efflux rate constants k_2 of VPM were increased as compared to the contralateral side. These asymmetries were most prominent in parahippocampal and ambient gyrus (k_1: –3.8 to +22.3%; k_2: –2.3 to +43.9%), amygdala (k_1: –17.1 to +31.3%; k_2: –18.0 to +38.9%), medial anterior TL (k_1: –8.3 to +14.5%; k_2: –14.5 to +31.0%) and lateral anterior TL (k_1: –20.7 to +16.8%; k_2: –24.4 to +22.6%). In contrast to TL VOIs, asymmetries were minimal in a region presumably not involved in epileptogenesis located outside the TL (superior parietal gyrus, k_1: –3.7 to +4.5%; k_2: –4.2 to 5.8%). In 5 out of 7 patients, ipsilateral efflux increases were more pronounced than ipsilateral influx increases, which resulted in prominent ipsilateral reductions (10–26%) of VPM distribution volumes. **Conclusion:** Our finding of increased VPM efflux ipsilateral to the epileptogenic focus supports the 'transporter' hypothesis that enhanced P-gp activity is a mechanism involved in drug resistance in TLE.

K8

The Effects of Intravenous Histamine and Histamine Receptor Blockade on Ocular Blood Flow

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Introduction: To investigate the effect of intravenous administered histamine on ocular blood flow and to identify the receptors mediating the histamine-induced blood flow response. **Methods:** In study A placebo or histamine was administrated intravenously. In study B and C histamine was infused in the absence or presence of the H_1 receptor antagonist diphenhydramine or the H_2 receptor antagonist cimetidine, respectively. In all three studies, choroidal blood flow was assessed by laser Doppler flowmetry. Retinal blood flow was calculated based on measurements of retinal vessel diameters using a retinal vessel analyzer and retinal blood cell velocity was assessed by laser Doppler velocimetry. **Results:** Histamine significantly increased retinal arterial and venous diameters, whereas retinal blood flow was not affected. Choroidal blood flow increased after administration of histamine. Co-administration of cimetidine did not modify the effects of histamine on ocular blood flow. In contrast, co-administration of diphenhydramine significantly reduced histamine-induced blood flow effects. **Conclusions:** Histamine induces an increase in choroidal blood flow as well as a vasodilatation in retinal arteries and veins. This effect can be blunted by co-administration of an H_1 receptor antagonist. Our results strongly suggest that in the human retina and choroid histamine-induced vasodilator effects are mediated by H_1 receptors.

K9

Novel Cholesterol Derivative for Selective Targeting of Gastric Cancer

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Introduction: Gastric cancer is the fourth most common cancer worldwide. Since the prognosis at advanced stage of disease is still poor, new agents for gastric cancer therapy are desperately needed. Here we investigated the potential of a novel water-soluble oxysterol derivative (7β-hydroxycholesterol-bishemisuccinate-diethanolaminoate, 7β-EA) for gastric cancer therapy. **Methods:** MKN45 and NCI-N87 gastric cancer cell lines were assessed for cell viability, cell death and modulation of the cell cycle by electronic cell counting and FACS analysis, respectively. Expression of cell cycle proteins was quantified by Western blotting. For combination therapy, 7β-EA was combined with cisplatin and analyzed for cell viability, cell death and cell cycle distribution. **Results:** 7β-EA inhibited cell growth of gastric cancer cell lines in a dose-dependent manner. Experiments using the pan-caspase inhibitor zVAD-FMK revealed that 7β-EA induces cell death via caspase-independent mechanisms. Notably, 7β-EA treatment resulted in a nearby complete G_1 cell cycle arrest. This cell cycle arrest coincided with down-regulation of CDK6 (–27%), CDK2 (–60%) and AKT (–63%) as well as up-regulation of p21 (+423%). Combination therapy of 7β-EA and cisplatin displayed synergistic anti-tumor effects. **Conclusion:** 7β-EA has pronounced anti-tumor activity as single agent and shows synergistic effects in combination with cisplatin against gastric cancer cells, thus warranting further studies in vivo.

