BOOK OF ABSTRACTS

4th Conference on Experimental and Translational Oncology

Kranjska gora, Slovenia,
March, 22-26, 2006

Co-chairs of the conference: *Tamara Lah Turnšek*, National Institute of Biology, Ljubljana, Slovenia
Gregor Serša, Institute of Oncology, Ljubljana, Slovenia
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March, 22-26, 2006

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19.00 – 20.15	Dinner
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8.55 – 9.20	Mueller Margareta, German Cancer Research Center; Heidelberg: Growth factors as paracrine und autocrine mediators of tumor progression and stroma modulation
9.20 – 9.45	Magdolen Viktor, Clinical Research Unit, TU München: Biochemical characterization and clinical relevance of human tissue kallikreins
9.45 – 10.10	Nathan Ilana, Soroka University MedicalCenter and Ben-Gurion University of the Negev, Beer Sheva: Triarylethylenes and related derivatives as novel antileukemic agents
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	Differential effects of Cathepsin L deficiency on multistage tumorigenesis of epidermal carcinomas in K14-HPV16 mice as compared to islet cell tumors in the RIP1-Tag2 mouse tumor model
11.50 – 12.15	Pilkington Geoffrey J., University of Portsmouth, Portsmouth: The role of tricyclic drugs in selective triggering mitochondrially-mediated apoptosis in neoplastic glia: a therapeutic option in malignant glioma?
12.15 – 12.40	Turk Boris, Jožef Stefan Institute; Ljubljana: Cysteine cathepsins pathways to apoptosis: can one use them in cancer treatment?
12.40 – 12.55	Vasiljeva Olga, Albert-Ludwigs-University Freiburg, Freiburg: Tumor cell- and macrophage-derived Cathepsin B promotes progression and lung metastasis of mammary cancer
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14.55 – 15.20	Christensen Ib Jarle, Hvidovre Hospital, Hvidovre: The selection of serological markers, for the determination of a prognostic profile predicting cancer-specific death in colorectal cancer
15.20 – 15.45	Høyer-Hansen Gunilla, Finsen Laboratory, Copenhagen: Cleavage of uPAR; mechanism and prognostic significance
15.45 – 16.10	Strojan Primož, Institute of Oncology, Ljubljana: Cathepsins and their inhibitors in head and neck cancer
16.10 – 16.25	Decock Julie, University Hospital Gasthuisberg, Leuven: Matrix metalloproteinase expression analyses of human primary breast cancer tissue
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17.25 – 17.50	Kirschfink Michael, University of Heidelberg, Heidelberg: Complement resistance impairs anti-tumor therapy

17.50 – 18.15	Kotnik Vladimir, University of Ljubljana, Ljubljana: The humoral and cellular mechanisms effective for killing tumor cells in vitro
18.15 – 18.30	Novaković Srdjan, Institute of Oncology, Ljubljana: Classical tumor vaccine potently stimulates dendritic cells for the activation of naive T lymphocytes and forming of memory cell pool
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17.50 – 18.15	Rols Marie-Pierre, IPBS CNRS (UMR 5089), Toulouse: Electropermeabilization: a non viral method for the delivery of DNA into cells
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11.50 – 12.15	Mackay Andrew R., University of L'Aquila, L'Aquila: TrkAIII: A tumor promoting switch of potential importance in neuroblastoma and glioblastoma mutliforme
12.15 – 12.40	Durán Alonso María Beatriz , National Institute of Biology, Ljubljana: Antiprotease therapy in cancer: hot or not?
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15.45 – 16.10	Levičar Nataša, Imperial College London, London: Stem cell therapy for liver insufficiency
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L2. *Noel Agnes*: Proteases and their inhibitors during tumoral angiogenesis: lessons from knock out mice

L3. *Mueller Margareta*: Growth factors as paracrine und autocrine mediators of tumor progression and stroma modulation

L4. *Magdolen Viktor*: Biochemical characterization and clinical relevance of human tissue kallikreins

L5. *Nathan Ilana*: Triarylethylenes and related derivatives as novel antileukemic agents

L6. *Premzl Aleš*: Assessment of Cathepsin B activity in tumour invasion and angiogenesis

L7. *Edwards Dylan R.*: The Cancer Degradome: New insights into protease function in cancer biology

L8. *Reinheckel Thomas*: Differential effects of Cathepsin L deficiency on multistage tumorigenesis of epidermal carcinomas in K14-HPV16 mice as compared to islet cell tumors in the RIP1-Tag2 mouse tumor model.

L9. *Pilkington Geoffrey J.*: The role of tricyclic drugs in selective triggering of mitochondrially-mediated apoptosis in neoplastic glia: a therapeutic option in malignant glioma?

L10. *Turk Boris*: Cysteine cathepsins pathways to apoptosis: can one use them in cancer treatment?

