ECDO 2014

22nd Euroconference on Apoptosis

Cell Death & Rejuvenation

1-4 October, Hersonissos, Crete, Greece

Programme &
Book of Abstracts

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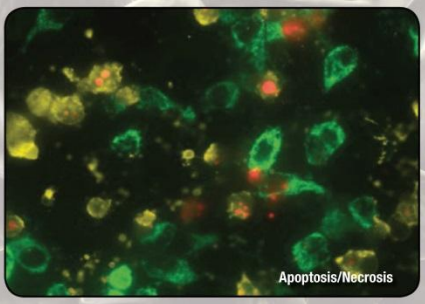

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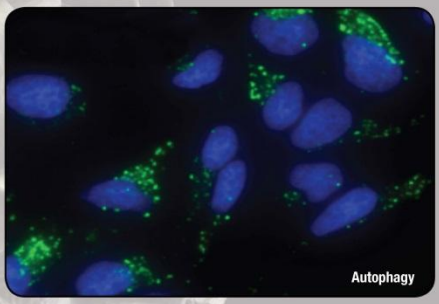

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Wellcome Address

Dear Speakers, Chairpersons and Participants,

It is a great pleasure to welcome you to the 22nd Conference of the European Cell Death Organization, titled "Death and Rejuvenation", in Hersonissos, a picturesque village on the northern coast of the island of Crete, Greece.

The Municipality of Hersonissos extends from the north coastline of central Crete to the imposing mountain chains of Lasithi. Hersonissos is known as one of the most well-organized tourist destination in Crete. It is also known for its high quality hotels, conference infrastructures and the natural beauty of its coasts. Its other side, which is less acknowledged, includes the pristine mainland, where many historical monuments and landscapes of exceptional beauty await to be discovered. At the modern settlement of Hersonissos is the site of the ancient town of Chersonesos, an important seaport from Classical Greece through Byzantine times that served the city of Lyttos. The contemporaneous pleasure port is built over the remains of the Roman port. Some traces of those remains, most of them submerged, are still visible in some places. On the seaside street there is a pyramidal Roman fountain with mosaics of fishing scenes. The vicinity of Hersonissos is noted for its prehistoric archaeological finds. Hersonisos boasts a number of sandy beaches all along the coast, while a number of lovely small coves can be found both to the west as well as to the east of the village.

The ECDO Conference on Cell Death has a long tradition and is established as a scientifically excellent and intense meeting. The focus of the conference, the special attention to poster presentations and short oral communications, coupled with the common lunches and social programs create an atmosphere of stimulating discussions and interactions. Therefore, the ECDO conference is considered as one of the best meetings in the field of cell death research. This year's specific focus on ageing and regeneration exemplifies the ECDO trend of highlighting cutting-edge developments in the field.

With this opportunity, I would like to thank all invited speakers, poster presenters and participants who have contributed to the outstanding quality of the meeting. I would also like to express my sincere gratitude to the ECDO Board, for an exceptional scientific program and thematic focus, this year. I

would also like to thank the ECDO Secretary Veronique Vandevoorde for her valuable help in preparing the conference. Finally, I wish to gratefully acknowledge the hard work of Georgia Houlaki and the local team of people from the Institute of Molecular Biology and Biotechnology, who have, most skilfully and selflessly, taken care of all logistics matters pertinent to the conference.

On behalf of ECDO and the local organizers, I wholeheartedly wish you a very pleasant and productive stay in Crete!

Nektarios Tavernarakis
Chair of the 22nd ECDO Conference on Cell Death

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ECDO 2014 - Invited Speakers

Darren Baker
Dept. Pediatric & Adolescent Medicine, Mayo Clinic, Rochester

Jiri Bartek
Genomi Integrity, Danish Cancer Society, Kopenhagen

Patricia Boya
Centro de Investigaciones Biológicas, CIB-CSIC, Madrid

Patrice Codogno
ISERM, Univ. Paris-Sud 11, Chatenay-Malabry

Gerald Cohen
MRC, Toxicology Unit, Liverpool

Ivan Dikic
Institut für Biochemie II, Goethe-Universität, Frankfurt

Brigitte Galliot
Dept. Genetics & Evolution, Univ. Geneva

Carlos Ibanez
Dept. Neuroscience, Karolinska Institute, Stockholm

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Institute Oncology-Pathology, Karolinska Institute

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Guido Kroemer
INSERM Cordeliers Research Center, Univ. Paris Descartes

Thomas Langer
Institute for Genetics, Univ. Cologne

Xin Lu
The Ludwin Institute for Cancer Research, Oxford

Frank Madeo
Institute Molecular Biosciences, Univ. of Graz

Seamus Martin
Dept. of Genetics, Trinity College, Univ. of Dublin

Gerry Melino
MRC, Toxicology Unit, Univ. of Leicester

Manolis Pasparakis
Institute of Genetics, Univ. of Cologne

Kodi Ravichandran
Dept. Microbiology, Immunology & Cancer Biology, Univ. of Virginia, Charlottesville

David Rubinsztein
Cambridge Inst. Medical Research, Univ. of Cambridge

Kevin Ryan
Beatson Institute for Cancer Research, Glasgow

Luca Scorrano
Medical School,
Univ. of Padova

Nektarios Tavernarakis
IMBB-FORTH &
Univ. of Crete,
Heraklion

Boris Turk
Dept. Biochemistry & Molecular Biology, Stefan Institute, Ljubljana

Peter Vandenabeele
VIB Inflammation Research Center,
Univ. of Gent Gent

David Wallach
Dept. Biological Chemistry,
Weizmann Inst. of Science, Rehovot

Ding Xue
Molecular, Cellular &
Developmental Biology,
Univ. of Colorado, Boulder

Junying Yuan
Dept. Cell Biology, Univ. of Harvard Medical School, Boston

ECDO 2014
22nd Euroconference on Apoptosis "Cell Death & Rejuvenation"

• PROGRAMME •

WEDNESAY, October 1st

15:00 - 19:00 **Registration**

Official Opening & ECDO Honorary Lecture

Chair: **Nektarios TAVERNARAKIS,** IMBB-FORTH, University of Crete, Heraklion, Greece

19:00 - 19:15 **Welcome Address**

19:15 - 20:00 ECDO Honorary Lecture
Gerry COHEN [University of Liverpool, UK]
BCL-2 Inhibitors and Cancer Chemotherapy

20:00 **Welcome Reception**

THURSDAY, October 2nd

Session 1: Cell Death Molecular Mechanisms and Pathways

Chair: **Marie-Lise GOUGEON** [Institut Pasteur, Paris, France]

09:00 - 09:30 **Gerry MELINO** [MRC Toxicology Unit, Leicester, UK]
TAp73 Regulates Tumour Angiogenesis by Promoting Hypoxia-inducible Factor-1α Degradation

09:30 - 10:00 **Jiri BARTEK** [Danish Cancer Society, Kopenhagen, Denmark]
Cellular Responses to Genotoxic Stress: Mechanisms and Relevance for Tumorigenesis and Cancer Treatment

10:00 - 10:30 **Boris TURK** [Stefan Institute, Ljubljana, Slovenia]
Lysosomes: at the Crossroad between Cell Death and Survival

10:30 - 11:00 Coffee Break

11:00 - 11:30 **Kodi RAVICHANDRAN** [University of Virginia, Charlottesville, USA]
Benefits of Apoptotic Cell Recognition and Cell Clearance

11:30 - 11:45 ***Short Talk 1:***
Liming SUN [Institute of Biochemistry and Cell Biology, Shanghai, China]
Necrosome Core Machinery: MLKL

11:45 - 12:00 ***Short Talk 2:***
Guy BROWN [University of Cambridge, UK]
Caspace-8 Inhibition Induces Necroptosis Specifically in Activated Microglia, Preventing their Phagoptosis of Neurons

12:00 - 12:30 **Peter VANDENABEELE** [VIB Inflammation Research Center, Gent, Belgium]
Molecular Mechanisms of Necroptosis: Old Paradigms Come Back

12:30 - 13:00 **Carlos IBANEZ** [Karolinska Institute, Stockholm, Sweden]
Genetic Dissection of Death Receptor Signalling

13:15 - 14:30 Lunch Break

Session 2: Inflammation

Chair: **Peter VANDENABEELE** [VIB Inflammation Research Center, Gent, Belgium]

14:30 - 15:00 **Seamus MARTIN** [Trinity College, Dublin, Ireland]
Cell Death and Inflammation

15:00 - 15:30 **George KOLLIAS** [BSRC "Alexander Fleming", Athens, Greece]
TNFR Signalling in the Mesenchyme and Physiological Roles in Chronic Inflammatory Disease

15:30 - 15:45 ***Short Talk 3:***
Geert VAN LOO [Ghent University, Belgium]
A20 in Inflammatory Signaling and Pathology

15:45 - 16:15 **Manolis PASPARAKIS** [CECAD Research Center, Cologne, Germany]
RIP Kinases in Cell Death and Inflammation

16:15 - 16:45 **Junyin YUAN** [Harvard Medical School, Boston, USA]
Regulation of the RIP1 Kinase at the Crossroad of Cell Death and Inflammation

17:00 - 18:30 **POSTER SESSION 1**

17:00 - 18:00 **ECDO Board Meeting**

20:00 **Gala Dinner**

FRIDAY, October 3rd

Session 3: Degradation Processes and Cell Death

Chair: **Mauro PIACENTINI** [University of Rome, Italy]

09:00 - 09:30 **Ding XUE** [University of Colorado, Boulder, USA]
Targets of Caspases -- Death by A Few Cuts

09:30 - 10:00 **David WALLACH** [Weizmann Institute of Science, Rehovot, Israel]
Anti-inflammatory Functions of Caspase-8

10:00 - 10:30 **Ivan DIKIC** [Goethe University School of Medicine, Frankfurt, Germany]
Linear Ubiquitination in Cell Death Pathways

10:30 - 11:00 Coffee Break

11:00 - 11:30 **Kevin RYAN** [Beatson Institute for Cancer Research, Glasgow, UK]
Autophagy in Cell Death and Cancer

11:30 - 11:45 ***Short Talk 4:***
Luizi LEANZA [University of Padua, Italy]
Targeting and Ion Channel for Selective Elimination of Cancer Cells in vivo

11:45 - 12:00 ***Short Talk 5:***
Joseph OPFERMAN [St. Jude Children's Research Hospital, Memphis, USA]
Identifying Strategies to Inhibit Anti-Apoptotic MCL-1 in Philadelphia Chromosome Acute Lymphoblastic Leukemia

12:00 - 12:30 **Bertrand JOSEPH** [Cancer Centrum Karolinska, Stockholm, Sweden]
The Return of the Nucleus: Epigenetic Control of Autophagy

12:30 - 14:00 Lunch Break

Session 4: Autophagy

Chair: **Alexander PINTZAS** [National Hellenic Research Foundation, Athens, Greece]

14:00 - 14:30 **Guido KROEMER** [INSERM, Paris, France]
Therapeutic Modulation of Autophagy

14:30 - 15:00 **David RUBINSZTEIN** [Cambridge Institute for Medical Research, Cambridge, UK]
Autophagy - from Neurodegeneration to the Plasma Membrane

15:00 - 15:30 **Patrice CODOGNO** [INSERM, Paris, France]
STK38 Kinase is a New Beclin 1 Partner required for Autophagosome Formation

15:30 - 15:45 ***Short Talk 6:***
Andreas VILLUNGER [Medical University Innsbruck, Austria]
Combined Loss of the BH3-only Protein Bim and Bmf Restores B Cell Development and Function in TACI-Ig Mice

15:45 - 16:00 ***Short Talk 7:***
Sylvie LEFEBVRE [Inflammation Research Center, VIB, Ghent, Belgium]
The Kinase RIPK1 Acts as a Guardian of Skin Homeostasis

16:00 - 16:30 **Patricia BOYA** [Spanish National Research Council, Madrid, Spain]
Autophagy, Cell Death and Aging

16:30 - 18:30 **POSTER SESSION 2**

ECDO Keynote Lecture & General Assembly

Chair: **Guido KROEMER** [University Paris Descartes, Paris, France]

18:30 - 19:15 ECDO Keynote Lecture
Xin LU [The Ludwig Institute for Cancer Research, Oxford, UK]
From Cell Death to Sudden Death: Cardiotoxicity Revisited

19:15 - 19:30 **Poster Awards**

19:30 - 20:00 **ECDO General Assembly**

SATURDAY, October 4th

Session 5: Ageing and Regeneration

Chair: **Boris ZHIVOTOVSKY** [Karolinska Institute, Stockholm, Sweden]

09:00 - 09:30 **Frank MADEO** [University of Graz, Austria]
From Dying Yeasts to Longevity Drugs

09:30 - 10:00 **Daren BAKER** [Mayo Clinic, Rochester, USA]
Targeted Apoptosis to Improve Animal Healthspan

10:00 - 10:30 **Brigitte GALLIOT** [University of Geneva, Switzerland]
Cell Death, a Program to Regenerate in Hydra

10:30 - 11:00 Coffee Break

11:00 - 11:30 **Luca SCORRANO** [University of Padova, Italy]
Keeping Mitochondria in Shape: A Matter of Life and Death

11:30 - 12:00 **Thomas LANGER** [University of Cologne, Germany]
Mitochondrial Quality Control and Neurodegeneration

12:00 - 12:15 ***Short Talk 8:***
Anne HAMACHER-BRADY [German Cancer Research Center, Heidelberg, Germany]
Canonical BH3-only Proteins Trigger Macroautophagy-independent, Ubiquitin-mediated Intramitochondrial Processing

12:15 - 12:45 **Nektarios TAVERNARAKIS** [IMBB-FORTH, University of Crete, Heraklion, Greece]
Coordination of Mitophagy and Mitochondrial Biogenesis during Ageing

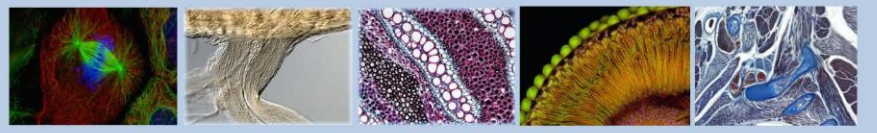
12:45 - 13:00 **Closing Remarks**

Abstracts
Oral Presentations

The abstracts are in
chronological order according to the programme
of the Conference

• ORAL PRESENTATIONS •

Scientific Imaging & Microscopy



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BCL-2 Inhibitors and Cancer Chemotherapy

Gerald M Cohen
Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, UK

Key observations of Kerr, Wyllie and Currie in the 1970s described the importance of programmed cell death and apoptosis in cancer. Subsequent work by Bob Horvitz and colleagues recognised the critical importance of three genes, CED-3, CED-4 and CED-9, in developmental cell death in the nematode *C. elegans.* This was followed by the identification of the mammalian homologues of these genes and a rapid explosion of our understanding of fundamental mechanisms of apoptosis. Resistance to apoptosis is recognised as one of the hallmarks of cancer. Subsequent studies in numerous laboratories in academia and industry have tried to utilise this knowledge to treat various diseases including cancer. Many avenues have been pursued including stimulation of death receptors with their cognate ligands, such as TRAIL, activation of p53 or caspases, SMAC mimetics and inhibitors of the BCL-2 family of proteins although the magic bullet still seems to allude us. Some of the progress and pitfalls of the development of BCL-2 inhibitors will be discussed.

Tap73 Regulates Tumour Angiogenesis by Promoting Hypoxia-Inducible Factor-1α Degradation

Ivano Amelio[1], Satoshi Inoue[2], Tak Wah Mak[2], and **Gerry Melino[1,3]**
[1] *MRC Toxicology Unit, LeicesterLE1 9HN, United Kingdom*
[2] *The Campbell Family Cancer Research Institute, Toronto, Ontario M5G 2M9, Canada*
[3] *University of Rome Tor Vergata, Rome, Italy (melino@uniroma2.it)*

p73 is a member of the p53 family, transcribed as two distinct isoforms TAp73 and ΔNp73, containing or not the N-terminal transactivation domain. P73 is involved in female infertility and maternal reproduction as well as in cancer formation. TAp73 knockout mice show high tumor incidence with hippocampal dysgenesis. Conversely, ΔNp73 knockout mice show a very low incidence of cancer, with sign of moderate neurodegeneration with a significant loss of cellularity in the cortex. This indicate a tumor suppressor role for TAp73 and an oncogenic role for ΔNp73. We identified several transcriptional targets, the mechanisms of regulation of cell death, and the protein degradation pathway, regulated by c-Abl, or by the Ub E3 ligases ITCH and FBXO45.

Here, we demonstrate that the transcription factor TAp73 opposes HIF-1 activity through a non-transcriptional mechanism, thus affecting tumour angiogenesis. TAp73-deficient mice have an increased incidence of spontaneous and chemically induced tumours that also display enhanced vascularisation. Mechanistically, TAp73 interacts with HIF-1α, promoting HIF-α polyubiquitination and consequent proteasomal degradation. In human lung cancer, TAp73 strongly predicts good patient prognosis, and its expression is associated with low HIF-1 activation and angiogenesis. These findings demonstrate a novel mechanism for HIF-1 regulation and provide an additional explanation for the molecular basis of the growth, progression, and invasiveness of human cancers.

Cellular Responses to Genotoxic Stress: Mechanisms and Relevance for Tumorigenesis and Cancer Treatment

Jiri Bartek
Danish Cancer Society Research Center, Copenhagen, Denmark

Biological response to DNA damage is a fundamental biological mechanism ensured through a complex network of DNA damage signaling and effector pathways, the latter including cell-cycle checkpoints, DNA repair and many other aspects of cell physiology. Malfunction of this network predisposes, or contributes to development of life-threatening pathologies including cancer, neurodegeneration, immunodefficiency and premature aging. The lecture will present recent data from our laboratory on both the basic mechanisms of DNA damage response (DDR) including links with cell death and autophagy pathways, and its involvement in tumorigenesis and treatment. Highlights from our multiple high-throughput siRNA-based and SILAC-proteomic screens for novel DDR factors, and their functional role in genome maintenance, will also be presented.

Furthermore, our recent results on the role of the DDR machinery and its relationship with the ARF-p53 pathways in protection against oncogenes and loss of tumor suppressors, as well as the key role of DNA replication stress in oncogenesis and genetic instability, aneuploidy and hence tumor heterogeneity, will be discussed. In terms of the ARF pathway, our data on a novel function of this important tumor suppressor in regulation of mitochondrial metabolism and interplay with the anti-apoptotic protein BCL-XL, and the significance of this function for melanomagenesis, will be presented.

Exploitation of the DDR defects in tumors as targets for innovative treatments, and their value as predictive markers to guide individualized cancer therapy, and potential vulnerabilities of cancer stem cells, will be presented. The DDR-targeted therapy will be illustrated by responses of human glioblastoma stem cells, and breast cancer to PARP inhibitors. In the latter scenario, we have discovered several factors whose loss causes 'synthetic viability' (enhanced fitness of cancer cells) when occurring in BRCA1-defective tumors, thereby also resulting in acquired resistance to PARP inhibitors.

Selected references: Jackson SP, Bartek J. *Nature*, 461, 1071-8 (2009); Bartkova J, et al. *Nature*, 434: 864-70 (2005); Doil C et al. *Cell*, 136, 435-446 (2009); Lukas C et al. *Nature Cell Biol*; 13, 243-253 (2011); Bartkova J, et al. *Nature*, 444: 633-7 (2006); Halazonetis, T. D., Gorgoulis, V. G., and Bartek, J. *Science***,** 319, 1352-1355 (2008); Bouwman P et al. *Nature Struct Mol Biol.*17, 688-95 (2010); Lukas J, Lukas C, Bartek J. *Nature Cell Biol.* 13, 1161-1169 (2011); Takacova S. et al. *Cancer Cell* , 21: 517-31 (2012); Gudjonsson T et al., *Cell* 150: 697-709, (2012), Burrell R. et al. *Nature* 494: 492-496 (2013); Velimezi G. et al. *Nature Cell Biol*. 15:967-77 (2013), Watanabe S. et al. *Nature Struct. Mol. Biol*, 20:1425-33 (2013); Toledo L. et al., *Cell*, 155: 1088-103 (2013); Burrell R. et al. *Nature* (insight review) 501:338-45 (2013). Kumar A. et al. *Cell*, 158: 633-646 (2014).

Lysosomes: at the Crossroad between Cell Death and Survival

Urska Repnik[1], Marusa Hafner Cesen[1], **Boris Turk[1,2,3]**
[1] *Department of Biochemistry, Molecular and Structural Biology, Jozef Stefan Institute, Jamova cesta 39, SI-1000 Ljubljana, Slovenia*
[2] *Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Jamova cesta 39, SI-1000 Ljubljana, Slovenia*
[3] *Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia*

Lysosomes, late endosomes, and hybrid organelles are collectively known as late endocytic compartments. In their acidic lumen, cargo material derived from endocytosed and phagocytosed extracellular material and autophagy-derived intracellular material is degraded by an arsenal of over 50 hydrolases. However, during lysosomal membrane permeabilization (LMP), the function of endo/lysosomal compartment is affected and the luminal contents including lysosomal proteases are released into the cytosol to various extents. LMP was thus shown to initiate the lysosomal apoptotic pathway and using the lysosomotropic detergent L-leucine-L-leucine-OMe (LLOMe), but not siramesine, we could identify lysosomal cysteine cathepsins as the critical players in the pathway. Following their release into cytosol, they activate Bid and degrade several antiapoptotic members of Bcl2 family leading to subsequent mitochondrial membrane permeabilization, which was found to be a critical downstream event in apoptosis. We have also investigated the effects of a subapoptotic LLOMe concentration on the endosomal/lysosomal compartment. We could demonstrate that LLOMe causes reversible damage to lysosomes which include short-lasting lysosomal membrane destabilization with a rise in pH and the inactivation and degradation of cysteine cathepsins that have not been translocated into the cytosol and remained inside the vesicles. The functional consequence includes the accumulation of enlarged late endosomes/endolysosomes. Within 48 hours HeLa cells replenish the arsenal of cysteine cathepsins and thereby their degradative capacity so that they are able to process the content of late endosomes and restore a steady-state autophagic flux. Newly synthesized cysteine cathepsins are delivered to the enlarged late endosomes, which indicates their role as a central digestive organelle of the cell. The mechanism of lysosomal cysteine cathepsin release itself excludes a protective role of autophagy at the early stage of lysosomal apoptotic pathway. However, such attenuated lysosomal degradation represents a therapeutic window for the application of other cytotoxic agents to counteract the cytoprotective role of autophagy.

Benefits of Apoptotic Cell Recognition and Cell Clearance

Chang Sup Lee[1,2], Ignacio J. Juncadella[1,2], Nozomi Takahashi[3], Tom Vanden Berghe[3], Peter Vandenabeele[3] and **Kodi S. Ravichandran[1,2]**

Department of Microbiology, Immunology, Cancer biology[1], and the Center for Cell clearance[2], University of Virginia, Charlottesville, Virginia 22908, USA. Molecular Signaling and Cell Death Unit[3], Inflammation Research Center (IRC), VIB, University of Ghent, Ghent, Belgium.

Abstract withdrawn by authors from online

Necrosome Core Machinery: MLKL

Liming Sun
Institute of Biochemistry and Cell Biology, SIBS, CAS

Genetic studies in *C. elegans* showing that the caspase CED-3 functions at the most downstream position in a linear apoptotic pathway suggested this enzyme is the executioner of programmed cell death. Moreover, biochemical studies in mammalian cells demonstrating the requirement of caspase activities for most of morphological and biochemical changes associated with apoptotic death further established such a role of caspases in apoptosis. Although **apoptotic cell death could be inhibited by a caspase inhibitor, such a treatment could not save the cells from dying but rather shifted the balance towards necrotic death**.

Necrosis is a kinase initiated death pathway. The RIP kinases respond to and link the death signals to the down stream substrate, MLKL. However, within the past few years, it has becoming clear that even one single step forward for the **signal transduction from RIP3 to MLKL built on one of the most formidable engine of regulation of all.** MLKL is a pseudo kinase, which doesn't have the bona fide kinase activity. Apart from the notion that MLKL receives death signal from its kinase RIP3 by the direct phosphorylation modification, it was not at all obvious how MLKL transduces signals to its down stream effectors. Recent studies on the signal transduction **using chemical tools and biomarkers support the idea that MLKL is able to make sense of the core machinery of necrosome.** The key role of MLKL in necrosis signaling sheds light on the logic underlying this unique cell death pathway.

It has been known that downstream necrosis signals works through MLKL. Accordingly, phosphorylated MLKL would be expected to transduce death signals. Both in vivo and in vitro biochemical analyses characterized the oligomerization nature of MLKL. Our recent data demonstrated that **phosphorylation of MLKL turns on its oligomerization formation.** Biochemical fractionation revealed that MLKL translocate to plasma membrane after necrosis was induced. Using the classical liposome leakage assay, **MLKL was found to translocate to the PIPs containing membranes and disrupt the membrane integrity in a dose dependent manner.** And this lipid binding property depends on the phosphorylation modification of MLKL. Besides, necrosis inhibitor **NSA could block MLKL membrane association.** Since NSA target the N-terminal MLKL Cys86, this discovery is consistent with the finding that the N-terminal domain mediates liposome damage and cell death.

Summary:
Our recently progress on the necrosis signaling advanced our knowledge on the core machinery, MLKL activation and highly ordered organization on liposome structures disruption.

Caspase-8 Inhibition Induces Necroptosis Specifically in Activated Microglia, Preventing Their Phagoptosis of Neurons

Guy C. Brown, Anna Vilalta, Aviva M. Tolkovsky, Jonas Neher & Michael Fricker
Department of Biochemistry, University of Cambridge, UK

Microglia are resident brain macrophages, which can cause neuronal loss when activated in infectious, ischaemic, traumatic and neurodegenerative diseases. Caspase-8 has both pro-death and pro-survival roles, mediating apoptosis and/or preventing RIPK1-mediated necroptosis depending on cell type and stimulus. We found that inflammatory stimuli (LPS, LTA or TNF-alpha) caused an increase in caspase-8 IETDase activity in primary rat microglia without inducing apoptosis. Inhibition of caspase-8 with either zVAD-fmk or IETD-fmk resulted in necrosis of activated microglia. Inhibition of caspases with zVAD-fmk did not kill non-activated microglia, or astrocytes and neurons in any condition. Necrostatin-1, a specific inhibitor of RIPK1, prevented caspase inhibition-induced microglial death, indicating death was by necroptosis.

It is generally assumed that microglia only phagocytose dead or dying neurons, however, we find that under inflammatory conditions microglia can also phagocytose live neurons and thereby kill them, a form of cell death called 'phagoptosis'. We find that microglial phagocytosis of stressed-but-viable neurons is mediated by the exposure of the 'eat-me' signal phosphatidylserine (PS) on neurons, which is bound by the adapter protein MFG-E8, which induces phagocytosis via the vitronectin receptor on microglia. Reversible PS exposure on viable neurons can be induced by low levels of glutamate, oxidants, of activated microglia, but in the presence of activated microglia this neuronal PS exposure induces their phagocytosis.

We found inflammatory activation of co-cultures of microglia and neurons results in progressive loss of neurons (without any apparent cell death), which is accompanied by microglial phagocytosis of neurons, and is prevented by blocking phagocytosis or phagocytic signalling. Neuronal loss induced by nanomolar amyloid-beta or LPS is absent in cultures from MFG-E8 knockout mice, but is reconstituted by adding wild-type MFG-E8. LPS-induced neuronal loss in vivo is reduced by co-injection of phagocytosis inhibitors or in MFG-E8 knockout mice. MFG-E8 knockout mice and MerTK mutant rats are strongly protected against

brain damage induced by transient ischaemia.

Inhibition of caspase-8, resulting in microglial necroptosis, prevented the neuronal loss/death caused by activated microglia, but this loss was restored by rescue of microglia with necrostatin-1. We conclude that the activation of caspase-8 in inflamed microglia prevents their death by necroptosis, and thus caspase-8 inhibitors may protect neurons in the inflamed brain by selectively killing activated microglia, which otherwise eat stressed-but-viable neurons resulting in their death by phagoptosis. Thus caspase-8 inhibition is a potential therapy to prevent inflammatory neuronal loss in the brain.

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Regulation and Execution of Necroptosis: Molecular Mechanisms *in vitro* and *in vivo*

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Until recently necrosis was generally considered as an accidental form of cell death due to physicochemical injury leading to swelling of the cell (oncosis), plasma membrane rupture and release of intracellular content. Recently a regulated form of necrosis has been identified, coined necroptosis, in which Receptor Interacting Protein Kinase 1 (RIPK1), RIPK3, and Mixed Lineage Kinase Like (MLKL) protein play a crucial role by the formation of the so-called necrosome complex. Many factors can regulate this necrosome complex formation and in this way sensitize or desensitize the necrotic cell death pathway. Genetic ablation of these genes as well as the development of RIPK1 inhibitors, the so called necrostatins, has provided crucial tools to study necroptosis in experimental disease models. These studies reveal that necroptosis can be targeted and that it may play a crucial role in many different important inflammatory, infectious, ischemia-reperfusion injury (IRI) and neurodegenerative diseases such as kidney and heart transplantation, cardiac infarction and brain trauma, acute pancreatitis, inflammatory bowel disease, ophthalmology diseases, skin inflammation, systemic inflammatory response syndrome (SIRS) diseases following TNF administration or bacterial infection, artherosclerosis and viral infection. Recent studies revealed that RIPK1, independent of its kinase activity, acts as a crucial survival factor in epithelial cells. By this way it functions as a guardian in the protection of epithelial cells against apoptotic and necroptotic cell death in intestine and skin, respectively.

Little is known about the precise execution mechanism of necroptosis. These involve lysosomal membrane permeabilizaton, complex I mediated ROS production, phospholipase A2 activity. Activation of receptor interacting kinase 3 (RIPK3) and subsequent phosphorylation of mixed-lineage kinase domain-like (MLKL) by RIPK3 represent a convergence point of many necroptotic pathways, highlighting the central role of MLKL in the induction of this cell death modality. However, the function of MLKL during necroptosis and the final execution

mechanism of necroptosis are still poorly understood. Here, we demonstrate that the full 4-helical bundle domain (4HBD) in the N-terminal region of MLKL is absolutely required for the induction of necroptosis. Furthermore, we present the recruitment mechanism of MLKL to the plasma membrane and suggest a model how MLKL contributes to the final execution mechanism of necroptosis, and the possible implication of lysosomal proteases in that.

Genetic Dissection of Death Receptor Signaling

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The p75 neurotrophin receptor (p75NTR), a member of the death receptor family, is a key regulator of trophic and injury responses in the nervous system. We have recently described a genetic approach to dissect p75NTR signaling and decipher its underlying logic. p75NTR was found to be activated by conformational rearrangement of disulphide linked dimers. Mutation of a highly conserved transmembrane cysteine selectively precluded the ability of the receptor to signal in response to neurotrophins but not to myelin-derived ligands, suggesting the existence of distinct, ligand-specific mechanisms of activation in p75NTR. Structural determinants important for regulation of cell death, NF-kB and RhoA pathways were identified in the p75NTR death domain. Pro-apoptotic and pro-survival pathways mapped onto non-overlapping epitopes, demonstrating that different signaling outputs can be genetically separated in p75NTR. These results provide new insights into the logic of p75NTR signaling and pave the way for a genetic dissection of p75NTR function and physiology.

Cell Death and Inflammation: Revisiting the Danger Model

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Inflammation is the set of reactions seen in response to infection or tissue damage and is critical for the recruitment of cells of the innate immune system to the correct location, as well as for the initiation of adaptive immune responses. The inflammatory response also initiates the process of tissue repair and the restoration of normal tissue integrity. Apoptosis is typically considered to be a non-inflammatory mode of cell death whereas Necrosis, as well as a recently described mode of programmed necrosis called Necroptosis, are considered to be pro-inflammatory. However, things may not be quite so simple as they might seem. Many physiological triggers of apoptosis, such as TNF, Fas and TRAIL, can elicit the production of pro-inflammatory cytokines and the effects of Necroptosis on the production of such cytokines is unclear. I will discuss the role of cell death stimuli as modulators of inflammation and the effects of apoptosis and necroptosis on these inflammatory signals. In contrast to the prevailing view, I will present data to argue that Necroptosis may be an anti-inflammatory mode of cell death in certain settings and that Apoptosis is not necessarily non-inflammatory.

TNFR Signalling in the Mesenchyme and Physiological Roles in Chronic Inflammatory Disease

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Abstract withdrawn by authors from online

A20 in Inflammatory Signaling and Pathology

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Different mechanisms control the dynamics of NF-κB activation assuring a tight regulation of inflammatory responses, and several autoregulatory feedback loops terminating the NF-κB response have been described. The ubiquitin-editing protein A20 (also known as TNFAIP3) is well known for its anti-inflammatory and protective activities, and genetic studies in humans identified polymorphisms in the A20/TNFAIP3 locus associated with multiple inflammatory and auto-immune pathologies including inflammatory bowel diseases (IBD) and rheumatoid arthritis.

To assess the role of A20 in intestinal inflammation and IBD development, we generated mice that are specifically deficient for A20 in intestinal epithelial cells (IECs). These mice develop normal intestinal epithelium without spontaneous inflammation, but are hypersensitive to experimental colitis due to increased IEC sensitivity to apoptosis (Vereecke et al., J. Exp. Med., 2010). These findings identify A20 as an essential protective factor for epithelial barrier integrity in inflammatory conditions. Mice in which A20 was specifically deleted in myeloid cells spontaneously develop a severe erosive polyarthritis (Matmati et al., Nat. Genet., 2011; Vande Walle et al., Nature, 2014) due to high levels of circulating inflammatory cytokines in their serum caused by a sustained NF-κB activation in macrophages. Through the combined deletion of A20 in both IECs and myeloid cells by intercrossing both mouse lines, we now developed a spontaneous mouse model of colitis characterized by intestinal epithelial apoptosis, Paneth and goblet cell loss, epithelial hyperproliferation and intestinal microbiota dysbiosis (Vereecke et al., unpublished). Together these data clearly define A20 as a crucial inhibitor of NF-κB-dependent inflammation and tissue protective factor *in vivo*.

RIP Kinases in Cell Death and Inflammation

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Programmed cell death has important functions in embryonic development, tissue homeostasis, immunity and inflammation. Apoptosis constitutes the most well studied form of programmed cell death. Recently, RIP kinase 3 (RIPK3) mediated necrosis, termed necroptosis, has been identified as another form of regulated cell death. RIPK1 is implicated in inflammatory and cell death signalling and its kinase activity is believed to drive RIPK3-mediated necroptosis. To study the role of RIPK1 in inflammation and cell death we generated mice with conditional and kinase-inactive RIPK1 alleles. Lack of RIPK1 kinase activity in RIPK1D148N knock-in mice did not cause any spontaneous pathology demonstrating that RIPK1 kinase activity is not essential for mouse development and normal tissue homeostasis. Intestinal epithelial cell (IEC)-specific RIPK1 knockout caused IEC apoptosis, villus atrophy, loss of goblet and Paneth cells and premature death in mice. This pathology developed independently of the microbiota and MyD88 signalling but was partly rescued by TNFR1 deficiency. Epithelial FADD ablation inhibited IEC apoptosis and prevented premature death of $RIPK1^{IEC\text{-}KO}$ mice, yet $RIPK1^{IEC\text{-}KO}/FADD^{IEC\text{-}KO}$ mice displayed RIPK3-dependent IEC necroptosis, Paneth cell loss and focal erosive inflammatory lesions in the colon. Moreover, a RIPK1 kinase inactive knock-in delayed but did not prevent inflammation caused by FADD deficiency in IECs or keratinocytes, showing that RIPK3-dependent necroptosis of FADD-deficient epithelial cells only partly requires RIPK1 kinase

activity. Epidermis-specific RIPK1 knockout triggered keratinocyte apoptosis and necroptosis and caused severe skin inflammation. Importantly, RIPK3 or MLKL deficiency fully prevented skin inflammation in mice with epidermis specific RIPK1 knockout while FADD deficiency had no effect, showing that keratinocyte necroptosis and not apoptosis triggers skin inflammation in these mice. Therefore, RIPK1 prevents skin inflammation by inhibiting RIPK3/MLKL-mediated necroptosis in keratinocytes *in vivo*. Taken together, these results show that kinase-independent scaffolding RIPK1 functions regulate homeostasis and prevent inflammation in barrier tissues by inhibiting epithelial cell apoptosis and necroptosis.