K10

Levels of Lipid-Based Amphotericin B in Epithelial Lining Fluid and in Human Monocytes

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Introduction: Concentrations of amphotericin B (AmB) in epithelial lining fluid (ELF) were determined and compared with simultaneous levels in plasma of patients on treatment with liposomal AmB (LAMB, AmBisome®), AmB colloidal dispersion (ABCD, Amphocil®) and AmB lipid complex (ABLC, Abelcet®). The penetration into human THP-1 monocytes was evaluated in vitro. **Patients and Methods:** Samples of twenty-two patients were obtained during routine bronchoscopy and measured using HPLC. Intracellular levels of amphotericin B were measured in THP-1 cells after incubation with lipid formulated AmB. **Results:** The three therapeutically used formulations of amphotericin B display remarkable differences in their pharmacokinetic behaviour. The highest concentrations in ELF were found after treatment with LAMB (mean 2.22 μg/ml), the best penetration ratio

was exhibited by ABLC (mean 550%) and high intracellular levels of ABCD were measured in monocytes. **Conclusions:** The concentrations in different compartments may depend on the administered formulation. Whether these differences have an impact on the therapeutical outcome, will have to be investigated in further clinical trials.

K11

MicroPET Imaging with the β-Amyloid Probe [^{18}F]FDDNP in a Transgenic Mouse Model of Alzheimer's Disease

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Introduction: Mouse models transgenic for the human amyloid precursor protein (APP) are commonly used in the development of new therapeutics against Alzheimer's disease (AD). Small animal positron emission tomography (microPET) with radiotracers that bind specifically to β-amyloid plaques (Aβ) in the AD brain potentially allow for performing longitudinal studies to assess in vivo the efficacy of new Alzheimer therapeutics. We evaluated the usefulness of microPET with the Aβ probe [^{18}F]FDDNP (2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malonitrile) for detection and quantification of Aβ in the brains of Tg2576 mice. **Methods:** Dynamic [^{18}F]FDDNP microPET scans were performed with a Siemens microPET Focus 220 scanner in a group of 6 elderly transgenic mice (Tg2576; age: 14 ± 1 months) and a control group of 6 elderly wild-type mice from the same litter (age: 15 ± 1 months). Brain retention of [^{18}F]FDDNP in frontal cortex, parietal cortex and hippocampus was quantified in terms of the distribution volume (DV) as calculated by Logan's graphical analysis using PMOD software (version 2.6.1, PMOD Technologies, Switzerland). **Results:** Significant differences between groups were observed for [^{18}F]FDDNP DVs in the frontal and parietal cortex but not in other brain regions, e.g. the hippocampus. **Conclusions:** [^{18}F]FDDNP PET might become a useful translational tool for the development of novel anti-AD drugs.

K12

Inhibition of HIF-1α by RAD001 (Everolimus) in Combination with Antiangiogenic Chemotherapy in a Gastric Cancer Model

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Introduction: Cytotoxic drugs can be used as antiangiogenic agents, thereby inducing hypoxia in tumors. Activation of HIF-1α by hypoxia leads to increased tumor angiogenesis, thus potentially limiting the activity of antiangiogenic strategies. Inhibition of mTOR has been reported to suppress HIF-1α expression. It was hypothesized that mTOR inhibition in combination with antiangiogenic chemotherapy may show synergistic anti-tumor activity. **Methods:** In vitro, effects of RAD001 on mTOR signalling, proliferation, cell cycle, HIF-1α and VEGF were evaluated in gastric cancer cells. In vivo, the effects of cyclophosphamide at two antiangiogenic schedules in combination with RAD001 were studied in a gastric cancer xenograft model. Ki-67 expression, activation of caspase 3, HIF-1α expression, microvascular density (MVD) and levels of circulating endothelial progenitors (CEPs) were assessed. **Results:** In vitro, RAD001 decreases proliferation and attenuates production of HIF-1α and VEGF. In vivo, RAD001 treatment inhibits tumor xenograft growth and suppresses HIF-1α expression. The combination of RAD001 with antiangiogenic cyclophosphamide reduces levels of CEPs and decreases MVD. Long-term tumor growth control exerted by combination of RAD001 with antiangiogenic cyclophosphamide is striking and highly significant. **Conclusions:** Combination of RAD001 with antiangiogenic cyclophosphamide might be a promising approach for gastric cancer therapy.

Grants: Hochschuljubiläumsstiftung der Stadt Wien (D.C.), Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien (V.W.).