L11. *Vasiljeva Olga*: Tumor cell- and macrophage-derived Cathepsin B promotes progression and lung metastasis of mammary cancer

L12. *Brünner Nils*: Plasma Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) predicts survival in patients with metastatic colorectal cancer receiving irinotecan in combination with 5FU and folinic acid

L13. *Christensen Ib Jarle*: The selection of serological markers for the determination of a prognostic profile predicting cancer-specific death in colorectal cancer

L14. *Høyer-Hansen Gunilla*: Cleavage of uPAR; mechanism and prognostic significance

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L19. *Kotnik Vladimir*: The humoral and cellular mechanisms effective for killing tumor cells in vitro

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L21. *Zavašnik-Bergant Tina*: Microscopic techniques approach in studying protease inhibitor cystatin in immune cells

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L26. *Miklavčič Damijan*: Treatment planning for effective electroporation

L27. *Cegnar Mateja*: Nanoscale carriers facilitate delivery of protein drugs into tumour cells

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L32. *Mencej Matjaž*: Visible light microscopy, efficient use of techniques and solutions to common complications

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L34. *Kanthou Chryso*: Tumour cell-endothelial interactions: signal pathways and therapeutic targets

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L36. *Foekens John A.*: Gene-expression and DNA-methylation profiling in breast cancer

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L38. *Bruschi Carlo V.*: Modelling of genomic aberrations responsible for neoplastic transformation and tumor progression in yeast.

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L44. *Bjerkvig Rolf*: Angiogenesis independent growth mediated by glioma stem cells

L45. *Moin Kamiar*: Tumor proteolysis: a multidimensional approach

L46. *Levičar Nataša*: Stem cell therapy for liver insufficiency

L47. *Strojnik Tadej*: Experimental model and immunohistochemical analyses of U87 human glioblastoma cells xenografts in the immunosuppressed rat brains

L48. *Černe Darko*: Increased plasma DNA concentration in patients with lung cancer

L49. *Platika Doros*: Leveraging a flat world: novel models for effective translational research, product generation and commercialization in oncology

Abstracts of lectures

Fluorescence in vivo imaging for oncology

Justin Teissié

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Following and quantifying the expression of reporter gene expression *in vivo* is very important to monitor the expression of therapeutic genes in targeted tissues in disease models, to follow the fate of tumour cells, and/or to assess the effectiveness of systems of gene therapy delivery. Gene expression of fluorescent proteins can be detected directly on living animals by simply observing the associated optical signals by mean of a cooled charged-coupled device (CCCD) camera. More accurate resolution can be obtained with more sophisticated technologies. Time- course and quasi-quantitative monitoring of the expression can be obtained on a given animal and followed on a large time window. The present lecture describes the physical and technological methodologies and associated problems of *in vivo* optical imaging. Several examples of *in vivo* detection for oncology purposes are described.

Proteases and their inhibitors during tumoral angiogenesis: lessons from knock out mice

Agnes Noel

Laboratory of Tumor and Development Biology, University of Liège, Sart Tilman, Belgium

Pathological angiogenesis are associated with extracellular matrix remodelling and involve various proteases such as the plasminogen (Plg)/plasminogen activator (PA) system and matrix metalloproteinases (MMPs). The specific functions of individual proteases and their inhibitors as anti- or pro-angiogenic mediators remain to be elucidated. Proteases and their inhibitors are believed to play a key role in pathological angiogenesis by mediating extracellular matrix degradation, by shedding cell surface molecules and/or controlling the biological activity of growth factors, angiogenic factors, chemokines and/or cytokines. Recent findings revealed paradoxical functions of proteases and their inhibitors. We assessed the impact of single or combined MMP deficiencies, or single inhibitor deficiency in *in vivo* and *in vitro* models of angiogenesis: (1) tumoral angiogenesis induced by malignant keratinocyte transplantation, (2) choroidal neoangiogenesis induced by a laser burn and mimicking the age-related macular degeneration (AMD), and (3) the aortic ring assay.

Our recent findings highlight the importance of gelatinases (MMP-2 and MMP-9), membrane type MMPs (MT1-MMP and MT4-MMP) and the inhibitor of plasminogen activator-1 (PAI-1) as positive regulator of pathological angiogenesis. In sharp contrast, MMP-19 appears as a negative regulators of tumoral angiogenesis. We will discuss the individual functions of proteases and their inhibitor during sprouting angiogenesis, vasculogenesis and lymphangiogenesis. The dissection of the molecular mechanisms of action of these proteolytic systems is mandatory to develop new anti-angiogenic strategies.

Growth factors as paracrine und autocrine mediators of tumor progression and stroma modulation

Wiltrud Lederle, Silvia Vosseler, Claudia Gutschalk, Norbert E. Fusenig, Margareta M. Mueller

Group Tumor and Microenvironment, German Cancer Research Center (DKFZ), INF 280, D-69120 Heidelberg

The altered expression of growth factors and receptors has a pivotal role in the interaction between the tumor and its stroma. Growth factors induce the generation of a tumor-permissive stromal surrounding that facilitates tumor growth and progression. In the HaCaT model for SCCs of the skin we could demonstrate that the kinetic and extend of stromal activation and angiogenesis critically contributes to the establishment of a benign or malignant tumor phenotype. We identified the expression of a number of growth factors to be associated with tumor progression and could verify the functional contribution of several factors to tumor progression in vivo.