Regulation of Necroptosis

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Regulated cell death has been recognized as a normal physiological process that functions to sculpt tissues during development and maintain tissue homeostasis by eliminating unnecessary or harmful cells. Apoptosis is an important regulated cell death mechanism. However, cells may activate alternative cell death mechanisms when apoptosis is blocked. Necroptosis is a novel RIP1 kinase-dependent necrotic cell death pathway that can be activated under apoptotic deficient conditions. In an effort to understand the mechanisms of necroptosis, we have conducted an extensive screen of small molecule library of ~500,000 compounds. This screen led to the identification of 33 highly potent small molecule inhibitors of necroptosis, termed necrostatins. Necrostatins target RIP1, a key kinase involved in the signaling of necroptosis, as well as regulators of RIP1 and downstream steps. Using necrostatins, we demonstrate that necroptosis plays an important role in mediating acute neurological injuries in vivo. Necrostatins provide powerful tools for exploring the mechanisms of necroptosis and the possibility to target necroptosis as therapeutics for human diseases involving neuronal cell death. Our recent works focus on a new necrostatin, necrostatin-29 (Nec-29), that acts by blocking a signal transduction event in necroptosis downstream from RIP1 kinase. Our work reveals a molecular mechanism involving a redox-regulated event that controls the formation of complex IIb in necroptosis.

Targets of Caspases -- Death by A Few Cuts

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Anti-inflammatory Functions of Caspase-8

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Caspase-8 was discovered in exploring the mechanisms of death induction by cytokines of the TNF family. Studies in transgenic mice models indicated that it serves several different functional roles including inhibition of inflammation. Control of inflammation can be mediated both via protein-synthesis dependent effects and through induction of necrotic cell death that occurs in a protein-synthesis independent manner (yielding release of Damage Associated Molecular Patterns). The finding that caspase-8 – a signaling protein controlling cell death induction – also has pronounced effects on inflammation, poses a challenge to our ability to define the relative contribution of these protein synthesis-dependent and –independent mechanisms to inflammation in distinct in vivo situations. The approaches that we are taking to explore this distinction will be briefly overviewed.

Linear Ubiquitination in Regulation of Cell Death and Inflammation

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Linear Ubiquitin chain Assembly Complex (LUBAC) is an E3 ligase complex that specifically generates linear (Met1-linked) ubiquitin chains. LUBAC consistis of a catalytic protein, HOIP, and two other critical subunits, Sharpin and HOIL-1. Mice with Sharpin deficiency develop spontaneously chronic proliferative dermatitis (cpdm) characterized by keratinocyte apoptosis and severe inflammatory skin lesions accompanied by inflammation in other organs including the lung and the liver. Epidermal keratinocyte-restricted ablation of TNFR1 fully prevents the development of skin lesions in Sharpin-deficient mice, demonstrating that keratinocyte-intrinsic TNFR1 signalling triggers skin inflammation. In addition, keratinocyte-specific ablation of TNFR1 associated death domain protein (TRADD), an adapter that is required for TNFR1-mediated apoptosis but not necroptosis, also prevented skin lesion formation in Sharpin-deficient mice. Keratinocyte restricted ablation of Fas associated death domain protein (FADD) in combination with systemic RIP3 deficiency fully prevented keratinocyte apoptosis and skin inflammation. Mechanistically, LUBAC modifies FADD with linear ubiquitin chains and linear di-ubiquitin fused to FADD is sufficient to block activation of caspase-8 in response to Fas ligand and TNF stimulation. In contrast, rapid apoptosis was induced in cells expressing a FADD lysine-less mutant that cannot be ubiquitinated. Collectively, these results suggest that Sharpin prevents inflammation by inhibiting TNFR1-mediated apoptosis via the linear ubiquitination of FADD.

Autophagy in Cell Death and Cancer

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Targeting an Ion Channel for Selective Elimination of Cancer Cells in vivo

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Ion channels are emerging oncological targets. In particular, potassium-selective channels show a de-regulated expression in different tumor cells/tissues compared to healthy ones, representing thus possible targets for the development of new chemotherapeutic drugs. Furthermore, pharmacological targeting of ion channels in mitochondria proved to be a promising strategy, in accordance with the essential role of ion channels for the regulation of bioenergetics in these organelles and with the crucial role of mitochondria in apoptotic signaling.

Pharmacological inhibition of the a mitochondrial potassium channel (mtKv1.3) by membrane permeant blockers, Psora-4, PAP-1 and clofazimine, indeed triggered apoptotic cell death in different cancer cell lines, even in the absence of Bax and Bak, by inducing mitochondrial membrane potential depolarization, production of mitochondrial ROS and release of cytochrome c. Apoptosis upon incubation with the drugs did not occur when expression of Kv1.3 was downregulated by siRNA.

Importantly, the Kv1.3 inhibitor clofazimine, a drug already used in clinic to treat leprosy and autoimmune diseases, reduced melanoma volume up to 90% compared to the untreated mouse in an orthotopic mouse melanoma model. Furthermore, Psora-4, PAP-1 and clofazimine were able to selectively kill also primary Chronic Lymphocytic Leukemia B cells (B-CLL), without affecting normal blood cells of the same patients and independently of the currently used prognostic factors. An increased ROS production together with an increased Kv1.3 expression in B-CLL cells seems to account for the selective apoptosis-inducing ability of the drugs.

Recently, we obtained promising in vivo data also in a Pancreatic Ductal Adenocarcinoma (PDAC) mouse model: treatment of mice with clofazimine reduced PDAC tumor weight by 50%.

Since membrane permeant Kv1.3 inhibitors are characterized by poor water solubility, in order to increase their solubility as well as to increase their bioavailability, we have recently synthesized new PAP-1 derivatives. In vitro experiments using different cancer cell lines demonstrate the ability of these novel derivatives to kill tumor cells at significantly lower concentrations with respect to the precursor, still maintaining the specificity toward Kv1.3-expressing cells. The newly synthesized derivatives have been tested also ex-vivo (B-CLL cells) and in vivo tumor models, yielding promising results.

Identifying Strategies to Inhibit Anti-Apoptotic MCL-1 in Philadelphia chromosome Acute Lymphoblastic Leukemia

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The response of Philadelphia chromosome (Ph+) acute lymphoblastic leukemia (ALL) to treatment by BCR-ABL tyrosine kinase inhibitors (TKIs) often results in short remissions typified by rapid outgrowth of drug-resistant clones. In a mouse model of Ph+ B-lineage ALL (B-ALL), MCL-1 expression is dysregulated by the BCR-ABL oncofusion protein and TKI treatment results in loss of MCL-1 expression prior to the induction of apoptosis. Using a mouse model in which conditional allele(s) of Mcl-1 can be deleted either during leukemia transformation or later after the establishment of leukemia, we have demonstrated that endogenous MCL-1's anti-apoptotic activity is essential for promoting survival during BCR-ABL-transformation and in established BCR-ABL+ leukemia.

Small molecule inhibitors (BH3-mimetics) have been designed to target anti-apoptotic proteins as a way to specifically targeting malignant cells. A number of inhibitors are in various stages of development with specific or broad activities against anti-apoptotic BCL-2 family members. To investigate the efficacy, specificity, and biochemical events mediated by small molecule inhibitors, we have developed a mouse genetic system in which we have "reprogrammed" BCR-ABL-transformed B-lineage acute lymphoblastic leukemia (B-ALL) cell lines to render them dependent on exogenous human MCL-1, BCL-2, BCL-xL, BFL-1, or BCL-w. We have used these BCR-ABL B-ALL cell lines to define, in a genetically-controlled setting, how various small molecule BH3-mimetics mechanistically induce cell death and to validate their spectrum of inhibition and specificity of action. Therefore, we submit that this panel of cell lines is an excellent in vitro and in vivo screening tool to test the efficacy and potency of small molecule inhibitors of anti-apoptotic BCL-2 family members.

MCL-1 plays an essential role in promoting the survival of many normal cellular lineages including myeloid cells, lymphocytes, cardiomyocytes, and neurons; therefore, a potential consequence of effective MCL-1 inhibition may be unwanted

toxicities. To circumvent such potential side effects associated with MCL-1 inhibition, we screened for agents that reduce MCL-1 expression in our model BCR-ABL+ leukemia cell lines. Since these agents decrease MCL-1 expression, we hypothesized that they might alter the sensitivity of the cells to other BH3 mimetic therapies such as ABT-263 or ABT-199. Indeed, down modulation of MCL-1 expression synergizes with non-MCL-1 targeted small molecule therapies to induce cell death. Thus, we have revealed a number of methods by which MCL-1's protective effects can be partially antagonized to render previously resistant cell lines sensitive to targeted therapies. This finding may expand the capabilities of BH3 mimetic drugs and may reveal methods to overcome resistance mechanisms.

The Return of the Nucleus: Epigenetic Control of Autophagy

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Autophagy is a conserved process by which cytoplasmic components are degraded by the lysosome/vacuole. Autophagy is commonly seen as a cytoplasmic event, and, hitherto, nuclear events have not been considered of primary importance for autophagy. We uncovered that epigenetic covalent modification of histones regulates the outcome of autophagy. Induction of autophagy, from yeast to mammalian cells, is coupled to Atg5- and Atg7-dependent reduction of H4K16ac through downregulation of the histone acetyltransferase hMOF. H4K16ac chromatin immunoprecipitation (ChIP-Seq) and global run-on sequencing (GRO-Seq) reveal on a genome-wide level that H4K16 deacetylation is associated predominantly with the downregulation of autophagy-related genes. Overexpression of hMOF or antagonizing the activity of SIRT1, a H4K16ac deacetylase, resulted in an upregulation of H4K16ac, alteration in the autophagic flux and the promotion of cell death in cancer cells. Our findings establish that alteration in a specific histone posttranslational modification during autophagy, affects the transcriptional regulation of autophagy-related genes and initiates a regulatory feedback loop, which serves as a key determinant of survival versus death responses upon autophagy induction.

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Therapeutic Modulation of Autophagy

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Caloric restriction (CR), be it constant or intermittent, is reputed to have health promoting, cancer-preventive and lifespan extending effects. Caloric restriction mimetics (CRMs) are compounds that mimic the biochemical and functional effects of CR. Here, we propose a unifying definition of CRMs as compounds that favor the deacetylation of cellular proteins as they stimulate autophagy. This effect can be achieved by three classes of compounds that (i) deplete acetyl coenzyme A (AcCoA), the sole donor of acetyl groups, (ii) inhibit acetyl transferases, a group of enzymes that acetylate lysine residues in an array of proteins, or (iii) stimulate the activity of deacetylases and hence reverse the action of acetyl transferases. We have accumulated evidence that CRMs can be used to induce autophagy in vivo, in mice, and that AcCoA depletion, inhibition of acetyl transferases or stimulation of deacetylases can prolong life span in model organisms through the induction of autophagy. In addition, we have found that artificial replenishment of AcCoA by providing cell-permeable precursors of α-ketoglutarate can inhibit adaptive autophagy induced by starvation, as well as maladaptive autophagy induced by pressure overload in the heart. Our findings point to the possibility of therapeutically modulating autophagy by manipulating the acetylation levels of cellular proteins.

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Autophagy: From Neurodegeneration to the Plasma Membrane

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Intracellular protein aggregation is a feature of many late-onset neurodegenerative diseases, including Parkinson's disease, tauopathies, and polyglutamine expansion diseases (like Huntington's disease (HD)). Many of these mutant proteins, like that causing HD, cause disease via toxic gain-of-function mechanisms. Therefore, the factors regulating their clearance are crucial for understanding disease pathogenesis and for developing rational therapeutic strategies.

The two major intracellular protein degradation pathways are the ubiquitin-proteasome system and (macro)autophagy. Autophagy is initiated by double-membraned structures, which engulf portions of cytoplasm. The resulting autophagosomes ultimately fuse with lysosomes, where their contents are degraded.

I will briefly describe the basic biology of autophagy before outlining its roles in neurodegeneration. We showed that the autophagy inducer, rapamycin, reduced the levels of mutant huntingtin and attenuated its toxicity in cells, and in *Drosophila* and mouse HD models. We have extended the range of intracellular proteinopathy substrates that are cleared by autophagy to other related neurodegenerative disease targets and have provided proof-of-principle in cells, Drosophila and mice. In order to induce autophagy long-term, we have been striving to identify safer alternatives to the mTOR inhibitor, rapamycin. To this end, we have been trying to discover novel components of the autophagy machinery and new signalling pathways and drugs that impact on autophagy. While autophagy induction is protective in models of various neurodegenerative diseases, certain other conditions, including lysosomal storage disorders, are associated with compromised autophagy. Finally, I will describe our recent studies that implicate trafficking events from the plasma membrane as being crucial for autophagosome biogenesis. I will discuss how some of these events are compromised by disease-associated variants.

KEYWORDS: Autophagy, lysosome, neurodegeneration, proteinopathy

STK38 Kinase is a New Beclin 1 Partner required for Autophagosome Formation

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Autophagy plays key roles in development, oncogenesis, cardiovascular, metabolic and neurodegenerative diseases. Hence, understanding how autophagy is regulated can reveal opportunities to modify autophagy in a disease-relevant manner. Here, we describe STK38 as a conserved regulator of autophagy. STK38 interacts with Beclin1, a key regulator of autophagy. By combining molecular, cell biological and genetic approaches, we show that STK38 promotes autophagosome formation in human cells and *Drosophila* larvae. STK38 supports Exo84/Beclin1 and Exo84/RalB interactions required for initiation of autophagosome formation. Upon autophagy induction, STK38 is stimulated in a MOB1- and exocyst-dependent manner. In contrast, RalB depletion triggers hyperactivation of STK38 resulting in STK38-dependent apoptosis, suggesting that STK38 and RalB assist the co-ordination between autophagic and apoptotic events upon autophagy induction. Collectively, our data establish STK38 as a conserved regulator of early autophagic events, further proposing a role for STK38 in determining cellular fate in response to autophagic conditions.

Canonical BH3-Only Proteins Trigger Macroautophagy-Independent, Ubiquitin-Mediated Intramitochondrial Processing

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Pro-apoptotic BH3-only proteins activate mitochondrial outer membrane permeabilization (MOMP), leading to mitochondrial release of proteins into the cytosol essential for activation and execution of caspase-mediated cell death. Damaged mitochondria can be degraded by macroautophagy (mitophagy), suggesting a role for mitophagy in regulating apoptosis. However, mitophagy occurs slower than apoptosis signaling, and caspases can inhibit macroautophagy. To date, for most intrinsic apoptosis scenarios it remains unknown if mitophagy is engaged, and whether mitophagy can influence the mitochondrial capacity to activate or enhance apoptosis. Employing high- and super-resolution microscopy we investigated the interplay of autophagy, lysosomal and ubiquitylation pathways in response to BH3-only protein expression. Thereby, we demonstrate that in parallel to intrinsic apoptosis signaling, the canonical BH3-only proteins tBid, Bim_{EL}, Bik and Bad induce intramitochondrial processing associated with lysosomal and proteasomal activities, in the absence of macroautophagy. We show that upon mitochondrial depolarization XIAP (X-linked inhibitor of apoptosis protein) rapidly translocates to all mitochondria where its E3 ligase activity triggers Bax-mediated MOMP. Concomitantly, mitochondrial ubiquitylation by XIAP directs entry of the endolysosomal machinery into mitochondria, and degradation of the endogenous XIAP inhibitor, Smac. Our findings reveal a functional integration between lysosomes and mitochondria, mediated by XIAP E3 ligase activity, and independent of macroautophagy, that constitutes a novel cellular mechanism with the potential to regulate mitochondrial apoptosis.

The Kinase RIPK1 Acts as a Guardian of Skin Homeostasis

Sylvie Lefebvre, Barbara Gilbert, Giel Tanghe, Linde Duprez, Kirsten Leurs, Corinne Urwyler, Jolien De Munck, Amanda Gonçalves, Riet De Rycke, Saskia Lippens, Vera Goosens, Peter Vandenabeele and Wim Declercq.
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Tumor necrosis factor (TNF) signaling can either induce apoptosis, necrosis or cell survival and inflammation via the transcription factor NF-κB. One major switch between these outcomes is the receptor interacting protein kinase 1 (RIPK1), which has kinase-dependent and kinase-independent functions. Consistent with RIPK1's important role in cell survival, RIPK1 full knock-out mice die at day 3 from extensive cell death. To understand the role of RIPK1 in skin homeostasis and inflammation we developed and analyzed the phenotype of mice lacking RIPK1 in the epidermis ($RIPK1^{EKO}$) by crossing $RIPIK1^{FL/FL}$ with K5-Cre mice. $RIPK1^{EKO}$ mice were born with the expected Mendelian ratio. We confirmed specific ablation of RIPK1 in the epidermis by means of western blotting. After 3 weeks of age, $RIPK1^{EKO}$ mice spontaneously develop severe skin lesions. Macroscopic analysis of the skin revealed areas of normal epidermis along with lesional regions. Microscopic analysis showed that the mutant had thicker and hyperproliferative epidermis in macroscopically normal-looking areas compared to $RIPK1^{FL/FL}$ and in lesional skin. $RIPK1^{EKO}$ skin was also characterized by increased immune cell infiltration in the dermis. Skin samples also revealed increased levels of apoptotic and necrotic keratinocytes in $RIPK1^{EKO}$ mice, suggesting that increased cell death lies at the basis of skin inflammation. Consequently, RIPK1 deficient keratinocytes were more sensitive to TNF-induced cell death compared to wild-type keratinocytes. However, neutralization of TNF by Etanercept or crossing RIPK1EKO mice with TNFR1 deficient mice did only delay the appearance of skin lesions, but did not prevent them. Importantly, RIPK1 kinase-dead knock-in mice did not develop any skin phenotype, suggesting that RIPK1-mediated protection resides in its kinase-independent platform function. These data highlighting a new and unexpected pro-survival platform-dependent function of RIPK1 in skin homeostasis.

Autophagy, Cell Death and Aging

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Apoptosis and autophagy are physiological processes implicated in the maintenance of cell and tissue homeostasis. Multiple signalling mechanisms control the adequate levels of both processes in normal physiology, whereas they appear to be deregulated in various pathological conditions, including neurodegenerative diseases. A decrease in autophagic activity with age observed in many tissues has been proposed to contribute to the aggravation of age-related diseases. We have found a marked reduction in macroautophagic activity in the retina with age, which coincides with an increase in chaperone-mediated autophagy. This increase in chaperone mediated autophagy is also observed during retinal neurodegeneration in the *Atg5flox/flox*; nestin-*Cre* mice, a mouse model with downregulation of macroautophagy in neuronal precursors. The age-related increase in retinal chaperone mediated autophagy markers noticeable contrasts with the decreased activity of this pathway that occurs in other organs with age, and may indicate a prominent role for this pathway in the retina when macroautophagy is compromised. Our results show a tissue-specific cross-talk of different lysosomal proteolytic systems during normal aging.

From Cell Death to Sudden Death: Cardiotoxicity Revisited

Professor Xin Lu
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Molecules that maintain normal cardiac function through p53-dependent and -independent pathways are potential targets of chemotherapy-induced cardiotoxicity. Here, we identify iASPP, an inhibitor of p53, as one such candidate. iASPP deficiency-causing spontaneous mutations are associated with cardiocutaneous disorders in CWH calves and Wa3 mice. In humans, cardiocutaneous disorder often associates with desmosomal dysfunction. We show here that iASPP deficiency specifically induces apoptosis and right ventricular dilation in E16.5 mouse embryos. iASPP$^{\Delta 8/\Delta 8}$ mice die of sudden death, displaying features of Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC). iASPP deficiency causing excessive cardiomyocyte loss was largely rescued in iASPP$^{\Delta 8/\Delta 8}$;p53$^{-/-}$ hearts. Moreover, iASPP anchors desmoplakin and desmin at the intercalated discs and binds desmoplakin, forming a trimeric complex with desmin or keratin to maintain the integrity of desmosomes and intermediate filament networks in cardiomyocytes and keratinocytes, respectively. iASPP, therefore, prevents cardiocutaneous disorder through its ability to inhibit p53-induced apoptosis and maintain desmosome function.

From Dying Yeasts to Longevity Drugs

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Spermidine is a ubiquitous polycation that is synthesized from putrescine and serves as a precursor of spermine. Putrescine, spermidine and spermine all are polyamines that participate in multiple known and unknown biological processes. Exogenous supply of spermidine supresses necrosis and prolongs the life span of several model organisms including yeast (*Saccharomyces cerevisiae*), nematodes (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*) and significantly reduces age-related oxidative protein damage in mice, indicating that this agent may act as a universal anti-aging drug. Spermidine induces autophagy in cultured yeast and mammalian cells, as well as in nematodes and flies. Genetic inactivation of genes essential for autophagy abolishes the life span-prolonging effect of spermidine in yeast, nematodes and flies. These findings complement expanding evidence that autophagy mediates cytoprotection against a variety of noxious agents and can confer longevity when induced at the whole-organism level. We hypothesize that increased autophagic turnover of cytoplasmic organelles or long-lived proteins is involved in many age associated diseases.

Targeted Apoptosis to Improve Animal Healthspan

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Mammalian cells have developed a variety of stress response mechanisms to deal with damage that has occurred within a cell. If the damage is too severe, cells have the potential to undergo programmed cell death.

Alternatively, cells have developed a mechanism to prevent further proliferation of these damaged cells and enforce a permanent cell cycle arrest, known as cellular senescence. Senescent cells are known to negatively impact the cells in the surrounding environment through the secretion of a variety of cytokines, chemokines and matrix metalloproteinases and are observed to increase in frequency with age. We have developed novel tools to trigger a programmed cell death in senescent cells and have found that the elimination of these cells results in healthspan extension in mice. These data suggest that stimulation of apoptosis of senescent cells may have multiple beneficial impacts to organismal health.

Injury-Induced Cell Death, a Program to Promote Regeneration?

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Our laboratory is using *Hydra* a freshwater cnidarian polyp to investigate the mechanisms of animal regeneration as this little cnidarian polyp easily regrows any missing part after bisection, the rather complex head region from the basal side, and the simpler basal disk from the apical side. We previously showed that an immediate wave of cell death is taking place in head-regenerating tips, the dying cells releasing signaling molecules that promote head regeneration (Chera et al. Dev Cell 2009). Regeneration in *Hydra* relies on three stem cell populations that continuously self-renew in the body column and are found terminally differentiated at the extremities. Surprisingly the epithelial cells are highly resistant to signals promoting cell death, whereas the interstitial cells and their derivatives are prone to cell death. We will address here two questions: First what are the injury signals and the signaling cascades that trigger cell death in head-regenerating tips? Second what does regulate the sensitivity to cell death in *Hydra* tissues. On one side we have characterized ROS signals immediately produced upon injury, which activate the MAPK pathway and lead to cell death. On the other side, by investigating the cycling regulation of epithelial and interstitial stem cells, we could show that G2 pausing as well as G2 arrest protects the epithelial cells from cell death. We will discuss the role of injury-induced cell death in animal regeneration.

Keeping Mitochondria in Shape, a Matter of Life and Death

Luca Scorrano
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Mitochondrial morphology changes occur during apoptosis and autophagy, but whether they are relevant in vivo for tissue response to damage is unclear.

Here we show that the inner mitochondrial membrane shaping protein Optic atrophy 1 (OPA1) protects multiple tissues from apoptotic, necrotic and pro-autophagic stimuli. Targeted insertion of one copy of Opa1 isoform 1 in a permissive X chromosome locus does not interfere with mouse development, but protects from muscular atrophy, from ischemic heart and brain damage, as well as from hepatocellular apoptosis. Mechanistically, OPA1 stabilizes mitochondrial cristae, increasing mitochondrial respiratory efficiency and blunting mitochondrial dysfunction, cytochrome c release and reactive oxygen species production caused by the pathological stimuli tested.

Our results indicate that the OPA1-dependent cristae remodeling pathway is a crucial, targetable determinant of cell damage in vivo.

Mitochondrial Quality Control and Neurodegeneration

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Mitochondria execute essential metabolic functions and are intimate part of cellular signaling cascades. The release of cytochrome c from the mitochondrial intermembrane space triggers a fatal cascade culminating in apoptosis. Apoptotic cell death is associated with mitochondrial fragmentation and the remodeling of mitochondrial cristae, facilitating cytochrome c release from intramitochondrial stores. The susceptibility of cells towards apoptosis depends also on cardiolipin in the mitochondrial inner membrane. This mitochondrion-specific phospholipid binds cytochrome c and retains it within mitochondria. Lower levels or oxidation of cardiolipin facilitates cytochrome c release and renders cells more susceptible to apoptosis.

Mitochondrial fusion and cristae morphogenesis depend on the dynamin-like GTPase OPA1 in the inner membrane. Proteolytic cleavage by the inner membrane peptidases YME1L and OMA1 at distinct sites leads to the balanced accumulation of long and short forms of OPA1. Various stress conditions and mitochondrial dysfunction activate the OMA1 peptidase and trigger the complete conversion of OPA1 into short forms inhibiting fusion. Ongoing fission events lead to the fragmentation of the mitochondrial network, which allows segregation of dysfunctional mitochondria and is associated with mitophagy and apoptosis. Experiments examining the role of stress-induced OPA1 processing for neuronal survival will be discussed.

Combined Loss of the BH3-Only Proteins Bim and Bmf Restores B Cell Development and Function in TACI-Ig Mice

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Terminal differentiation of B cells depends on two interconnected survival pathways, elicited by the B cell receptor (BCR) and the BAFF receptor (BAFF-R), respectively. Loss of either pathway impairs B cell development and causes B cell deficiency. While BCR-dependent survival depends mainly on the activation of the AKT/PI3-kinase network, BAFF-R-mediated survival engages non-canonical NF-kB signalling as well as MAPK/ERK and AKT/PI3-kinase modules to allow proper B cell development. Ultimately, these complex signalling events culminate in increased expression of anti-apoptotic Bcl2 family proteins. How lack of BAFF-R mediated signalling triggers B cell apoptosis remains largely unexplored. Here, we show that two pro-apoptotic members of the "BH3-only" subgroup of the Bcl2 family, Bim and Bmf, mediate apoptosis upon BAFF-depletion caused by TACI-Ig overexpression. Surprisingly, although Bcl2 overexpression triggers B cell hyperplasia exceeding the one observed in *Bim*$^{-/-}$*Bmf*$^{-/-}$ mice, Bcl-2 transgenic B cells remained susceptible to BAFF-depletion *in vivo*, leading to ameliorated pathology in *Vav-Bcl2* transgenic mice. Together, our findings shed new light on the molecular machinery restricting B cell survival during development and under pathological conditions. Our data further suggests that Bcl2 antagonists might improve the potency of BAFF-depletion strategies in B cell driven pathologies.

Coordination of mitophagy and mitochondrial biogenesis during ageing

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Aberrant accumulation of mitochondria in disparate cell types is a shared hallmark of many human pathologies and ageing. How mitochondrial biogenesis coordinates with the removal of damaged or superfluous mitochondria to maintain cellular homeostasis is not well understood. Here, we show that mitophagy, a selective type of autophagy targeting mitochondria for degradation, interfaces with mitochondrial biogenesis to regulate mitochondrial content in *Caenorhabditis elegans*. We found that DCT-1 is a key mediator of mitophagy and longevity assurance under conditions of stress in *C. elegans*. Impairment of mitophagy compromises stress resistance and triggers mitochondrial retrograde signalling through the SKN-1 transcription factor that regulates both mitochondrial biogenesis genes and mitophagy by enhancing DCT-1 expression. Our findings reveal a homeostatic feedback loop that integrates metabolic signals to coordinate the biogenesis and turnover of mitochondria. Uncoupling of these two processes during ageing contributes to overproliferation of damaged mitochondria and decline of cellular function.

Abstracts
Poster Presentations

The abstracts are in
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• POSTER PRESENTATIONS •

Unravelling New Tumour-Immunogenic Features of Necroptosis

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Effective anti-cancer therapies rely to a great extent on the immunogenic properties of the cancerous cells. Immunogenic apoptosis, which can be induced by various anthracyclines, has in the recent years been thoroughly described in anti-cancer therapeutic models. However, cancer cells often carry mutations that protect them from apoptosis induction. Characterizing alternative immunogenic cell death modalities is therefore of great need in order to improve current anti-cancer therapies

To date, the immunogenicity of programmed cell death forms other than apoptosis is poorly described. Regulated necrosis (referred to as necroptosis) is another programmed cell death modality, which depends on RIPK1/3 kinase activities. It has been shown that damage-associated molecular patterns (DAMPs) are released during necroptosis, but whether this form of cell death is immunogenic or has any potential for cancer therapy is currently unknown.

In order to investigate these points, we have generated a Tet-On inducible system, by which we trigger necroptosis in a mouse colon carcinoma (CT26) cell line. In this system, necroptosis is associated with ROS production, but independent of ER stress; and various DAMPs (such as ATP and HMGB1) and chemokines are released during the cell death progression. We observed that cancerous cells undergoing necroptosis are engulfed by bone marrow-derived dendritic cells *in vitro*, as well as induce their maturation and activation – seen by the up-regulation of the cell surface markers CD11c, MHCII, CD86 and CD80. Furthermore, necroptotic cancer cells elicit an effective anti-tumour immune response *in vivo* that protects the mice from tumour growth in a prophylactic tumour vaccination model. The splenocytes deriving from such immunised mice produce high amounts of interferon gamma, when stimulated with a CT26-associated tumour antigen *ex vivo*. Finally, we have found that depletion of $CD8^+$ T cells or inhibition

of the purinergic receptors does not abolish the immunisation effect, thereby excluding the role of $CD8^+$ T cells and ATP in the anti-tumour immune response achieved by necroptotic cells *in vivo*.

With these results, we are bringing novel insights into the field of immunogenic cell death by showing that necroptosis is highly immunogenic and proves to be very promising as a way of killing tumours in anti-cancer therapy.

Poster #**001** / Session **I**

Construction of Regulatory Cell Death Networks Linked to Early and Late Stages of Ischemia-Reperfusion Injury in the Retina

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It is widely acknowledged that complex diseases, including retinal disorders, are more often than not, linked to groups of genes, gene modules or gene pathways rather than a single gene. The transcriptional regulation of these genes is mediated by transcription factors (TFs), while their post-transcriptional regulation is mediated by small non coding RNAs, a prominent class of which are microRNAs (miRs). Despite their different levels of regulation, both transcriptional and post-transcriptional regulatory interactions are not isolated but interact with each other to execute complex regulatory programs which, in turn, modulate cellular functions. Cellular and tissue functions rely on well-coordinated molecular interactions between genes, TFs and miRs, all integrated within gene regulatory networks. Such networks drive most if not all biological processes.

Our lab has generated mRNA and miRNA microarray expression data to investigate time-dependent changes in gene expression, following induction of ischemia-reperfusion (IR) injury in the rat retina. Data from three reperfusion time points following retinal IR-injury (0h, 24h and 7d) were analyzed. Paired expression of miRNAs- mRNAs was used to identify regulatory motifs, where miR targets a TF and both co-regulate the expression of a co-targeted gene. The expressions of all three molecular components in these motifs were altered by the IR-injury paradigm. The motives were further integrated into larger regulatory sub-networks. In our preliminary results four sub-networks corresponding to the early (24h) time point and three sub-networks corresponding to the late (7d) time point were generated. The top molecular and cellular functions associated with the highest number of regulatory loops in early, as well as late periods following IR were related to cell death. The cell death sub-network at the early stage contained 1691 regulatory motifs, while their number in the late stage was 454. Interestingly, only 34 motifs, consisting of 28 miRs, 19 mRNA and 1 TF were shared between both stages. These data lead us to suggest that different cell death mechanisms are executed by time point-specific regulatory motifs. Further analyses are in progress to identify if the diversity in the regulatory motifs is associated with different modes of cell death and/or if the various retinal cell types follow different cell

death pathways. It is also possible that there is a switch in the cell death mechanism depending on the time-dependent microenvironment and the disease progression.

Poster #**002** / Session **II**

Cerebral Cortex and Cerebellum Mitochondria Exert Different Sensitivity to Ischemia and Cyclosporine A

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Ischemia leads to inhibition of mitochondrial respiration rate and opening of mitochondrial permeability transition pore (MPTP). It has been shown, that inhibition of MPTP by cyclosporine A (CsA) reduced ischemia-induced cell death in the brain, but some controversies still exist. Brain regions have a different composition of neurons and astrocytes, and this may cause different sensitivity to ischemia-induced damages or functions of mitochondria. In this study, we evaluated ischemia-effect of ischemia on mitochondrial respiration rate and calcium retention capacity (CRC) and necrotic cell death (lactate dehydrogenase activity) in the 6 month rats cerebellum characterised as neurons rich region and cerebral cortex as astrocytes rich region. We found that 90 min ischemia caused similar level of necrosis in both regions of the brain – cortex and cerebellum. However, mitochondrial respiratory dysfunction was found to be different in these regions of the brain after 90 min ischemia: mitochondrial respiration was inhibited by 50-70 % in astrocyte-rich cortex whereas respiration of mitochondria isolated from cerebellum was not affected by ischemia. This suggests that neuronal mitochondria may be more resistant to ischemia-induced damages than astrocyte mitochondria. CRC of normal, non-ischemic mitochondria isolated from cortex and cerebellum was similar and ischemia decreased CRC by about 30 %. Interestingly, mitochondria from different regions of the brain exhibited different sensitivity to CsA, the inhibitor of MPTP. In cortical mitochondria 70% increase in RCR was observed at 0.5 μM CsA concentration whereas 10 folds higher CsA concentration was necessary to achieve the same increase in CRC of cerebellum mitochondria. Rotenone, an inhibitor of mitochondrial complex I, was found to increase CRC by 100-115% in mitochondria from cortex and cerebellum. Moreover, the effect of rotenone on CRC was additive to CsA in both types of mitochondria (up to 170-180%).

In conclusion, our data show that 90 min ischemia causes similar level of necrosis in cortex and cerebellum though mitochondrial dysfunction is observed only in cortex. Cortex mitochondria are more sensitive to ischemia and cyclosporine A compared to cerebellum mitochondria. Calcium-induced MPTP can be blocked by CsA and rotenone in synergistic manner in both cortical and cerebellum mitochondria, suggesting that for therapeutic purposes against ischemic brain damage CsA should be combined with inhibitors of mitochondrial complex I.