T1

Alkyl- and Aryl-Substituted Spin Traps: Free Radical Adduct Formation and Toxicity Studies

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Several lipophilic spin traps have been investigated, in which the methyl group of EMPO is replaced by either a phenyl or an n-pentyl substituent. Superoxide radical adducts from EPhPO ($t_{1/2}$ = 16.1 min) and from the two pentyl compounds having an extra methyl group at either position 3 ($t_{1/2}$ = 12.1 min) or 4 ($t_{1/2}$ = 25 min) are relatively stable. Except for the phenyl derivatives, relatively stable hydroxyl radical adducts were formed ($t_{1/2}$ = 15–20 min). We also detected spin adduct formation from various other oxygen-centered as well as carbon-centered radicals (e.g. derived from methanol or linoleic acid hydroperoxide). From these results it can be concluded that the phenyl-substituted compounds EPhPO and PPhPO have better spin-trapping properties than their n-pentyl-substituted counterparts, EPtPO and PPtPO. Methyl groups in either position 3 or 4 also increase the stability of the spin adducts. Toxicity studies of a series of recently described spin traps were performed with cultured human colon carcinoma cells (SW480), human hepatocarcinoma cells (HepG2) and primary rat hepatocytes. A concentration-dependent decrease of cell number and an increase of LDH release was detected. Our data indicate necrotic cell death possibly due to membrane toxicity. The following toxicity ranking was obtained: 4,5-DPPO > 3-BEMPO ~ 4-BEMPO ~ trans 3,5-EDPO > 4,5-DiPPO ~ 3,5-DPPO > cis 3,5-EDPO ~ 3,5-DiPPO ~ 4,5-EDPO.

T2

Isolated bc_1 Complex as a Screening System for Potential Anti-Malaria Drugs

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Malaria is responsible for 1–2 million deaths per year. Therefore, the development of new and inexpensive anti-malaria drugs is required. The mitochondrial bc_1 complex in protozoa was identified as a suitable drug target for malaria antibiotics. The mechanism is based on a stronger inhibition of the bc_1 complex in plasmodia than in mammalian bc_1 complex. Aim of this study was to set up a model system for the screening of compounds with possible anti-malaria activity. Due to the sequence homology of the bc_1 complex in plasmodia and *Saccharomyces cerevisiae*, its bc_1 complex can be used as a model. Therefore, the membrane-bound bc_1 complex was isolated via a detergent-based solubilization and chromatographic purification from bovine heart mitochondria and from yeast mitochondria as models for mammals and plasmodia, respectively. The measurement of the quinol : cytochrome c oxidoreductase activity in both complexes gave turnover numbers of about 200 s^{-1} indicating a functional enzyme preparation. Titration of the enzymatic activity with stigmatellin revealed an IC_{50} of 2.15 ± 0.28 nM for bovine and 1.80 ± 0.19 nM for the yeast bc_1 complex indicating the non-selective toxicity of this compound. However, if the only available anti-malaria drug for this target atovaquone was used, an IC_{50} of 12.4 ± 1.08 μM for bovine and 4.52 ± 0.35 μM for yeast bc_1 complex was obtained. In contrast to stigmatellin, the drug atovaquone inhibits the yeast complex three times more than the mammalian bc_1 complex. These basic biochemical data indicate that the model consisting of isolated bovine and yeast bc_1 complex is suitable for the identification of new anti-malaria drugs.

T3

Upregulation of FGF-18 in Chemically Induced Rodent Hepatocarcinogenesis and in Human Hepatocellular Carcinoma

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The fibroblast growth factors (FGF) FGF-8, -17 and -18 are down-stream targets of the wnt-pathway, which often is deregulated in hepatocellular carcinoma. We therefore sought to determine in detail the role of these FGFs for carcinogenesis in the liver. Hepatocarcinogenesis was induced in rats by the genotoxic carcinogen N-nitrosomorpholine (NNM) and subsequent promotion by phenobarbital. In half of the hepatocellular adenoma and carcinoma studied we found pronounced upregulation of FGF-18, and marginally enhanced expression of the other FGFs and the corresponding FGF receptors. Human hepatocellular carcinoma showed similar alterations. For functional studies we chose primary co-cultures of normal and NNM-induced premalignant (GSTp+) rat hepatocytes. DNA-synthesis was significantly higher in GSTp+ than in normal hepatocytes indicating an inherent growth advantage of the premalignant cell population. FGF-18 stimulated DNA synthesis in normal cells and even more pronounced in the GSTp+ hepatocytes. Conclusions: FGF-18 induced preferential growth of premalignant hepatocytes and is highly upregulated in liver tumors of two mammalian species indicating auto- and/or paracrine stimulation of tumor formation. This is also evidence that the molecular mechanisms underlying chemically induced rat hepatocarcinogenesis and human hepatocarcinogenesis are often identical, which may facilitate interpretation of results obtained from rodent bioassays for chemical carcinogenicity.