Transfection of non-tumorigenic HaCaT cells with VEGF induced a persistent stromal ctivation and angiogenesis and as a consequence progression to a low grade malignant tumor phenotype with very strong angiogenesis and invasive growth into the surrounding host stroma. Similarly, induction of a persistent recruitment of inflammatory cells to the tumor microenvironment that is followed by persistent angiogenesis and can be induced by the expression of IL-6 or G-CSF and GM-CSF in previously benign tumor cells promotes tumor progression to a malignant phenotype. Interestingly this growth factor induced tumor progression is associated with the expression of a specific set of MMPs in tumor and stromal cells. Reciprocally blocking stromal activation and angiogenesis with a neutralizing antibody to VEGFR-2 inhibits malignant tumor growth via a phenotypic reversion to a pre malignant tumor phenotype. This is associated with an abrogation of the persistent angiogenesis, a down regulation of stromal MMPs, a maturation of blood vessels and a normalization of the tumor stroma. Thus our system demonstrates the importance of the stromal activation and angiogenesis in determining the benign or malignant tumor phenotype and provides promising new targets for the control of tumor growth and progression through the normalization of the tumor microenvironment.

Biochemical characterization and clinical relevance of human tissue kallikreins

Viktor Magdolen

Clinical Research Unit, Department of Obstetrics and Gynecology, TU München, D-81675 München, Germany

The human tissue kallikrein gene family (*KLK*1 to *KLK*15) encodes a group of 15 serine proteases (hK1 to hK15), several of which have been implicated to play a role in cancer-related processes. Many tissue kallikrein genes/proteins are either under- or overexpressed in certain carcinomas, especially in breast, prostate, testicula, and ovarian cancer. Thus, the tissue kallikreins represent interesting tumor biomarkers and are considered novel therapeutic targets in cancer.

In collaboration with several groups in Germany, The Netherlands, Greece, France, and the USA, we have set up a series of projects to learn more about the tumor biological role of tissue kallikreins:

1. Most of the human tissue kallikreins were cloned, expressed in *E. coli*, refolded, activated, and purified. Subsequently, the recombinant proteins were biochemically characterized and also several structures (among them hK4 and hK7) experimentally solved. The specificity profiles and the structural data will assist in identifying their physiological protein substrates as well as in designing selective inhibitors of individual tissue kallikreins.

2. Accumulating evidence suggests that certain tissue kallikreins are part of an enzymatic cascade pathway which is activated in ovarian cancer. We therefore have analyzed the effects of overexpression of hK4, 5, 6, and 7 in ovarian cancer cells *in vitro* and *in vivo*. Our results strongly support the view that tumor-associated overexpression of tissue kallikreins contributes to ovarian cancer progression.

3. In contrast to the generally observed overexpression of tissue kallikreins in ovarian cancer, the expression of many tissue kallikrein genes, *e.g.* *KLK*5, *KLK*6, *KLK*8, and *KLK*10, is downregulated in breast cancer tissue. In our own studies, we established a specific assay for quantification of full length *KLK*7 mRNA excluding amplification of its exon 2 deletion splice variant (the latter does not encode a functional protease), and evaluated full length *KLK*7 mRNA expression (normalized to h-G6PDH) in tumor tissue specimens of 155 breast cancer patients. High *KLK*7 mRNA expression was significantly associated with a better patient outcome in both univariate and multivariate Cox survival analysis. Thus, full length *KLK*7 mRNA expression may represent a new favorable prognostic marker in breast cancer disease.

I4

Triarylethylenes and related derivatives as novel antileukemic agents

E. Levy [1], Z. Rappaport [2], D. Arad [3], O. Shpilberg [4], I. Levy [1], I. Nathan [1]

[1] Institute of Hematology, Soroka University Medical Center and Ben-Gurion University of the Negev, Beer Sheva, Israel; [2] The Hebrew University of Jerusalem, Department of Organic Chemistry, Israel; [3] NLC Pharma; [4] Institute of Hematology, Medical Center Rabin, Campus Belinson, Petach Tikva, Israel

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In our search for novel derivatives whose structure consists of an arylethylene moiety which can serve as anticancer agents, we found two groups of compounds that have an antitumoral effect that is stronger than that of the known derivatives, namely, substituted 9- arylideneanthrones and members of the triarylvinylic systems. Newly synthesized compounds based on a structure-function relationship and drugs commonly used in clinics were studied for their antitumoral efficacy and mode of action. Leukemia cell lines and primary cells obtained from chronic lymphoblastic leukemia (CLL) and acute myeloid leukemia (AML) patients were used in the study. Various substituted derivatives displayed marked antitumoral activity and exhibited a high degree of selectivity for leukemic cells and low toxicity against normal blood cells and hematopoietic progenitors. Both groups of compounds studied were found to act through different signaling pathways which have not yet been fully elucidated**.** However, both pathways involve the production of reactive oxygen species (ROS) that leads to cell death cell with apoptotic characteristics. Specific translocation of protein kinase C-ε (PKC-ε) from the cytosol to the plasma membrane was observed, indicating that PKC-ε is activated and takes place prior to ROS formation. PKC activation is an important step in cell death induced by members of the triarylvinylic systems. In order to identify the enzyme/system responsible for the formation of ROS, we used various inhibitors of candidate enzymes. It was found that MK886, an inhibitor of lipoxygenase, was able to inhibit apoptosis induction by members of the triarylvinylic systems but not that of the substituted 9- arylideneanthrones group. The mitochondrial uncoupler CiCCP inhibited the activity of substituted 9- arylideneanthrones, suggesting that the mitochondria is involved in this process. Studies with isolated rat liver mitochondria showed protection against Ca^{2+} induced swelling, which indicated that these compounds have an effect on mitochondrial permeability transition. The results from ex-vivo experiments with cells obtained from CLL and AML patients showed that they were sensitive to the action of these two groups of compounds. On the basis of these results, we conducted a phase I-II clinical trial to evaluate the potential therapeutic effect of triarylvinylic systems in advanced and refractory CLL and AML patients. The findings from these studies suggest that the derivatives of these compounds are potential agents for the treatment of cancer and for the identification of cellular targets responsible for cancer eradication.