Poster #**003** / Session **I**

Exploiting Cannabinoid-Induced Cytotoxic Autophagy to Drive Melanoma Cell Death

Jane Armstrong[1,2], David Hill[1], Christopher McKee[1], Marco Corazzari[3] Sonia Hernandez-Tiedra[4], Mar Lorente[4], Israel Lopez-Valero[4], Maria Eleni Anagnostou[1], Guillermo Velasco[4,] and **Penny Lovat[1]**

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Metastatic melanoma remains largely untreatable, likely due to apoptotic resistance partly reflected by the ability of tumours to promote pro-survival signalling mechanisms including autophagy, the principle lysosomal mechanism for the degradation and recycling of damaged organelles and excess proteins. Coupled with observations that many chemotherapeutic drugs activate autophagy as a compensatory mechanism to counteract apoptotic signals, current therapeutic strategies have focussed on the combined use of the lysosomal inhibitor, hydroxychloroquine (HCQ) to inhibit pro-survival autophagy. However, the capacity for sensitization to HCQ varies, and includes sensitization of normal cells to the cytotoxic effects of chemotherapy. Since blocking autophagy may also promote secondary tumour development, the alternative use of autophagy-inducing drugs able to promote cell death such as Δ^9-Tetrahydrocannabinol (THC), the main biologically active component of cannabis may represent a better therapeutic strategy. To this aim, the present study determined the contribution of autophagy to the cytotoxic effect of THC and a laboratory mimic (sativex-like) of the clinical cannabinoid preparation, sativex, in melanoma both *in vitro* and *in vivo.*

THC-induced autophagic flux as shown by immunofluorescence/Western blotting of LC3 I-II conversion and caspase-dependent apoptosis of metastatic A375, CHL-1 and SK-MEL-28 melanoma cells as demonstrated by significant inhibition of cell viability, increased caspase 3 cleavage and prevention of cell death by ZVAD-FMK ($P < 0.001$). Conversely, THC demonstrated little cytotoxicity to normal melanocytes. Treatment with chloroquine or knockdown of Atg7, but not Beclin-1 or Ambra1, prevented THC-induced autophagy and cell death, suggesting THC activates a non-canonical autophagic mechanism leading to apoptosis of melanoma cells. *In vitro*, Sativex also substantially inhibited melanoma viability, and *in vivo*, both treatment with THC or the Sativex-like agent resulted in a marked reduction in xenograft tumour volume and cell proliferation, alongside an increase in

apoptosis and autophagy compared with treatment with gold standard temozolomide. Our findings thus suggest activation of autophagy in response to cannabinoid treatment is cytotoxic in melanoma cells and that Sativex warrants clinical evaluation as a cytotoxic autophagy therapy for metastatic melanoma.

Poster #**004** / Session **II**

Preserved Cognitive Function after Radiation Exposure Is Associated With BIRC5 Gene Expression and Higher Micronuclei Counts

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Whether a postradiation cognitive impairment following exposure to low doses of ionizing radiation has a distinct molecular background is not clear. Aim of the study was to estimate the role of gene regulation of cell cycle, differentiation and telomere length in a preservation of mental health after harmful influence of such environmental factor as radiation exposure.

Patients. Subjects (n=150) were selected randomly among the Chernobyl cleanup workers cohort. Methods. Relative telomere length (RTL) was studied by Flow-FISH assay with Telomere PNA

Kit/FITC using K562 cell line as a standard. Cellular phenotype, p53, bcl2 regulatory proteins expression and apoptosis in Annexin-V test, CD95+ fraction were studied by flow cytometry, gene expression by RT-PCR. MN counts were performed using PI staining. Several psychometric scales were used to estimate behavioral and cognitive functions including Mini Mental State Examination (MMSE). Patients with MMSE index higher than 27 where compared with the group of patients with different stages of cognitive deficit (MMSE < 27).

Results. Absence of cognitive deficiency was associated with increased TR53 and TP53I3 genes expression and normal or increased numbers of CD4+, CD4+25+ cells. No correlation was found between BAX expression and CCND1 gene and bcl-2 expression and radiation dose. Telomere length changes were insignificant. In cognitive deficit patients telomere length shortening, TERF1, TERF2, NFK2B and DDB2 genes hyperexpression, high oxidative stress markers, low MDA and GSH and an increased T-cell number under G1-cell cycle arrest were demonstrated. BIRC5 and MKNK2 expression positively correlated with MMSE score as well as MN counts and serve possibly as protective, also depending on age. Summary: This study supports the relationship between the preserved cognitive function and cell survival lymphocyte genes and proteins at a late period after radiation exposure.

Poster **#005** / Session **I**

VE-821 and VE-822, Selective Inhibitors of Ataxia Telangiectasia and Rad3 Related Kinase, Potentiate Cell Death after PUVA Treatment

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PUVA (psoralen + UVA) treatment, used in early stages of mycosis fungoides (MF), consists of a pretreatment with a photosynthesizing agent (such as 8-methoxypsoralen) and subsequent ultraviolet A (UVA) irradiation. It leads to DNA crosslinking, DNA double strand breaks and cell death via apoptosis.

Since genotoxicity is the principle of PUVA treatment we reasoned, that the therapeutic response may be augmented by pharmacological inhibition of DNA repair mechanisms. Using MyLa2000, cell line derived from an MF patient, we tested a range of inhibitors of proteins involved in DNA damage response, belonging to phosphatidylinositol 3-kinase-related kinases (PIKKs) family. We selected Ataxia Telangiectasia and Rad3 related kinase (ATR) inhibitors VE-821 and VE822 for further analysis.

VE-821 and VE-822, when combined with PUVA, increased cell death by up to 60%. They also released cells from the G2/M block, induced by PUVA treatment. Both VE-821 and VE-822 significantly decreased phosphorylation of Rad17, factor required for G2/M arrest and for recruiting Rad1-Rad9-Hus1 checkpoint protein complex. Surprisingly, both ATR inhibitors increased rather than decreased phosphorylation level of histone H2AX and of Chk1, a well-known ATR target.

Poster #**006** / Session **II**

Caspase-8 Inhibition Induces Necroptosis Specifically in Activated Microglia, Preventing Their Phagoptosis of Neurons

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Microglia are resident brain macrophages, which can cause neuronal loss when activated in infectious, ischaemic, traumatic and neurodegenerative diseases. Caspase-8 has both pro-death and pro-survival roles, mediating apoptosis and/or preventing RIPK1-mediated necroptosis depending on cell type and stimulus. We found that inflammatory stimuli (LPS, LTA or TNF-alpha) caused an increase in caspase-8 IETDase activity in primary rat microglia without inducing apoptosis. Inhibition of caspase-8 with either zVAD-fmk or IETD-fmk resulted in necrosis of activated microglia. Inhibition of caspases with zVAD-fmk did not kill non-activated microglia, or astrocytes and neurons in any condition. Necrostatin-1, a specific inhibitor of RIPK1, prevented caspase inhibition-induced microglial death, indicating death was by necroptosis.

It is generally assumed that microglia only phagocytose dead or dying neurons, however, we find that under inflammatory conditions microglia can also phagocytose live neurons and thereby kill them, a form of cell death called 'phagoptosis'. We find that microglial phagocytosis of stressed-but-viable neurons is mediated by the exposure of the 'eat-me' signal phosphatidyserine (PS) on neurons, which is bound by the adapter protein MFG-E8, which induces phagocytosis via the vitronectin receptor on microglia. Reversible PS exposure on viable neurons can be induced by low levels of glutamate, oxidants, of activated microglia, but in the presence of activated microglia this neuronal PS exposure induces their phagocytosis.

We found inflammatory activation of co-cultures of microglia and neurons results in progressive loss of neurons (without any apparent cell death), which is accompanied by microglial phagocytosis of neurons, and is prevented by blocking phagocytosis or phagocytic signalling. Neuronal loss induced by nanomolar amyloid-beta or LPS is absent in cultures from MFG-E8 knockout mice, but is reconstituted by adding wild-type MFG-E8. LPS-induced neuronal loss in vivo is reduced by co-injection of phagocytosis inhibitors or in MFG-E8 knockout mice. MFG-E8 knockout mice and MerTK mutant rats are strongly protected against brain damage induced by transient ischaemia.

Inhibition of caspase-8, resulting in microglial necroptosis, prevented the neuronal loss/death caused by activated microglia, but this loss was restored by rescue of microglia with necrostatin-1. We conclude that the activation of caspase-8 in inflamed microglia prevents their death by necroptosis, and thus caspase-8 inhibitors may protect neurons in the inflamed brain by selectively killing activated microglia, which otherwise eat stressed-but-viable neurons resulting in their death by phagoptosis. Thus caspase-8 inhibition is a potential therapy to prevent inflammatory neuronal loss in the brain.

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Poster #**007** / Session **I**

Identifying Strategies to Inhibit Anti-Apoptotic MCL-1 in Philadelphia chromosome Acute Lymphoblastic Leukemia

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The response of Philadelphia chromosome (Ph+) acute lymphoblastic leukemia (ALL) to treatment by BCR-ABL tyrosine kinase inhibitors (TKIs) often results in short remissions typified by rapid outgrowth of drug-resistant clones. In a mouse model of Ph+ B-lineage ALL (B-ALL), MCL-1 expression is dysregulated by the BCR-ABL oncofusion protein and TKI treatment results in loss of MCL-1 expression prior to the induction of apoptosis. Using a mouse model in which conditional allele(s) of Mcl-1 can be deleted either during leukemia transformation or later after the establishment of leukemia, we have demonstrated that endogenous MCL-1's anti-apoptotic activity is essential for promoting survival during BCR-ABL-transformation and in established BCR-ABL+ leukemia.

Small molecule inhibitors (BH3-mimetics) have been designed to target anti-apoptotic proteins as a way to specifically targeting malignant cells. A number of inhibitors are in various stages of development with specific or broad activities against anti-apoptotic BCL-2 family members. To investigate the efficacy, specificity, and biochemical events mediated by small molecule inhibitors, we have developed a mouse genetic system in which we have "reprogrammed" BCR-ABL-transformed B-lineage acute lymphoblastic leukemia (B-ALL) cell lines to render them dependent on exogenous human MCL-1, BCL-2, BCL-xL, BFL-1, or BCL-w. We have used these BCR-ABL B-ALL cell lines to define, in a genetically-controlled setting, how various small molecule BH3-mimetics mechanistically induce cell death and to validate their spectrum of inhibition and specificity of action. Therefore, we submit that this panel of cell lines is an excellent in vitro and in vivo screening tool to test the efficacy and potency of small molecule inhibitors of anti-apoptotic BCL-2 family members.

MCL-1 plays an essential role in promoting the survival of many normal cellular lineages including myeloid cells, lymphocytes, cardiomyocytes, and neurons; therefore, a potential consequence of effective MCL-1 inhibition may be unwanted

toxicities. To circumvent such potential side effects associated with MCL-1 inhibition, we screened for agents that reduce MCL-1 expression in our model BCR-ABL+ leukemia cell lines. Since these agents decrease MCL-1 expression, we hypothesized that they might alter the sensitivity of the cells to other BH3 mimetic therapies such as ABT-263 or ABT-199. Indeed, down modulation of MCL-1 expression synergizes with non-MCL-1 targeted small molecule therapies to induce cell death. Thus, we have revealed a number of methods by which MCL-1's protective effects can be partially antagonized to render previously resistant cell lines sensitive to targeted therapies. This finding may expand the capabilities of BH3 mimetic drugs and may reveal methods to overcome resistance mechanisms.

Poster #**008** / Session **II**

Dual Role of the MAPK/Erk Pathway in the Regulation of TRAIL Sensitivity in Human Breast Epithelial Cells

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Increased activation of the epidermal growth factor receptor (EGFR) is frequently observed in tumors, and inhibition of the signalling pathways originated in the EGFR normally renders tumor cells more sensitive to different apoptotic stimuli, including TRAIL. However, despite all the available evidences in tumor cells, the regulation of TRAIL sensitivity by EGFR signalling in non-tumor cells remains to be investigated in detail. We have recently demonstrated that inhibition of EGFR signalling in various non-transformed breast epithelial cells by long-term EGF deprivation or gefitinib treatment, causes the up-regulation of the long isoform of caspase-8 inhibitor FLICE-inhibitory protein ($FLIP_L$) and makes these cells more resistant to TRAIL. Hereby, we have examined the role of signalling pathways downstream of EGF receptor in the regulation of TRAIL-induced apoptosis in human breast epithelial cells. We show that the prolonged inhibition of the MAPK/Erk pathway prior to TRAIL addition leads to the up-regulation of FLIP and a marked inhibition of TRAIL-induced apoptosis. In contrast, simultaneous treatment with the MAPK/Erk inhibitor U0216 and TRAIL synergistically induces apoptosis in breast epithelial cells. Moreover, inhibition of both the MAPK/Erk pathway and Akt further sensitizes breast epithelial cells to TRAIL-induced DISC formation, caspase-8 activation and apoptosis. Interestingly, TRAIL promotes the early activation of the MAPK/Erk pathway in a caspase-8-dependent manner. Collectively, our results suggest a dual role of the MAPK/Erk pathway in the regulation of TRAIL-induced apoptosis in EGF-dependent human breast epithelial cells. Firstly, activation of the MAPK/Erk pathway by EGF maintains low levels of FLIP in cells and makes these cells sensitive to TRAIL. On the other hand, the early activation of the MAPK/Erk pathway by TRAIL is an obstacle to the formation of the TRAIL DISC and activation of apoptosis by TRAIL. We are currently investigating the mechanism underlying the feedback inhibition of early TRAIL apoptotic signalling by the MAPK/Erk pathway.

Poster #**009** / Session **I**

Lack of Collagen VI Induces Neuronal Degeneration by Impairing Autophagy and Promoting Apoptosis

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Collagen VI is an extracellular matrix (ECM) protein with a broad distribution in different tissues, where it is largely deposited at the close periphery of the cell surface [1]. Mutations of collagen VI genes in humans cause several muscle diseases, including Bethlem myopathy and Ullrich congenital muscular dystrophy. Collagen VI null (*Col6a1*$^{-/-}$) mice display an early onset myopathic phenotype characterized by organelle defects, mitochondrial dysfunction, spontaneous apoptosis and defective autophagy [2,3]. Recently, we demonstrated that collagen VI is abundantly expressed by Schwann cells and that ablation of collagen VI leads to increased myelin thickness in the peripheral nervous system, indicating that this extracellular matrix molecule is an essential component of nerves involved in modulating peripheral nerve structure and function [4]. Although some studies showed that collagen VI is able to protect neurons from the toxicity of amyloid β-peptides and from UV-induced damage [5], the physiological role of this protein in the central nervous system (CNS) remains unknown.

In order to investigate the role of collagen VI in CNS, we established primary neural cultures from murine cortex and hippocampus and carried out *in vivo* and *in vitro* studies in wild-type and *Col6a1*$^{-/-}$ mice. *Col6a1*$^{-/-}$ neural cultures displayed a remarkable incidence of spontaneous apoptosis and higher vulnerability to oxidative stress. These defects were accompanied by altered regulation of autophagy in *Col6a1*$^{-/-}$ neural cultures, with increased p62 protein levels and decreased LC3 lipidation. Characterization of fluorescent GFP-LC3 puncta in neural cells derived from wild-type and *Col6a1*$^{-/-}$ mice allowed establishing that the autophagic flux is impaired in the absence of collagen VI. Analysis of brain sections confirmed increased apoptosis and abnormal regulation of autophagy in the CNS of collagen VI-deficient animals. To investigate the *in vivo* physiological consequences of these CNS defects, we carried out functional studies and found that motor and memory task performances were reduced in aged *Col6a1*$^{-/-}$ mice.

These findings indicate that lack of collagen VI leads to defective autophagy and spontaneous apoptosis in neural cells, and suggest a protective role for collagen VI in the CNS during physiological aging.

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Poster #**010** / Session **II**

Iridovirus CARD Protein Inhibits Apoptosis through Intrinsic and Extrinsic Pathways

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Abstract withdrawn by authors from online

Poster #**011** / Session **I**

Developing Micro-Molecular Agonists of NGF Receptors with Anti-Apoptotic Effects on Nervous System: Mechanism of Action

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Endogenous neurotrophic factors control neuronal cell fate and function during development and adulthood. They act through Trk and $p75^{NTR}$ receptors, exerting potent neuroprotective effects. However, neurotrophins' therapeutic usefulness is compromised by their polypeptidic nature and limited penetrance to the blood-brain barrier (BBB). We have synthesized analogs of neurosteroid dehydroepiandrosterone with anti-apoptotic properties, deprived of androgenic/estrogenic actions. In the present study, we provide evidence that derivative BNN27 binds to NGF receptors, namely TrkA and $p75^{NTR}$, at nanomolar concentrations (Kd: 1.86±0.4nM and 3.9±1.2nM respectively). Mutagenesis assays have shown that binding of BNN27 to TrkA receptors does not require its extracellular, NGF binding domain, in contrast to mutated $p75^{NTR}\Delta ECD$ receptor where no binding is observed. BNN27 induced TrkA tyrosine phosphorylation in all three tyrosine residues (490, 675 and 785), affecting downstream signaling of Akt and MAPKs in primary sympathetic neurons. However, BNN27 differentially regulated TrkA internalization: it induced internalization and fast return of the receptor into the membrane through activation of rab5 protein and docking of the receptor in the early endosomes. Using cholesterol depletion agents and separating lipid raft microdomains, we showed that BNN –in contrast to NGF- caused TrkA to effectively segregate into lipid raft fractions, presenting differential membrane localization of the receptor. BNN27 significantly reversed apoptosis of NGF-dependent embryonic sensory neurons of NGF null mice and of sympathetic neurons in primary culture. Moreover, BNN27 was effective in promoting the interaction of $p75^{NTR}$ receptors with its interactors RhoGDI, RIP2 and TRAF6. Interestingly, BNN27 was ineffective by itself in inducing differentiation and neurite outgrowth of PC12 cells. However, it enhanced the effects of NGF at low

concentrations in both phenomena. NMR studies with BNN27 and recombinant NGF receptors suggest that it facilitates binding of NGF to its receptors. In conclusion, BNN27 exerts strong anti-apoptotic, neuroprotective actions via NGF receptors, differentially activating prosurvival signaling, and thus it may serve as a lead molecule to develop BBB permeable, neurotrophin-like small molecules (microneurotrophins) with potential applications in the treatment of neurodegenerative diseases.

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Poster #**012** / Session **II**

Inhibition of mitochondrial transcription causes germline hyperplasia in *C. elegans*

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Mitochondria are semi- autonomous organelles mainly relying on nuclear gene products for their function. Nevertheless they have retained their own genome which encodes twelve electron transport chain components. Mitochondrial activity has been extensively linked with the onset of aging, however little is known about the effect of mitochondrial transcription on ageing at the cellular and organismal level. Genetic inhibition of *rpom-1*, the homologue of the mammalian mitochondrial RNA polymerase in *C. elegans*, causes a profound reduction in brood size usually accompanied by a protruding vulva phenotype after the cessation of egg production. Interestingly, RPOM-1 depletion causes hyperplasia in the gonadal syncytium region, while germline apoptosis is significantly increased. RPOM-1 is expressed in a punctuate pattern almost in all tissues including the intestine, vulva and germline. Collectively, our findings suggest that mitochondrial transcription is crucial for fecundity, while its impairment can lead to the manifestation of tumor-like phenotypes. We are currently investigating the molecular circuitry that links mitochondrial transcription with fate decisions such as the transition from mitosis to meiosis in the nematode gonad.

Poster #**013**/ Session **I**

The Effects of Selected Inhibitors of Histone Modifying Enzyme on Autophagy in Glioma Stem Cells

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Glioblastoma (GBM) is the most frequent adult primary malignant brain tumor that remains incurable despite aggressive treatment. GBM harbors hierarchical tumor cells called glioma stem-like cells (GSCs) that maintain tumor growth, drive tumor progression and cause tumor relapse due to their increased resistance to therapy. The metabolic pathway - autophagy, traditionally considered as a cellular homeostatic and recycling mechanism, has been implicated in the regulation of survival in GBM. However, the role of autophagy in GSCs biology is currently unclear. Recent studies demonstrate that epigenetic modifications may regulate the occurrence of autophagy, however the exact mechanism of epigenetic influence remains unknown. Here, we focused on the regulation of autophagy in GSCs and the effects of selected inhibitors of histone modifying enzyme on this process .

GSCs were isolated from a human glioblastoma LN18 cells and primary cultures of human glioblastoma. These cells were characterized by higher expression levels of stem/progenitor cell transcription factors: *NANOG*, *OCT3/4*, *SOX2*, *CD133* as compared to parental glioma cell cultures or differentiated cells. Our preliminary results have shown that GSCs isolated from cancer spheres express a significantly lower level of LC3-II (an autophagy marker) and the reduced level of autophagy related proteins (Beclin1, ULK1, Atg5, Atg7) than the parental glioma cell cultures. It suggests reduced levels of autophagy in GSCs. We assessed the effect of selected epigenetic inhibitors on cell viability, histone modifying enzyme activities, and the genes encoding stem/progenitor and autophagy markers in parental glioma cell cultures and GSCs. The compounds included in this study were: two inhibitors of HDACs: valproic acid (VPA) and trichostatin A (TSA), the inhibitor of H3K27 methyltransferase EZH2: 3-deazaneplanocin (3DZNep), the inhibitor of H3K9 methyltransferase SUV39: chaetocin and the inhibitor of G9a methyltransferase that methylates H3K9 and H3K27: BIX01294. The results show a considerable impact of BIX01294 on LC3 expression and lipidation in bulk/differentiated and GSC cultures.

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Poster #**014** / Session **II**

Stress Induced Premature Senescence of colon cancer cells treated with 5-fluorouracil

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Discovery of stress induced premature senescence (SIPS) in cancer cells in response to chemotherapeutics gave hope for alternative approach to anticancer therapy. The main characteristic of senescent cells is that they remain viable and metabolically active whereas their growth is permanently arrested. Recent studies, including ours (Sliwinska et al, 2009), suggest that chemotherapeutic induced senescence leads to the appearance of transiently senescent cell population.

The main purpose of our project is to study the influence of clinically used chemotherapeutic 5-fluorouracil (5-FU) on induced senescence of colon cancer cells *in vitro*. We established protocols that imitate chemotherapy in cancer patients therefore we treated HCT116 cells with repeated 5-FU cycles. After such treatment cells show hallmarks of senescence, especially: growth arrest, enlarged size and flattened shape, increased granularity, augmented β-galactosidase activity, elevated level of p21 protein and increased secretion of VEGF and IL-8. Shortly after final drug withdrawal a subpopulation of not senescent – small and highly proliferating cells appears.

Overall, we conclude that colon cancer cells treated with 5-FU undergo SIPS. Drug removal leads to the appearance of highly proliferative progeny, which can be one of the mechanisms responsible for tumor regrowth.

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Poster #**015** / Session **I**

Anoikis Induced by Nitric Oxide Can Be Modulated by an EGFR Independent Mechanism

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Introduction: Nitric oxide (NO) and other reactive nitrogen species are molecules that play decisive roles in controlling cell proliferation and cell death. Cell proliferation is commonly associated with low/physiological concentrations of NO. In contrast, NO generated in high concentrations induce apoptotic cell death in adhered cells. We reviewed the accumulated experimental evidence on the role of NO as an inducer of extracellular matrix detachment and of modifications to the cell cytoskeleton. Based on that, we suggested a role for NO in cell death induced by detachment from the substrate (anoikis).

Objective: In this report, we compared the effects of increasing concentrations of the NO donor sodium nitroprusside (SNP) in HeLa cells cultured adhered to a Petri dish surface or kept in suspension. We evaluated the EGFR and Bim expression levels, activation of caspase-3, and cell viability in adhered cells and in cells kept in suspension.

Methods: HeLa human cervical cancer cells were seeded in adherent and non-adherent surfaces (poly -2-hydroxyethyl methacrylate - treated Petri dishes surfaces) and exposed to increasing concentrations of SNP. After 24 h treatment, we performed westernblot analysis to determine EGFR and Bim expression levels, cleaved caspase-3 levels, and determine cell viability by the XTT assay. Statistical analysis was performed using the Student´s *t*-test.

Results: We observed a basal decrease on cell viability in cells kept in suspension during 24 h. After treatment with low concentrations of SNP (50 – 100 μM) we observed a significant and additional decrease on cell viability and activation of caspase-3. Bim expression levels increase upon cell detachment. No further increase on Bim expression levels was detected in suspended cells treated with SNP (50μM). Upon treatment with low concentrations of SNP, there were no changes in EGFR expression levels. Interestingly, the use of high concentrations of SNP (500μM) increased the cell viability, decreased caspase-3 activation, and decreased Bim expression levels. However the EGFR expression levels, significantly decreased under these experimental conditions.

Conclusions: We demonstrate that SNP-induced cell death, when cells are kept in suspension, occurred upon treatment with low concentrations of the NO donor. NO-mediated cell death induced by detachment from the substrate, occurred without changes on the EGFR expression levels, an important marker of anoikis. On the other hand, in HeLa cells maintained in suspension, high concentrations of SNP promoted cell survival and a decrease on EGFR expression levels. Our findings suggest that NO-mediated cell death by anoikis is a process independent on EGFR expression levels.

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Poster #**016**/ Session **II**

Compounds Enhancing Intracellular cAMP Levels Increase the Expression of Transglutaminase 2 in Mouse Thymocytes

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Transglutaminase 2 (TG2) is a multifunctional protein which plays a role in multiple biological processes including apoptosis. Previously our research group has shown that TG2 is induced during in vivo, but not in vitro apoptosis of thymocytes indicating that factors present in the tissue environment are required for the process. Recently our laboratory has found that retinoids and transforming growth factor beta (TGF-β) released by engulfing machrophages are involved in this process. Since adenosine and prostaglandin E2 (PGE2), which are also produced by macrophages during engulfment of apoptotic cells, trigger the adenylate cyclase pathway, we decided to investigate the possible involvement of the adenylate cyclase pathway in the TG2 induction. Our data indicate that compounds increasing intracellular cAMP level, alone or in combination with retinoids and TGF-β can contribute to TG2 expression in dying thymocytes.

Poster #**017** / Session **I**

Coupling of mitochondrial metabolism with mRNA turnover influences cell death during ageing

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Ageing is associated with marked alterations in protein synthesis and turnover rates in diverse species ranging from yeast to humans. Importantly, interventions that lower the rate of mRNA translation extend the lifespan of different organisms. Recent studies suggest that mRNA translation and degradation pathways are often regulated by coupled mechanisms. Cell survival relies on the biogenesis and function of mitochondria, the energy generating organelles in eukaryotic cells. Molecular mechanisms underlying mitochondrial function comprising ATP production, oxygen consumption and ROS release are confoundedly examined with several of their regulatory components already revealed. Accumulating evidence suggests that mitochondrial dysfunction is strongly connected to cell death in ageing and age-associated diseases. Apoptosis and necrosis are the two major modes of cell death. Apoptosis, or programmed cell death, is important for normal development and morphogenesis. Apoptosis can be triggered by either extrinsic stimuli detected by cell surface death receptors or by intrinsic stimuli through the mitochondrial signaling pathway. We are using the nematode *Caenorhabtitis elegans* in an effort to investigate the crosstalk between the mRNA turnover pathway and the intrinsic apoptosis pathway- the most important components of which are the: EGL-1, CED-9, CED-4 and the caspase CED-3-. Our working hypothesis is that mRNA metabolism is implicated in the regulation of the intrinsic pathway also affecting mitochondrial morphology and function as well as lifespan. To address this issue, we apply genetic tools or *C. elegans* strains that either lack or overexpress known modulators of mRNA decay such as the decapping enzyme, DCAP-2. We find that RNAi knockdown of the *dcap-2* gene increases the mRNA levels of *hsp-60*, which encodes a mitochondrial associated protein chaperone of the mitochondria-specific unfolded protein response pathway. Similarly, CED-9, which is the mammalian homologue of the anti-apoptotic protein Bcl-2, is transcriptionally upregulated in wild-type nematodes subjected to RNAi against *dcap-2*. Moreover, the mitochondrial network is altered in wild-type animals grown on *dcap-2* RNAi, as evidenced by staining with the mitochondrial

specific dye tetramethylrhodamine ethyl ester (TMRE). Elucidation of the intricate interplay between the mRNA turnover pathway and the intrinsic apoptotic signaling pathway would be crucial for developing novel intervention strategies to treat or prevent numerous human diseases and improve healthspan.

Poster #**018** / Session **II**

Hallmarks of Cornification, Apoptosis and Autophagy in Photodynamically Treated Primary Keratinocytes Heka Vs Carcinoma A-431 Cells

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Hallmarks of cell death triggered by tetrahydroxyphenyl chlorine (mTHPC)-mediated photodynamic treatment (PDT) were followed in primary human epidermal keratinocytes HEKa and human epidermoid carcinoma cell line A-431 *in vitro.* PDT induced the similar decline in cell viability in either of cell lines. Cornification was assessed by expression of cornification markers: keratin 10, involucrin and procaspase 14. Apoptosis was followed by caspase-3 activation. Autophagy was registered as accumulation of the autophagy marker LC3-II, and autophagic flux, which was studied using inhibitors of lysosomal peptidases.

mTHPC–PDT induced apoptosis in human primary epidermal keratinocytes HEKa and epidermoid carcinoma A-431 cells. At 24 h post-exposure, cornification overrules apoptosis in normal keratinocytes. In cancer cells, no signs of cornification were observed. mTHPC-PDT increased the rate of autophagy in both HEKa and A-431 cell lines..

Poster #**019** / Session **I**

Inhibition of Necroptosis through Hydantoins

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Besides its capacity to inhibit necroptosis, Necrostatin-1 has been suggested to exert off-target effects. Some of these off-target effects may be involved in the prevention of regulated cell death (RCD). As Necrostatin-1 is a hydantoin derivative, we screened such compounds for putative effects on RCD. Two compounds, Phenytoin and 5-Benzyl-Hydantoin strongly inhibit TNFα/zVAD mediated necroptosis *in vitro*. In order to examine the *in vivo* protective function of Phenytoin and 5-Benzyl-Hydantoin, we used both inhibitors in a standard renal ischemia-reperfusion injury (IRI) model. As with Necrostatin-1, also Phenytoin and 5-Benzyl-Hydantoin treated mice were prevented in comparison to untreated control mice. We assessed potential toxicity by serial i.p. applications over 8 days (200 µg daily) and did not find significant differences in any of the investigated serum markers.

In conclusion, these observations lead us to speculate that hydantoins may prevent necroptosis *in vitro* and *in vivo*. Given our clinical experience with patients treated on Phenytoin for decades for anticonvulsive therapy, Phenytoin might be easily applied in situations where we anticipate necroptosis to contribute to the pathophysiology.

Poster #**020** / Session **II**

An NF-Kb-Independent Role of IKKs in Regulating RIPK1 Killing Potential

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TNF is a pleiotropic cytokine that can paradoxically induce both cell survival and cell death. In most cell types, activation of TNFR1 by TNF does not induce cell death but instead leads to the NF-kB-dependent transcriptional upregulation of genes encoding pro-survival molecules that inhibit activation of the death pathway. Accordingly, when the NF-kB response is inhibited, either by expression of a proteasomal degradation-resistant mutant of IkBa or by the use of the general translation inhibitor cycloheximide (CHX), TNFR1 activation switches from a pro-survival to a pro-apoptotic response. Under these conditions, TNF-mediated death was shown not to depend on RIPK1. Cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) are required for TNF-dependent NF-kB activation and, as a consequence, their depletion also induces a switch to apoptosis. However, under these conditions, TNF-mediated death was shown to rely on RIPK1 kinase activity, highlighting existence of another cell death checkpoint in the TNFR1 apoptotic pathway. It has been postulated that the contribution of RIPK1 to the killing process was controlled by its ubiquitylation status at the receptor complex, a process regulated by cIAP1/2. However, we demonstrate in this study that depleting IKKa/b also induces RIPK1-dependent apoptosis without affecting RIPK1 ubiquitylation status. We show that IKKa/b directly phosphorylate RIPK1, and that inhibiting IKKa/b kinase activities allows assembly of a caspase-8/RIPK1 death complex. Importantly, we demonstrate that the role of IKKa/b in regulating RIPK1 killing potential is NF-kB-independent, therefore demonstrating an unexpected new function of IKKa/b.

Poster #**021** / Session **I**

Photodynamic Therapy: a Promising Alternative to Induce Massive Cell Death in Human Breast Cancer Cells

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Breast cancer is a devastating disease with global impact on health, where metastasis remains the main cause of death. Photodynamic therapy (PDT), which cause tissue destruction by visible light in the presence of a photosensitizing substance and oxygen, appears as a promising alternative for cancer treatment. However, the mechanisms of the cells death as well as the efficacy of PDT on mammary tumors remain unclear.

Therefore, in this work we set out to evaluate the cytotoxic effects of PDT by using methylene blue as a photosensitizer (MB-PDT) in two (2D) and three (3D) dimension cultures of human breast tumor cells with different invasive properties and to study the signaling pathways involved in cell death as possible mediators of the cytotoxic effects of MB-PDT.

Invasive MDA-MB-231, non-invasive MCF-7 and non-tumorigenic MCF-10A cells were used. The cells were incubated for 2h in the absence or presence of different MB concentrations and irradiated (λ=640nm) or not at 4,5J/cm^2 dose rate. Cell viability was evaluated by fluorescence microscopy analysis using Hoechst 33342 and Propidium Iodide staining. Autophagy was evaluated by acridine orange staining and western blotting. The role of autophagy after MB-PDT was investigated by viability analysis using chloroquine (CQ) and LY294002 (LY) as inhibitors and rapamycin (RAPA) as activator of this pathway.

MB-PDT was able to significantly increase cell death from 1h to 24h following cell irradiation of both tumoral cell lines kept under either 2D or 3D culture (98,6% ± 0,5%). MB-PDT effect was significantly lower in MCF-10A (52,2% ± 3,8%) cells despite the higher intracellular concentration of MB. No significant cell death induction was observed in the presence of MB or light alone. Hoechst and Propidium Iodide staining presented no signs of apoptotic nuclei. Moreover, at early time points following irradiation, a significant increase of both the LC3-II/LC-I ratio and acidic vesicle formation was observed only in MDA-MB-231

cells upon MB (2μM) treatment. After cotreatment with CQ or LY the effect of MB-PDT was increased and after cotreatment with RAPA it was lowered, showing that autophagy could be displaying a citoprotective function upon MB-PDT.

We showed for the first time, that MB-PDT was able to induce massive cell death in two human breast cancer cell lines in a model that recapitulates the morphology of glandular epithelium. Our observations contribute to indicate PDT as an alternative and effective therapy displaying minimal side effects for invasive breast tumors.

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Poster #**022**/ Session **II**

Neuronal Apoptosis, EGLN3 and Cancer: Novel Activation of TNFR1 Mediated Cell Death?