T4

Alterations of the Ras Pathway in Hepatocarcinogenesis: Species Comparison and Implications for Risk Assessment

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The molecular mechanisms underlying carcinogenesis often reveal considerable species differences complicating the interpretation of results obtained from rodent bioassays for chemical carcinogenicity; e.g., in human hepatocellular carcinoma (HCCs) the frequency of Ras mutations is low, while chemically-induced rodent liver tumors frequently harbour activating point mutations in the Ras oncogenes. NORE1B, NORE1A and RASSF1A are part of a regulatory mechanism that antagonizes the growth-enhancing effects of the proto-oncogene Ras. We could show that epigenetic inactivation of NORE1B occurrs in 62% of the human HCCs due to considerable promoter hypermethylation of the gene. Hypermethylation of the RASSF1A promoter was also frequently detected resulting that epigenetic silencing of at least one of the genes occured in 97% of the HCCs studied. We also found that NORE1B interacts via its RA- and SARAH-domain with the known tumor suppressor RASSF1A, that it leads to a cell cycle delay and suppresses transformation of cells. It therefore meets several criteria required to be addressed as a tumor suppressor gene. Our data suggest that epigenetic silencing of NORE1B and RASSF1A appears to be significant for human hepatocarcinogenesis and may exert effects similar to activating mutations of Ras in rodent liver tumors.

T5

Linoleic Acid Hydroperoxides and the Induction of Antiapoptotic Genes in Cultured Hepatocarcinoma Cells

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Hydroperoxides of polyunsaturated fatty acids are formed in dietary oils in the presence of atmospheric oxygen or internally during inflammation. Since liver cancer almost always arises upon chronic hepatitis, we investigated the possible impact of lipid hydroperoxides for the progression of liver cancer. Linoleic acid is one of the major components of cellular fatty acids. A mixture of its hydroperoxides (LOOH) was synthesized. Detection of radicals from LOOH was carried out by the ESR spin trapping technique. Alkyl, superoxide and carbon-centred radicals were identified. LOOH was also applied to cultured human (HepG2, HCC-1.2) and rat (H4IIE) hepatocarcinoma cells. LOOH toxicity to all investigated cells (LD_{50} = 35–80 μM, 24 h incubation) was found to be dose-dependent. Increased LDH release was observed in the presence of LOOH at 10 μM and above indicating membrane damage. Non-oxidized linoleic acid was not toxic. LOOH caused an increased expression of the stress indicator and antiapoptotic survival factor heme oxygenase 1 in HepG2 and HCC-1.2 cells. Early response gene *c-fos* and antiapoptotic gene *bcl-2* were upregulated by LOOH, whereas proapoptotic *bax* was down-regulated by this compound. In summary, the present results indicate that lipid hydroperoxides induce an antiapoptotic programme, which may be relevant for cancer progression.

T6

Aquatic Ecotoxicity of Quaternary Ammonium Compounds

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Introduction: Quaternary ammonium compounds (QACs) are strong cationic surface-active industrial chemicals, primarily used as disinfectants and detergents, typically released from point sources into waste water treatment plants, characterized by their high potential to act as biocides on both target and non-target organisms. Based on product and application profiles, QACs form a group of potentially hazardous substances. The goal of this study was a broad based environmental risk assessment (ERA) for selected QACs (benzalkonium chlorides, dialkyldimethylammonium chlorides) in Austrian rivers. **Methods:** A modular study design was employed involving environmental exposure characterization, QAC fate and effect analysis in the waste water, ecotoxicological effect characterization (based on a set of microbiotests conducted, and a critical literature review), and environmental risk evaluation. **Conclusions:** QAC concentrations in waste water were below effect concentrations for microorganisms in waste water treatment plants. Pollution sources were related to two single source polluters: hospitals and laundries. Sediment concentrations were well below PNEC values. For surface water, environmental risk could not be excluded, as PEC:PNEC ratio was above 1 for one river with low discharge and high content of treated waste water. The overall dataset applicable in ERA for QACs is still fragmentary.

Grants: BM Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft, Vienna, Austria.