Assessment of cathepsin B activity in tumour invasion and angiogenesis

Aleš Premzl [1] *and Janko Kos* [2]

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Lysosomal cysteine protease cathepsin B is implicated in degradation and remodelling of extracellular matrix (ECM), creating a local environment that facilitates progression of solid tumours and formation of new blood vessels. Besides pericellular proteolyitic activity of this enzyme the evidence about the contribution of intracellular cathepsin B to these processes has emerged in recent years.

The aim of our study was to investigate the role of both extracellular and intracellular cathepsin B activity in the invasion of *ras*-transformed human breast epithelial MCF-10A neoT cells and in the formation of capillary-like tubes by HUVEC endothelial cells in angiogenesis *in vitro*. We used general and specific cysteine protease inhibitors and cathepsin B neutralising monoclonal antibody to assess the role of both fractions of cathepsin B in selected cell models. Additionally, fluorescent and confocal microscopy was used to (co-)localise total proteolytic activity detected by degradation of quenched fluorescent protein substrate DQ-collagen IV and intracellular cathepsin B activity detected by degradation of Z-Arg-Arg-cresyl violet substrate in living tumour and endothelial cells. We have shown that in MCF-10A neoT cells activity of both extracellular and intracellular cathepsin B contributes to their invasive potential, and that inhibitors acting on both fractions of the enzyme are more effective in preventing tumour cell invasion. However, contrary to our expectations, in HUVECs only intracellular cathepsin B activity showed significant effect on the formation of capillary-like tubes by cells grown on Matrigel, despite the fact that the total extracellular proteolysis was more intense when compared to the total intracellular proteolysis, as observed by degradation of DQ-collagen IV.

Our results clearly demonstrate that intracellular and extracellular cathepsin B activity differs in its contribution to the degradation of ECM in tumour cell invasion and angiogenesis *in vitro*. Further, they also indicate that localisation of target protease activity should be taken into consideration when planning therapeutic strategies for treatment of cancer with protease inhibitors.

The Cancer Degradome: new insights into protease function in cancer biology

Dylan R. Edwards

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There is a long history that implicates secreted proteases such as the matrix metalloproteinases and the serine proteases of the plasminogen activation cascade as mediators of the genesis and spread of cancer. However, synthetic MP inhibitors proved disappointing in clinical trials and there is a growing awareness that proteases can in some instances antagonize rather than promote tumourigenesis. It is therefore necessary to unravel the contributions of components of the "degradome" – the repertoire of proteases, substrates and inhibitors employed in malignant tumours. Knowledge of the cancer degradome may lead to new diagnostic markers and therapeutic agents, and also to improved imaging of tumours. The talk will review recent progress from bioinformatic analysis of the degradome, and expression profiling and functional studies concerning the involvement of proteases in breast and prostate cancer. In particular, our data have identified *MMP8* and *ADAMTS15* as novel markers of good prognosis in breast cancer, whereas *MMP11* and *ADAMTS8* are associated with poor outcome. The rationale for the design of novel, selective agents that target proteolysis will also be highlighted.

Differential effects of Cathepsin L deficiency on multistage tumorigenesis of epidermal carcinomas in K14-HPV16 mice as compared to islet cell tumors in the RIP1-Tag2 mouse tumor model

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To assess the functional significance of cathepsin L (CTSL) in epidermal carcinogenesis, we adopted a genetic approach utilizing $ctsl^{-/-}$ mice bred with transgenic Tg(K14-HPV16) mice, which express the human papillomavirus type 16 oncogenes under the control of the keratin 14 promoter and reproducibly show multistage development of invasive squamous cell carcinomas of the epidermis. During the observation period of 52 weeks the overall survival of Tg(K14-HPV16);$ctsl^{-/-}$ mice is significantly shorter than the survival of Tg(K14-HPV16);$ctsl^{+/+}$,and Tg(K14-HPV16);$ctsl^{+/-}$ mice. Development of dysplastic lesions and average tumor onset of Tg(K14-HPV16);$ctsl^{-/-}$ mice occurs 2 months earlier than in Tg(K14-HPV16);$ctsl^{+/+}$ or Tg(K14-HPV16);$ctsl^{+/-}$ mice ($p<0.01$). In addition, Tg(K14-HPV16);$ctsl^{-/-}$ develop a final tumor size of 1 cm^3 significantly earlier than the control groups ($p<0.01$). Grading of $1cm^3$ squamous cell carcinomas revealed an increased proportion of undifferentiated grade 3 and grade 4 cancers in Tg(K14-HPV16);$ctsl^{-/-}$ group as compared to the Tg(K14-HPV16);$ctsl^{+/+}$ animals ($p<0.01$), and Tg(K14-HPV16);$ctsl^{-/-}$ mice develop a higher frequency of lymph node metastasis ($p<0.05$). Immuno-histochemical staining and FACS-quantification CD31-positive endothelial cells did not reveal CTSL genotype-specific differences in the angiogenic switch that is characteristic for tumor progression in Tg(K14-HPV16) mice.