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Apoptosis sculpts the developing peripheral nervous system (PNS), culling excess neurons in response to competition for neurotrophins, such as nerve growth factor (NGF). Mutations affecting NGF-dependent neuronal survival are associated with neuroblastoma and later-developing malignancies of neural-crest derived origin, such as paraganglioma and pheochromocytoma. Our previous work demonstrated that familial pheochromocytoma lesions effect a pathway that regulates the pro-apoptotic activity of the oxygen-sensing prolyl-hydroxylase EGLN3 and that EGLN3 activity is required for NGF-deprivation induced apoptosis. We hypothesise that inhibition of EGLN3 during development may predispose to certain types of cancers that arise from the neural crest and aim to describe EGLN3 function during developmental apoptosis. We performed a genome-wide RNAi screen for genes required for EGLN3-induced apoptosis. This screen revealed that Tumour necrosis factor receptor one (TNFR1) is required for EGLN3-induced apoptosis and that TNFR1 effects EGLN3-induced cell death via a Caspase-8 dependent mechanism. Deletion or epigenetic inactivation of Caspase-8 occurs in the majority of neuroendocrine tumours. The activation of TNF receptor mediated apoptosis observed here occurs in the absence of known TNFR1 ligands, suggesting a novel mechanism for TNFR1 activation. Our current work is focussed on investigating this mechanism and the importance of TNFR1 and Caspase-8 during developmental culling of sympathetic neurons *in vivo* and in mouse models of neuroblastoma.

Poster #**023** / Session **I**

Neuronal Apoptosis by EGLN3: A New Way to Provoke TNFR1 Mediated Cell Death?

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During normal embryological development neuronal progenitor cells migrate from the neural crest to the nascent ganglia, competing with each other for growth factors such as nerve growth factor (NGF). Only cells that obtain a sufficient amount of NGF can survive and undergo full differentiation, the rest die by apoptosis. Mutations affecting NGF-dependent neuronal survival have been associated with neuroblastoma and later-developing malignancies of neural crest-derived origin, such as paraganglioma and pheochromocytoma.

Our previous work showed that oxygen-sensing prolyl-hydroxylase EglN3 is critical for apoptosis during sympathetic neural development. We hypothesised that failure of EglN3-mediated apoptosis during sympathetic development can predispose to certain types of cancers that arise from the neural crest. To establish the role of EGLN3 during developmental apoptosis, a genome-wide RNAi screen for genes required for EGLN3-induced cell death was performed. The screen revealed that Tumour necrosis factor receptor 1 (TNFR1) is required for EGLN3-induced apoptosis and TNFR1 effects EGLN3 induced cell death via a Caspase-8 dependent mechanism. The observed activation of TNF receptor-mediated apoptosis occurs in the absence of known TNFR1 ligands, suggesting a novel mechanism for TNFR1 activation. Our current work is focused on investigating this mechanism and the importance of TNFR1 and Caspase-8 during developmental culling of sympathetic neurons *in vivo*.

Poster #**024** / Session **II**

Increased Resistance to Proteasome Inhibitors in Multiple Myeloma Mediated By Ciap2 Overexpression - Implications for a Combinatorial Treatment

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The important discovery of the use of proteasome inhibitors like bortezomib for use in treatment of multiple myeloma (MM) has significantly improved patient outcome. Despite the advances in treatment strategies, MM is still, however, considered incurable due to the development of resistant tumor clones. To optimize current treatment strategies there is therefore an urgent need to unravel the important proteins and mechanisms underlying emerging drug resistance and to describe the use of these as new targets for combinatorial treatment options. Alterations of the IAP proteins are frequently found in tumors as an underlying cause for chemoresistance and poor patient prognosis. In MM, cIAP2 is part of the gene signature being a hallmark of aberrant NF-kB signaling. Using lentiviral transduction increased expression of the cellular Inhibitor of apoptosis 2 (cIAP2) was induced in TRAF3 deleted/mutated MM cells lines. In these MM cell lines, predominantly using the canonical NF-KB pathway, we evaluated the role of cIAP2 as a regulator of cell death and survival. We found that increased survival and decreased activation of caspases was associated with cIAP2 overexpression in MM cell lines treated with proteasome inhibitors in clinical use including bortezomib, MG132 and carfilzomib as compared to the vector control. The cIAP2 protein increase apoptosis tolerance to proteasome inhibitors by enhanced viability and decreased caspase activation. Gene expression analysis reveal 809 genes differentially regulated in the cIAP2 overexpressing cells during treatment with proteasome inhibitors among these genes involved in signaling pathways of NF-κB signaling, DNA damage response, autophagy and metabolism. Importantly, the use of combinatorial treatment of the IAP inhibitor AT-406 and bortezomib was shown to have a synergistic effect on MM cell lines with cIAP2 expression. Taken together, our results show that cIAP2 is an important factor mediating bortezomib resistance in MM cells with TRAF3 deletion/mutation and therefore should be considered as a target for combinatorial treatment to reduce the emergence of drug resistant MM clones.

Poster #**025** / Session **I**

High Hydrostatic Pressure Induces Immunogenic Cell Death In Human Tumor Cells

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Recent studies have identified molecular events characteristic of immunogenic cell death. These include surface exposure of CRT (calreticulin), HSP70 and HSP90, release of intranuclear HMGB1 and secretion of ATP from dying cells. Several chemotherapeutic agents (anthracyclines, oxaliplatin and bortezomib) and hypericin-based photodynamic therapy have been described to induce the immunogenic cell death in human tumor cells. We investigated the potential of high hydrostatic pressure (HHP) to induce immunogenic cell death in human tumor cells.

Prostate and ovarian cancer cell lines and primary tumor cells were treated by HHP and we analyzed the kinetics of the expression of immunogenic cell death markers. HHP killed tumor cells expressing immunogenic cell death markers were tested for their ability to activate dendritic cells (DCs), to induce tumor specific T cells and regulatory T cells (Tregs).

HHP induced rapid expression of HSP70, HSP90 and CRT on the cell surface of all tested cell lines and primary tumor cells. The expression of these molecules was 1,5-2 fold higher compared to idarubicin. HHP also induced release of HMGB1 and ATP from treated cells. The interaction of DCs with HHP-treated tumor cells led to the faster rate of phagocytosis, significant upregulation of CD83, CD86 and HLA-DR and release of IL-6, IL-12p70 and TNFα. The ability of HHP-killed tumor cells to promote DCs maturation was cell contact dependent. DCs pulsed with tumor cells killed by HHP induced high numbers of tumor-specific T cells even in the absence of additional maturation stimulus. DCs pulsed with HHP treated tumor cells also induced the lowest number of Tregs.

High hydrostatic pressure is a reliable and very potent inducer of immunogenic cell death in the wide range of human tumor cell lines and primary tumor cells.

Poster **#026** / Session **II**

The Histone H4 Lysine 16 Acetyltransferase hMOF regulates the Outcome of Autophagy

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Autophagy is an evolutionarily conserved catabolic process involved in several physiological and pathological processes. Although primarily cytoprotective, autophagy can also contribute to cell death; it is thus important to understand what distinguishes the life or death decision in autophagic cells. Here we report that induction of autophagy is coupled to reduction of histone H4 lysine 16 acetylation (H4K16ac) through downregulation of the histone acetyltransferase hMOF (also called KAT8 or MYST1), and demonstrate that this histone modification regulates the outcome of autophagy. At a genome-wide level, we find that H4K16 deacetylation is associated predominantly with the downregulation of autophagy-related genes. Antagonizing H4K16ac downregulation upon autophagy induction results in the promotion of cell death. Our findings establish that alteration in a specific histone post-translational modification during autophagy affects the transcriptional regulation of autophagy-related genes and initiates a regulatory feedback loop, which serves as a key determinant of survival versus death responses upon autophagy induction.

Poster #**027** / Session **I**

Met Dependence Receptor Amplifies Apoptosis via the Intrinsic Pathway

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Met is the receptor tyrosine kinase for HGF/SF (Hepatocyte Growth Factor/Scatter Factor) ligand. Met-HGF/SF couple is important during embryonic development, and its aberrant expression/activity is associated with the progression of many different tumors. Whereas ligand-activated Met promotes cell survival, Met can be cleaved by caspases under stress conditions. The caspase-generated p40Met fragment amplifies apoptosis. This dual activity of Met receptor is a hallmark of the dependence receptor family. Although the survival signaling pathways induced by Met have been thoroughly investigated, the pro-apoptotic mechanisms triggered by p40Met remain more unclear.

We show that, although the caspase-generated p40Met fragment contains the entire kinase domain, it accelerates apoptosis independently of kinase activity. This pro-apoptotic activity is dependent on p40Met localization tightly associated with the mitochondria. *In vivo* hepatic apoptosis induction also leads to the generation of p40Met found in the mitochondrial fraction. In agreement with its localization, the fragment favors mitochondrial permeabilization and cytochrome C release, which is inhibited both by Bak silencing or Bcl-xL overexpression. Moreover, Met silencing delays mitochondrial permeabilization induced by an apoptotic treatment, which illustrates the control by the dependence receptor Met of the apoptotic intrinsic pathway. We are now taking profit of a knock-in mouse model in which Met caspase cleavage site has been mutated. MEFs (Mouse Embryonic Fibroblasts) derived from mutant mice are less prone to undergo apoptosis in response to anisomycin, when compared to MEFs from wt mice. Our mice model should thus be a precious tool to study and understand the importance and the conditions in which Met can trigger apoptosis *in vivo*.

Poster #**028** / Session **II**

Sex Steroids Regulate the Expression of Autophagy-Related Genes in Bovine Mammary Epithelial Cells

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Sex steroids: 17β-estradiol (E2) and progesterone (P4) belong to the key hormones regulating normal development of the mammary gland. E2 is necessary for proper formation and elongation of mammary ductal system, whereas P4 stimulates the development of lobule-alveoli during the final stages of mammogenesis which occurs at the time of gestation. In dairy cattle consecutive pregnancies are typically overlapped by a period of mammary gland involution that serves to rebuild the secretory tissue before next lactation. Our previous studies have shown that in the presence of E2 and P4 bovine mammary epithelial cells (MECs) enhance autophagy, induced as a survival mechanism during stress connected with temporal malnutrition. Reduction of foetal bovine serum (FBS) content from standard 10% to 0.5% in growth medium of BME-UV1 bovine MECs resulted in decreased phosphorylation of mTOR kinase, and increased level of LC3-II autophagic marker. In the presence of E2, or progesterone P4 the level of LC3-II was further increased. Furthermore, *in silico* analysis of potential binding sites for steroid receptors on promoters of bovine autophagy-related genes (*ATGs*) revealed that promoter regions of bovine *ATG5*, and *LC3B* genes potentially contain estrogen response element (ERE), whereas *ATG3* shows the presence of androgen response element (ARE). Thus, the present study aimed to evaluate the role of E2 and P4 in regulation of expression of chosen autophagic genes: *ATG5, ATG3, LC3B, BECN1* and corresponding proteins in BME-UV1 cells. The expression of chosen *ATGs* was evaluated using real time RT-PCR after 2, 4, 6, 12 and 24h of culture in experimental medium (0.5% FBS) with or without addition of sex steroids (E2, 1nM; P4, 5ng/ml). Protein levels were determined by immunoblotting in the same experimental conditions after 2, 6, 12, 24 and 48h of incubation. Additionally, in order to determine the binding capacity of nuclear proteins to DNA probes containing consensus sequences of ERE and progesterone response element (PRE) EMSA was performed on lysates isolated from cells treated with E2 or P4.

We observed increased binding of nuclear proteins to DNA probes containing ERE, or PRE 30-60 min. after addition of E2, or P4 into experimental medium. The expression of *BECN1, ATG5, ATG3, LC3B* increased after 2h incubation in

0.5%FBS medium. Addition of E2 further elevated the expression of these genes at the 2nd, 12th, and 24th h of incubation, and increased the levels of Atg5, LC3-II proteins in comparison to control (0.5% FBS), especially in the later time points of incubation (between 12 and 48 h), although the results were less pronounced. Progesterone stimulated the expression of *ATG5* and *LC3B*, but this effect was noted only at the12th h of incubation, and no significant changes in the levels of autophagic proteins were observed. These results indicate that 17β-estradiol and progesterone are not only involved in MECs proliferation during development of bovine mammary gland, but also actively regulate the induction of autophagy in these cells. The role of 17β-estradiol in regulation of autophagic genes is more significant. Thus, in cattle, autophagy may serve as an important process in preventing extensive cell death during development and remodelling of the mammary gland, and its induction by E2 may enable easier regeneration of the mammary gland during gestation-lactation cycle.

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Poster #**029** / Session **I**

HspB11, Mitochondrial Network And Apoptosis Resistance

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AlphaB-crystallin homology, heat stress induction and chaperone activity suggested that a previously encloned gene product is a novel small heat shock protein (HSPB11). Suppression of HSPB11 by siRNA sensitized cells to hydrogen peroxide or taxol induced cell-death, while its over-expression protected cells against stress stimuli by inhibiting cytochrome c release from the mitochondria, nuclear translocation of AIF and endonuclease G, and caspase 3 activation. Recombinant HSPB11 protected mitochondrial membrane potential against calcium induced collapse in vitro indicating that it stabilizes mitochondrial membrane systems. HSPB11 formed self-aggregates and bound to Hsp90. Inhibition of Hsp90 by geldanamycin diminished the cytoprotective effect of HSPB11 indicating that this effect was Hsp90-mediated. HSPB11 over-expression increased lipid rafts formation as demonstrated by increased cell surface labeling with fluorescent cholera toxin B, and increased Akt phosphorylation. The inhibition of PI-3-kinase-Akt pathway by LY-294002 or wortmannin significantly decreased the protective effect of HSPB11. Progressive cytoplasmic expression of HSPB11 also correlated with brain tumor malignancy based upon the findings on 91 diagnosed brain tumor cases. To study how cytoplasmic abundance of HSPB11 augments tumor malignancy, we overexpressed HSPB11 in NIH3T3 fibroblasts and silenced its expression in HeLa cervix carcinoma cells. Endogenous HSPB11 expression is low in NIH3T3 and high in HeLa cells. Both cell lines and their untransfected control counterparts were exposed to 150 nM paclitaxel, a known inducer of apoptotic and necrotic death. We determined type of cell death, association of HSPB11 with mitochondria, mitochondrial fission and deactivation of dynamin-like protein-1 (DRP-1). We found that HSPB11 increased inhibitory phosphorylation of DRP-1, attenuated mitochondrial fission and cell death, which was exclusively apoptotic in NIH3T3 and predominantly apoptotic in HeLa cells. Furthermore, paclitaxel did not increase mitochondrial association of HSPB11. All these data suggests that DRP-1 dependent mitochondrial network stabilisation could contribute to elevated apoptosis resistance and increased malignancy in HSPB11 overexpressing tumors.

Poster #**030** / Session **II**

Immunogenic Cell Death-based DC Vaccines Induce Rejection of Immunoprivileged Glioblastoma, Prompting a Prognostically Favorable "Treg-to-Th1/ Th17/CTL Shift" in Brain Immunocontexture

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Abstract withdrawn by authors from online

Poster #**031** / Session **I**

Nitric Oxide – Associated 1 is Part of The Opa1 Complexes Targeted during Cell Death

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Mitochondria are key regulators of programmed cell death, during which their shape and ultrastructure change, contributing to the mobilization of the cristae-endowed cytochrome c to the intermembrane space and eventually to the cytosol. This process of cristae remodeling is controlled by Optic Atrophy 1 (OPA1), an inner mitochondrial membrane protein, whose complexes are disrupted early during apoptosis. Three dimensional BlueNative - BlueNative - SDS PAGE followed by LC/MS analysis of normal and apoptotic mitochondria revealed a list of candidate proteins that significantly increase or decrease in OPA1 complexes in the course of apoptosis among which a newly characterized protein named nitric oxide-associated 1 (NOA1) was identified. NOA1 is a mitochondrial GTPase, but its precise function is poorly understood. Our studies demonstrate that NOA1 is a peripheral membrane protein, located within the inner compartments of mitochondria, interacting with OPA1. We suggest a functional interplay between OPA1 and NOA1, since OPA1 is up-regulated in *Noa1* deficient MEFs, but also, because NOA1 is lacking in *Opa1* deficient MEFs. Deficiency of NOA1 leads to a dramatic cristae disorganization, indicating an important role of the protein in mitochondrial ultrastructure. Last but not least, upon apoptotic stimulation, NOA1 protein levels decrease, likely due to a proteolytic cleavage.

Poster #**032** / Session **II**

Influence of Autocrine Factors on CTLL-2 Cytotoxic Lymphocyte Survival in a Model of Cell Hypoxia

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In multicellular organisms cell survival is controlled by numerous cytokines of various origins including autocrine survival factors (AFs). We studied the influence of AFs on intracellular ATP content and survival of cytotoxic IL-2-dependent CTLL-2 cells in a model of hypoxia using cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) as the chemical inducer of hypoxia. Our results demonstrated that intracellular level of ATP in CTLL-2 cells incubated in culture medium containing 200 μM $CoCl_2$ was significantly lower as compared with that in the cells cultivated in normal conditions. Addition of AFs (50 % of conditioned medium obtained from a special CTLL-2 cell culture, CM) to the cells incubated in conditions of $CoCl_2$ –induced hypoxia restored intracellular concentration of ATP in CTLL-2 cells up to the normal level. Flow cytometry analysis of the cells cultivated with $CoCl_2$ for 20 hrs showed that more than 60% of the cells were dead (apoptotic or necrotic type of the cell death). The addition of CM to the cell cultures decreased the percentage of dead cells after cultivation under chemical hypoxia conditions up to 10%. To identify AFs that protected the cells from the death in our model of chemical hypoxia we separated different fraction of CM using gel filtration of the conditioned supernatant by a column with Bio-Gel P-10. The gel filtration resulted in isolation of three primary fractions corresponding to the three distinct peaks on obtained chromatogram. The isolated fractions of CM showed different activity in the model of chemical hypoxia. In particular one of fractions with low molecular masses had a considerable effect on restoration of intracellular level of ATP in $CoCl_2$ treated cells. Besides, significant but not strong protective effects were observed for two low molecular mass fractions of CM in experiments with evaluation of their influence on CTLL-2 survival in conditions of the chemical hypoxia.

Poster #**033** / Session **I**

Comparison of the Mechanism of Different Lysosomotropic Agents

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Lysosomotropic detergents have an important potential in cancer treatment, because they trigger lysosomal membrane permeabilization (LMP), leading to lysosomal apoptotic pathway and inhibition of autophagic protective function. However, the mechanism of numerous such compounds is often poorly understood or the evidence for LMP is not convincing. Therefore it is crucial to choose the appropriate methodological approaches to evaluate LMP and its consequences [1]. Furthermore, not all lysosomotropic agents are lysosomotropic detergents. Recently, we have shown that siramesine, which was previously reported to act as lysosomotropic detergent [2], induces cell death through destabilization of mitochondria and that cell death is independent of LMP and the release of cathepsins into the cytosol, however, the increase in lysosomal pH was detected [3]. Unlike siramesine, well-studied compound LeuLeuOMe is a true lysosomotropic detergent [4; 5; Repnik et al., in preparation].

In this study we have thoroughly analyzed the mechanism of sphingosine, which was reported to be a lysosomotropic detergent [6], and N-dodecylimidazole (NDI), the mechanism of which is controversial [7; 8; 9]. In addition, we compared these results to the results of previously studied siramesine and LeuLeuOMe. In contrast to previous reports of NDI and sphingosine, we have shown that neither of the two agents triggered LMP, since there was no cysteine cathepsin activity detected in the cytosol over a wide range of concentrations used, although both agents induced rapid increase in lysosomal pH, similar as we have shown before for siramesine. Our results indicate that NDI and sphingosine are not lysosomotropic detergents as it was reported previously. Furthermore, we have shown that the damage to mitochondria rather than lysosomes is the cause of cell death induced by NDI and sphingosine.

This study presents several new findings in the field of lysosomotropic agents. Understanding the mechanism of such compounds is significantly important for cancer treatment and designing new analogues with even higher anticancer potential. Furthermore, the study also shows the importance of choosing the appropriate methodology and how the interpretation of the results can affect the outcome.

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Poster #**034** / Session **II**

Targeting Autophagy, an Achilles' Heel Of Cancer Stem Cells

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Breast tumors contain a small population of tumor-initiating cells known as cancer stem-like cells (CSCs). Breast CSCs appear to be responsible for metastasis and tumor recurrence. Moreover, CSCs are highly resistant to conventional therapy. We have shown that autophagy is essential for the self-renewal and the tumorigenicity of breast CSCs (Gong et al. 2013 *Oncogene* 32, 2261-72). A high-throughput screen was used to identify drugs that inhibit breast CSC self-renewal and trigger cell death (Gupta et al. 2009 *Cell* 138, 645-59). One of these is the $K^{(+)}/H^{(+)}$ ionophore salinomycin (Sal). Recently, we demonstrated that Sal blocks autophagic flux by inhibiting the lysosomal proteolytic activity (Yue and Hamai et al. 2013 *Autophagy* 9, 714-29). However, although Sal efficiently targets breast CSCs it cannot be used in cancer therapy because of its neurotoxic activity. Consequently, we have developed analogues of Sal. Two of these analogues are 50-100 times more efficiently than Sal to inhibit breast CSC self-renewal and to trigger cell death. Using a click reaction-based fluorescence-staining method, we observed that these analogues accumulate in the lysosomal compartment and block autophagic flux. The rational for this preferential targeting of breast CSC lysosomes remains to be investigated. However, we have identified a new class of of lysosomotropic agents with potential therapeutic development that targets breast CSCs and blocks their autophagy-dependent self-renewal.

Poster #**035**/ Session **I**

Canonical BH3-Only Proteins Trigger Macroautophagy-Independent, Ubiquitin-Mediated Intramitochondrial Processing

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Pro-apoptotic BH3-only proteins activate mitochondrial outer membrane permeabilization (MOMP), leading to mitochondrial release of proteins into the cytosol essential for activation and execution of caspase-mediated cell death. Damaged mitochondria can be degraded by macroautophagy (mitophagy), suggesting a role for mitophagy in regulating apoptosis. However, mitophagy occurs slower than apoptosis signaling, and caspases can inhibit macroautophagy. To date, for most intrinsic apoptosis scenarios it remains unknown if mitophagy is engaged, and whether mitophagy can influence the mitochondrial capacity to activate or enhance apoptosis. Employing high- and super-resolution microscopy we investigated the interplay of autophagy, lysosomal and ubiquitylation pathways in response to BH3-only protein expression. Thereby, we demonstrate that in parallel to intrinsic apoptosis signaling, the canonical BH3-only proteins tBid, Bim_{EL}, Bik and Bad induce intramitochondrial processing associated with lysosomal and proteasomal activities, in the absence of macroautophagy. We show that upon mitochondrial depolarization XIAP (X-linked inhibitor of apoptosis protein) rapidly translocates to all mitochondria where its E3 ligase activity triggers Bax-mediated MOMP. Concomitantly, mitochondrial ubiquitylation by XIAP directs entry of the endolysosomal machinery into mitochondria, and degradation of the endogenous XIAP inhibitor, Smac. Our findings reveal a functional integration between lysosomes and mitochondria, mediated by XIAP E3 ligase activity, and independent of macroautophagy, that constitutes a novel cellular mechanism with the potential to regulate mitochondrial apoptosis.

Poster #**036** / Session **II**

Interactions between Psycho-Emotional Stress and Apoptosis Regulatory Network in Prostate Cancer

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Stress/epinephrine signaling via ADRB2 has been recently connected with prostate cancer pathophysiology and beta blockers were proposed as means of therapy for advanced prostate cancer. Yet mechanisms that mediate the effects of stress/epinephrine remain poorly defined. We used systems approach to examine signal transduction pathways that control apoptosis and apoptosis regulatory proteins in prostate cancer cells. Topology analysis revealed convergent signaling network that connects active PI3K/Akt, receptor tyrosine kinases of EGFR family and G-protein coupled receptors with BAD and Mcl-1. Experiments with shRNA knockdown and ectopic expression identified BAD dephosphorylation and decreased Mcl-1 expression as key events that induce rapid apoptosis in a subset of prostate cancer cells. The impact of BAD dephosphorylation and decreased Mcl-1 expression on apoptosis is determined by the expression pattern of other proteins of Bcl2 family. Analysis of the mechanisms identified epinephrine/ADRB2/ adenylylcyclase/PKA signaling pathway responsible for increased BAD phosphorylation and increased expression of Mcl-1 in prostate tumors of mice subjected to stress.

Experiments in prostate cancer cells, luminescent xenograft tumors C42Luc and Hi-Myc mouse model of prostate cancer will be presented.

Poster #**037** / Session **I**

JNJ-26481585, a Potent Second-Generation Histone Deacetylase Inhibitor, Promotes Intrinsic Apoptosis in Rhabdomyosarcoma and Sensitizes Them to Small Molecule Inhibitors of Antiapoptotic Bcl-2 Proteins

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HDAC inhibitors (HDACI), a promising class of new anti-cancer agents, induce apoptosis via epigenetic or non-epigenetic regulation in many cancer entities. JNJ-26481585 is a second-generation class-I-HDAC inhibitor that has displayed improved efficiacy in preclinical studies compared to the established HDACI Vorinostat. Therefore, we evaluated the effects of JNJ-26481585 on human rhambdomyosarcoma (RMS) cell lines.

Indeed, JNJ-26481585 promotes histone H3 acetylation and induces apoptosis even at low nanomolar concentrations in alveolar and embryonal RMS cell lines characterized by activation of the caspase cascade, PARP cleavage and DNA fragmentation. Caspase-dependent apoptotic cell death is confirmed by the use of the broad-range caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk), which significantly decreases JNJ-triggered DNA fragmentation and completely abrogates JNJ-induced loss of cell viability and cell density. However, zVAD.fmk fails to inhibit JNJ-26481585-induced cleavage of caspase-9 and rather promotes accumulation of the inactive p19 fragment of caspase-3, while formation of the p17/p12 active intermediate cleavage fragments of caspase-3 and caspase-8 cleavage is profoundly attenuated. Furthermore, JNJ-26481585 significantly inhibits tumor growth of RMS cells in an *in vivo* chicken chorioallantoic membrane (CAM) tumor model, supporting the notion that JNJ-26481585 hampers tumor maintenance.

Mechanistically, JNJ-induced apoptosis is mediated via the intrinsic apoptotic pathway, since we observed increased loss of mitochondrial membrane potential and induction or activation of the proapoptotic Bcl-2 family members Bim_{EL}, Bmf, Puma, Bax and Bak. JNJ-26481585-initiated activation of Bax and Bak can't be prevented by additional treatment with zVAD.fmk, suggesting that JNJ-26481585 first disrupts the mitochondria and subsequently activates the caspase-cascade. JNJ-26481585 treatment triggers induction of Bim, Bmf and Puma on mRNA

level, pointing to altered transcription as important event for apoptosis induction. Bak, Bim and Puma are crucial for JNJ-induced apoptosis, since RNAi mediated silencing of Bim, Bak or/and Puma significantly impedes JNJ-induced DNA fragmentation. Furthermore, ectopic overexpression of Bcl-2 profoundly impairs JNJ-mediated apoptosis, abrogates caspase cleavage and reduces activation of Bax and Bak as well as cleavage of Bid into tBid, underlining the hypothesis that JNJ-26481585 initially disrupts the mitochondria and then activates caspase-8 and Bid.

Intriguingly, we found that JNJ-26481585 synergistically cooperates with small molecule inhibitors (SMI) of antiapoptotic Bcl-2 proteins like ABT-737 and Obatoclax (Combination Index $\leq$ 0,639). This underscores the requirement of the mitochondrial apoptotic pathway for the induction of apoptosis by JNJ-26481585.

Taken together, we provide evidence that JNJ-26481585 alone or in combination with SMIs of antiapoptotic Bcl-2 proteins represent a promising new therapeutic approach for the treatment of RMS.

Poster #**038** / Session **II**

Proteomic Analysis of Autophagy Network

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Abstract withdrawn by authors from online

Poster #**039** / Session **I**

Deciphering a Novel Caspase-8 Activation Mechanism to Induce Apoptosis in Chemo-Resistant Cancer Cells

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We recently identified that cancer cells which are resistant to genotoxic chemotherapeutics can be eliminated by apoptosis upon initiation of a non-canonical caspase-8 activation pathway in response to proteasome inhibition. Here, we identified molecular mechanisms which regulate caspase-8 activation in this treatment scenario and describe the subcellular location and isolation of the caspase-8 activation platform and a number of its components.

We identified that caspase-8 is activated as an apical caspase upon proteasome inhibition and that, in contrast to genotoxic agents, caspase-8 potently induces apoptotic death in cells which are incompetent to activate the mitochondrial apoptosis pathway. The availability of glucose critically determines the capacity of proteasome inhibition to trigger this non-canonical mode of caspase-8 activation, and changes in the amounts of cFLIP variants modulate the timing and efficiency of caspase-8 activation in this treatment modality. Our imaging studies identified that caspase-8 is activated on intracellular platforms rather than at the plasma membrane. The caspase-8 activation platform was isolated by subcellular fractionation and size exclusion chromatography, revealing the formation of high molecular weight complexes that activate caspase-8 in the cytosol. These complexes contain FADD but not RIP1, ruling out ripoptosomes as the activation platforms. We also found Atg5 to be required for caspase-8 activation and large increases in the amount of LC3I and II. However, imaging and ultrastructural analyses failed to detect notable increases in autophagosomes when compared to serum starvation scenarios. Instead, cells presented with an excessively dilated ER and aggresome accumulations, and these features may be functionally linked to caspase-8 activation in this context. Further studies investigated the contribution of non-apoptotic cell death modalities, including RIP1-dependent and independent necroptosis, ROS-induced cell death and ferroptosis. Together, our data provide detailed mechanistic insight into cell death responses and the caspase-8 activation processes upon proteasome inhibition.

Poster #**040** / Session **II**

Regulators of Cell Death, Survival and Proliferation Are Combinatorial Biomarker Candidates in Stage III Colorectal Cancer

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Colorectal cancer (CRC) is one of the most frequent cancers, causing >390,000 deaths annually worldwide. Adjuvant 5-fluorouracil-based chemotherapy is the current standard treatment for stage III CRC, but unfortunately approximately 50% of patients experience disease recurrence within 5 years. Despite numerous large scale studies, reliable protein markers for predicting recurrence-free survival have not been found. To address this problem, we combined technologies for the hyperplexed and quantitative analysis of *in situ* protein expression at single cell level in formalin-fixed paraffin embedded (FFPE) cancer tissues (MultiOmyx®)[1] together with systems biological processing and analysis of biologically relevant groups of multiplexed protein data (SYS-ACT)[2]. The combination of both approaches aims to overcome shortcomings of classical immunohistochemistry and statistical analysis.

Using MultiOmyx®, 28 key regulatory proteins involved in the control of cell death, survival and proliferation were imaged in an iterative process of staining, imaging and signal inactivation in intact FFPE sections of 140 stage III CRC patients. Protein data were then analysed within the SYS-ACT modeling environment, employing algorithms from the fields of multivariate statistics, data-driven modeling, and pattern recognition. From our analyses, we constructed a multi-dimensional space into which patients could be positioned according to their individual protein expression profiles. Of note, within this modeling space we identified a region that is predominantly occupied by patients free from disease recurrence (n = 77). Similar trends were reproducibly seen in cross-validation analysis (n=10) in which data from randomly selected patients were used. Importantly, such reduced data sets were sufficient to predict recurrence free

survival in the remaining patient population with a specificity of >80% (evaluated from n = 140 predictions from 10 separate test runs).

Our proof-of-concept study therefore demonstrates that the expression of regulators of cell death, survival and proliferation, when measured at cellular level and analyzed by systems biological methods, can be a powerful novel approach to predict recurrence free survival in stage III CRC.

Previous Studies

1. Gerdes, M.J., et al., *Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue.* Proc Natl Acad Sci U S A, 2013. **110**(29): p. 11982-7.

2. Passante, E., et al., *Systems analysis of apoptosis protein expression allows the case-specific prediction of cell death responsiveness of melanoma cells.* Cell Death Differ, 2013. **20**(11): p. 1521-31.

Poster #**041** / Session **I**

Bid Chimeras Indicate that Most BH3-only Proteins Can Directly Activate Bak and Bax, but Show No Preference for Bak Versus Bax

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The mitochondrial pathway of apoptosis is initiated by BH3-only members of the Bcl-2 protein family. Upon upregulation or activation, certain BH3-only proteins can directly bind and activate Bak and Bax to induce conformation change, oligomerization and pore formation in mitochondria. BH3-only proteins, with the exception of Bid, are intrinsically disordered and have hydrophobic membrane targeting domains, so functional studies often utilize peptides based on the BH3 domain. Thus, to generate each BH3-only protein as a recombinant protein that could efficiently target mitochondria we developed recombinant Bid chimeras in which the BH3 domain was replaced with that of other BH3-only proteins (Bim, Puma, Noxa, Bad, Bmf, Bik and Hrk). The chimeras were stable following purification, and each immunoprecipitated with full-length Bcl-x_L according to the specificity reported for the related BH3 peptide. When tested for activation of Bak and Bax in mitochondrial permeabilization assays, Bid chimeras were around 1000-fold more effective than the related BH3 peptides. BH3 sequences from Bid and Bim were the strongest activators, followed by Puma, Hrk, Bmf and Bik, while Bad and Noxa were not activators. Notably, chimeras showed no preference for activating Bak or Bax. In addition, within the BH3 domain, the h0 position recently found to be important for Bax activation was important also for Bak activation. Together, our data with full-length proteins indicate that most BH3-only proteins can directly activate both Bak and Bax.

Poster #**042** / Session **II**

Overcoming Smac Mimetic Resistance in Breast Cancer Cell Lines

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Objective

Smac is a pro-apoptotic protein that facilitates apoptosis through binding inhibitor of apoptosis proteins (IAPs). The relevance of Smac in stimulation of apoptosis can be shown through a class of compounds called Smac mimetics that mimic the IAP-binding functions of Smac. They facilitate cell death in cancer cells and are currently investigated in clinical trials as anti-cancer agents. Sensitivity of cancer cell lines to Smac mimetics has been reported to be due to induction of TNF-alpha production. However, many cancer cell lines show insensitivity to Smac mimetics.

Our objective is to study the functional mechanisms behind Smac mimetic resistance in breast cancer cell lines with the long-term goal to heighten the efficiency of treatment. This is pursued by elucidating the molecular factors determining the TNF-alpha response and by studying if other death-inducing ligands such as TRAIL can be used to overcome resistance.

Methods

The efficiency of the Smac mimetic LCL-161 to induce cell death alone or in combination with TNF-alpha or TRAIL was studied by WST-1 assay on different breast cancer cell lines after a period of treatment. TNF-alpha mRNA levels were measured by qRT-PCR.

Results

Treatment of a panel of breast cancer cell lines with the Smac mimetic LCL-161 showed that MDA-MB-231 cells had a significant ~30% reduction in cell viability after 30 h of treatment as shown by WST-1 assay whereas MDA-MB-468 and MCF-7 cells were unaffected. Combination treatment with LCL-161 and TNF-alpha gave an almost complete loss of cell viability for the MDA-MB-231 cell line, whereas the MDA-MB-468 and MCF-7 cell lines had a ~60% and ~30% reduction in cell viability respectively. qRT-PCR experiments showed that TNF-alpha is not induced by LCL-161 in MCF-7 cells. Combination treatment of MDA-

MB-468 cells with LCL-161 and TRAIL decreased cell viability more than TRAIL alone (36% resp. 96% cell viability) and addition of z-VAD-fmk completely rescued the cell death, showing that the cells undergo caspase-dependent cell death. LCL-161 and TRAIL showed no effect on cell viability in MCF-7 cells compared to TRAIL alone, however, the cells underwent a change in cell morphology where the cells rounded up and decreased in size with the combination treatment.