T7

Hepatocarcinogenesis by Diethylnitrosamine (DEN) in NADPH Oxidase Knock-Out Mice and Their Wild-Type Counterparts

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Nitrosamines occur in traces in food or may be generated endogenously. They are one chemical factor involved in hepatocarcinogenesis, a process mediated by genotoxic and cytotoxic events. Ethanol is a widely consumed hepatotoxic agent and has been shown to increase hepatocarcinogenesis in humans. Cytotoxic reactive oxygen species (ROS) and TNF-α production, and DNA damage increased after DEN treatment in wild-type (wt) mouse liver but not in p47-NADPH oxidase knockout (phox-ko) mice [Teufelhofer et al: Carcinogenesis 2005;26:319–329]. Therefore we examined if phox-ko mice might be protected from hepatocarcinogenesis. Two models were employed: (1) treatment of neonatal mice with a single dose of DEN and later with three cytotoxic doses of DEN at 7 week intervals. (2) Initiation of carcinogenesis in 6 week old mice and subsequent promotion by either feeding a phenobarbital diet or providing ethanol in the drinking water. Here we present the macroscopic findings: (1) was a powerful regimen and produced tumors in almost all wt mice after 30 weeks. However, some phox-ko mice also developed tumors. (2) Phenobarbital was a strong tumor-promoting agent producing more tumors in wt than in phox-ko. Ethanol proved to be much weaker but it enhanced tumorigenesis in wt and less so in phox-ko. Oxidative DNA lesions were higher in wt than in phox-ko liver DNA. Thus, ROS generation appears to be an important mechanism in DEN carcinogenesis but other factors seem to be involved, as exemplified by tumor development in phox-ko mice.

Grant support by Herzfelder'sche Familienstiftung is gratefully acknowledged.

T8

The *Fusarium* Toxin Enniatin Exerts Cytotoxic Activities via Apoptosis Induction

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The cyclic hexadepsipeptide enniatin (ENN), produced by various strains of the genus *Fusarium*, has been reported to possess antibiotic activity, immunmodulatory properties and ionophoric activities. Furthermore, it is well known as an inhibitor of mammalian cholesterol acyl-transferase. In this study we investigated its cytotoxic activity against human tumor cells. A 24 h treatment of several tumor cells with 5 and 10 μM ENN induced massive cell death, identified as apoptosis by demonstrating cell shrinkage, chromatin condensation, and nucleic fragmentation into apoptotic bodies. Correspondingly, cleavage of PARP was detectable. Induction of cell death was accompanied by a massive loss of mitochondrial membrane potential. Additionally, no signs of necrosis like release of lactate dehydrogenase (LDH) were detectable after treatment with ENN for 24 h. Referring to apoptosis- and cell cycle-regulating proteins, no major influence of p53, p21 and bax on the cell death induction by ENN was detected. Nevertheless, ENN induced a significantly more efficient stop of DNA synthesis and cell cycle arrest in p53 wildtype as compared to knock-out cells. In summary, we demonstrated a very strong cytotoxic effect of the mycotoxin and natural drug ENN on several cancer cell lines based on induction of apoptosis. Further studies are under way to clarify the molecular mechanisms underlying the induction of apoptotic cell death by ENN.

T9

Impact of Staining Procedures on the Results of the Micronucleus Assay with Exfoliated Cells as Biomarkers for Human Cancer Risk

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Micronuclei (MN) in exfoliated epithelial cells (EC) are widely used as a biomarker for genomic instability to detect cancer risk caused by occupational and life style factors. Strongly conflicting results were obtained in studies with smokers who are at increased risk for cancer in mouth, oesophagus, lungs and other organs. In the present study we investigated if and to which extent the use of different staining procedures affects the outcome of MN experiments with oral EC collected from heavy smokers (n = 20, more than 30 cigarettes per day) and non-smokers (n = 10). With DNA non-specific stains such as Giemsa and May-Grünwald-Giemsa significant increases were found in EC of smokers compared to non-smokers (circa 5.0-fold over control), but no significant effects were detectable with DNA-specific stains such as Feulgen, DAPI and acridine orange. The evaluation of cells of the two study groups with Feulgen stain showed that EC from smokers had significantly increased levels of nuclear anomalies (NA) other than MN. These NA are consequences of cell injury found in EC and are paralleled by formation of keratin bodies in the cytoplasm which resemble MN. Correlation analyses showed that MN frequencies scored in Giemsa-stained slides correlated significantly with NA, whereas no such correlations were found with DNA-specific stains. These findings indicate that NA (and possibly keratin bodies) may be misinterpreted as MN with non-specific DNA stains and lead to false-positive results in studies with EC. Furthermore, our results show that exposure of oral cells to genotoxic carcinogens contained in tobacco smoke does not lead to induction of MN in EC.