In striking contrast to the K14-HPV16 model are the findings on Cathepsin L deficiency in the RIP1-Tag2 pancreatic islet cell carcinogenesis. In this model, progression to (micro-) invasive carcinomas is impaired in Tg(RIP1-Tag2);$ctsl^{-/-}$ mice, since these mice show a lower number of malignant islet cell cancers while the encapsulated tumor stages exhibit a higher frequency in the CTSL deficent animals as compared to the controls. Further, total tumor volume is reduced in Tg(RIP1-Tag2);$ctsl^{-/-}$ mice as compared to control mice ($p<0.001$). This is probably due to higher rates of apoptosis (TUNEL-staining) and a lower proliferation index (Brdu-uptake) in CTSL deficient RIP1-Tag2 tumors. As in the K14-HPV16 model, tumor-angiogenesis, i.e. the number of angiogenic islets and microvascular density, was not different in CSTL knockout and control RIP1-Tag2 tumor mice.

In summary, the results show that deficiency of CTSL promotes progression and metastasis of epidermal cancers but inhibits formation of pancreatic islet cell carcinomas. These contradicting findings clearly illustrate, that the functions of cathepsin L in cancers depend on the tissue/cell types from which the tumor originates. These considerations are highly important with respect to the general consent that inhibition of cathepsins could be a therapeutic measure.

The role of tricyclic drugs in selective triggering of mitochondrially-mediated apoptosis in neoplastic glia: a therapeutic option in malignant glioma?

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We previously demonstrated that the tricyclic antidepressant, Clomipramine, exerts a concentration-dependant, tumour cell specific, pro-apoptotic effect on human glioma cells *in vitro* and that this effect is not mirrored in non-neoplastic human astrocytes (Rooprai *et al Acta Neurochirurgica*: 145: 145-148 2003). Moreover, the drug acts by triggering mitochondrially-mediated apoptosis by way of complex 3 of the respiratory chain. Here, through reduced reactive oxygen species and neoplastic cell specific, altered membrane potential, cytochrome c is realeased thereby activating a caspase pathway to apoptosis (Daley *et al* 2005). Similarly, the tricyclic analog, desipramine, has also been reported to induce mitochondrially-mediated apoptosis in C6 glioma cells via increased caspase-3 gene expression and intracellular calcium homeostasis changes (Qui *et al Acta Pharmacol Sin* 23: 803-807, 2002). In addition, while we and others have shown that further antidepressants, including those of the selective serotonin reuptake inhibitor (SSRI) group, also mediate cancer cell apoptosis in both glioma and lymphoma, clomipramine appears to be most effective in this context (Meredith *et al* The *FASEB Journal* (2005; published online May 2005). Very recently, Levkovitz *et al J Mol Neurosci* 27: 29-42 (2005) independently reported that clomipramine, in a comparative study between SSRIs and clomipramine in C6 rat glioma and human neuroblastoma cells, caused apoptosis preceded by a rapid increase in p-c-Jun levels, cytochrome c release from mitochondria and caspase-3-like activity. Significantly lower sensitivity to the drug's pro-apoptotic activity was demonstrated in primary mouse brain and neuronal cultures. The authors therefore concluded – as we had previously - that the high sensitivity of cancer cells to the drug suggested that clomipramine may have potential in the treatment of brain tumours. In addition to clomipramine we have investigated the possible pro-apoptotic activity of a range of further tricyclic drugs. Only two such agents (amitriptyline and doxepin) showed a similar, or better, effect when compared with clomipramine. Since both orally administered clomipramine and amitriptyline are metabolised to desmethyl clomipramine (norclomipramine) and nortriptyline respectively it is necessary for testing at a tumour cell level to be carried out with both the parent tricyclic and the metabolic product. In addition, reversal of multidrug resistance in a number of solid cancers following treatment with both clomipramine and amitriptyline has been reported. This additional role for tricyclics may, albeit at differing concentrations, be of some significance in the treatment of primary and secondary brain tumours. Since a substantial number of patients with malignant glioma have already received and are receiving clomipramine, both

anecdotally and within a clinical trial at King's College Hospital, London we have carried out two pilot experiments. One to determine the CYP (P450) gene expression of individuals and the other to determine blood plasma levels of clomipramine and norclomipramine, in order to determine whether differences in individual patient metabolism influences clinical outcome.

Cysteine cathepsins pathways to apoptosis: can one use them in cancer treatment?