Conclusion

Our data indicates that sensitivity to the Smac mimetic LCL-161 can be increased by co-treatment with TNF-alpha or TRAIL with varying efficiency in different breast cancer cell lines. The mechanisms behind these discrepant effects on cell death as well as the significance in the change in cell morphology observed in MCF-7 cells during co-treatment with TRAIL will be pursued in further studies.

Poster #**043** / Session **I**

LEI/L-DNase II a Caspase-Independent Cell Death Pathway and its Interaction with Other Cell Death Effectors

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LEI (Leukocyte Elastase Inhibitor) is an ubiquitous cytoplasmic protein belonging to the serpin family. In its native form it bears an antiprotease activity that confers anti-apoptotic properties. When apoptosis is induced by certain agents, LEI can be cleaved by serine proteases or by cathepsins, and is transformed into an acid endonuclease: L-DNase II. It is then translocated to the nucleus where it is responsible for DNA degradation into oligonucleosomes.

In a large-scale interactomic study aiming to identify LEI/L-DNase II partners, we found several intersting potential regulators of cell death. In this work we present the interaction and biological effets of this system with: 1) AIF (Apoptosis-Inducing Factor), a mitochondrial flavoprotein also involved in a caspase-independent cell death pathway. Upon apoptosis induction, AIF can be released in the cytosol and translocates to the nucleus where it induces chromatin condensation. 2) PARP-1 (Poly(ADP-ribose)polymerase-1), an enzyme belonging to the DNA reparation machinery that has been pointed out as being essential for AIF release. 3) Bax and Bcl-2: two members of the Bcl-2 family with opposite and well known effects on caspase-dependent apoptosis; 4) PkC zeta, an atypical protein kinase C, involved in cell death and in cell proliferation. 5) The activation of L-DNase II by the lysosomal enzyme Cathepsin D led us to the investigation of lysosomal permeabilization mechanism, also discussed in this paper.

Poster #**044** / Session **II**

Cleavage of Bcl-2 by Cathepsin D: A Novel Mechanism of Cathepsin D-Mediated Regulation of Apoptosis

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Cathepsin D (CTSD) is a member of the subfamily of lysosomal aspartic proteases. However, its enzymatic function is not restricted solely to the acidic milieu of lysosomes. It can facilitate cancer cell migration and invasion by digesting the basement membrane, extracellular matrix, and connective tissue. Because of its mitogenic and proteolytic activities, CTSD has been suggested to act as prognostic marker in many tumour types, especially breast cancer. CTSD has been also implicated in positive and negative regulation of apoptosis. In order to clarify the role of CTSD and its enzymatic activity in the Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) -induced apoptotic signaling, MDA-MB-231 breast carcinoma cells were transfected with cDNA coding for CTSDwt and its enzymatically inactive counterpart (CTSDmut). Control and CTSDwt/CTSDmut overexpressing stable transfected cell lines were then exposed to TRAIL and frequency of apoptotic cell death was determined by analysis of nuclear morphology by fluorescence microscopy and immunodetection of poly-ADP-rybose polymerase (PARP) cleavage and activation of caspases. In this study we describe that CTSD facilitates the TRAIL-induced apoptosis of breast cancer MDA-MB-231 cells in enzymatic activity-dependent manner. Analysis of the potential substrates specifically cleaved by CTSD during apoptotic cell death progression provided a new evidence of the CTSD-mediated cleavage of the Bcl-2 protein. The Bcl-2 cleavage occurred both in MDA-MB-231 cells and *in vitro* in a range of pH values as detected by immunoblotting. As Bcl-2 is prominent anti-apoptotic regulator, its cleavage may represent novel mechanism of cathepsin D-mediated regulation of apoptosis.

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Poster #**045** / Session **I**

Molecular Mechanisms of Cell Death Induction by Classical and Novel Taxanes in Breast Cancer Cells

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Background: The classical taxanes paclitaxel (Taxol®) and docetaxel (Taxotere®) are routinely used in therapy of solid tumors. Unfortunately, cancer cells often become resistant to taxanes. Therefore, new taxanes were prepared and tested for using in the therapy of resistant cancer cells. As for mechanisms of cell death induction, taxanes are known to block depolymerization of tubulin and to induce programmed cell death because of mitotic failure. However, the exact mechanisms of cell death induction are still not understood. To help to elucidate this issue, we investigated breast cancer cells after application of classical and novel taxanes.

Results: We studied taxane-induced cell death in two breast cancer cell lines SK-BR-3 and MCF-7. We used paclitaxel and novel taxanes SB-T-1216 and SB-T-12854 (being more efficient against resistant cancer cells) at a death inducing concentration. Programmed cell death was observed in both tested cell lines after application of classical as well as novel taxanes. The roles of autophagy and some signaling pathways were tested but they probably have no direct effect on taxane induced cell death. Next we studied the role of proteins of Bcl-2 family. We confirmed the importance of proteins Bad and Bim in cell death induction described previously and in addition we found that proteins Bik and Bok also seem to function in cell death induction. The translocation of cytochrome C and decrease of mitochondrial membrane potential were observed only in SK-BR-3 cells and not in MCF-7 cells, so the role of mitochondria is questionable. On the other hand, caspase -2, -8, -9, -7 and -3 (caspase-3 only in SK-BR-3 cells) were activated in SK-BR-3 as well as MCF-7 cells 36 h after application of taxanes. As previously reported, caspase-2 seems to be the most apical caspase in both cell lines. Using of specific siRNA showed that caspase-3 and caspase-7 had also important function in apoptotic cascade. On the contrary, caspase-8 and caspase-9 are probably only the members of apoptotic amplification loops.

Conclusions: We found that classical and novel taxanes induced cell death very similarly in breast cancer cells. Proteins from Bcl-2 family, (Bad, Bim, Bik and Bok) and caspase-2, -3 and -7 probably play the most important role in cell death

induction. On the other hand, mitochondria and other caspases do not seem to have relevant function there. In summary, new taxanes could be used in future anticancer therapy of resistant tumor cells.

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Poster #**046** / Session **II**

Role of Caspase-3 during TNF-alpha Induced CD30 and CD45 Shedding from K-562 Cells

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Caspases are a large family of evolutionary conserved proteases. Although caspases act mainly within the cell, their activity is triggered and modulated by external signals, including cytokines. Cytokine and caspase networks are closely inter-connected in various ways at different levels including multiple biological effects. In our study correlation between caspase-3 activation, degree of apoptosis and necrosis with changes of CD30 and CD45 on cell surface and their appearances in supernatant was estimated in kinetics study in eritroleukemia K-562 cells, after treatment with diverse dose of TNF-alpha. Apoptosis, necrosis and cell surface expression of CD30 and CD45 was analyzed by Flow cytometry (FACS Calibur using Cell Quest Pro Software), while intracellular caspase-3 activity, intracellular CD30, CD45 in cell lysate as well as and soluble forms of CD45 and CD30 in supernatante of cultures cells where analyzed by Western blotting methods. The quantification was performed by densitometry analysis at GS-710 Imaging Densitometer, using Image J software. Kinetics study indicated that caspase-3 activities were increased in dose and time depending manner, which correlates with apoptosis rate. However, appearances of soluble CD30 and CD45 in supernatants of TNF-alpha treated K-562 cells are not uniform. Soluble CD30 is rapidly increased in supernatant of treated cells with effects after 4h without strong caspase-3 activation, while soluble CD45 in supernatant, correlates with caspase-3 activity and rate of apoptosis with maximal effects at 24h. Our results indicate that TNF-alpha and caspase-3 inter reaction have been associated in regulation of diverse cell effects including shedding of cell surface molecules in K-562 tumor cells.

Poster #**047** / Session **I**

Death of Human Colon Adenocarcinoma COLO 205 Cells

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In this study we investigated the molecular mechanism of verapamil-induced autophagy in human colon adenocarcinoma COLO 205 cells. Verapamil is a well-known L-type calcium channel blocker, although it also retards glucose transport. By affecting calcium fluxes and glucose uptake verapamil might lead to endoplasmic reticulum (ER) stress. Involvement of clusterin protein, which is known to regulate cell death and survival processes, was also investigated in this study.

COLO 205 cells were cultured in sterile conditions in 75 cm2 bottles, 96-well plates or petri dishes, depending on type of experiment. Experiments were performed during 6, 12, 24, and 48 hours. Viability, proliferation and lysosome activity were evaluated by MTT, crystal violet, and neutral red assay, respectively. Intracellular Ca^{2+} level was measured by FURA-2 fluorescence. Ultrastructural analysis was performed using transmission electron microscopy (TEM). Protein expression levels were evaluated by Western blot (WB). Percentage of the apoptotic and necrotic cells was calculated via flow cytometry analysis (FACS).

Viability and proliferation of COLO 205 cells dropped up to 64.29 % ± SEM and 42.16 % ± SEM ($p<0.001$) upon verapamil [100 uM, 48h] treatment. Moreover, verapamil [100 uM, 48h] decreased the intracellular calcium level to 63.68 %. ± SEM ($p<0.001$). In contrast, the lysosome activity was increased to 211.8% ± SEM ($p<0.001$) after verapamil treatment [200 uM, 12h]. The TEM analysis revealed typical hallmarks of autophagy such as autophagic vacuoles, numerous lysosomes and endosomes and multilamellar bodies. WB analysis showed increased expression of MAP LC3, BECLIN 1, CASPASE 3, CHOP and GRP78/BiP along with decreased BCL-2 and secretory clusterin (sCLU). Presence of apoptotic (40.96% ± SEM, $p<0.01$) and necrotic (26.43% ± SEM, $p<0.01$) cells was confirmed through FACS analysis after verapamil treatment [200 uM, 24h]. Addition of glucose [2, 4 g/L] to the medium restored cell viability affected by verapamil [100 uM] and elevated the expression of sCLU.

The molecular mechanisms of verapamil-induced autophagy possibly occur either via blockage of glucose transport and/or calcium entry. Furthermore, it is possible that ER stress could be mediator of this process. Obviously clusterin plays an important role in control of viability and autophagy in cancer cells. Finally induction of autophagy contributes in verapamil-dependent cell death in COLO 205 cells.

Poster #**048** / Session **II**

1,4-naphthoquinones Inhibit MAPK/ERK Signaling and Induce Apoptotic Cell Death in Breast Cancer Cells

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The mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway is constitutively activated in various cancers, including breast cancer. The activation of this pathway is associated with inhibition of apoptotic cell death via the up-regulation of anti-apoptotic Bcl-2 proteins. Research has shown that 1,4-naphthoquinones are potent inducers of apoptosis and inhibitors of the Bcl-2 protein. The objective of our research was to select structures from the group of 1,4-naphthoquinones with the highest inhibitory activity toward the ERK1/2 protein. Molecular modeling distinguished several 1,4-naphthoquinones with potent inhibitory activity. Cell culture-based analysis, using various breast cancer cell lines, confirmed inhibition of MAPK signaling by these compounds through suppression of ERK1/2 phosphorylation. The 1,4-naphthoquinones induced apoptotic cell death as determined by Annexin V-PE staining, cell cycle analysis and caspase activity analysis. Moreover, they increased the expression of pro-apoptotic Bcl-2 proteins and down-regulated anti-apoptotic Bcl-2 in breast cancer cells.

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Poster #**049** / Session **I**

Atg5-Independent Autophagy Promotes Sorafenib-Induced Necroptosis

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Autophagy is a survival mechanism that cancers employ to alleviate the cytotoxic damage induced by therapeutic drugs. Treatment of DU154 prostate cancer cells with sorafenib, a multi-tyrosine kinase inhibitor, leads to mitochondrial disruption, autophagy and cell death. Despite the fact that these cells are Atg5-deficient, lack LC3 lipidation, and have high levels of p62 (SQSTM1); treatment with sorafenib results in autophagy as demonstrated by electron microscopy, LC3-GFP and LC3-GFP/RFP foci. Silencing of ULK1 and Beclin1 or re-expression of Atg5 in DU145 or in Atg5-/- MEFs inhibit sorafenib-induced cell death indicating that these cells die in an autophagy-dependent manner. Development of resistance to sorafenib in this prostate model system associates with the reactivation of the Src/PI3K/AKT/mTOR pathway leading to inhibition of autophagy. Treatment with sorafenib induces the interaction of RIPK1 with p62, as demonstrated by immunoprecipitation and a proximity ligation assay, and sorafenib-induced cell death is blocked by the RIPK1 inhibitor, necrostatin-1. Knockdown of p62 dramatically decreases RIPK1 protein levels and renders necrostatin-1 ineffective in blocking sorafenib-induced cell death. Thus, Atg5-deficient autophagy induced by mitochondrial damage, leads to the accumulation of p62 protein and promotes the activation of necroptosis.

Poster #**050** / Session **II**

Autophagic Properties Induced By Oncogenic Signalling: Towards Exploitation for Novel Therapeutic Protocols

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Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes. Autophagy plays an important role in cancer – both in protecting against tumour progression by isolating damaged organelles, also, depending on the tumour, by potentially contributing to cancer growth in promoting survival of tumour cells that have been starved.

The differential impact of autophagy in RAS induced transformation and apoptosis has been shown, and still remains to be further analysed, depending on the tumour cell context. Notably, resistance of colorectal neoplasms has been observed for anti-cancer agents against components of these pathways. Resistance pathways are currently under investigation, among which autophagy has attracted a major interest.

In the present study, the role of KRAS/BRAF/PK3CA oncogenic pathways on the autophagic cell properties and expression of main components of the autophagic machinery in colon tumour cells has been analysed. Here, oncogene regulated autophagic markers have been analysed and search of novel combinatorial antitumour therapeutic protocols has been performed for further exploitation.

Poster #**051** / Session **I**

Inhibition of Mitochondrial Complex II by Thenoyltrifluoroacetone Potentiates the Efficacy of Anticancer Therapies

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Cancer and apoptosis are generally regarded as antagonistic processes. Apoptosis is involved in spontaneous regression of tumors, whereas defects in apoptotic pathways contribute to tumor progression and resistance to treatment. Therefore, stimulation of cell death is the most widely used tool in cancer therapy. Considering the pivotal role of mitochondria in essential physiological processes such as generation of ATP for metabolic demands, and in various forms of cell death, steps directed to mitochondrial destabilization, permeabilization of the outer mitochondrial membrane (OMM) and release of pro-apoptotic proteins represent a promising strategy to combat cancer.

Cisplatin, an alkylating agent, is widely used for treatment of a variety of cancers, such as neuroblastoma (NB), head and neck, bladder and testicular cancers. Unfortunately, chemotherapy often fails due to development of cisplatin resistance or from toxicity associated with therapy. This highlights an unmet clinical need for a safe and effective cisplatin dose-sparing co-treatment. In this study, the mitochondrial complex II inhibitor thenoyltrifluoroacetone (TTFA) was combined with cisplatin to determine whether mitochondrial destabilization can potentiate the therapeutic effectiveness of the drug.

Incubation of NB cell lines, e.g Tet21N and SK-N-BE(2), with 5 μg/ml cisplatin for 24 h induced very minor manifestations of apoptosis, assessed by morphological changes, release of cytochrome *c* from mitochondria, stimulation of caspase-3-like activity, and cleavage of poly (ADP-ribose) polymerase (PARP). However, when combining cisplatin and TTFA at cell-type specific sub-lethal

doses (5μg/ml and 50-100 μM, respectively), a significant synergistic increase in the apoptotic response was observed in a dose- and time-dependent manner. Further, our results demonstrate that TTFA's chemopotentiating effect is reactive oxygen species (ROS) -dependent. Mitochondria are a major site of ROS-production, which participates in different signaling pathways, but might cause cell damage if produced excessively. Blocking respiratory complexes stimulates leakage of electrons with formation of ROS, contributing to OMM permeabilization. Using live cell imaging and the superoxide-sensitive dye MitoSOX™ Red we confirmed that TTFA increases ROS production induced by cisplatin, and that its chemopotentiating effect can be abrogated by the antioxidant N-acetyl-cysteine (NAC).

Thus, these findings suggest that destabilizing mitochondria by TTFA shows potential to increase the effectiveness of anticancer therapies and thereby ultimately improve patients' quality of life.

Poster #**052** / Session **II**

The *C. elegans* homologue of the human iron-sulphur domain protein CISD1, mediates a pro-longevity response through the intrinsic apoptosis pathway

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NEET proteins are recently discovered proteins, implicated in several pathologies, including cancer, diabetes, neurodegenerative diseases and the Wolfram syndrome 2, however their mode of action remains largely enigmatic. They are mainly localized on mitochondria and the endoplasmic reticulum. They are suggested to play important roles in apoptosis, autophagy and mitochondrial homeostasis. Our aim is to develop a genetic model towards characterizing NEET proteins *in vivo* and identifying the mechanisms that link them to apoptosis and mitochondrial function. To this end, we are characterizing the *C. elegans W02B12.15* gene, which encodes a functional homologue of CISD1, a member of the human NEET protein family. We find that the W02B12.15 protein is localized on the outer mitochondrial membrane, in body muscle, intestinal and neuronal cells. Knockdown of the *W02B12.15* gene triggers apoptosis, UPR^{ER} under endoplasmic reticulum stress, increases mitochondrial membrane potential, oxygen consumption and ATP production, while it reduces UPR^{mito}. In addition, we examined the effects of *W02B12.15* knockdown on the lifespan of diverse genetic backgrounds (wild type; *daf-2* and *ife-2* mutants as major modulators of lifespan; *ced-9*, *ced-4*, *ced-3* and *egl-1* mutants as key modulators of apoptosis and *hsp-6*, *mev-1*, *isp-1*, *clk-1* mutants deficient in mitochondrial homeostasis) and find that W02B12.15 is an important determinant of physiological aging. Overall our data suggest that W02B12.15 may serve as a regulator of mitochondrial homeostasis, with impact on lifespan *via* mechanisms that integrate ROS production, stress responses and the intrinsic apoptosis pathway.

Poster #**053** / Session **I**

Examining Conditional Caspase-6 Deficiency as a Therapeutic Proof of Concept for Huntington Disease

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Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor, cognitive, and psychiatric symptoms. HD is caused by a CAG repeat expansion in the huntingtin (HTT) gene resulting in the production of mutant huntingtin (mHTT). Caspase-6 (C6) is a cysteine aspartyl protease that plays a central role in apoptosis and has recently been implicated in several neurodegenerative diseases. Increased C6 activation is observed in early grade human HD brains and in mouse models of HD, and genetic or peptide inhibition of C6 activity in vitro protects neurons from degeneration. C6 is one of several proteases that cleaves mHTT, and our lab has previously demonstrated that preventing C6-mediated cleavage of mHTT at the 586 amino acid site ameliorates neuropathology and rescues cognitive and motor deficits in the YAC128 mouse model of HD. These data point to an essential role for C6 in the pathogenesis of HD and provide a rationale for the inhibition of C6 as a therapeutic approach.

The goal of this study is to investigate the effect of adult C6 deficiency on the YAC128 phenotype. This will be carried out by crossing a C6flox/CreERT2 mouse with the YAC128 mouse model of HD and inducing excision of the C6 gene by tamoxifen treatment. Treated mice will be examined for behavioural, neuropathological and biochemical endpoints. Results from this study will provide insight into the effect of the loss of C6 in adulthood on HD pathogenesis and will further validate C6 as a therapeutic target in HD.

Poster #**054** / Session **II**

Targeting an Ion Channel for Selective Elimination of Cancer Cells in vivo

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Ion channels are emerging oncological targets. In particular, potassium-selective channels show a de-regulated expression in different tumor cells/tissues compared to healthy ones, representing thus possible targets for the development of new chemotherapeutic drugs. Furthermore, pharmacological targeting of ion channels in mitochondria proved to be a promising strategy, in accordance with the essential role of ion channels for the regulation of bioenergetics in these organelles and with the crucial role of mitochondria in apoptotic signaling.

Pharmacological inhibition of the a mitochondrial potassium channel (mtKv1.3) by membrane permeant blockers, Psora-4, PAP-1 and clofazimine, indeed triggered apoptotic cell death in different cancer cell lines, even in the absence of Bax and Bak, by inducing mitochondrial membrane potential depolarization, production of mitochondrial ROS and release of cytochrome c. Apoptosis upon incubation with the drugs did not occur when expression of Kv1.3 was downregulated by siRNA.

Importantly, the Kv1.3 inhibitor clofazimine, a drug already used in clinic to treat leprosy and autoimmune diseases, reduced melanoma volume up to 90% compared to the untreated mouse in an orthotopic mouse melanoma model. Furthermore, Psora-4, PAP-1 and clofazimine were able to selectively kill also primary Chronic Lymphocytic Leukemia B cells (B-CLL), without affecting normal blood cells of the same patients and independently of the currently used prognostic factors. An increased ROS production together with an increased Kv1.3 expression in B-CLL cells seems to account for the selective apoptosis-inducing ability of the drugs.

Recently, we obtained promising in vivo data also in a Pancreatic Ductal Adenocarcinoma (PDAC) mouse model: treatment of mice with clofazimine reduced PDAC tumor weight by 50%.

Since membrane permeant Kv1.3 inhibitors are characterized by poor water solubility, in order to increase their solubility as well as to increase their bioavailability, we have recently synthesized new PAP-1 derivatives. In vitro experiments using different cancer cell lines demonstrate the ability of these novel derivatives to kill tumor cells at significantly lower concentrations with respect to the precursor, still maintaining the specificity toward Kv1.3-expressing cells. The newly synthesized derivatives have been tested also ex-vivo (B-CLL cells) and in vivo tumor models, yielding promising results.

Poster #**055** / Session **I**

Study of Cell Death Mechanisms in Spinocerebellar Ataxia Type 7 Retinal Degeneration

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Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disease characterized by a progressive neuronal loss in cerebellum, brain and retina leading to ataxia and progressive central visual loss resulting in blindness. SCA7 belongs to the polyQ disorders family, which also includes Huntington's disease and other spinocerebellar ataxias. Indeed, ATXN7, the only gene associated with SCA7, can present an abnormal CAG trinucleotide repeat expansion in its N-Terminal part. The polyQ tract gives to the protein a new aggregation property, which leads to toxicity, cellular stress and, finally the death of cells. So far, SCA7 is the only polyQ disorder that affects retina. The loss of photoreceptors occurs in two distinct waves of cell death going along with Müller cells «trans-differentiation».

We used a murine model of SCA7 in order to study polyQ-induced photoreceptors death. Our results show that the first wave of cell death is apoptotic but does not require caspases. AIF (Apoptosis Inducing Factor) and the LEI/L-DNaseII (Leucocyte Elastase Inhibitor) are activated in this model. These two cell death effectors, involved in caspase-independent apoptosis, can collaborate during the DNA degradation step, as previous studies in the lab had shown.

Concerning the second observed wave of death, it appears to be a dark degeneration but little is known about this form of cell stress. It has only being morphologically described but it is interesting to note that it has already been observed in other polyQ disorders as well as in excitotoxicity neuronal death. Due to the little amount of affected neurons, we set up an *in vitro* model. A photoreceptor cell line is treated with glutamate in order to activate excitotoxicity and to identify activated pathways. Proteomics changes are currently analysed by two-dimensional electrophoresis (2D-E) and mass spectrometry.

Poster #**056** / Session **II**

Deubiquitylation-mediated Stabilization of cIAP2 by USP11 Controls BV6-mediated Apoptosis

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Inhibitor of apoptosis proteins (IAPs) contribute to cell survival via direct inhibition of caspases or indirect control of NF-κB-related pathway. Here, we report that the stabilization of cellular IAP2 (cIAP2) is a major factor suppressing apoptosis induced by the IAP antagonist BV6 in certain cancer cell lines. Only cIAP2 depletion, but not cIAP1, sensitized cells to TNF/BV6-induced apoptosis. Ubiquitin-specific protease 11 (USP11) was identified as a deubiquitylating enzyme directly stabilizing cIAP2. USP11 ablation resulted in the proteasomal degradation of cIAP2, and accelerated BV6-induced cIAP2 degradation and apoptosis. Clinical data base search indicated that USP11 was overexpressed in colorectal cancer and melanoma. These cancer cell lines expressing high levels of USP11 exhibited high resistance against BV6-induced cIAP2 degradation and TRAIL/BV6-mediated apoptosis. Downregulation of USP11 sensitized these cells to TRAIL/BV6-induced apoptosis and suppressed tumor growth in xenograft mice model. Finally, USP11 induced via TNFα-JNK pathway was able to up-regulate cIAP2 stability. Collectively, our data reveal that cIAP2 stabilized by USP11 could be a barrier for IAP targeted clinical approach.

Poster #**057** / Session **I**

Bis Targeting Induces Cellular Senescence through the Regulation of 14-3-3zeta/STAT3/Skp2/p27 in Glioblastoma Cells

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Cellular senescence is an important mechanism for preventing tumor progression. The elevated expression of Bcl-2-interacting cell death suppressor (Bis), an anti-apoptotic and anti-stress protein, often correlates with poor prognosis in several cancers including glioma; however, the role of Bis in the regulation of senescence has not been well defined. Here, we describe for the first time that the depletion of Bis induces G1 arrest and cellular senescence through the accumulation of p27 that is independent of p53, p21 or p16. The increase in p27 expression in Bis-depleted cells was attributable to an impairment of the ubiquitin-mediated degradation of p27, which was caused by a decrease in S-phase kinase-associated protein 2 (Skp2) at the transcriptional level. As an underlying molecular mechanism, we demonstrate that the loss of activity of signal transducer and activator of transcription 3 (STAT3) was specifically linked to the suppression of Skp2 expression. Despite a reduction in phospho-STAT3 levels, total-STAT3 levels were unexpectedly increased by Bis depletion, specifically in the insoluble fraction. Our results show that 14-3-3ζ expression is decreased by Bis knockdown and that 14-3-3ζ depletion *per se* significantly induced senescence phenotypes. In addition, the ectopic expression of 14-3-3ζ blocked senescence caused by Bis depletion, which was paralleled with a decrease in insoluble STAT3 in A172 glioma cells. These findings indicate that the impairment of the protein quality control conferred by Bis and/or 14-3-3ζ is critical for Bis depletion-induced senescence. Moreover, Bis knockdown also induced senescence along with an accumulation of total STAT3 and p27 in several different cell types as well as embryonic fibroblasts derived from *Bis*-knockout mice with/without variations in 14-3-3ζ levels. Therefore, our findings suggest that a downregulation of Bis expression could serve as a potential strategy for restricting tumor progression via an induction of senescence through the regulation of STAT3/Skp2/p27 pathway.

Poster #**058** / Session **II**

Differential Expression of the Calcium-Sensing Receptor in the Ischemic Core and Penumbra after Transient Focal Cerebral Ischemia in Rats

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Calcium-sensing receptor (CaSR), a G-protein-coupled receptor (GPCR), was initially identified as protein playing a role in the maintenance of calcium homeostasis by regulating secretion of parathyroid hormone, but now is regarded as an important modulator of diverse cellular functions by sensing changes in the extracellular Ca^{2+} levels in the central nervous system. The present study aimed to provide a detailed characterization of the CaSR expression using a rat focal cerebral ischemia–reperfusion model induced by middle cerebral artery occlusion. Double-labeling study using CaSR and Fluoro-Jade B staining or the Tdt-dUTP terminal nick-end labeling (TUNEL) clearly demonstrated an overlapping regional distribution of CaSR and two markers for apoptotic and degenerating cells in the core region of ischemic injury within the first 24 hours. CaSR expression occurred in neurons and was associated with the vasculature, including microvasculature and larger caliber vessels. The temporal pattern of expression in the ischemic core region overlaps with the period during which neuronal cell death occurs, indicating that CaSR expression is induced in neurons undergoing cell death. CaSR expression was still associated with the vasculature, but not in neurons, in the ischemic core by day 3. This expression pattern was maintained on day 14, but there was a notable decrease in the intensity of CaSR expression in the ischemic core. Three days after ischemia, CaSR expression was observed in the peri-infarct region and was sustained for more than two weeks. Most of CaSR-positive cells included reactive astrocytes. Viable neurons sometimes revealed CaSR expression, but much less frequent than the astrocytes, and no significant induction of CaSR was observed in activated microglial cells. No staining was detected in adjacent undamaged tissues or in the contralateral hemisphere. Our data demonstrated characteristic time- and cell-dependent expression patterns for CaSR within the ischemic core and penumbra, indicating that the expression of CaSR might be regulated in response to altered extracellular levels of calcium following an ischemic insult. Thus, our data suggest that the regulation of CaSR expression is implicated to be likely in the pathophysiology of stroke.

Poster #**059** / Session **I**

The Kinase RIPK1 Acts as a Guardian of Skin Homeostasis

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Tumor necrosis factor (TNF) signaling can either induce apoptosis, necrosis or cell survival and inflammation via the transcription factor NF-κB. One major switch between these outcomes is the receptor interacting protein kinase 1 (RIPK1), which has kinase-dependent and kinase-independent functions. Consistent with RIPK1's important role in cell survival, RIPK1 full knock-out mice die at day 3 from extensive cell death. To understand the role of RIPK1 in skin homeostasis and inflammation we developed and analyzed the phenotype of mice lacking RIPK1 in the epidermis ($RIPK1^{EKO}$) by crossing $RIPIK1^{FL/FL}$ with K5-Cre mice. $RIPK1^{EKO}$ mice were born with the expected Medelian ratio. We confirmed specific ablation of RIPK1 in the epidermis by means of western blotting. After 3 weeks of age, $RIPK1^{EKO}$ mice spontaneously develop severe skin lesions. Macroscopic analysis of the skin revealed areas of normal epidermis along with lesional regions. Microscopic analysis showed that the mutant had thicker and hyperproliferative epidermis in macroscopically normal-looking areas compared to $RIPK1^{FL/FL}$ and in lesional skin. $RIPK1^{EKO}$ skin was also characterized by increased immune cell infiltration in the dermis. Skin samples also revealed increased levels of apoptotic and necrotic keratinocytes in $RIPK1^{EKO}$ mice, suggesting that increased cell death lies at the basis of skin inflammation. Consequently, RIPK1 deficient keratinocytes were more sensitive to TNF-induced cell death compared to wild-type keratinocytes. However, neutralization of TNF by Etanercept or crossing RIPK1EKO mice with TNFR1 deficient mice did only delay the appearance of skin lesions, but did not prevent them. Importantly, RIPK1 kinase-dead knock-in mice did not develop any skin phenotype, suggesting that RIPK1-mediated protection resides in its kinase-independent platform function. These data highlighting a new and unexpected pro-survival platform-dependent function of RIPK1 in skin homeostasis.

Poster #**060** / Session **II**

Calmodulin and Calcineurin are Required For KIF1B Beta-Induced Apoptosis via the Regulation of Drp1 Phosphorylation in Neuroblastomas

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The pediatric cancer neuroblastoma (NB) arises from the abnormal growth of cells of the neural crest, which form the peripheral nervous system and specialized cells found in different organs. KIF1Bb belongs to the family of kinesin motor proteins and is located on human chromosome 1p36.2, a region of the genome that is frequently deleted in NB and other forms of neural crest-derived tumors. Although it was shown that KIF1Bb is both necessary and sufficient for neuronal apoptosis, its mechanism of action remains unknown.

We have recently found that ectopic expression of KIF1Bb induces mitochondrial fragmentation by decreasing phosphorylation of mitochondrial fission protein DRP1. Dephosphorylation of DRP1 has been shown to localize to mitochondria to mediate mitochondrial division, and shown to regulate developmental apoptosis. We observed that KIF1Bb induces DRP1 mitochondrial localization through the regulation of interaction between CALM2 and phosphatase calcineurin. Both DRP1 and calcineurin are necessary for KIF1Bb apoptosis function.

We are currently investigating Drp1 phosphorylation levels in tumor tissue samples from patients with newly diagnosed neuroblastoma. All these results showed that Drp1 might be the new target for neuroblastoma therapies.

Poster #**061** / Session **I**

Synchronized Tubular Cell Death is Predominantly Mediated by Ferroptosis

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Receptor-interacting protein kinase 3 (RIPK3)-mediated necroptosis is thought to be the pathophysiologically predominant pathway that leads to regulated necrosis of parenchymal cells in ischemia reperfusion injury. Here, we demonstrate that freshly isolated renal tubules do not undergo necroptosis upon genetic ablation of a necroptosis-inhibiting complex which includes FADD and caspase-8, and that RIPK3-deficiency affects the blood flow of peritubular capillaries. In contrast, iron dependent ferroptosis directly causes synchronized necrosis of renal tubules, as evident in acute ischemia-reperfusion injury and chronic calciumoxalate-induced chronic kidney crystalopathy. We generated a novel third-generation ferrostatin (16-86) which we demonstrate to be much more stable and effective *in vivo*

compared to ferrostatin-1 (Fer-1), the first generation prototype compound. We previously detected a strong protective effect in IRI mediated by combination therapy which consists of necrostatins and compounds that inhibit mitochondrial permeability transition. 16-86 further improved this effect upon ultra-heavy IRI which has never before been survived in any murine setting. We conclude that necrotic tubular death occurs via ferroptosis, and that combination therapy targeting diverse pathways of regulated necrosis provides major protective effects in IRI.

Poster #**062** / Session **II**

Autophagy-Related Protein 5 (ATG5) Suppresses Early-Stage Melanoma Tumorigenesis by Inducing Senescence

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Cutaneous melanoma is a common malignancy of melanocytes. In its early stage malignant melanoma can be cured by surgical resection, but it becomes extremely difficult to treat once it progresses to the metastatic stage due to its high resistance to current chemotherapeutic drugs. Autophagy is a highly conserved cellular self-eating process, in which proteins and organelles are sequestered and subsequently degraded in a double-membrane structure called autophagosome. Autophagy-related proteins (ATGs) are the main players during this process. Autophagy has been shown to be involved in the pathogenesis of numerous diseases, including neural degenerative diseases, infections and cancer. We are interested in the role of ATG5 in melanoma. By studying the expression of ATG5 and microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy, in a relative large cohort of primary melanomas, we found that ATG5 and LC3 are decreased in patients with melanomas comparing with those having benign nevi. Melanoma patients having low level of ATG5 showed low disease-free survival. Using an in vitro model of melanoma tumorigenesis, in which the mutated B-rapidly growing fibrosarcoma (BRAF) oncogene was introduced into normal human epidermal melanocytes, we were able to show that down-regulation of ATG5 promoted proliferation of melanocytes by bypassing oncogene-induced senescence. Our work provide evidence that ATG5 may functions as a tumor suppressor of early-stage melanoma and could serve as a diagnostic marker to distinguish benign from malignant tumor of melanocytes.