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Late Abstracts of the 11th Scientific Symposium 2005

L1

Induction of Oxidative Stress by Acrylamide Is Related to the Differentiation Grade of Caco-2 Cells

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Objectives: Acrylamide is metabolized via cytochrome P450 2E1 (CYP2E1) to glycidamide (phase I), a chemically reactive epoxide that forms DNA adducts. This metabolite is detoxified via glutathione-S-transferase (GST) to N-acetyl-S-(3-amino-3-oxopropyl) cysteine (phase II). Caco-2 cells express both, CYP2E1 and GST, but expression of the latter is dependent on the degree of differentiation, for which apical alkaline phosphatase expression is a marker. **Methods:** In the presented study Caco-2 cells were grown until different stages of confluency (day 3, 7) and differentiation (day 3, 7, 14, 21). Cells were incubated for 24 h with 0.1, 0.5 and 1 mM of acrylamide dissolved in deionized water added directly into the culture medium. Thereafter activity of GST and alkaline phosphatase as well as reduced/oxidized glutathione GSH/GSSG ratio and lipid peroxidation were analyzed. **Results and Discussion:** GST and alkaline phosphatase activities increased during differentiation of the cells while addition of acrylamide showed no effects. Glutathione levels decreased until confluency was reached (day 7). At day 14 and 21 (day 21: cells are fully differentiated) acrylamide significantly decreased total glutathione levels and at day 14, also the GSH/GSSG ratio decreased in a concentration dependent manner. This indicates oxidative stress burden. However, regarding lipid peroxidation all samples analyzed were below the detection limit. **Conclusion:** The present data suggest that, by reducing glutathione levels, acrylamide or its metabolite might affect phase II detoxification in differentiating Caco-2 cells. Additional measurements of CYP2E1 expression are currently performed to assess the role of this CYP in acrylamide induced oxidative stress.

L2

Interference of Tocopheryl Quinone with Mitochondrial Electron Transfer

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Vitamin E functions as a radical-trapping antioxidant in mitochondria which are believed to release superoxide as a byproduct of respiration. Oxidized tocopherol which escapes recycling by ascorbate or ubiquinol is irreversibly converted to tocopheryl quinone (TQ). We analyzed the amounts of TQ in submitochondrial fractions from healthy rats and found ca. 10 pmol TQ per mg protein in mitoplasts and outer mitochondrial membranes, which translates into a ca. 10-fold smaller TQ:lipid ratio in the latter. This underscores the protective role of tocopherol at the site of cell respiration. Since TQ resembles ubiquinone (UQ), we studied the extent to which short-chain TQ_1 competes with UQ_1 in mitochondrial electron transport, using submitochondrial particles from beef heart. Complex I (NADH dehydrogenase) turned out to reduce TQ_1 in a rotenone-sensitive manner, although with smaller affinity (K_M 115 vs. 23 μM) and 10-fold reduced rate (v_{max} 23 vs. 260 nmol/mg/min). On the other hand, no reactivity was found for complex II (succinate dehydrogenase) due to its more specific quinone binding pocket. Complex III reduced TQ_1 in the course of the Q cycle. This antimycin A-sensitive reaction was 100 times slower compared to UQ_1, but the affinities of the two substrates were comparable (K_M 36 and 22 μM for TQ_1 and UQ_1, respectively). The results suggest a weak competition of TQ, especially under conditions of oxidative stress, and also provide a mechanism for the generation of reduced TQ which itself is a strong antioxidant.

The research was supported by the FWF, grant P16244-B11.