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Apoptosis is the major way of eliminating potentially harmfull and superfluous cells. The pathway is severely impaired in cancer and cancer cells generally fail to die. A number of anticancer therapies currently used are therefore aimed to restore the apoptotic program. There is increasing evidence that at least some of them induce cell death through lysosomal membrane permeabilization (LMP). Although cysteine proteases caspases are known to play a major role in the process, these findings suggest that lysosomal proteases are actively involved in apoptosis. The most studied cathepsins in this respect are cathepsin B, which is a cysteine protease, and the aspartic protease cathepsin D. In order to study the molecular mechanisms of lysosome-induced cell death, we have been using an artificial detergent (LeuLeu-OMe) to trigger LMP. We were able to show in several cellular models that selective lysosome disruption with Leu-Leu-OMe resulted in apoptosis, characterized by lysosome disruption, translocation of lysosomal proteases to the cytosol and subsequent activation of caspases indirectly through the cleavage of a proapoptotic Bcl-2 family member Bid and mitochondria disruption. In addition to Bid, some other cathepsin cellular targets were also identified and will be further discussed.

l10

Tumor cell- and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer

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Tumor progression is a multi-step process that is accompanied by invasive proteolytic activity. Numerous experimental and clinical evidence has linked cathepsin B with tumor invasion and metastasis through its remodeling of the extracellular matrix. To investigate the role of the cysteine protease cathepsin B in tumor progression and metastasis, we crossed cathepsin B-deficient mice (*ctsb*$^{-/-}$) with a mouse strain that develops mammary tumors due to the expression of the polyoma virus middle-T oncogene (PyMT). We showed that *PyMT;ctsb*$^{-/-}$ exhibited a significantly delayed onset and reduced growth rate of mammary cancers compared to wildtype PyMT mice. Lung metastasis volumes were significantly reduced in *PyMT;ctsb*$^{+/-}$, an effect that was not further enhanced in *PyMT;ctsb*$^{-/-}$ mice. Furthermore lung colonisation studies of PyMT cells with different CTSB genotypes injected into congenic wildtype mice and *in vitro* matrigel-invasion assays confirmed a specific role for tumor-derived CTSB in invasion and metastasis. Interestingly, cell surface labeling of cysteine cathepsins by the active site probe DCG-04 detected upregulation of cathepsin X on *PyMT;ctsb*$^{-/-}$ cells. Treatment of cells with a neutralizing anti-cathepsin X antibody significantly reduced matrigel-invasion of *PyMT;ctsb*$^{-/-}$ cells but did not affect invasion of *PyMT;ctsb*$^{+/+}$ or *PyMT;ctsb*$^{+/-}$ cells, indicating a compensatory function of cathepsin X in CTSB-deficient tumor cells. Finally, an adoptive transfer model in which *ctsb*$^{+/+}$, *ctsb*$^{+/-}$ and *ctsb*$^{-/-}$ recipient mice were challenged with *PyMT;ctsb*$^{+/+}$ cells was used to address the role of stroma-derived CTSB in lung metastasis formation. Notably, *ctsb*$^{-/-}$ mice showed reduced number and volume of lung colonies and infiltrating macrophages showed a strongly upregulated expression of CTSB within metastatic cell populations. These results indicate that both cancer cell-derived and stroma cell (i.e. macrophages) derived CTSB plays an important role in tumor progression and metastasis.

Plasma Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) predicts survival in patients with metastatic colorectal cancer receiving irinotecan in combination with 5FU and folinic acid

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Background: At present no biomarker is in routine use for the prediction of treatment effects in metastatic colorectal cancer (mCRC) patients receiving systemic chemotherapy. TIMP-1 protects cancer cells against apoptosis. We raised the hypothesis that elevated plasma levels of TIMP-1 in mCRC patients would predict resistance to apoptosis-inducing chemotherapy. Accordingly, these patients would have a shorter survival than mCRC patients with low plasma TIMP-1 levels despite chemotherapy. The objective of the present study was to test this hypothesis.

Patients and Methods: 89 patients with mCRC were included in the study. Plasma TIMP-1 was measured in samples obtained before the first cycle of chemotherapy. TIMP-1 was analysed by an ELISA that measures both uncomplexed and complexed TIMP-1.

Results: Plasma TIMP-1 scored as a continuous variable on the log scale ($\log_e$) was significantly associated to OS (HR=3.8, 95% CI: 2.4-6.0, p<0.0001). A similar result was demonstrated for time to progression (HR=1.6, 95% CI: 1.0-2.4, p=0.04). Multivariable analysis showed that plasma TIMP-1 was significant for OS when including routine clinical baseline covariates (HR=3.5, 95% CI: 2.1-5.8, p<0.0001). The only other significant covariate was performance score (HR=2.3, 95% p=0.01). A multivariable analysis of time to progression showed that only plasma TIMP-1 was retained in the model (HR=1.6). Analysis of best objective response scored as complete or partial response versus stable or progressive disease showed that patients with low plasma TIMP-1 had higher probability of response (OR=3.4, 95% CI: 1.4-8.3, p=0.007). The area under the receiver operating characteristic curve (AUC) was 0.67. Plasma TIMP-1 was the only significant covariate in a multivariable analysis of best objective response (HR=3.6, 95% CI: 1.4-9.5, p=0.01). A similar result was found for best subjective response (multivariable analysis HR=3.2, 95% CI: 1.2-8.7, p=0.02). Gender was also significant in this model (p=0.03). The AUC for best subjective response was 0.64.