Poster #**063** / Session **I**

p53 Tumour Suppressor Family Engages the Adenosine A2B Receptor Signalling to Prime Cancer Cells to Chemotherapy- and Metabolic Stress-Induced Death

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Metabolic reprograming is a common feature of cancer cells and is thought to be critical for disease development and progression. In light of this, the increasingly prominent role of p53 in regulating cellular metabolism has in recent years been considered to constitute its tumour suppressive function. In this study, we add an additional layer of knowledge to this emerging role of p53 in cancer prevention, by linking cancer-related metabolic alterations to the ability of p53 to execute tumour cell death. We demonstrate that p53 activates a distinct cell death pathway that respond to stress- and therapy-induced accumulation of the metabolite, adenosine – the backbone of ATP - by upregulating the A2B adenosine receptor. Stimulation of A2B signalling by p53 activates a caspase- and Puma-dependent apoptotic response, which can be rescued by the overexpression of anti-apoptotic Bcl-2 proteins. Similar to p53, we also found its closely-related member, p73, engages A2B receptor signalling to stimulate cancer cell death in response to chemotherapy. While p53 is mutated in almost half of human cancers, p73 is found to be rarely mutated and, surprisingly even upregulated in some tumours. Moreover, since extracellular adenosine is known to accumulate within the tumour microenvironment, these findings not only impact on our understanding of p53 family function, but also uncover an alternative cell death pathway that could be modulated for therapeutic benefit.

Poster #**064** / Session **II**

Mimicking the BCL2 Addiction of Cancer Cells

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Abstract withdrawn by authors from online

Poster #**065** / Session **I**

Salinomycin, a New Inducer of Apoptosis: Insights into the Mechanism of Action

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Salinomycin is a monocarboxyl polyether antibiotic isolated from *Streptomyces albus*, with anticoccidial and antimicrobial activity. This molecule carries cations through the lipid bilayer, displaying high affinity for potassium (K^+) ions, therefore it is considered to act as a K^+ ionophore, like valinomycin (1). Recently, salinomycin has been identified as a selective inhibitor of cancer stem cells (CSCs) (2), becoming a molecule of great interest, since CSCs drive growth and regeneration of the tumors. Moreover, there is growing evidence that salinomycin is able to kill also different types of non-CSCs tumor cells that usually display resistance to common therapeutic approaches (3) while leaving healthy cells alive. However the mechanism of action of this molecule is poorly understood so far.

Since salinomycin is universally defined as a valinomycin-like K^+ ionophore, we investigated the bioenergetic effects of this compound on mitochondria and explored whether its effects on mitochondria might contribute to its pro-apoptotic function. We compared the effect of salinomycin with that one of nigericin, a H^+/K^+ antiporter, and of valinomycin. On isolated mitochondria, salinomycin causes hyperpolarization of the inner mitochondrial membrane without causing swelling (similarly to nigericin). In intact cells, application of salinomycin does not result in short-term mitochondrial depolarization and ROS production, in contrary to what can be observed with valinomycin, but causes matrix acidification as revealed by the use of a fluorescent probe. Moreover, when measuring mitochondrial respiration in intact cells, salinomycin affects mitochondrial respiration and clearly behaves as nigericin and not as valinomycin. Thus, we conclude that salinomycin acts as a H^+/K^+ antiporter and its activity as ionophore decreases mitochondrial bioenergetic efficiency. The direct effect of salinomycin on respiratory chain complexes has also been investigated.

Furthermore, in accordance with the above data, the pro-apoptotic effect of salinomycin was found to be similar in B-cells isolated from patients with CLL (chronic lymphocytic leukemia) and in B-cells obtained from healthy subjects. This finding questions the specificity of salinomycin toward cancer cells (4). The lack of specificity was further confirmed in B-CLL cells cultured in the presence of mesenchymal stromal cells, a condition which mimics the in vivo tumor environment.

Thus, our results indicate that at the concentration range used in most studies, salinomycin exerts its effect also at the level of mitochondria, even in healthy cells.

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Poster #**066** / Session **II**

MPS1 as a Target for Anti-Cancer Therapy

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MPS1, a mitotic kinase that is overexpressed in several human cancers, contributes to the alignment of chromosomes to the metaphase plate as well as to the execution of the spindle assembly checkpoint (SAC). In this study, we successfully developed a novel anticancer strategy targeting MPS1. First, we demonstrated that the inhibition (by administering distinct pharmacological inhibitors) or the depletion (by transfecting cells with specific siRNAs) of MPS1 abolishes SAC function, both *in vitro* and *in vivo*, thus causing chromosome mis-segregation, aneuploidization/polyploidization and, ultimately, mitotic catastrophe via the activation of the mitochondrial pathway of apoptosis. In addition, we put in evidence that the inhibition or depletion of MPS1 can eliminate tetraploid cancer cells more efficiently than their parental diploid counterpart. Of note, tetraploidy constitutes a genomically metastable state that can lead to aneuploidy and genomic instability. Tetraploid cells have been observed in the early stages of multiple tumors as well as in pre-neoplastic lesions such as Barrett's esophagus. We surmise that the anti-tetraploid effect of MPS1 inhibition/depletion may be explained by the evidence that tetraploid cells are intrinsically more prone than their euploid counterparts to mitotic aberrations and mitotic catastrophe, as they require (or even are "addicted" to) a very robust mitotic machinery for correctly segregating their genetic material.

Altogether, these results suggest that MPS1 inhibitors may exert robust anticancer/antitetraploid activity.

Poster #**067** / Session **I**

Evolutionary Solution for Cancer Prevention: Normal Fibroblasts of Wild Hypoxia-Tolerant Subterranean Rodent *Spalax Ehrenbergi* Suppress Anchorage-Independent Cancer Cell Growth and Inhibit Cancer Cell Migration

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Biodiversity that evolved on Earth created solutions for various biological challenges. In this regard, the study of natural resistance to cancer observed in several wild animals seems to be a very promising and an important direction in anticancer and cancer prevention strategies. We recently reported that the hypoxia-resistant long-lived subterranean blind mole rat (*Spalax ehrenbergi)* possesses an outstanding tolerance to carcinogens and a direct inhibition of cancer cell growth by *Spalax*'s primary fibroblasts (1). Here we demonstrate that anchorage-dependent apoptosis is the predominant mode of cancer cell death in response to paracrine factors released by *Spalax* fibroblasts. The soft agar colony formation assay and Matrigel invasion assay were used to monitor anchorage-independent spreading of cancer cells originated from different species and tissues (human breast cancer MDA-MB-231 and prostate cancer PC-3cells, human hepatocellular carcinoma Hep3B and HepG2 cells, as well as newly isolated mouse and *Spalax*-derived fibrosarcomas). Transwell migration assay was employed to evaluate chemotactic activity. In addition, effects of conditioned media generated by *Spalax*, rat or mouse fibroblasts on viability and cell cycle distributions were investigated. *Spalax* fibroblasts monolayer strongly reduced anchorage-independent tumor growth while mouse and rat fibroblasts had no effects or stimulated colony formation. Similarly, *Spalax* fibroblasts suppressed cancer cells migration through the membrane pores and across the Matrigel. Medium conditioned by *Spalax* fibroblasts induced arrest of proliferation in different cancer cells, while normal cells continued to proliferate. Protein expression profiling in Hep3B cells exposed to *Spalax* fibroblast-conditioned medium revealed alterations in extracellular matrix signaling molecules and apoptotic regulatory proteins compared with control cells. Conclusion**:** The cancer microenvironment is an integral part of the tumor where cancer cells grow and invade. The main scenario of successful cancer progression is the dynamic repression of natural host cancer

tolerance. We propose *Spalax* as a natural cancer-resistant model in which pre-malignant or cancerous cells apparently do not receive support from adjacent stroma, and wherein tumors presumably would not be able to freely proliferate and metastasize.

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Poster #**068** / Session **II**

Autophagy Protects Against Age-associated Neurodegeneration

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Ageing, the progressive accumulation of damage to molecules, cells, tissues and organs, is accompanied by a marked decrease of neuronal function that eventually leads to cell death and increased vulnerability to neurodegeneration in diverse organisms, including *C. elegans*, *Drosophila*, mouse and humans. The molecular pathways affecting age-related neural deterioration remain largely obscure. Autophagy, an evolutionarily conserved process for lysosomal protein and organelle degradation and recycling, serves essential roles in development, survival and homeostasis at both the cellular and organismal level. Autophagic activity declines with age and deregulation of autophagy has been associated with accelerated ageing and various human pathologies such as cancer, neurodegenerative diseases, muscular disorders and microbial infection. Although caloric restriction that improves healthspan and lifespan in organisms ranging from the lowly nematode *Caenorhabtitis elegans* to mammals activates autophagy, a direct molecular link between autophagy and neurodegeneration has not been established. Furthermore, whether autophagy protects from or contributes to neurodegeneration is unclear. We exploit the unique advantages of the *C. elegans* model system to dissect the contribution of autophagy to the pathogenesis of various neurodegenerative disorders. To this end, we use reporter strains expressing fluorescent-tagged translational fusions of the *lgg-1* gene, which encodes an ubiquitin-like protein homologous to mammalian LC3 protein localizing to preautophagosomal and autophagosomal membranes. Our preliminary work suggests an essential link between autophagy and neurodegenerative proteotoxicity. We find that activation of autophagy can confer protection against a-synuclein-induced toxicity in a *C. elegans* model of Parkinson's disease. By contrast, autophagy inhibition appears to abrogate protection and increase the neurotoxicity of a-synuclein aggregates. We are now using LGG-1 reporter strains to uncover novel components of the autophagy pathway that act as modulators of cell death and neurodegeneration in *C. elegans*. Elucidation of the mechanisms underlying the neuroprotective effects of autophagy may contribute to the development of better intervention strategies aiming to ameliorate or prevent neurodegenerative disorders.

Poster #**069** / Session **I**

Simultaneous Induction of Apoptosis and Necroptosis by SMAC Mimetics in Acute Lymphoblastic Leukemia

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SMAC mimetics (SM) are an emerging class of drugs, which specifically target the inhibitor of apoptosis proteins (cIAP1, cIAP2 and XIAP), leading to the disinhibition of Rip1 kinase and activating cell death through caspase-dependent and -independent mechanisms. The high expression of IAP proteins in acute lymphoblastic leukemia renders SMs as interesting agents for anti-leukemic therapy. In a series of 51 primary samples derived from patients with acute lymphoblastic leukemia (ALL) we detected that approximately 20% of the cases were highly sensitive to *in vitro* treatment with pre-clinical SMAC mimetics, LCL-161 or Birinapant (IC50 in the nanomolar range). To determine the pathway of cell death activated in sensitive cells, we co-treated cells with SM and a pan-caspase inhibitor (zVAD) to block apoptosis, a RIP1 kinase activity inhibitor (necrostatin-1) to block necroptosis, or a combination of both inhibitors. Surprisingly, we found that SM treatment resulted in a mixture of both cell death modalities which varied greatly among patients, although the majority of samples showed the greatest rescue of viability after combined inhibition of caspases and Rip1 kinase. Analysis of caspase activation and loss of plasma membrane integrity by FACS and microscopy in SM treated cells showed that apoptosis and necroptosis occurred simultaneously within a given cell population. Using CRISPR gene targeting technology to specifically disrupt candidate regulators, we find that both modes of cell death are dependent on TNF-receptor and Rip1 kinase expression. Downstream, cell death can be shifted towards apoptosis by targeting FADD and towards necroptosis by targeting Rip3 expression.

On the mechanistic level, this data shows that apoptotic and necroptotic cell death pathways are not mutually exclusive phenomena, but can occur simultaneously within a population of cells. Overall, our data indicate that because SMs can induce multiple forms of cell death, these agents have a strong potential for anti-leukemic therapy in childhood ALL.

Poster **#070** / Session **II**

The Kinase GSK-3β and the E3 Ligases Mule and β-TrCP Contribute to Cytokine-mediated Mcl-1 Degradation and Pancreatic β-cell Apoptosis

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Type 1 diabetes (T1D) is an autoimmune disease characterised by selective destruction of the insulin-producing pancreatic β-cells via the intrinsic pathway of apoptosis. We have previously shown that cytokine-mediated down-regulation of myeloid cell leukaemia sequence 1 (Mcl-1) protein, an anti-apoptotic member of the Bcl-2 family, is an important contributory event for β-cell apoptosis. In line with this, overexpression of Mcl-1 or prevention of its degradation significantly decreases cytokine-mediated β-cell death. Importantly, decreased expression of Mcl-1 was observed in β-cells from T1D patients. The *in vitro* studies mentioned above were performed in rodent β-cells, and we have now evaluated the role of Mcl-1 in the well-differentiated human β-cell line EndoC-βH1 and also in primary human islet cells. Furthermore, the mechanisms by which cytokines regulate Mcl-1 turnover in β-cells were investigated.

As observed in rodent β-cells, exposure of the human β-cell line EndoC-βH1 to the pro-inflammatory cytokines IL-1β+IFN-γ and TNF+IFN-γ for 24 hours decreased Mcl-1 protein expression by 42 and 50%, respectively ($p<0.05$; n=4). Silencing Mcl-1 with specific siRNAs sensitised EndoC-βH1 cells to apoptosis induced by the different cytokine combinations (IL-1β+IFN-γ increased by 36%, $p<0.05$; TNF+IFN-γ increased by 48%, $p<0.01$; n=4). In mirror experiments, overexpression of Mcl-1 using adenovirus protected EndoC-βH1 cells (36% protection; $p<0.05$; n=4) and dispersed human islet cells (52% protection; $p<0.05$; n=6) against cytokine-induced apoptosis. Phosphorylation of Mcl-1 by the GSK-3β kinase has been shown to contribute to Mcl-1 degradation in other cell types, and inhibition of GSK-3β activity in INS-1E cells using two specific chemical inhibitors (SB-216763 and BIO) prevented Mcl-1 degradation after cytokine treatment by up to 40% as compared to DMSO-treated cells ($p<0.05$; n=5). Moreover, GSK-3β inhibition resulted in significant decrease IL-β+IFN-γ-mediated apoptosis in both INS-1E (40-50%, $p<0.005$; n=3) and in EndoC-βH1 cells (35%, $p<0.05$; n=5). Phosphorylated Mcl-1 was found to be ubiquitinated by the E3 ligases Mule, βTrCP and FBW7, leading to its proteosomal degradation.

Silencing of these E3 ligases by specific siRNAs increased basal Mcl-1 protein expression, and knockdown of either Mule or βTrCP also prevented Mcl-1 IL-β+IFN-γ-induced Mcl-1 decrease in INS-1E cells (1.8-fold siMule *vs* siCTR, $p<0.01$; 1.6-fold after siβTrCP *vs* siCTR, $p<0.05$; cytokine treated conditions; n=3-7).

In conclusion, our findings demonstrate that decreased Mcl-1 expression is also an important feature contributing to cytokine-mediated apoptosis in human pancreatic β-cells. Moreover, we show that the kinase GSK-3β and the E3 ligases Mule and βTrCP are important regulators of cytokine-mediated Mcl-1 turnover in these cells. This information is of relevance for the development of novel strategies to prevent Mcl-1 degradation and β-cell apoptosis in T1D.

Poster #**071** / Session **I**

Protein Phosphatase 4 Regulates Cell Survival, Proliferation and Responses to Chemotherapeutic Drugs in Breast Cancer Cell lines

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The activity of many of the proteins which regulate cell survival and proliferation, in common with those involved in controlling other cellular processes, is controlled by reversible phosphorylation. As a consequence, the relevant kinases and phosphatases play vital roles in the control of cell death and survival. Here we report the analysis of the role of the catalytic subunit of the serine/threonine protein phosphatase, protein phosphatase 4 (PP4c), in controlling the survival and proliferation of breast cancer cell lines, using the complementary techniques of gene over-expression and down-regulation through RNA interference. This analysis has demonstrated that PP4c regulates cell death, proliferation and anchorage independent growth in breast cancer cell lines, including the metastatic cells. Results also showed that down regulation of PP4c expression impaired the induction of cell death by certain chemotherapeutic drugs and other apoptotic stimuli in the breast cell lines.

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Poster #**072** / Session **II**

Molecular Characterization of Immunogenic Cell Death Triggered by the High Hydrostatic Pressure in Human Tumor Cells

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Immunogenic cell death (ICD) is a form of cell death caused by certain chemicals or physical modalities: cytostatic agents such as anthracyclines, oxaliplatin and bortezomib, or radiotherapy and photodynamic therapy. Typical molecular events characteristic for ICD are surface exposure of calreticulin, HSP70 and HSP90, release of intranuclear HMGB1 and the secretion of ATP from dying cells. We investigated the potential of high hydrostatic pressure (HHP) to induce ICD in human tumor cells and analyzed the key components of apoptotic pathways.

Prostate and ovarian cancer cell lines were treated by HHP, UV-B and anthracycline (idarubicin). The kinetics of ICD markers and key components of ER stress-mediated apoptotic pathway were analyzed by flow cytometry, western blotting and confocal microscopy.

HHP induced rapid expression of HSP70, HSP90 and calreticulin on the cell surface of early apoptotic cells. In cells treated by HHP an increased production of reactive oxygen species (ROS) was detected. Such production was suppressed in cells pretreated with the ROS scavengers (N-acetyl-L-cysteine (NAC) or L-glutathione (GSH)), followed by decreased calreticulin expression and deceleration of apoptosis. In addition, cells, in which the ROS production was inhibited, had reduced capacity to cleave caspases (-8, -3) and exhibited decreased levels of phosphorylated eIF2α upon HHP treatment. This finding links the oxidative stress with the ER stress response and downstream activation of caspases. The other key components of the ER stress-mediated apoptotic pathway, such as phosphorylation of the eIF2α, the activation of caspase-8 and caspase-8-mediated cleavage of the ER protein BAP31 were also detected. The importance of the apoptotic pathway induced by HHP treatment was confirmed using selective inhibitors, transient transfection (siRNA) or gene knockdown by shRNA.

We identified HHP as an additional modality inducing key characteristics of ICD, including the involvement of ER stress-mediated apoptotic pathway. HHP acts as a potent inducer of ICD leading to apoptosis and it complements already identified group of chemotherapeutics as well as physical modalities described to initiate ICD in tumor cells.

Poster #**073** / Session **I**

Analysis and Characterization of TRAIL-Induced, Receptor-Specific Signaling in Cancer Cells

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Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine that can effectively induce apoptosis in tumour cells via the activation of its pro-apoptotic cell surface receptors: TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and is being considered as a potential novel anticancer agent. TRAIL can also bind decoy receptors TRAIL-R3 (DcR1) or TRAIL-R4 (DcR2) that compete for TRAIL binding thus blunting TRAIL-induced activation of apoptosis. In addition to apoptosis, TRAIL can trigger several other signaling pathways such as activation of MAP kinases or canonical NF-κB signaling. Both receptors are ubiquitously expressed at various levels on normal and cancer cells, but the relative contribution of DR4 and DR5 to TRAIL-induced signaling is not well known.

We prepared Strep-tagged variants of recombinant human TRAIL with high affinity for either DR4- and DR5 receptors. With these receptor-specific ligands we examined contribution of individual pro-apoptotic receptors to apoptosis and other TRAIL-induced signaling pathways, especially NF-κB and JNK, p38, ERK1/2 and TAK1 in selected colorectal cell line. We found that in TRAIL-resistant HT-29 cells though DISC formation and activation of caspase-8 proceeds mainly via DR4-specific signaling, activation of NF-κB pathway is mainly triggered by DR5 selective ligand. In other analyzed signaling pathways both receptor-specific ligands triggered very similar responses. Our work provides the first systematic insight into DR4/DR5-specific signaling in human colorectal cell lines.

Poster #**074** / Session **II**

Necdin is a Novel Regulator of Mitochondrial Integrity and Stress Response Pathways in Mammalian Neurons: Insights from *C. elegans*

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Necdin, a member of the melanoma antigen (MAGE) superfamily, has pleiotropic effects in the function of the nervous system. Loss of a chromosomal locus that contains the Necdin gene invariably results in Prader Willi Syndrome, a neurodevelopmental disorder manifested by neurological and metabolic defects. Necdin is known to have specific interactions with proteins that regulate neuronal development and maturation, including in particular the neurotrophin Trk receptors, the p75 neurotrophn receptor, p53 and E2F1 among others. Selective neuronal populations, such as GABAergic and sensory neurons, undergo cell death in the absence of Necdin, while other populations display synaptic defects. We have identified and characterized the single *C. elegans* homologue of mammalian MAGE genes, named *mage-1*. MAGE-1 localizes to the mitochondria of neurons and intestinal cells and the loss of its function results in mitochondrial fragmentation, aberrant resistance to various forms of stress and increased cell death. Notably, we also found that a fraction of Necdin also localizes to mitochondria in mammalian neurons, where it safeguards mitochondrial integrity and controls resistance to stress. These findings have implications both in normal development but also in terms of neuropathology, linking for the first time MAGE proteins with mitochondrial function and stress resistance pathways.

Poster #**075** / Session **I**

Inhibitor of Apoptosis Proteins as Promising Therapeutic Targets in Chronic Lymphocytic Leukemia

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Inhibitor of apoptosis (IAP) proteins are expressed at high levels in CLL cells and may contribute to evasion of cell death leading to poor therapeutic outcome.

Of note, prognostic unfavourable cases with e.g. non-mutated VH-status and TP53 mutation responded significantly better to BV6 than samples with unknown or favourable prognosis e.g. 13q deletion. The majority of cases with 17p deletion (10/12) and Fludarabine refractory cases were sensitive to BV6, indicating that BV6 acts independently of the p53 pathway.

Importantly, BV6 dose-dependently induced cell death in 28 of 51 (54%) investigated patient samples while B cells from healthy donors were largely unaffected. BV6 also triggered cell death under survival conditions mimicking the microenvironment e.g. by adding CD40 ligand or in conditioned medium.

Gene expression profiling identified cell death- and NF-κB-signaling among the top pathways regulated by BV6 not only in CLL but also in core-binding factor (CBF) acute myeloid leukemia (AML). This was confirmed by data showing that BV6 causes degradation of cIAP1 and cIAP2 and NF-κB pathway activation.

BV6 induced cell death depended on production of reactive oxygen species, since addition of ROS scavengers significantly rescued BV6-triggerd cell death. In contrast, BV6 induced cell death independently of caspase activity, RIP1 activity or TNFα, since zVAD.fmk, necrostatin-1 or TNFα-blocking antibody Enbrel failed to protect against cell death. Of note, transcripts of ROS regulatory proteins were modulated by BV6.

Thus, these data have important implications for developing new therapeutic strategies to overcome cell death resistance in CLL especially in poor prognostic subgroups.

Poster **#076** / Session **II**

Long-Term Doxorubicin Treatment Stimulates Mitochondrial Damage and Suppresses Mitochondrial Biogenesis

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Cardiotoxicity induced by chemotherapeutic agents is a recognized side effect of a class of drugs known as anthracyclines (ANTs). These drugs are the most widely used antineoplastic agents; however their long term clinical use is limited due to the dose-dependent cardiomyopathy which can take years to clinically manifest. The molecular mechanisms that govern this event still remain a topic of controversy, but the leading hypothesis has emphasized the role of oxidative stress generated during intracellular metabolism, which triggers oxidative damage to the myocardium. Although numerous antioxidant-based clinical interventions have failed, this can be justified by the inadequate understanding of molecular events that influence ANT-induced cardiotoxicity, and the difficulty of accurately mimicking this condition *in vitro* or *in vivo*. Mitochondria are dynamic organelles essential for life and death. These structures have previously been demonstrated to be extensively damaged during conditions of cardiotoxicity and have thus become the main target for novel therapeutic interventions. Since mitochondrial morphology is often associated with crucial cellular functions, this study aimed to investigate the long-term effects of the ANT doxorubicin (DOX) on mitochondrial fission and fusion events. To simulate chronic cardiotoxicity, H9C2 cells were treated daily with 0.2 or 1.0 μM DOX for a total of 120 hrs. This resulted in cumulative doses of 1.0 or 5.0 μM respectively after the treatment duration. Our data suggests that chronic DOX treatment induces mitochondrial fission, and these damaged mitochondria are consequently removed *via* mitophagy. MARCH5, an E3 ubiquitin ligase was significantly upregulated and possibly mediated mitochondrial fusion protein degradation as these proteins were downregulated in this scenario. In addition, oxidative stress was elevated which correlated with increased intracellular and mitochondrial calcium. All these events culminated in apoptotic cell death reflected by augmented caspase 3/7 activity. These results suggest that oxidative stress need not be the main focus of ANT-induced cardiotoxicity but rather extensive mitochondrial damage and suppression of mitochondrial biogenesis as indicated by a decline in PGC1-α.

Poster #**077** / Session **I**

On The Implication of Alpha-Synuclein in Autophagy Modulation of Primary Human T Lymphocytes

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Alpha-synuclein has been demonstrated to aggregate and contribute to the pathogenesis of some neurodegenerative diseases, e.g. Parkinson disease. In neuronal cells it has been demonstrated as capable of hindering autophagy. We investigated the implication of α-synuclein in the autophagy process in primary human T lymphocytes. We provided evidence that: i) knocking down of the α-synuclein gene resulted in increased autophagy, ii) autophagy induction by energy deprivation was associated with a significant decrease of α-synuclein levels, iii) autophagy inhibition by 3-methyladenine or by ATG5 knocking down led to a significant increase of α-synuclein levels, and iv) autophagy impairment, constitutive in T lymphocytes from patients with Systemic Lupus Erythematosus, was associated with abnormal accumulation of α-synuclein aggregates. Alpha-synuclein can thus actually be considered as a key player in the mechanism of autophagy of primary human T lymphocytes. A clinical application of this molecule for monitoring autophagy level in freshly isolated T cells can also be taken into consideration.

Poster #**078** / Session **II**

Impact of Lysosomotropism and Lysosomes on Toxicity of Anti-Cancer Drugs

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Lysosomes are acidic organelles, the main function of which is degradation of cellular components. However, lysosomes are involved also in cell death mechanisms such as lysosomal-mediated cell death and/or autophagy. Lysosomotropism is accumulation of weakly basic anticancer agents (cationic amphiphilic drug (CAD) in lysosomes through ion trapping. Administration of lysosomotropic drugs such as analgetics and/or anxiolytics to cancer patients is often used as a side therapy.

To investigate possible interactions of such drugs with cytostatics, we studied the effect of one analgetic drug Lidocaine in combination with widely used cytostatic Doxorubicin (Doxo) on viability of several human cancer cell lines including multidrug resistant line.

We compared the effect of Lidocaine on Doxo toxicity on two promyelotic leukaemia cell line - HL60MDR, overexpressing P-glycoprotein and its parental cell line HL60, six melanoma cell lines (A375, MelJuSo, RPM 7951, IPC 298, MelHo and SkMel30), colon carcinoma (RKO) and non-small cell lung carcinoma (H1299). Our results show that Lidocaine can have a strong effect on toxicity of Doxo depending on the cell line. The antiproliferative activity of Doxo was singificantly reduced in resistant HL60MDR cells when Lidocaine was present, while in parental HL60 similarly as A375 melanoma cell lines Lidocaine potentiated Doxo cytotoxicity.

Interestingly in cases of other melanoma and non-melanoma cell lines (except HL60MDR), we did not observe any potentiation or significant reduction of Doxo toxicity. Thank to Doxo fluorescence, we could investigate the localisation of Doxo in small vesicle-like compartments inside of the A375 cells. These vesicles could be either lysosomes or melanosomes.

These preliminary results show that combining anticancer drugs with selected CAD could have a huge impact on cytotoxicity of cytostatics, in meaning of reducing their effect (HL60MDR), or potentiating it (A375). Nevertheless, this effect is strongly dependent on the cell type. It is highly probable, that interaction

of these drugs with cytostatics depends on properties of lysosomes (e.g. intralysosomal pH) in individual cell types and on possible flux of drugs between lysosomes and cytoplasm. Also lysosomal membrane permeabilisation and lysosomal-mediated cell death could contribute to cytotoxic effect. On the other hand trapping of cytostatics inside the lysosomes and lysosomes blebbing could moderate the CAD effect.

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Poster #**079** / Session **I**

Human Mutated Beta-Amyloid Precursor Protein Gene (*APP-Sw*) Leads to Reduced Viability, Autophagy-Like Process and Increased Expression and Secretion of Beta Amyloid in PC-12 Cells

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Pheochromocytoma PC-12 cells are immune to physiological stimuli directed to evoke programmed cell death. Besides, metabolic inhibitors are incapable to sensitize PC-12 cells to extrinsic or intrinsic apoptosis unless they are used in toxic concentrations. Surprisingly, these cells become receptive to cell deletion after human *APP-sw* gene expression.

We observed reduced cell viability in *GFP* vector + *APP-sw*-nucleofected cells (drop by 36%) but not in *GFP* vector-, or *GFP* vector + *APP-wt*-nucleofected cells. Lower viability was accompanied by higher expression of Aβ 1-16 and elevated secretion of Aβ 1-40 (in average 53.58 pg/mL). At the ultrastructural level autophagy-like process was demonstrated to occur in *APP-sw*-nucleofected cells with numerous autophagosomes and multivesicular bodies but without autolysosomes.

Human *APP-sw* gene is harmful to PC-12 cells and cells are additionally driven to incomplete autophagy-like process. When stimulated by TRAIL or nystatin, CLU protein expression accompanies early phase of autophagy.

Poster #**080** / Session **II**

Mitophagy and Neurodegeneration in *C. elegans*

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Mitochondria are essential for energy production and have vital roles in calcium signalling and storage, metabolite synthesis and apoptosis in eukaryotic cells. Neuronal cells are dependent, perhaps more than any other cell type, on proper mitochondrial function. Thus, maintenance of neuronal homeostasis necessitates a tight regulation of mitochondrial biogenesis, as well as, the elimination of damaged or superfluous mitochondria. Mitochondrial impairment has been implicated in several age-relarted neurodegenerative diseases. Mitophagy is a selective type of autophagy mediating elimination of damaged mitochondria, and the major degradative pathway, by which cells regulate mitochondrial number in response to metabolic state. However, little is known about the effects of mitophagy deficiency in neuronal physiology. To address this question, we developed an imaging system to monitor neuronal mitophagy *in vivo*. We used this system to investigate the involvement of mitophagy in *C. elegans* models of neurodegeneration. Autophagic activity is known to decline with age. We observed accumulation of neuronal mitochondria during ageing. Additionally, inhibition of mitophagy percipitates marked increased of mitochondrial mass in neurons of age-matched animals. Importantly, mitophagy-deficient mutants display spontaneous neurodegeneration. Our results indicate that mitophagy contribute to promote mitochondrial homeostasis and neuronal health.

Poster #**081** / Session **I**

mTNF-alpha Signaling Inhibits LPS-Induced Proinflammatory Cytokine Formation by Upregulating TGF-β in Macrophages

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Tumor necrosis alpha (TNFα) exists in two isoforms: the soluble sTNFα which is a trimer built up of 17 kDa sized subunits and the membrane-bound 26 kDa sized mTNFα. Increasing evidence suggests that following engagement with TNF receptor mTNFα initiates a reverse signaling pathway, but the details have not been described yet.

For characterization of TNFα production we examined the mRNA levels both in resting and in bacterial lipopolysaccharide (LPS) activated bone marrow derived macrophages by Q-PCR, the amounts of secreted sTNFα by ELISA technique, while the amount of mTNFα by flow cytometry. To study mTNFα signaling, mTNFα was crosslinked by coated antibodies and the signaling was studied by the Proteome Profiler Human Phospho-MAPK Array as well as the secreted cytokines were studied by Cytokine arrays.

Our results indicate that following LPS stimulation mTNFα is originated first from the stored cytosolic pool and the *de novo* synthesis contributes to the late expression of mTNFα. TNFα appears first in the membrane, but later it is cleaved by metalloproteases to form sTNFα resulting in a very little steady state mTNFα concentrations on the cell surface. mTNFα reverse signaling induces the production of TGF-beta in mouse bone marrow derived macrophages. In turn, TGF-beta acts back on macrophages and triggers the upregulation of the dual specific phosphatase 1 (DUSP1) and IL-10 via activating the MKK3/MKK6 signaling pathway. As a result triggering mTNFα leads to the downregulation of LPS-induced signaling and the consequent proinflammatory responses (IL-6 production) in macrophages.

Our data indicate that some of the neutralizing anti-TNFα antibodies used in human therapy which trigger mTNFα signaling might exert their anti-inflammatory effects via the mTNFα signaling pathway as well.

Poster #**082** / Session **II**

DNA Δamage-induced Necrotic Neurodegeneration and Ageing

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DNA damage is a major contributing factor in ageing and has been implicated in neurodegeneration. A critical question that emerges is whether intrinsic neuronal stress response pathways engage to protect against DNA damage-triggered neurodegeneration. Moreover, although it is well established that DNA damage induces apoptosis, the contribution of necrotic cell death to DNA damage-related pathology remains largely elusive. To address these questions we are developing a model of DNA damage-induced necrotic neurodegeneration in the nematode *Caenorhabditis elegans*. We used UV-C irradiation to trigger DNA damage in *C. elegans* neurons. Our initial observations revealed a marked increase in cytoplasmic calcium concentration upon treatment. To examine whether this acute elevation of cytoplasmic calcium triggers neurodegenerative necrotic cell death, we exposed *ercc-1* mutant animals carrying lesions in the nucleotide excision repair pathway (NER) to UV-C. These DNA repair-deficient animals are hypersensitive to UV irradiation and exhibit necrotic cell corpses upon exposure. Neurons are particularly affected. Additionally, we provide experimental evidence of spontaneous necrosis in these DNA repair-deficient worms during aging. To follow DNA damage responses and consequences in postmitotic neurons, we will investigate these neuronal effects in the NER-specific *csb-1* mutant animals which have been previously shown to exhibit UV sensitivity specifically in postmitotic somatic tissues. In addition, we are studying the involvement of other DNA repair pathways on the viability of specific neuronal cell types, their susceptibility to neurodegeneration and the consequent effects in behavior. Ultimately, we will examine the evolutionary conservation of the mechanisms involved by extending our analysis to mouse neurons, and mammalian models of neurodegenerative disease.

Poster #**083** / Session **I**

N-Cadherin Proteolysis in Apoptotic Bladder Cancer Cells

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Abstract withdrawn by authors from online

Poster #**084** / Session **II**

The Wnt Target PPAN is a Novel Anti-Apoptotic Factor

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Abstract withdrawn by authors from online

Poster #**085** / Session I

Targeting Opa1 to Increase Mitochondria-Dependent Apoptosis of Cancer Cells

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In addition to providing the majority of cellular ATP, mitochondria mediate intrinsic apoptosis, which is characterized by the release of apoptogenic factors including cytochrome c from the mitochondria into the cytosol. The majority of cytochrome c is stored in cristae formed by the inner mitochondrial membrane, which are tightened at the cristae junctions by the inner mitochondrial membrane protein optic atrophy 1 (Opa1). Thus Opa1, a multifunctional GTPase, not only promotes mitochondrial fusion, but it also controls cytochrome c release from mitochondria thus playing a regulatory role in subsequent apoptosis (1).

The upregulation of Opa1 in a number of solid and hematological tumors (2) and the necessity of the GTPase domain of Opa1 for its role in apoptosis (1) reveal the cristae remodeling pathway as a possible new drug target to induce cell death. We hypothesize that pharmacological inhibition of the GTPase activity of Opa1 can cause cytochrome c release by inducing cristae remodeling, and consequently sensitizing cells to apoptotic stimuli. Here we show the steps towards the identification of a pharmacological inhibitor of the GTPase activity of Opa1.