L3

Fine Particulates and Cardiopulmonary Incidents in Vienna

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In earlier smog episodes SO_2 proved to be the indicator of air pollution with highest correlation to daily mortality and morbidity [Wien Klin Wochenschr 2004;116(suppl 1):8–12]. After its successful reduction focus shifted to particulate pollutants and time series studies found dose-response relationships with fine particle mass without indication for a threshold. In Vienna daily hospital admissions of chronic pulmonary diseases (ICD-9: 490–496) in 1999/2000 increased in persons aged 65 years and older with $PM_{2.5}$ concentrations [Atmos Environ 2004;38:3971–3981]. The aim of this study was to analyse daily mortality in Vienna 2000–2004 with methods used in studies of other European cities [Am J Respir Crit Care Med 2004;169:1257–1258]. Polynomially distributed lag models for up to 14 days were used (Poisson GAMs), applying stringent convergence criteria and considering seasonal, daily meteorological influences and gaseous pollutants as confounders. Per 10 μg TSP/m^3 the increase of total daily mortality (lagged 0–14 days) was 1.2% and for elderly aged 65+ it was 1.5%. An increase of 3.7% (3.9% at age 65+) was found for ischemic heart disease (ICD-9: 410–414) and of 7.6% for chronic pulmonary disease (ICD-9: 490–496). The contributions of particle subfractions still have to be determined. NO_2 proved to be an independent indicator of urban air pollution increasing cardiopulmonary incidents. Reduction of both fine particulates and NO_2 need to be continued; outdoors mainly by control of motor traffic and indoors by tobacco control, cleanup of workplaces and ventilation of gas stoves.

L4

Coffee Protects Peripheral Human Lymphocytes Against Oxidative DNA-Damage

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Aim of the study was the investigation of the potential DNA-protective effects of coffee consumption in humans. DNA damage was monitored in lymphocytes of eight individuals with the single cell gel electrophoresis assay before and after consumption of 600 ml coffee/day over five days. Under standard conditions, no alteration of DNA migration was seen, but a pronounced reduction of DNA migration attributable to endogenous formation of oxidised purines and pyrimidines was detected with restriction enzymes; furthermore induction of DNA damage induced by hydrogen peroxide and by heterocyclic aromatic amines was significantly reduced after coffee consumption. Also in in vitro experiments with lymphocytes, inhibition of H_2O_2 induced DNA damage was found with coffee at low concentrations whereas coffee diterpenoids caused only marginal effects. These findings indicate that the effects in humans are partly due to scavenging effects of constituents other than diterpenoids. Enzyme measurements showed that also induction of antioxidant enzymes may play a role: while the activity of glutathione peroxidase in cytosols of peripheral lymphocytes was only marginally increased at the end of the intervention period, a significant increase of superoxide dismutase activity was detected. Comparisons of our findings with results of earlier studies support the assumption that coffee consumption prevents oxidative DNA damage to a higher extent than fruits and vegetables.

L5

Consumption of Brussels Sprouts Protects Peripheral Human Lymphocytes against 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]-Pyridine (PhIP): Results of a Controlled Human Intervention Trial

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Heterocyclic amines (HAs) are formed during cooking of meats and might be involved in the aetiology of various forms of human cancer. Due to the low exposure levels, it is not possible to monitor prevention of HA adduct formation in humans. As an alternative we developed a protocol in which we monitored the alterations of the sensitivity of peripheral human lymphocytes in single cell gel electrophoresis assays with PhIP (the most abundant HA in fried meat) and Trp-P-2 (which is less abundant but a stronger carcinogen in rodents). We used this model to study the effects of Brussels sprout consumption on HA-induced DNA damage. Eight volunteers consumed 300 g/day of steamed sprouts over five days. Lymphocytes were isolated at the beginning and at the end of the study and were exposed to the amines for 30 min. PhIP-induced DNA migration was inhibited significantly after consumption of the vegetables, whereas the sensitivity of the cells towards Trp-P-2 was not affected. Since genetically engineered cells showed that PhIP but not Trp-P-2 requires activation via sulfotransferases, we monitored the effect of Brussels sprout consumption on SULT (1A1, 1A3) in Western blots and enzyme assays in a further trial and found strong inhibition of these enzymes. Our findings provide an explanation for the prevention of PhIP DNA adduct formation seen by sprouts in animals and suggest that the consumption protects humans against PhIP induced DNA damage via inhibition of sulfotransferases.

L6

In silico Toxicology: Current Status and Future Perspectives

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The term 'in silico toxicology' generally stands for computer-assisted toxicological and ADME assessment of chemical substances as well as the development of corresponding computer models and software tools. Two general approaches can be identified: on the one hand fine-tuned expert systems exist that try to qualitatively guess substance effects on classical toxicological endpoints, on the other hand mechanistic models are increasingly developed that focus on quantitative prediction of individual aspects in the overall toxicological (or ADME) picture. While it would of course be highly desirable to apply mechanistically well-founded quantitative models also for 'global' toxicity prediction this is currently hampered by the extraordinary complexity of underlying phenomena. However, toxicogenomics, -proteomics and systems biology approaches increasingly contribute data on which refined mechanistic models can be built that also incorporate structural aspects of involved enzymes, receptors and other proteins. It can be estimated that in the future in silico toxicology will not only continue to gain importance in industrial drug development, but will also reach regulatory acceptance as a tool to assist in data evaluation as well as for prioritisation of experimental testing.