Conclusion: We have shown that plasma TIMP-1 levels independently predict TTP and OS in patients with mCRC receiving chemotherapy. Based on the knowledge that TIMP-1 is a strong protector of apoptosis, future studies should be designed to determine a clinically relevant cut-point and to validate whether plasma TIMP-1 measurements can be used to predict response to chemotherapy in patients with mCRC.

L12

The selection of serological markers for the determination of a prognostic profile predicting cancer-specific death in colorectal cancer

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Introduction: The prognostic value of serological markers sampled preoperatively in patients with colorectal cancer (CRC) has been established. This study combines a panel of these markers in order to select a prognostic profile independent of the clinical baseline covariates: stage, localization, gender and age at diagnosis. The markers selected for this study are plasminogen activator inhibitor-1 (PAI-1), soluble urokinase plasminogen activator receptor (suPAR), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), tetranectin, YKL-40, soluble vascular endothelial growth factor (VEGF), molecules of the mannan-binding lectin pathway of innate immunity MBL and its associated protease MASP-2, carcinoembryonic antigen (CEA) and the acute phase response marker C-reactive protein (CRP). In addition, cathepsin B and the cysteine proteinase inhibitors stefin A, stefin B and cystatin C have been analysed in a subset of this dataset.

Methods: Six-hundred and fifty-four CRC patients undergoing elective surgery have been followed for 7.9 years (median, 6.8-9.1 years). No patients received adjuvant chemotherapy or radiation therapy. The primary end-point was cancer-specific death and 308 patients were registered with CRC as primary cause of death. Multiple imputation was used to manage missing values and the time to cancer-specific death was subsequently analysed with the multivariable Cox proportional hazards model. Serological markers were scored as continuous variables on the log scale or dichotomised using previously determined cut-points. The selected model was assessed using cross validation techniques and Schoenfeld and martingale residuals.

Results: The mannan-binding lectin pathway molecules were not associated to any of the other markers. No pair of markers were shown to have a correlation higher than 0.7. Univariable analysis of the serological markers has shown all to be significant predictors of cancer-specific death with the exception of MBL and stefin B. All markers have been entered into the multivariable analysis and the final model was selected including only covariates which are significant in the analysis and independent of the baseline clinical characteristics. The final multivariable analysis showed that TIMP-1 ($p<0.0001$, hazard ratio (HR) = 2.3, 95% CI 1.6-3.3), PAI-1 ($p=0.001$, HR=0.8, 95% CI 0.7-0.9) as well as MASP-2 ($p=0.001$, HR=1.4, 95% CI 1.2-1.8) and CRP (dichotomized at 10 mgl^{-1}, $p=0.005$, HR = 1.4, 95% CI 1.1-1.7) were statistically significant.

Conclusion: The serological markers TIMP-1, PAI-1, MASP-2 and CRP are demonstrated to be predictors of cancer-specific death independent of localization, Dukes stage, gender and age. The selected serological markers may be the basis for a prognostic profile in CRC. Further studies are needed to validate the model, in particular, studies including patients receiving adjuvant therapy.

Cleavage of uPAR; mechanism and prognostic significance

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Urokinase (uPA) cleaves its receptor, uPAR, thereby inactivating the binding potential of this molecule both with regards to uPA and vitronectin. The N-terminal domain I, uPAR(I) is liberated and the cleaved uPAR(II-III) stays on the cell surface. The cleavage takes place in the linker region between domains I and II after R^{83} and R^{89}. The cleavage is greatly accelerated on the cell surface compared to in solution and this acceleration is dependent on the uPA-uPAR binding. uPA cleaves glycolipid anchored uPAR (GPI-uPAR) but not soluble uPAR (suPAR), which lacks the glycolipid anchor. This is due to a difference in the conformation of the linker region between domains I and II and not because of a general difference in proteolytic susceptibility, since GPI-uPAR and suPAR are cleaved with equal efficiency by plasmin. The collective amounts of all uPAR forms measured by ELISA in tumour lysates or blood correlates to prognosis in several forms of cancer. However, the amounts of uPAR(II-III) and uPAR(I) may be directly related to the uPA activity and therefore be even stronger prognostic markers. Using combinations of monoclonal antibodies we have designed 3 time-resolved fluoroimmunoassays for the specific measurements of uPAR(I-III), uPAR(I-III) + uPAR(II-III), and uPAR(I). The amounts of uPAR(II-III) can be calculated. Applying these assays on tumour extracts from 63 patients diagnosed with squamous cell lung carcinoma revealed a stronger prognostic impact of uPAR(I) compared to total uPAR as measured by ELISA.

L14

Cathepsins and their inhibitors in head and neck cancer

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Head and neck cancer comprises approximately 5% of newly diagnosed invasive malignancies. More than 95 % of tumors are of epithelial origins: histologically they belong to the group of squamous cell carcinomas. Alcohol and tobacco abuse are common etiologic factors for oral cavity, oropharyngeal, hypopharyngeal, and laryngeal cancers. Moreover, because the entire aerodigestive epithelium may be exposed to these carcinogens, patients with head and neck cancer are at risk for developing second primary neoplasms of the head and neck, lung, and esophagus.