For that, an enzymatically active recombinant form of Opa1 is used to perform a high-throughput screen of drug like compounds that affect Opa1 GTPase activity. We characterized the kinetic parameters K_m and k_{cat} of Opa1 to adjust an enzymatic-screening assay accordingly in which the free phosphate released during the hydrolysis of GTP by Opa1 will serve as readout (3). Concurrently, we are generating an *in silico* model of the GTPase domain of Opa1 that allows us to analyze the intermolecular interactions of potential inhibitors with Opa1 to optimize lead molecules. This progress shall lead to the identification of a pharmacological drug for a novel target, the GTPase Opa1.

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Poster #**086** / Session **II**

Targeting Nucleostemin Induces Autophagy, Cell-Cycle Arrest, and Differentiation in KG1a Leukemia Stem Cells

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Introduction: Nucleostemin (NS) is a nucleolar GTP-binding protein known to play a variety of biological functions in cell-cycle progression, self-renewal maintaining, apoptosis initiation and the genomic protection of normal and cancerous stem cells. Recently, high NS transcript levels have been found in leukemia acute myeloid leukemia (AML) patients with poor prognosis which is closely correlated with undifferentiated state of leukemia stem cells. However, the exact role of NS in leukemia cells is yet to be determined. To address this question, we knocked down NS by means of specific small interference RNAs (siNS) in KG1a cell line and studied its effects on different cell fates of this typical *in vitro* model of leukemia stem cells.

Methods and Results: Targeting NS decreased the rate of cells proliferation and induced G1 cell-cycle arrest in KG1a cells. Both flow cytometry and fluorescent microscopy demonstrated an increase in red fluorescence in NS depleted cells stained with acridine orange, indicating the presence of intracellular acidification as one of the hallmarks of autophagic response. NS depletion also stimulated LC3 conversion, ATGs expressions and p62 degradation in the cells, fully confirming occurrence of autophagy. Interestingly, profound morphological changes such as cell-to-cell and cell-to-plate adherence were appeared in NS depleted leukemia cells. Wright-Giemsa staining and expression of CD11b cell surface marker confirmed that NS silencing induced myeloid differentiation in KG1a cells. Time course studies revealed that LC3-II was detected at approximately 3 hours, whereas cell-cycle arrest and differentiation were occurred at 24 and 48 hours post-transfection, respectively. Inhibition of autophagy by pharmacologic means (3-MA band BafA) abolished morphological changes and differentiation markers but not cell-cycle arrest and caused a significant accumulation of apoptotic cells. Both western blotting and RT-PCR experiments revealed that NS targeting up-

regulated the p73 protein and mRNA levels, but not p53 or p63 transcript levels, after 3-6 hours siNS transfection of KG1a cells, pointing to involvement of this member of p53-family proteins in NS effects.

Conclusion: Our data demonstrate that NS is an important regulator of cell-cycle and differentiation of leukemia stem cells and that autophagy induction is the major molecular mechanism underlying functions of NS in p53-null KG1a cells. The functions of NS in KG1a cells are apparently through p53-independent pathways and seem to be mediated, at least in part, by induction of p73.

Poster #**087** / Session **I**

Combination of Indomethacin and Bcl2 Blockers Induces Cell Death in Lymphoma/ Leukemia Cell Lines

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Objective: In a previous study we showed that combination treatment with fenretinide and indomethacin resulted in Jurkat cell death, that is peculiar and differs from classical apoptosis. We provided evidence, that induced death was not accompanied by activation of effector caspase-3 but may go through different AIF-mediated pathway.

In this study we investigated the effects of indomethacin in combination with some Bcl2 protein blockers on the process of cell death in several human and canine leukemia/lymphoma cell lines.

Methods: The cells were treated with indomethacin and HA1-14, ABT263 and ABT737 alone or in combination. Early apoptotic events and DNA fragmentation were detected by flow cytometry using the Annexin V-FITC/PI and ethanol-fixed propidium iodide stained cells, respectively. Cell viability was determined using the MTs test. ROS induction was detected using H2DCFDA and the mitochondrial potential was measured using JC-1. The expression of some anti- and pro-apoptotic proteins was detected by Western blotting.

Results: The combination of indomethacin with Bcl2 protein blockers (HA1-14, ABT263, ABT767) had strong effect on induction of apoptosis of different leukemia/lymphoma cell lines (Jurkat, HL60, K562, Raji, HuT28, U937, CLBL-1, CL-1). ABT263 was the most effective. Indomethacin alone generated ROS induction after 1 hour of incubation, whereas the loss of mitochondrial membrane potential was changed after 24 hours of incubation with combination of indomethacin and ABT263. Cell death was accompanied by caspase 9 and 3 activation and DNA degradation.

Conclusion: In this study we show that the combination of indomethacin and blockers of Bcl2 proteins is able to induce a high level of cell death in some leukemia/lymphoma cell lines. The cell death is induced by classical caspase-dependent pathway.

Poster #**088** / Session **II**

In vitro and *ex vivo* Evaluation of Newly Designed 17-beta Estradiol Analogues as Potential Anticancer Agents

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Microtubules exert a pivotal role during mitosis; this attribute renders them attractive anticancer targets. Agents that suppress microtubule dynamics may lead to mitotic spindle disruption in dividing cells; inhibit angiogenesis and contribute to excessive reactive oxygen species signaling; thus sensitize hyper-proliferating cells to pro-death signaling.

The endogenous metabolite of 17-beta-estradiol, 2-methoxyestradiol (2ME) possesses antiproliferative activity *in vitro* and *in vivo*, but has limited biological accessibility and is rapidly degraded during metabolism.

Because of 2ME's limitations, estradiol analogues were *in silico*-designed and tested for tubulin and carbonic anhydrase (CA II, IX, XII) binding affinity. Lead compounds exerting potential antiproliferative activity *in silico*, namely 2-ethyl-3-*O*-sulphamoyl-estra-1,3,5(10),15-tetraen-17-ol (ESE-15-ol) and 2-ethyl-3-*O*-sulphamoyl-estra-1,3,5(10)16-tetraene (ESE-16) were synthesized by iThemba Pharmaceuticals (Modderfontein, Gauteng, SA).

The xCELLigence system and gene- and protein microarray techniques, as well as bioinformatics analysis for high throughput analysis of gene regulation and protein responses were conducted. Morphological influence and generation of reactive oxygen species by ESE-16 on erythrocytes and platelet samples by means of

scanning electron microscopy, transmission electron microscopy and flow cytometry were also investigated.

Newly synthesized novel compounds were five to eight times more potent *in vitro* on cell lines of national and international importance. Data demonstrate extremely low concentrations for a drug to have anticancer activity when compared to conventional current treatments. The poster will provide an overview of the antiproliferative mechanisms and cell signaling events of these newly *in silico*-designed analogues *in vitro* and *ex vivo*.

Poster #**089** / Session **I**

CD47-BNIP3 Complex: a Novel Pro-Tumorigenic Axis in Melanoma

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Melanoma exhibits a plethora of features that support its proliferation, invasion and metastasis. It exhibits unique immunological characteristics coupled with immunoevasive activity and potent ability to suppress cell death. Thus, simultaneous targeting of modulators of immunosurveillance (e.g. proteins that function at the phagocytic interface of cancer cell and immune cell), as well as modulators of cell death that support pro-survival (e.g. pro-autophagic proteins) becomes an attractive avenue.

BNIP3, an atypical BH3-only member of the BCL-2 protein family with both pro-death and pro-autophagic roles, has been found to be a context-dependent modulator of tumor growth. We have recently identified a pro-survival role for BNIP3 in melanoma. In line with this, knockdown of BNIP3 in melanoma cells reduced autophagy, cell migration and vasculogenic mimicry and blunted long-term clonogenic growth. Interestingly, BNIP3 has been found to physically interact and cross talks with CD47, a potent "don't eat me" signal and well-established negative prognostic marker and suppressor of phagocytic immunosurveillance.

Consistently, we found that the presence of BNIP3 in melanoma cells was required to maintain the expression levels of CD47, as knockdown of BNIP3 favored the degradation of CD47. Interestingly, a statistically significant clinical correlation was also observed between BNIP3 and CD47 transcript levels which, in turn was associated with reduced overall survival in melanoma patients, thus highlighting a relevant role for this interaction in melanoma progression. Based on these observations, we hypothesized that BNIP3 could affect cancer cell-immune cell phagocytic interactions. In line with this, our preliminary data shows that BNIP3 regulates the phagocytosis of melanoma cells by macrophages, a function that is independent of its pro-autophagic activity. Therefore BNIP3 is endowed with the

ability to sustain both melanoma cell-intrinsic survival (due to its pro-autophagic activity) and melanoma cell-extrinsic immunoevasion function (by sustaining the surface expression of CD47 'don't eat' me signal), disclosing BNIP3 as an attractive therapeutic target in melanoma.

Ongoing analysis is addressing the molecular / signaling mechanisms underlying BNIP3-CD47 interaction, its modulation by metabolic stress found in the tumor microenvironment and its impact on the phagocytic activity, within the context of tumor immunoevasion.

Poster #**090** / Session **II**

The Generation of Neutrophils in the Bone Marrow is Controlled by Autophagy

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Autophagy is a self-degradation lysosomal pathway that plays a vital role in cell growth, development and homeostasis. It has recently been shown that it is also involved in non-metabolic functions that particularly concern blood cells and their differentiation during erythropoiesis and lymphopoiesis. To investigate the role of autophagy in neutrophil granulopoiesis, we studied the effect of myelo-/monocyte-specific knockout of the essential autophagy-related gene 5 (*Atg5*) in $Atg5^{flox/flox}$.$Lyz2^{Cre/Cre}$ mice. No abnormalities in survival and spontaneous apoptosis of ATG5-deficient neutrophils were observed. However, we observed an increased proliferation rate in neutrophil precursor cells of the bone marrow as well as an accelerated process of neutrophil differentiation, resulting in an accumulation of mature neutrophils in the bone marrow, blood, spleen, and lymph nodes. To further analyze the ATG5-regulation of granulopoiesis, we used the *in vitro* model of SCF-^{cond}Hoxb8 promyelocytes. These are 4-hidroxy tamoxifen-regulated immortalized neutrophil progenitors that execute normal differentiation and innate immune function upon Hoxb8 inactivation. The modulation of ATG5 expression in these cells revealed an accelerated maturation of myeloid progenitors with reduced ATG5 levels, whereas the ATG5-overexpression delayed the differentiation process *in vitro*. Moreover, pharmacological inhibition of p38 MAPK or mTORC1 induced autophagy in neutrophilic precursor cells and blocked their differentiation, suggesting that autophagy is negatively controlled by the p38 MAPK – mTORC1 signaling pathway. Taken together, these findings show that, in contrast to erythropoiesis and B-cell differentiation, autophagy is not essential for neutrophil granulopoiesis, having instead a negative impact on the generation of neutrophils.

Poster #**091** / Session **I**

Reactive Oxygen Species Induce Necroptosis in MCF-7, MDA-MB-468 and T-47D Breast Cancer Cell Lines

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Objectives: Oxidative stress is a well-known old enemy that plays crucial roles in pathogenesis of human disease but it might be a new opportunity to therapeutics for cancers too. Hence, better understanding of the relationship between oxidative stress and cell fate will help us to use the beneficial side of it. The present study was designed to elucidate the mechanism by which oxidative stress alters the fate of cells in human breast cancer cell lines.

Material and methods: MCF-7, MDA-MB-468 and T-47D were treated with Shikonin as ROS inducer. The levels of ROS, cell proliferation, expression levels of RIP1 and RIP3 and mitochondrial membrane potential were studied using appropriate methods.

Results: Induction of ROS was proved in the treated cells and dose- and time-dependent cell death was also observed. It was found that cell death was mainly occurred through necroptosis with significant increase in RIP1 and RIP3 expression and characteristic morphological changes. The mitochondrial membrane potential was reduced in cells treated with shikonin.

Conclusions: These findings implicate that oxidative stress alters breast cancer cell fate from proliferation to cell death that mainly goes through induction of RIP1 and RIP3 kinases dependent necroptosis. Hence, it could be concluded that tailored application of ROS inducers might be an effective approach to target breast cancer therapy.

Poster #**092** / Session **II**

Targeting Refractory T-ALL by Manipulation of Reactive Oxygen Species Homeostasis

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Acute Lymphoblastic Leukemia of T-cell subtype (T-ALL) represents an important clinical problem, as about 20% of the patients are resistant to therapy. The present study was aimed at targeting refractory T-ALL by manipulation of reactive oxygen species (ROS) homeostasis.

In previous studies we showed that the p13 protein of human T-cell leukemia virus type 1 (HTLV-1) is targeted to the inner mitochondrial membrane and produces an inward K+ current that results in an increased production of ROS by mitochondria. This has distinct consequences on cell turnover depending on the cell's inherent ROS levels, with activation predominating in normal resting T-cells and death-promoting effects in HTLV-1-transformed T-cells and the T-ALL cell line Jurkat. These observations imply that tumor cells might be particularly vulnerable to cell death in response to agents that increase ROS.

Building on these studies, we employed pharmacological agents that raise the ROS set-point in cancer cells to selectively kill them while sparing normal cells. Experiments were carried out in a panel of T-ALL cell lines and explants of pediatric T-ALL cells grown in NOD/SCID mice. Cultures of resting and stimulated peripheral blood mononuclear cells (PBMC) obtained from healthy blood donors were used as controls. Our results showed that T-ALL cell lines and primary T-ALL explants have a higher ROS set-point compared to normal PBMC. Combined treatment with a K+ channel opener and a drug that interferes with the pentose phosphate pathway induced apoptotic cell death in neoplastic cells, while no significant effect was observed in primary T-cells.

These data suggest common core perturbations of ROS homeostasis in T-cell neoplasms. The potential synergism of ROS-modulating agents with drugs engaging key apoptotic pathways may provide new leads into strategies aimed at overcoming drug resistance.

Poster #**093** / Session **I**

Cell Type-Specific Differences in Activity-Dependent Postnatal Apoptosis in Neocortical Cultures

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A wave of neuronal apoptosis occurs in all mammals, ensuring the proper development of the nervous system. This physiological process, which triggers the loss of up to 50% of the neurons in certain brain areas, happens mostly during the last trimester in humans and the first two postnatal weeks in rodents.

Here, we studied the developmental profile of apoptosis in different neuronal subpopulations using immunochemistry in dissociated cortical cultures from neonatal mice. We were particularly interested in a transient neuronal subpopulation, the Cajal-Retzius neurons (CRNs), which disappear by the end of the second postnatal week *in vivo*. Our current data suggest that the time course and the rate of postnatal apoptosis vary between different neuronal subpopulations *in vitro*. Whereas about 60% of GABAergic neurons undergo apoptosis between DIV 6 and 12, the majority of CRNs (80 %) has already undergone apoptosis by DIV 9. Moreover, about 30% of the overall population of neurons is lost from DIV 3 to DIV 14.

While the regulation of early apoptosis is well understood in the peripheral nervous system, the mechanisms underlying apoptosis in central neurons remain rather elusive. Previous work from our group and others suggest that electrical activity is a major regulator of neuronal cell death in the brain. Based on that we first studied how chronic pharmacological treatments (e.g. TTX, gabazine and bumetanide) affect electrical activity patterns of our dissociated cortical cultures using multi-electrode array (MEA) recordings. We then investigated the effect of these activity changes on the rate of cell death in two different neuronal subpopulations (CRNs and NeuN-positive neurons). Our data suggest that the subpopulations are not similarly affected by modulations in the electrical activity patterns. Thus, distinct mechanisms seem to control cell survival and cell death in the different subpopulations of neurons.

To conclude, this *in vitro* study can provide further insights into the mechanisms underlying the neurodevelopmental activity-dependent apoptosis occurring in rodents as well as in humans.

Poster #**094** / Session **II**

Dual Role of Anthocyanins in Protection against Heart Ischaemia-Induced Apoptosis and Necrosis

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It is well established that mitochondria mediate cell death during heart ischaemia by releasing cytochrome c to cytosol where it participates in formation of apoptosome and caspase activation. On the other hand, loss of cytochrome c from mitochondria results in mitochondrial dysfunction and ATP depletion leading to necrosis. Anthocyanins are a class of the widespread plant flavonoids which are known to exert cardio-, neuroprotective effects. There is a lot of evidence, that anthocyanins are strong antioxidants. However, antioxidants are generally strong reductants, and some reductants have been found to block apoptosis by changing the redox state of cytosolic cytochrome c.

In this study, we sought to investigate the mechanism by which anthocyanins protect against ischaemia-induced caspase activation, cell death and mitochondrial dysfunction in the perfused rat heart.

We tested the ability of five common anthocyanidin glucosides: delphinidin (Dp3G), cyanidin (Cy3G), pelargonidin (Pg3G), peonidin (Pn3G), and malvidin (Mv3G) to reduce cytochrome c in vitro. We found that Dp3G and Cy3G were able to reduce extramitochondrial cytochrome c directly and rapidly, whereas Pg3G, Mv3G and Pn3G had relatively low cytochrome c reducing activities. At 10-40μM concentrations no one of investigated anthocyanins had an effect on state 3 respiration rate in normal, non-ischaemic mitochondria. Dp3G and Cy3G but not Pg3G supported mitochondrial state 4 respiration in the presence of exogenous cytochrome c. We also found that Cy3G and Dp3G increased activity of complex I in ischemia-damaged mitochondria measured in the presence or absence of CoQ_1 whereas in non-ischaemic mitochondria the effect was observed only in the absence of CoQ_1. Dp3G and Cy3G but not Pg3G increased State 3 respiration and ATP synthesis in mitochondria after ischemia. Pre-perfusion of the hearts with Cy3G but not Pg3G prevented ischaemia-induced caspase-3 activation, cardiomyocyte apoptosis and necrosis, though the ischaemia-induced release of cytochrome c from mitochondria was not prevented by anthocyanins.

The results suggest that: 1) anthocyanins with high reducing activity can protect against ischaemia-induced caspase activation and apoptosis possibly by reducing cytosolic cytochrome c; 2) certain anthocyanins can act as electron acceptors at complex I and bypass ischaemia-induced inhibition resulting in increased ATP production at reperfusion after ischaemia therefore reducing necrotic cell death.

Poster #**095** / Session **I**

Apoptosis, Necroptosis or Autophagy, Who is the Killer?

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The cell death mechanism upon treatment with quaternary benzo[c]phenanthridine alkaloids (QBA) was studied. QBA are natural products with significant anti-proliferative activities, therefore they are considered as agents promising for cancer therapy.

In this study antiproliferative and pro-apoptotic activity of sanguirubine (SR), chelirubine (CHR), sanguinarine (SA), chelerythrine (CHE), and sanguilutine (SL) were investigated using MTT assay, PI exclusion assay and Western blot analysis. Inhibitors of apoptosis (z-VAD-fmk), necroptosis (necrostatine; Nec-1), ROS (N-acetyl cysteine, NAC) and/or autophagy (3-MA) were used to identify the cell death mechanisms. The experiments were performed on various human cancer cells, i.e. A375 (melanoma), HeLa (cervix carcinoma) and HL60 (promyelotic leukaemia).

Despite the profound similarity of QBA molecular structures, we demonstrated that the mechanism of the cell death is different at the case of each alkaloid and dependent on the cell type. SR and CHR as well as SA and CHE were able to induce both apoptosis and necroptosis on cell line A-375. Since Nec-1 completely but z-VAD-fmk only partially prevented the cell death, necroptosis appeared to be predominant cell death pathway. Interestingly, only Nec-1 reduced cell death in HeLa cells. NAC decreased cell death in similar way as Nec-1, proving the fact that necroptosis is accompanied by ROS production. In the case of SL, Nec-1 completely reversed the cell death, while z-VAD-fmk was non-functional in both A375 and HeLa cells. Protective role of autophagy upon all QBA treatment was demonstrated in both A-375 and HeLa cell lines.

It is apparent that SL induces exclusively caspase-independent cell death and could therefore be appropriate molecule for study of the mechanism of necroptosis. Therefore we have compared the effect of SL with Camptothecin, which was used

as a control inducer of apoptosis. In the case of Camptothecin, z-VAD-fmk was partially effective in cell death reduction, while Nec-1 had no effect.

The results present QBA as interesting compounds not only in cancer biology point of view, but also as molecules applicable to study of individual cell death pathways and their interconnections.

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Poster #**096** / Session **II**

Regulation of Necroptosis by the Ubiquitylation- and Lysosome-Dependent Degradation of RIPK3

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Poster #**097** / Session **I**

Involvement of Mitochondria in the Development of the Mitotic Catastrophe

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Mitotic catastrophe is a process preceding cell death, which might occur via necrosis, apoptosis or senescence, depending on the molecular profile of the cell. Mitochondria play a crucial role in cell life and death. One of the major mitochondria function is an energy supply, which is important to maintain the viability of for giant cells, usually observed during the mitotic catastrophe. Substantial involvement of mitochondria in cell death allows suggesting their role in the development of the mitotic catastrophe. Nowadays the role of mitochondria in mitotic catastrophe as well as in the switch between different modes of cell death occurring after mitotic catastrophe is unclear.

Doxorubucin (600 nM) and colcemid (0,1 mg/ml) treatment caused the development of the mitotic catastrophe in colorectal carcinoma cell lines: wild type HCT116 cells and HCT116 cells lacking 14-3-3σ, also known as epithelial cell marker protein-1 or stratifin (14-3-3$\sigma^{-/-}$).This effect was the most pronounced after colcemid administration. Lack of 14-3-3σ protein significantly increased the percentage of nuclei with morphology of the mitotic catastrophe. Analysis of sub-G1 population showed that doxorubicin and colcemid stimulated apoptotic cell death especially in HCT116 14-3-3$\sigma^{-/-}$ cells. It should be noted that colcemid administration significantly increased the percentage of cells in sub-G1 population in comparison with doxorubucin. Transfection with anti-apoptotic proteins Bcl-xL-GFP and Mcl-1-YFP practically did not influence apoptosis induced by doxorubicin but significantly inhibited apoptotic cell death caused by colcemid. At the same time overexpression of Mcl-1-YFP prevented the development of the mitotic catastrophe whereas transfection with Bcl-xL-GFP promoted this process after treatment with used DNA-damaging drugs in both HCT116wt and HCT116 14-3-3$\sigma^{-/-}$ cells.

Mitotic catastrophe caused by colcemid accompanied by the formation of reactive oxygen species (ROS), mainly in HCT116 14-3-3$\sigma^{-/-}$ cells. Interestingly, a basal level of ROS in HCT116 14-3-3$\sigma^{-/-}$ cells was significantly higher. Synchronization of cells in S-phase increased ROS level only in HCT116wt cells. Addition of N-

acetyl cysteine (NAC) reduces ROS level to the initial. NAC inhibited apoptosis induced by doxorubicin but stimulated apoptosis caused by colcemid administration. In combination with doxorubicin NAC significantly increased the percentage of nuclei with mitotic catastrophe morphology but had no effect on the mitotic catastrophe induced by colcemid. To clarify this collision assessment of oxygen consumption was carried out. While doxorubicin did not influence the respiration in both cell lines colcemid significantly inhibited this process in HCT116wt cells, suggesting different mechanisms of action of these drugs. Obtained data allow us to suggest that pathways of the mitotic catastrophe stimulation depend on an acting agent and can be affected by the activity of mitochondria.

Poster #**098** / Session **II**

Early Manifestations of Peplicative Aging in the Yeast *Saccharomyces cerevisiae*

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The yeast Saccharomyces cerevisiae is successfully used as a model organism to find genes responsible for lifespan control of higher organisms. As functional decline of higher eukaryotes can start as early as one quarter of the average lifespan, we asked whether *S. cerevisiae* can be used to model this manifestation of aging. It appeared that resistances to certain stresses start to decrease much earlier than the average replicative life span. Looking into the mechanism, we found that knockouts of genes responsible for mitochondria-to-nucleus signaling, RTG1 or RTG3, significantly decrease the resistance of cells that generated more than four daughters, but not of the younger ones. We also found that even young mother cells frequently contain mitochondria with heterogeneous transmembrane potential and that the percentage of such cells correlates with replicative age. Together, these facts suggest that retrograde signaling starts to malfunction in relatively young cells, leading to accumulation of heterogeneous mitochondria within one cell. Independently, we found that the ability for activation of HO-promoter (mating type switching) also starts to decline relatively early.

It is known that functional decline of humans also starts at relatively early age. Therefore, our data suggest that yeast can serve as a model to study not only lifespan-regulating mechanisms, but also the mechanisms of the early age-associated decline.

Poster #**099** / Session **I**

The Role of p38 MAPK and ERK Signalling Pathways in Apoptosis Regulation by Fatty Acids in Pancreatic β-Cells NES2Y

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Background: There is increasing experimental evidence that saturated stearic acid (SA) exposure induces apoptosis in pancreatic β-cells, whereas unsaturated oleic acid (OA) is well tolerated and is even capable of inhibiting pro-apoptotic effect of SA. Although precise molecular mechanisms of apoptosis induction and inhibition by fatty acids are still unclear, there are some indications that the p38 MAPK and ERK signalling pathways could be involved.

Results: We have found in the NES2Y human pancreatic β-cells that saturated SA at apoptosis-inducing concentration (1mM) activated the p38 MAPK signaling pathway (MKK3/6→p38 MAPK→MAPKAPK-2) and inhibited the ERK signaling pathway (c-Raf→MEK1/2→ERK1/2→p90RSK). Unsaturated OA (0.2mM) was able to inhibit these effects of stearic acid. Application of p38 MAPK inhibitor SB202190 led to ERK pathway activation and had no effect on the cleavage of PARP, caspase-7, -8, and -9 as well as on cell viability during SA-induced apoptosis. p38 MAPK silencing resulted in no effect on ERK pathway, the cleavage of caspase-8, -9 and PARP and on the cell viability during SA treatment, however, it increased caspase-7 cleavage. Inhibition of MEK1/2 by specific inhibitor U0126 led to certain caspase-7, -8, -9 and PARP cleavage and decrease of the cell number; activation of p38 MAPK pathway did not seem to be affected.

Conclusions: We have demonstrated that stearic acid activates p38 MAPK and inhibits ERK signalling pathways. These effects of stearic acid were inhibited by oleic acid co-application. According to this finding, the point of inhibitory intervention of oleic acid in SA-induced signaling lies upstream from the p38 MAPK and ERK pathways. Studied pathways are probably not involved in pro-apoptotic signaling induced by stearic acid, however, they may have some pro-survival function in the NES2Y β-cells.

The ERK pathway also may be involved in the regulation of cell proliferation. On the basis of our data we suggest that SB202190 can activate the ERK pathway; however, it is not clear if this pathway can also be regulated by p38 MAPK. The ERK pathway does not seem to affect the p38 MAPK pathway in NES2Y β-cells.

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Poster #**100** / Session **II**

BITC Treatment of U937 Cells Induce Apoptosis without Cell Disintegration

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Over the past three decades research has confirmed the efficacy of various ITCs against cancer in preclinical models. One of such dietary chemopreventive agent is a benzyl isothiocyanate (BITC), which is characterize with ability to inhibit the growth of various chemically induced cancer cells by causing apoptosis. Exposure of different cells to BITC resulted in increase in apoptosis with major of its characteristic biochemical changes e.g. caspase activation, cytochrome c release, nuclear apoptosis-inducing factor (AIF) accumulation, Bcl2-associated X protein (Bax) translocation.

U937 cell line cells treated with apoptosis inducers is characterized with disintegration to apoptotic bodies. This process is accompanied with other classic apoptotic changes e.g. decrease of mitochondrial potential, blebbing, phosphatidylserine exposure on outer leaflet of cell membrane. Benzyl isothiocyanate treated cells dye with most of apoptotic mechanism characteristics but fragmentation of nucleus and cell disintegration. The morphology of dying U937 cells were very similar to the morphology of U937 subline (U937v) described earlier.

During our study we have been trying to confirm that high concentration of BITC is initializing the apoptotic mechanism of cell death in the U937 cell line. We were also comparing the basic apoptotic features in cells treated with BITC and etoposide.

The results show that two and half higher concentration of BITC comparing to EC 50 is still initiating the apoptosis of U937 cells. That particular mechanism of apoptosis lack nucleus fragmentation and disintegration to apoptotic bodies. Other apoptosis characteristics are present e.g. PS exposure on the outer leaflet of cell membrane, decrease of mitochondrial potential, presence of active caspases products and preserved integrity of cell membrane are present.

Dying cells in the other hand shows untypical cell death morphology very similar to the morphology described for the cell death of U937v cells. We also observed differences in the IL-8 and MCP-1 concentration in the supernatant above dying cells.

Poster #**101** / Session **I**

A New Mechanism for the Regulation of Mitochondria Related Cell Death by PARP-1 Inhibition in Oxidative Stress.

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In this report, we provide a novel mechanism by which reactive oxygen species-activated PARP-1 regulated the activation of JNK and p38 MAP kinase. Inhibition of PARP-1 by pharmacons, small interfering RNA mediated PARP-1 gene silencing, or transdominant expression of enzymatically inactive PARP-1 resulted in the inactivation of these MAPKs. This regulation was achieved by increased expression and increased cytoplasmic localization of MAPK phosphatase-1 (MKP-1) upon PARP-1 inhibition in oxidative stress. Changes in MKP-1 expression were reflected in the phosphorylation states of JNK and p38. Furthermore, we found that in MKP-1-silenced cells, PARP inhibition was unable to exert its protective effect. These results indicated the pivotal role of JNK and p38 in mediating the oxidative-stress-induced cell death as well as that of increased MKP-1 expression in mediating the protective effect of PARP inhibition. We identified a transcription factor poly-ADP-ribosylated thereby inactivated by PARP-1 that was responsible for upregulating MKP-1/Dusp1 expression. This novel mechanism explains how PARP-1 inhibition could prevent poly-ADP-ribosylation mediated inactivation of the transcription factor responsible for MKP-1/Dusp1 expression thereby inactivate JNK and p38 MAPK that eventually leads to protection against mitochondria mediated cell death in oxidative stress.

Poster #**102** / Session **II**

Necrosome Core Machinery: MLKL

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Genetic studies in C. elegans showing that the caspase CED-3 functions at the most downstream position in a linear apoptotic pathway suggested this enzyme is the executioner of programmed cell death. Moreover, biochemical studies in mammalian cells demonstrating the requirement of caspase activities for most of morphological and biochemical changes associated with apoptotic death further established such a role of caspases in apoptosis. Although **apoptotic cell death could be inhibited by a caspase inhibitor, such a treatment could not save the cells from dying but rather shifted the balance towards necrotic death.**

Necrosis is a kinase initiated death pathway. The RIP kinases respond to and link the death signals to the down stream substrate, MLKL. However, within the past few years, it has becoming clear that even one single step forward for the **signal transduction from RIP3 to MLKL built on one of the most formidable engine of regulation of all.** MLKL is a pseudo kinase, which doesn't have the bona fide kinase activity. Apart from the notion that MLKL receives death signal from its kinase RIP3 by the direct phosphorylation modification, it was not at all obvious how MLKL transduces signals to its down stream effectors. Recent studies on the signal transduction **using chemical tools and biomarkers support the idea that MLKL is able to make sense of the core machinery of necrosome.** The key role of MLKL in necrosis signaling sheds light on the logic underlying this unique cell death pathway.

It has been known that downstream necrosis signals works through MLKL. Accordingly, phosphorylated MLKL would be expected to transduce death signals. Both in vivo and in vitro biochemical analyses characterized the oligomerization nature of MLKL. Our recent data demonstrated that **phosphorylation of MLKL turns on its oligomerization formation.** Biochemical fractionation revealed that MLKL translocate to plasma membrane after necrosis was induced. Using the classical liposome leakage assay, **MLKL was found to translocate to the PIPs containing membranes and disrupt the membrane integrity in a dose dependent manner.** And this lipid binding property depends on the phosphorylation modification of MLKL. Besides, necrosis inhibitor **NSA could block MLKL membrane association.** Since NSA target to the N-terminal MLKL Cys86, this discovery is consistent with the finding that the N-terminal domain mediates liposome damage and cell death.

Summary:

Our recently progress on the necrosis signaling advanced our knowledge on the core machinery, MLKL activation and highly ordered organization on liposome structures disruption.

Poster #**103** / Session **I**

Mitochondrial pro-inflammatory signalling links apoptosis to necroptosis

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Poster #**104** / Session **II**

Autophagy in Prolactin-Treated Beta-Cells

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Autophagy is a conserved physiological system of intracellular degradation which can be considered as a cytoprotective mechanism; however, it is also able to promote cell death. It has also been proposed that deregulation of this process may have important roles in several diseases. While its role in diabetes is still obscure, but recent observations suggest that autophagy may have important roles in the development and prevention of diabetes.

We have already showed that prolactin promotes significant cytoprotection against cytokines and serum starvation induced beta-cells apoptosis in human islets. In order to further analyze the cytoprotective capacity of prolactin (PRL) after other cell death-inducing mechanisms, we set out to investigate whether recombinant human PRL (rhPRL) would have a role upon autophagy induction.

ATG5 knockdown was performed through treatment with specific siRNAs and confirmed by Western Blotting in INS-1E beta-cell cultures. Viability and acidic vesicles formation were analyzed through immunofluorescence and autophagy markers expression and/or phosphorylation were accessed by Western Blotting in INS-1E cells or primary cultures of human islets treated under different conditions.

While no alteration in beta-cell apoptotic cell death was seen upon rapamycin treatment, we observed an increase in beta-cell apoptosis in ATG5 silenced cells. Serum starvation, as well as treatment with either rapamycin, cloroquine or ionomycin induced an increase in acidic vesicle formation in beta-cells; however, prolactin cotreatement was able to inhibit this induction mediated by serum starvation and ionomycin treatment. Furthermore, rapamycin-induced decrease in phosphorylated mTOR levels was restored upon rhPRL pre-treament. Moreover, ionomycin incubation lead to an increase in LC3-II/LC3-I ratio in beta-cells that was reverted to control levels upon cotreatment with rhPRL. Moreover, LC3-II/LC3-I ratio was also decreased in primary cultures of human islets treated with rhPRL after serum starvation. Interestingly treatment with pharmacological inhibitors for either JAK/STAT, PI3K or MEK1/2, inhibited ionomycin-induced autophagy in beta-cells.

These findings support the hypothesis that autophagy plays a protective role in beta-cells and provide a deeper characterization of the PRL prosurvival mechanisms in beta-cells. We hypothesize that the reason PRL is able to restaure autophagy markers in beta-cells is because it prevents general cell damage.

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Poster #**105** / Session **I**

Mice Transgenic Approaches Shake to Their Foundations

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Targeted mutagenesis in mice is a powerful tool for the analysis of gene functions. However, genetic variation between donor (embryonic stem cells) and recipient strains typically results in so-called passenger mutations that flank the targeted gene and potentially influence the phenotype of the transgenic mice. Here we exemplify this highly underestimated issue by keynoting some case studies we discovered using our newly developed web tool: "Passenger Mutation Finder" (PaMuFinder). Comparative genomic analysis of 129 and C57BL/6 mouse strains reveals SNPs/indels in 1150 genes that are predicted to affect the protein sequence. This entails that > 98% of the 129-derived transgenic mice that were backcrossed to C57BL/6 still contain these passenger mutations that might affect the phenotypic outcome. We found that the 129-derived inactivating Casp4 mutation, not only affects CASP1- and cIAP1-null animals, but also knockout mice, among > 15 predicted others, targeting pannexin 1 and several members of the matrix metalloproteinase (MMP) family. Consequently, these mice conferred protection against LPS-induced shock due to this Casp4 passenger mutation. This illustrates how one passenger mutation can muddle up phenotypic outcomes and result in potentially false positive phenotypes. Considering that 1150 genes are predicted to be aberrantly expressed in 129 mice, our case studies only illustrate the top of the iceberg. The PuMaFinder will allow researchers to pinpoint and subsequently verify potential 129-derived passenger mutations in any 129-derived transgenic null animal. Our findings also highlight the importance of (re)generating transgenic mice in one defined genetic background.