L7

Genotoxic Effects of Common Mycotoxins Cause DNA Damage in Human-Derived Hepatoma (HepG2) Cells

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It is known that certain mycotoxins (ochratoxin A and B, OTA and OTB; fumonisin B_1, FB_1; citrinin, CIT) which occur in human foods cause tumors in laboratory rodents, but data on their genotoxic effects in human cells are scarce. In most conventional in vitro genotoxicity tests mainly negative results were obtained with these compounds. Therefore we studied the effects of these fungal toxins in a human-derived hepatoma cell line (HepG2) with the micronucleus (MN) and the single cell gel electrophoresis (SCGE) assay. These cells retained the activity of phase I and phase II enzymes. Additionally, also bacterial (Salmonella/microsome) assays were carried out with enzyme homogenate prepared from HepG2 cells. In MN assays clear dose-dependent effects were found with OTA ($\geq$5 μg/ml), CIT ($\geq$2.5 μg/ml) and FB_1 ($\geq$25 μg/ml). Also in SCGE experiments positive effects were seen with OTA and FB_1, but not with CIT. Subsequent experiments with pancentromeric probes showed that the MN induction of CIT is due to aneugenic effects which cannot be detected in SCGE assays. Bacterial mutagenicity tests with and without enzyme homogenate from HepG2 cells (which contains only active phase I enzymes) failed to detect mutagenic effects of the mycotoxins. OTB was devoid of activity in all three tests. Our results show that the different fungal toxins cause DNA-damage in human-derived cells. Therefore consumption of mouldy foods might lead to DNA damage and increased cancer risk in humans.

L8

Application of DNA Microarrays for Immunotoxicological Testing

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The goal of our present research is the development of a DNA microarray as an in vitro toxicological test system to reveal the sensitising potential of chemicals. Dendritic cells from human donors are used to analyse gene expression after treatment with different potential allergens. We developed a DNA microarray containing 65 immune genes, housekeeping genes, negative controls and external spike controls for normalization. Different labelling techniques were tested, especially taking into account the limited amount of immune cells and RNA available. THP-1 cells were stimulated with LPS and gene expression in LPS-treated cells was compared to gene expression in untreated cells. Applying a direct labelling protocol, increased expression of several immune relevant genes could be shown with either 10 μg or only 2 μg of total RNA. However, more upregulated genes could be detected with an indirect labelling method, again using only 2 μg of RNA. Based on these results, we now use the indirect labelling protocol. In order to find candidate genes for the identification of immunotoxic chemicals, different model allergens are applied to dendritic cells and gene expression is compared to gene expression in untreated cells. To establish a general treatment procedure, expression patterns at different time points after application of allergens are compared. Our preliminary results indicate that the microarray technology could provide an in vitro alternative to animal test methods currently available to predict the sensitizing effects of chemicals.

L9

The Prediction of Respiratory Sensitizing Potential of Chemicals in a Modified Local Lymph Node Assay

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The local lymph node assay (LLNA) is a validated method to identify chemical allergens. The test substance is applied topically and the immunogenic potential of the chemical is evaluated by the proliferative response of T cells in the draining lymph nodes. Though the skin is known to be the relevant route of exposure for contact sensitization, experiments have shown that, at least in rodents, dermal application of chemical allergens may also be relevant for the development of respiratory sensitization. Cytokine profiling has been suggested to differentiate between contact and respiratory sensitizers in the LLNA. The dichotomy of T helper cells is a well known phenomenon. Naïve T cells either differentiate to TH1 or TH2 cells characterized by their specific cytokine profile. TH2 cells promote humoral immune function while TH1 cells promote DTH reactions. We used a slightly modified LLNA and a cytometric bead array for the determination of cytokines. Flow cytometric bead arrays consist of bead populations with discrete fluorescence intensity, conjugated with capture antibodies against a set of cytokines. The fluorescence measurement of a second antibody allows the quantitative analysis of cytokines in the test sample. Three contact, three respiratory sensitizers and the irritant SDS have been tested. No difference in TH1 cytokines after ex vivo ConA stimulation was found, but in all animals a high amount of interleukin-4 was induced with respiratory compared to contact sensitizers, SDS or the solvent control thus allowing a clear identification of the sensitizer.