Poster #**106** / Session **II**

Targeting Mitochondrial Potassium Channel Kv1.3 to Reduce Glioblastoma

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Glioblastoma (GBM) is the most common and aggressive tumor among glia neoplasms. Current treatments include surgery and chemotherapy in combination with irradiation, but the median survival only reaches 14 months (1). Recently, a new role of K^+ channels in cancer therapy was discovered (2). Indeed, the inhibition of a mitochondrial K^+ channel, mtKv1.3, with membrane permeant compounds PAP-1, Psora-4 and clofazimine has been demonstrated to be able to induce the intrinsic pathway of apoptosis, in a Bax/Bak independent manner, *in vitro* in different cancer cell lines and *in vivo* in an orthotopic mouse melanoma model (3).

Here, we used clofazimine and three newly synthetized different PAP-1 derivatives to trigger apoptosis in three glioma cell lines. We demonstrated that the treatment with these compounds induced an increase of reactive oxygen species (ROS), a depolarization of the inner mitochondrial membrane (IMM), and the release of cytochrome c, finally leading to apoptotic cell death. The ablation of Kv1.3 expression by siRNA abolished these effects, demonstrating the specificity of the treatment.

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Poster #**107** / Session **I**

A20 in Inflammatory Signaling and Pathology

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Different mechanisms control the dynamics of NF-κB activation assuring a tight regulation of inflammatory responses, and several autoregulatory feedback loops terminating the NF-κB response have been described. The ubiquitin-editing protein A20 (also known as TNFAIP3) is well known for its anti-inflammatory and protective activities, and genetic studies in humans identified polymorphisms in the A20/TNFAIP3 locus associated with multiple inflammatory and auto-immune pathologies including inflammatory bowel diseases (IBD) and rheumatoid arthritis.

To assess the role of A20 in intestinal inflammation and IBD development, we generated mice that are specifically deficient for A20 in intestinal epithelial cells (IECs). These mice develop normal intestinal epithelium without spontaneous inflammation, but are hypersensitive to experimental colitis due to increased IEC sensitivity to apoptosis (Vereecke et al., J. Exp. Med., 2010). These findings identify A20 as an essential protective factor for epithelial barrier integrity in inflammatory conditions. Mice in which A20 was specifically deleted in myeloid cells spontaneously develop a severe erosive polyarthritis (Matmati et al., Nat. Genet., 2011; Vande Walle et al., Nature, 2014) due to high levels of circulating inflammatory cytokines in their serum caused by a sustained NF-κB activation in macrophages. Through the combined deletion of A20 in both IECs and myeloid cells by intercrossing both mouse lines, we now developed a spontaneous mouse model of colitis characterized by intestinal epithelial apoptosis, Paneth and goblet cell loss, epithelial hyperproliferation and intestinal microbiota dysbiosis (Vereecke et al., unpublished). Together these data clearly define A20 as a crucial inhibitor of NF-κB-dependent inflammation and tissue protective factor *in vivo*.

Poster #**108** / Session **II**

State of Oncomarker Proteins Nucleophosmin and UBF during Apoptosis

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Tumor cells are characterized not only by hyperactivity and pleomorphism of the nucleoli, but also by sharply increased content of some nucleolar proteins, such as protein B23/nucleophosmin and UBF, leading to resistance of tumor cells to apoptosis. Toxicity of many apoptotic agents for tumor cells correlates with their ability to induce translocation of these proteins from the nucleolus into the nucleoplasm or to activate their proteolysis, but in none of these studies the sites of protein splitting were not characterized. It was shown that cell malignization is accompanied by structural changes of nucleophosmin resulting in appearance of unique oligomeric forms resistant to SDS-treatment, but at present there are no data on changes of monomer-oligomer state of nucleophosmin during apoptosis.

The purpose of the present work is analysis of the monomer-oligomer state of nucleophosmin and structural changes of nucleophosmin and UBF on protein level during apoptosis induced by alpha-TNF/emetine in human cervical carcinoma cells (HeLa). Immunoblotting analysis of samples containing 25, 45 and 100% of cells with apoptotic nuclei showed that UBF was cleaved by caspase giving a stable 76-kDa fragment. Increasing of its content during apoptosis correlated with the percent of apoptotic cells and with a decrease level of full-sized UBF. We determined site of UBF-splitting and showed that the proteolysis resulted in detachment of the N-terminal fragment, containing domain important for both UBF dimerization and functioning as transcription factor and one of six HMG-boxes. Nucleophosmin did not undergo proteolysis during apoptosis and its content was unchanged even in the sample containing 100% of cells with apoptotic nuclei. However in these cells the balance between monomeric and oligomeric forms of nucleophosmin changed due to depletion of monomeric forms and increasing in content of oligomers with appearance of two additional oligomers. This work was supported by the gtants of the Program "Fundamental Sciences for Medicine, 2012-2014".

Poster #**109** / Session **I**

TOR Complex 2 Signaling Promotes the General Amino Acid Control Response and Autophagy

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Autophagy is an evolutionarily conserved process that facilitates the recycling of cytoplasmic contents in eukaryotic cells facing energetic stress. Autophagy serves as both a rejuvenating mechanism for maintaining cellular homeostasis as well as an adaptive response during nutrient deprivation. As such, impaired regulation of autophagy has been linked to a number of metabolic and aging related disorders as well as cell death. A central regulator of autophagy is the conserved TOR (Target of Rapamycin) kinase, which couples the cellular nutritional status to growth in eukaryotic organisms. TOR forms two structurally and functionally distinct complexes, TORC1 and TORC2, where TORC1 is a well-established negative regulator of autophagy. Here we demonstrate TOR also operates independently through the TORC2 signaling pathway to promote autophagy upon amino acid limitation. Under these conditions, TORC2, through its downstream target kinase Ypk1, inhibits the Ca2+ dependent phosphatase, calcineurin, to enable the activation of the general amino acid control response required for autophagy induction. Thus TORC2 signaling regulates autophagy in a pathway distinct from TORC1 to provide a tunable response to the cellular metabolic state.

Poster #**110** / Session **II**

TRAIL-Induced Necroptosis as a Novel Approach to Eliminate Tumor Cells

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We have previously identified the sphingolipid ceramide (generated by acid sphingomyelinase (A-SMase)) as one of the pivotal mediators in death receptor-mediated necroptosis. Necroptosis is a mode of programmed cell death that operates via yet not fully understood molecular pathways, which are however clearly distinct from apoptosis. Therefore, necroptosis potentially constitutes a novel (yet virtually unexplored) option for the elimination of apoptosis-resistant tumor cells. Here, we have investigated the impact of necroptosis elicited by tumor necrosis factor related apoptosis inducing ligand (TRAIL) in a panel of tumor cell lines of wide-ranging origin. Eight out of 14 tumor cell lines proved spontaneously sensitive to TRAIL/ceramide-induced necroptosis. Furthermore, clonogenic survival was reduced in five sensitive and one resistant cell line. Substantiating the role of ceramide as a key element of death receptor-induced necroptosis also for the examined tumor cell lines, all five sensitive tumor cell lines but not a resistant cell line displayed a clear accumulation of intracellular ceramide after induction of necroptosis. Moreover, Arc39, a potent and specific inhibitor of A-SMase clearly inhibited programmed necrosis in all five sensitive cancer cell lines. In addition, our data point to expression of the kinase RIPK3 as a primary determinant for resistance or susceptibility of the analyzed tumor cells, revealing RIPK3 as a potential predictive marker. Finally, we found that TRAIL synergizes with chemotherapeutics in killing tumor cell lines by necroptosis, enhancing their effect in eight out of 10 tested tumor cell lines and in 41 out of 80 chemotherapeutic/TRAIL combinations. Altogether, our results corroborate the concept that TRAIL/ceramide-mediated necroptosis may represent a novel option for the development of future combination therapies and thus the treatment of cancer patients.

Poster **#111** / Session **I**

Prevention of Memory Loss and Neuronal Death in Olfactory Bulbectomized Mice Immunized with Fragments of Neurotrophin Receptor

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Neurotoxic beta-amyloid peptide (beta-A) plays an important role in the pathology of Alzheimer's disease (AD). It is known that beta-A binds p75 neurotrophin receptor and mediates neuronal apoptosis. We proposed that induction of antibodies against potential binding sites of p75 with beta-A can be a promising approach to prevention of neuronal death. Eight potentially immunoactive fragments of p75 were chosen and synthesized. Investigation of immunoprotective effect of the peptide fragments was carried out in mice with experimentally induced form of AD - bulbectomized mice. We have revealed two fragments effectively preventing of murine memory from impairment. Results obtained by ELISA showed that immunization with fragments led to decrease in beta-amyloid level in the brain of the experimental mice. Morphological characterization of brain neurons showed that immunization with these peptides protects neurons in cortex and hippocampus from neuronal death and from significant increase of markers of neurodegeneration, including pyknosis, cytolysis, and vacuolization. Supported by RFBR Grant No. 13-04-40106-H.

Poster #**112** / Session **II**

Antiapoptotic HSPB1 Mediates Prolactin-Induced Pro-survival Effects on Pancreatic β-Cells

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Transplantation of pancreatic islets constitutes an alternative treatment for type 1 diabetes (DM1). However, it is limited by the shortage of organ donors. Ex-vivo expansion of islet cell cultures appears as an attractive strategy; however, the islet fate in culture is determined, at least in part, by the balance between pro- and anti-apoptotic mediators. We have previously shown that prolactin (PRL) inhibits beta-cell apoptosis, and recently that PRL induced the up-regulation of the anti-apoptotic Heat Shock Protein B1 (HSPB1) in human islets. Since the function of HSPB1 in beta-cells has not been directly studied, we set out to explore the role of HSPB1 in PRL-induced beta-cell cytoprotection.

For this purpose**,** we used parental and HSPB1 knocked-down Min6 cells. Apoptosis was evaluated by DNA fragmentation and quantified by flow cytometry. We studied anti- and pro-apoptotic protein levels by western blotting. Caspase-3 and -8 activities were studied by fluorimetric assays. In order to evaluate whether the cell responses were specific of the PRL treatment, cells were pre-treated with either a specific inhibitor of JAK2 or vehicle.

Our data showed that upon cytokines and rhPRL treatment, the proportion of fragmented nuclei was increased in HSPB1 silenced cells ($p<0.05$) when compared to control cells. In addition, the inhibition of cytokine-induced capase-3 and caspase-8 activities as well as Bcl-2/Bax ratio and caspase-9 protein levels mediated by rhPRL in wild type cells was significantly reverted in knocked-down cells ($p<0.05$). In conclusion, we demonstrated a key role for HSPB1 in PRL-induced cytoprotective effects, since the lack of this protein abolished the beneficial effects induced by PRL in β-cells. Furthermore, our results could lead to the mitigation of β-cell death through the up-regulation of an endogenous protective pathway which is not dependent on the modulation of the immune system.

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Poster #**113** / Session **I**

Isoflurane Preconditioning in the Myocardium Requires RhoA-Cell Adhesion Signaling Mediated by Reactive Oxygen Species

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The cellular and molecular events that link reactive oxygen species (ROS) and isoflurane-conferred preconditioning in cardioprotectio remain to be explored. In this study the embryonic rat heart-derived cell line H9c2 and primary neonatal Sprague-Dawley (SD) rat ventricular myocytes were subjected to oxygen/glucose deprivation followed by reoxygenation to mimic ischemia/reperfusion. The ischemia/reperfusion was also performed in SD rat and C57 mouse models. Isoflurane pretreatment resulted in cardioprotective effects both in vitro and in vivo. Upon isoflurane exposure, a transient and modest increase of ROS was detected, and a marked enhancement of cell adherence was observed in cultured cells following activation of RhoA. A rapid increase in ROS and activated RhoA was also detected in rat hearts exposed to isoflurane. Isoflurane promoted the assembly of vinculin with F-actin to form focal adhesions in cultured cells and enhanced the recruitment of vinculin to intercalated discs in rat myocardium. The increases in adhesion ability and viability of cardiomyocytes by isoflurane were

reversed by either an antioxidant or the pharmacological inhibitor of Rho-associated kinase. Finally, a mouse model with a cardiac-specific knockdown of RhoA showed that RhoA is essential for the ultrastructural integrity of the fascia adherens in intercalated discs and for the cardioprotective effect of isoflurane. Taken together, we concluded that the precondioning effects of isoflurane on cardiomyocytes are mediated by the activation of RhoA and cell adhesion signaling, and ROS act as an upstream trigger of this signaling pathway.

Poster **#114** / Session **II**

CD4+ T Cell Deficient in GIMAP1 Die by Extrinsic Apoptosis

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GIMAPs (GTPases of the Immunity Associated Proteins) are a family of small GTPases related to septins and dynamins that are predominantly expressed in lymphoid tissue, where they play an important but poorly characterised role in the regulation of lymphocyte survival. We have previously shown that a conditional knockout of family member *Gimap1* results in a dramatic loss of both T and B lymphocytes in the periphery, despite near-normal development of these cells in primary lymphoid organs. In order to understand the role that GIMAP1 plays in peripheral lymphocyte survival, we have generated a mouse line: *Gimap1*$^{flox/flox}$*; ER*^{T2}*Cre* (*Gimert1*), in which *Gimap1* can be knocked-out inducibly by the synthetic oestrogen 4-hydroxytamoxifen (4-OHT). Using this model system we have been able to dissect the pathways of death engaged when GIMAP1 is absent.

Using an *in vitro* model of CD4+ T cell survival, we show that peripheral CD4+ T cells require continued expression of GIMAP1 for their survival. Following addition of 4-OHT, purified *Gimert1* CD4+ T cells cultured in IL-7 lost GIMAP1 expression and started to die within 48 hours. Cell death was associated with Annexin V binding, activation of Caspase 3, and loss of mitochondrial membrane potential – all indicators of apoptosis. In addition, we found that GIMAP1-deficient cells expressed active Caspase 8 (but not active Caspase 9), indicative of activation of the extrinisic apoptosis pathway. Apoptosis of GIMAP1-deficient cells was inhibited by addition of the Caspase 8-specific inhibitor, Z-IETD-FMK.

Together, these data suggest that continuous expression of GIMAP1 is required to prevent activation of the extrinisic apoptosis pathway in resting peripheral CD4+ T lymphocytes.

Poster #**115** / Session **I**

Combined Loss of the BH3-Only Proteins Bim and Bmf Restores B Cell Development and Function In TACI-Ig Mice

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Terminal differentiation of B cells depends on two interconnected survival pathways, elicited by the B cell receptor (BCR) and the BAFF receptor (BAFF-R), respectively. Loss of either pathway impairs B cell development and causes B cell deficiency. While BCR-dependent survival depends mainly on the activation of the AKT/PI3-kinase network, BAFF-R-mediated survival engages non-canonical NF-kB signalling as well as MAPK/ERK and AKT/PI3-kinase modules to allow proper B cell development. Ultimately, these complex signalling events culminate in increased expression of anti-apoptotic Bcl2 family proteins. How lack of BAFF-R mediated signalling triggers B cell apoptosis remains largely unexplored. Here, we show that two pro-apoptotic members of the "BH3-only" subgroup of the Bcl2 family, Bim and Bmf, mediate apoptosis upon BAFF-depletion caused by TACI-Ig overexpression. Surprisingly, although Bcl2 overexpression triggers B cell hyperplasia exceeding the one observed in *Bim*$^{-/-}$*Bmf*$^{-/-}$ mice, Bcl-2 transgenic B cells remained susceptible to BAFF-depletion *in vivo*, leading to ameliorated pathology in *Vav-Bcl2* transgenic mice. Together, our findings shed new light on the molecular machinery restricting B cell survival during development and under pathological conditions. Our data further suggests that Bcl2 antagonists might improve the potency of BAFF-depletion strategies in B cell driven pathologies.

Poster **#116** / Session **II**

Familial Parkinsonism with *G2019S* LRRK2 Mutation Displays a Susceptibility to The MPP^+ Neurotoxin by a Mtor-Dependent Autophagy

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by mitochondrial dysfunction, oxidative stress and later neuronal death. Several genetics and environmental factors have been implicated in the pathogenesis of PD.

MPP^+ is a neurotoxin widely used to induce parkinsonian cellular model, it is responsible for cellular damage at different levels, apoptotic death, depletion of mitochondrial potential membrane and deregulation of cellular recycling machinery.

In this study, we characterized the MPP^+-induced toxicity in fibroblasts from PD patients with G2019S LRRK2 and control individuals without this mutation. Obtained results show that MPP^+ induces a mTOR-dependent autophagy in both fibroblasts cells. Further, cell death to MPP^+ was higher in mutant fibroblasts which exhibited a basal level of mTOR-independent autophagy due to the *G2019S* LRRK2 mutation.

Inhibition of autophagosome-lysosome fusion by Bafilomycin A1 exacerbated the response to MPP^+ exposure in both cell lines, but inhibition of early state autophagy by 3-methyladenine lessened this difference between both cell types.

This finding confirms the important implication of the interaction of genetics and environmental factors in the PD etiology and may help to get a better understanding in the pathogenic mechanism of this disease.

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Poster #**117** / Session **I**

Caspase-2-Mediated Ku80 Cleavage Facilitates DNA Damage Repair

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Caspases-2 belongs to a family of caspase, highly conserved aspartate-specific cysteine proteases. The apoptotic role of Caspases is well established in the last two decades. Importantly, caspase-2 has been shown to function in non-apoptotic tumor suppression as well as in apoptosis. Caspase-2 is the only caspase that constitutively localizes to the nucleus as well as other cellular compartments. However, the functional significance of this nuclear localization is unknown. Caspase-2 has some unique features as initiator as well as executor caspase. However, in contrast to other known caspases, among the more than 250 proteins presently known to be cleaved by different caspases, only very few substrate serve as target proteins for caspase-2, few substrate involved in tumor-suppression is either discovered.

In this study, firstly, we found that caspase-2 deletion increases DNA damage upon etoposide pulse treatment without obvious apoptosis. Similarly, caspase-2-deficient cells display a significantly higher level of DNA damage compared with normal cells over time following H_2O_2 and IR. And then we observed that loss of caspase-2 impaired DNA repair foci. Then the effect of caspase-2 on NHEJ (non-homologue end-joinning) in caspase-2-deficient cells was followed up using in vivo end-joinning assay via transfection with a pEGFP-N1 plasmid linearized with the restriction enzyme BsrGI that cleaves the plasmid within the GFP coding region. Caspase-2-deficient cells displayed a reduction in end-joining activity. Loss of Caspase-2 also decreased Ku80/DNA-PKcs complex formation which is major complex responsible for NHEJ. Meawhile, caspase-2 can interact with NHEJ complex confirmed by FRET anylysis. Notely, caspase-2 activity is required for NHEJ repair, Ku80 is just interacted with activated caspase-2 showing the possibility of Ku80 as substrate of Caspase-2. Taken together, Caspase-2 mediated-Ku80 cleavage might facilitate Ku80/DNA-PKcs complex formation and contributes to the DNA repair by NHEJ pathway which is implied that Caspase-2 tumor suppressor function and genomic stability via DNA repair upon mild genotoxic stress.

Keywords: Caspase-2; DNA damage repair; cleave; ku80.

Poster #**118** / Session **II**

Multiprotein Activation Platform of Caspase-2 upon Genotoxic Stress

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The key step of apoptosis induction is the formation of the high molecular weight caspase-activating complexes. Caspase-2 was shown to be an initiator caspase of apoptotic cell death in ovarian carcinoma cells. Caspase-2 knockout (KO) mice are characterized by the defects in ovary development and, furthermore, tumors that are formed in ovaries are resistant towards conventional chemotherapy. Therefore, the studies of the mechanisms of caspase-2 activation in ovarian carcinoma cells and the platforms of its activation are essential for understanding the resistance mechanisms of ovarian carcinoma cells towards chemotherapy. Here we analyzed cell death in the ovarian carcinoma cell line Caov-4 treated with DNA-damaging agent cisplatin.

Using wt Caov-4 and Caov-4 caspase-2-deficient cells (shRNA-Caspase-2) we showed that DNA damage induced by cisplatin treatment leads to caspase-2-dependent cell death with apoptotic and necroptotic features. Analysis of the kinetic of caspase-2 processing after genotoxic stress revealed the appearance of cleavage form of caspase-2 after 2-4h incubation with cisplatin while substantial caspase-2 activation was observed after 18-24h incubation. The processing of caspase-3 followed caspase-2 activation. Expression of RAIDD siRNA in Caov-4 cells did not influence caspase-2 activation and apoptosis. Gel-filtration analysis of cisplatin-treated and control cells identified caspase-2 only in the high molecular weight (HMW) fractions isolated from cisplatin-treated cells. Immunoprecipitation (IP) via caspase-2 antibody from HMW gel-filtration fractions allowed us to isolate the complex containing caspase-2. The highest caspase-2 activity was detected in IP samples from HMW fraction. The analyses of these samples by Western blot revealed PIDD- and RAIDD-independent activation of caspase-2.

The composition of this complex is currently analyzed by Mass Spectrometry analysis. Collectively, we have demonstrated the formation of the HMW complex that activates caspase-2 in ovarian carcinoma cells upon genotoxic stress. Importantly, this platform does not contain proteins PIDD and RAIDD that were reported earlier to be essential for caspase-2 activation within PIDDosome. Our view on the caspase-activating platform in ovarian carcinoma cells will be presented.

Poster #**119** / Session **I**

Autophagy and Apoptosis Regulation in Mammary Gland during Lactation Cycle - Transcriptomic Profile of Bovine Milk Somatic Cells

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The secretion of the mammary gland contains heterogeneous cell population, which profile differs depending on the animal species. Predominant are the immune system cells, protecting the mammary gland against infections, but there is also significant epithelial cells subpopulation. In cattle and goats somatic cells count (SCC) changes in the lactation cycle could be noticed.

For the research purposes somatic cells were isolated from milk during the lactation cycle and used as material for genomic studies. Milk was sampled from Polish Holstein-Friesian (min. 87.5% HF) cows in first lactation, kept in a herd belonging to IGAB Jastrzębiec. Tests samples (1L) were collected from healthy individuals (no clinical signs of mastitis, SCC $<250 \times 10^3$/ml), during the lactation cycle, on days 10, 60, 120 and 240. Methodology of RNA isolation from SC, was based on the method presented in the work of Boutinaud et al. (2008), with own modifications. Transcriptomic analyses with microarrays usage were performed on RNA pools (8 individuals in group). Statistical analysis was performed using ANOVA test ($p < 0.05$ level of significance) with a 5% FDR correction. The presence of SC subpopulations was checked by flow cytometry.

The difference in the expression of 6884 genes has been shown. Changes in gene expression were strongest at day 10th of lactation, the strong similarity between the expression profiles of the last phases of lactation could be seen. The statistically significant changes in gene expression between the two groups of cows, with high (10-12, 000) and low (6-8, 000) milk yield had not been established - this is probably due to the relatively small differences in productivity

in the tested herd. An analysis of signaling pathways and for the effect of significantly regulated genes in the biological processes was performed (Panther and KEGG). Genes important for regulation of apoptosis and autophagy processes have been also found. Number of differently expressed genes involved in apoptosis process: 60v10:35, 120v10:58, 240v10:68. Changes in expression of genes from signaling pathways were noticed, among other: apoptosis signaling pathway, regulation of autophagy, PI3K-Akt, MAPK, mTOR, TGF-β1. One of the interesting identified genes is Runx1, which new insights in epithelial stem cell biology are currently researched.

Poster #**120** / Session **II**

Glucagon-Like Peptide-1 Protects Pancreatic Beta-Cells From Programmed Cell Death: A Role For Autophagy

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Worldwide, more than 371 million people have type 2 diabetes mellitus (T2DM) and the condition is becoming increasingly common. Loss of pancreatic β-cell mass is central to the pathogenesis of T2DM. Accordingly, strategies promoting β-cell survival offer an attractive therapeutic approach. Understanding the cellular signalling mechanisms by which pancreatic beta cell survival is mediated is hence critical to the development of novel pharmacological targets.

The aim of the current study was to identify the mechanisms by which the glucagon-like peptide-1 (GLP-1) analog exendin-4 protects β-cells from cell death after a diabetic stimulus, and the contribution of autophagy.

Treatment of the pancreatic β-cell line INS-1E with T2DM glucolipotoxic stresses (high glucose and palmitate) increased apoptotic cell death as evidenced by increased caspase 3 cleavage ($p<0.001$) and also increased LC3-I to LC3-II flux and p62 puncta. Co-treatment with exendin-4 protected INS-1E cells from glucolipotoxic- induced cell death via apoptosis ($p<0.05$) and also exacerbates LC3-I to LC3-II conversion, potentially stimulating autophagy and promoting β-cells survival.

Collectively these data reveal a novel role for autophagy in exendin-4 mediated regulation of β-cell mass, as well as a potential therapeutic strategy through which to protect β-cells in T2DM.

Poster #**121** / Session **I**

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Conference Info

❖ TRANSFER TO HERSONISSOS & THE CONFERENCE SITE

BY BUS

There is a regular public bus service from Heraklion to Hersonissos and there is a bus stop outside Heraklion International Airport [HER]:

The bus goes by every 15 minutes, the ticket price is 3,80 € and the travel time is about 1 hour.

You should notify the bus driver of your exact destination to drop you off and help you find your way. For those of you staying at Creta Maris Hotel (or around) the bus station is about 250 meters away from the hotel.

More detailed information together with timetables can be found here: http://bus-service-crete.com/timetabledet.php?line=13&lg=2 (roughly add 15 minutes to the indicated departure time from Heraklion to estimate the arrival time at the airport).

BY TAXI

There is a TAXI station outside Arrivals, on the left. The TAXI drive from the airport to Hersonissos costs around 35 € depending on the taximeter and the distance (your hotel locations vary).

We have an offer from our partner organizer CCBS-Greece for 34 €. With this price, the TAXI driver will wait for you holding a sign with your name on it. The price is fixed from Heraklion to any part of Hersonissos. If you wish to take advantage of the service you are kindly requested to send an e-mail to sales@ccbsgreece.gr, subject: "ecdo-taxi", indicating your name, your arrival flight number and your arrival date and time in Heraklion airport.

BY CAR

If you rent a car at the airport, you should get into the Highway, heading east to Agios Nikolaos; you should look for signs to Hersonissos after 25 km drive.

❖ REGISTRATION

Wednesday, 1 October @ 15.00
Please register at the Conference Secretariat in the Secretariat Office of the Conference Center (see hotel plan page 275 and Conference Room plan, page 276).
Upon registration you will get your conference material and the gala dinner voucher.
You are kindly requested to wear your name badge during all events of the meeting in order to justify your access to all sessions, welcome reception, lunches, etc.

- Gala dinner vouchers will be available at the Conference Secretariat at the price of 55,00 €.
- Lunch package vouchers for accompanying persons will be available at the Conference Secretariat at the price of 50,00 €. Vouchers on a per lunch basis are not available.

❖ SPEAKERS

There will be a data projector connected to a PC (Windows - Microsoft Office), so kindly prepare your presentation file(s) accordingly. There will be assistance inside the conference room; members of the local organizer laboratories will be at your disposal for any technical assistance. You will meet them during registration. You should not forget to contact them during the break prior to your presentation's session and hand over to them your presentation storage unit or device (CD, memory stick, notebook/laptop). Assistants will wear a yellow name badge.

If you are a Mac user, please don't forget to bring the cable required to connect your machine to the projector.

All speakers will have to stick strictly to the time allocated for their talk:

Short Talks : 15 minutes, discussion included

Invited Speakers : 25 minutes + 5 minutes discussion

❖ POSTER PRESENTERS

Posters should be mounted from the beginning of the meeting and be displayed throughout the meeting.

There are two fixed poster sessions:

Poster Session I (odd numbers): Thursday, 2 October @ 17.00

Poser Session II (even number): Saturday, 3 October @ 16.30

Posters should be prepared according to the guidelines in portrait format with up to 90 cm width and up to 180 cm height.

This year's Poster Prizes:

1. Free online subscription to ***Cell Death & Differentiation***

2. Free registration to the next year conference – ECDO 2015

You should have removed your poster by Sunday, October 4th, at the end of the meeting. The organizers or the hotel hold no responsibility for any posters not removed by then.

❖ INTERNET ACCESS

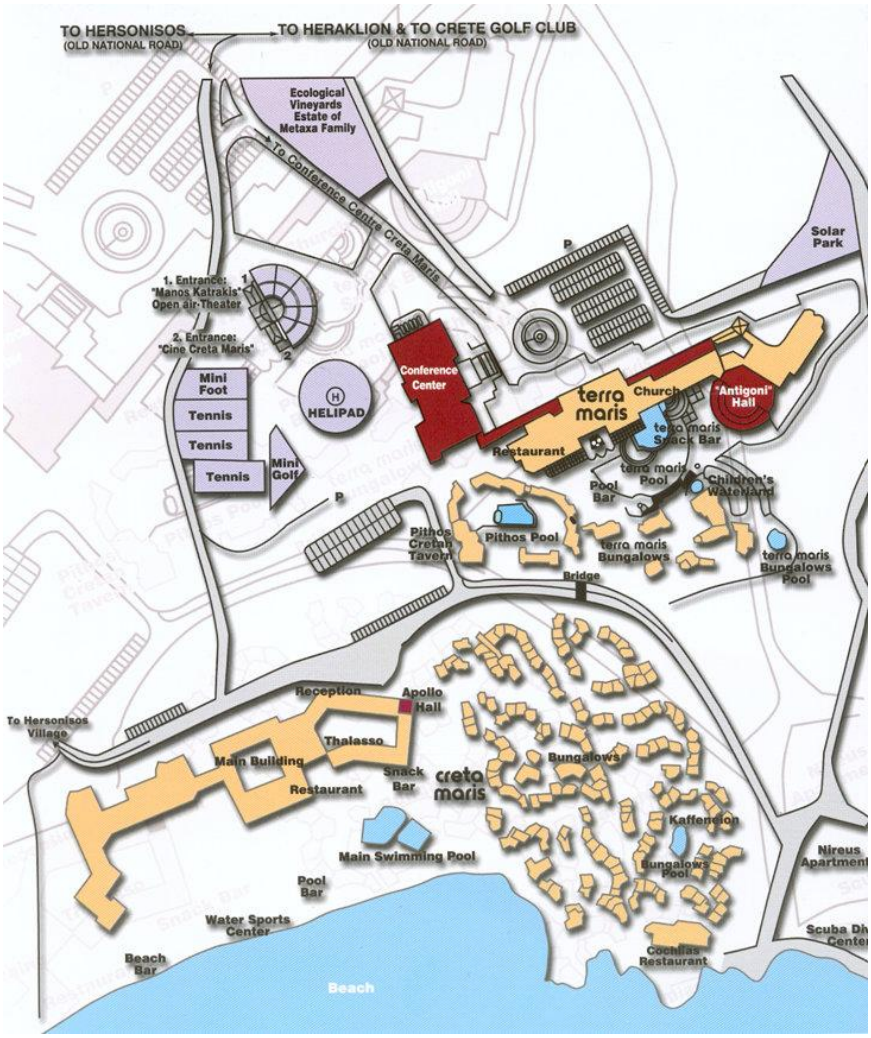
Wireless Internet access will be available in the Conference Room and the surrounding area, free of charge.

5 PC's with internet access will be available to the conference participants inside the poster room at the conference venue.

❖ USEFUL PRACTICAL INFO

- ❖ Our partner organizer - CCBS-Greece – will have two representatives on site: Ms. Koronaiou and Ms. Kyriazi. They will be happy to advice you on short visits or day schedules all around Crete and will be more than willing to arrange such off-conference activities for you and/or your escorts.
- ❖ Provided that you have made your reservation through the meeting organizers, if you wish to extend your stay or keep your room after 12:00 on departure day, you should notify the representative of CCBS-Greece.

Last but not least, the weather in early October should normally be really great (although, of course we are reluctant to bet our heads on it!). Check the links on the meeting web site for more information.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(OLD NATIONAL ROAD)
TO HERAKLION & TO CRETE GOLF CLUB
(OLD NATIONAL ROAD)
Ecological Vineyards Estate of Metaxa Family
To Conference Centre Creta Maris
1. Entrance: "Manos Katrakis" Open air-Theater
2. Entrance: "Cine Creta Maris"
Mini Foot
Tennis
Tennis
Tennis
Mini Golf
HELIPAD
Conference Center
P
Solar Park
terra maris
Church
"Antigoni" Hall
terra maris Snack Bar
Restaurant
terra maris Pool
Pool Bar
Children's Waterland
Pithos Cretan Tavern
Pithos Pool
terra maris Bungalows
terra maris Bungalows Pool
Bridge
Reception
Apollo Hall
Te Hersonisos Village
Thalasso
Main Building
Snack Bar
Restaurant
creta maris
Bungalows
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Main Swimming Pool
Bungalows Pool
Nireus Apartment
Pool Bar
Water Sports Center
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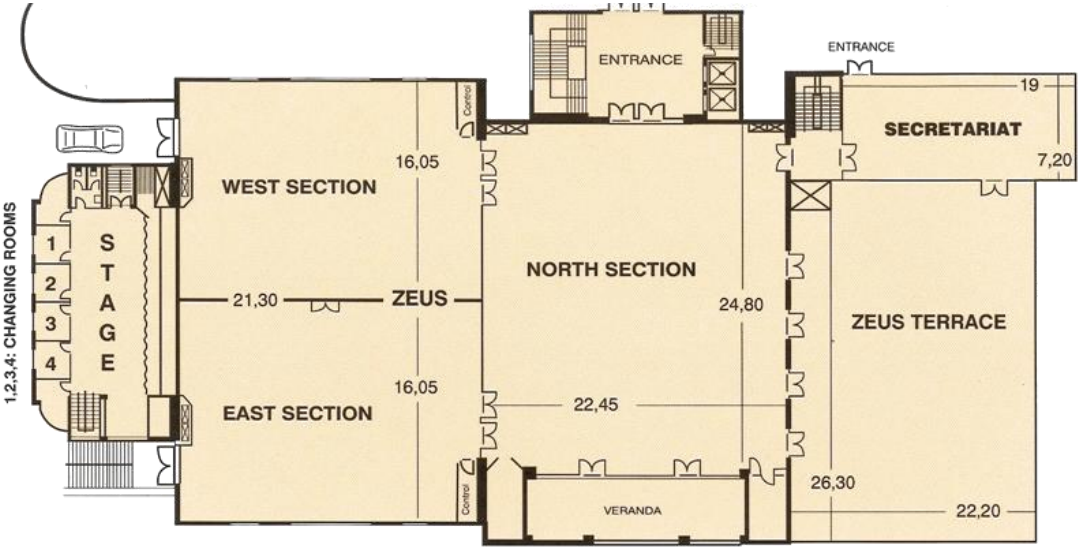

Zeus Conference Hall

West – East Sections:	Conference Room
North Section:	Poster Room / Exhibition
Secretariat:	Registration
Zeus Terrace:	Coffee Breaks / Lunches

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