Successful treatment of head and neck cancer requires accurate risk stratification to determine the type and extent of therapy needed and the expected clinical outcome. Physical examination, diagnostic imaging studies, and pathologic review are aimed to determine the size and extent of the primary tumor (T-stage), the status of regional (cervical) lymph nodes (N-stage), and the likelihood of systemic metastases (M-stage). In addition to TNM staging, other clinical and pathological factors have been shown to influence response to therapy and outcome.

Cathepsins are a group of lysosomal cysteine (cathepsins B, H, L and others) or aspartic proteases (cathepsin D) that are involved predominantly in phagolysosomal protein degradation. Most of the cathepsins are expressed ubiquitously, but a few demonstrate strict tissue specificity. Endogenous inhibitors of cysteine cathepsins belong to cystatins, which are subdivided into three families, i.e. stefins, cystatins, and kininogens, and thyropins, whereas the naturally occurring inhibitor of aspartic protease, cathepsin D, has not been found yet in men.

During the last decades it has been shown that pericellular proteolysis has multiple roles in virtually all aspects of the normal life of a cell, such as protein degradation, antigen presentation, bone resorption and hormone processing. The same process is involved in the degradation of extracellular matrix barriers during invasion and metastasizing.

The predictive and prognostic value of individual cathepsins and their inhibitors was widely investigated in breast, lung, and colorectal carcinoma. Compared to these tumors, the squamous cell carcinoma of the head and neck fall into much less investigated group of cancers. Apart from the studies focused to the activity or level of cathepsins and their inhibitors as determined in matched pairs of tumor tissue and normal mucosa, there are only a limited number of reports in the literature assessing their predictive or prognostic value in this H&N cancer. There are several reasons, the two most important being:

- head and neck cancers appear to be a heterogeneous group of tumors consisting of multiple primary sites inside the upper aerodigestive tract, each with its own natural history and treatment outcome

- the overall incidence of head and neck cancer is much lower compared to breast, lung or colorectal cancer. Consequently, it is difficult to conduct a study with sufficient number of patients included to obtain statistically meaningful results.

The studies on cathepsins and their inhibitors in head and neck cancers may be categorized as follows:

- markers for diagnosis (to identify patients during the earliest and most amenable stages for treatment and cure – screening)
- predictive markers for lymph node metastasis (to predict the presence of cervical lymph node metastases in clinically/radiologically negative necks or false-positive nodes in patients with palpable/radiologically determined neck metastases)
- predictive markers for response to therapy and for recurrent disease (to assessing the efficiency of particular therapy by monitoring the presence of tumor cells in the body)
- markers for prognosis (to predict survival probability).

To date, no factor within the wide spectrum of novel biochemical or molecular factors has yet been identified as reliably predicting the natural course of the disease or its response to therapy. Although the investigations on clinical utility of cathepsins and their endogenous inhibitors in the management of SCCHN are limited, it is already evident that they exhibit potential in the clinical setting, particularly.

Matrix metalloproteinase expression analyses of human primary breast cancer tissue

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Background: Matrix metalloproteinases play a key role in many aspects of tumor invasion and metastasis. Previous studies have determined the expression of individual MMPs, in particular the gelatinases MMP2 and MMP9. The current study aimed to define a profile of various MMPs in primary breast tumor tissue, to study the correlation of their expression with clinical prognostic markers as to better understand the role of MMPs in breast cancer.

Methods: We performed a quantitative real time RT-PCR analysis of a panel of MMPs (MMP2, MMP8, MMP9, MMP10, MMP11 and MMP13) in the primary tumor tissue of 30 breast cancer patients. To define their diagnostic and/or prognostic value, we evaluated these data against the most important classical clinicopathological parameters including tumor size and grade, steroid hormone receptor status, lymph node status and menopausal status,. Survival analyses were performed to study whether any of the individual MMP expressions were correlated with disease-free survival.

Results: In the overall population, MMP2 RNA expression was inversely correlated with the PR expression in the primary tumor. Moreover, postmenopausal patients with a PR positive tumor tended to have lower MMP2 RNA levels. An inverse correlation was found between MMP2 RNA expression and lymph node involvement in the whole patient group as well as in the subgroup of postmenopausal patients. A borderline significant correlation was found between MMP10 RNA expression and estrogen receptor status. None of the MMP RNA expressions were associated with disease-free survival. We found an indication of co-regulation of MMP2 and MMP10, and MMP8 and MMP9, respectively.

Conclusions: In summary, out of the 6 MMPs investigated MMP2 might be the most important in breast cancer. Interestingly, an inverse association was found between MMP2 RNA expression and PR expression or lymph node involvement in postmenopausal patients. Further, the present data suggest that MMP2 and MMP10, MMP8 and MMP9 respectively might be co-regulated.

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t16

Tumor-reactive memory T cells and their activation in cancer patients

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Antigen-specific memory T cells could be an ideal source for effective immunotherapy of cancer since they show higher frequencies and exert stronger immune responses than naïve T cells. Our previous studies revealed that tumor reactive memory T cells are enriched in the bone marrow (BM) of mice and humans. Such cells have cell-surface markers that characterize them as either central or effector memory T cells. They can be restimulated *ex vivo* by autologous dendritic cells loaded with tumor-associated antigens (TAA) to produce interferon-γ and to become cytotoxic. Restimulated human memory T cells but not naïve T cells infiltrated autologous tumor but not normal skin transplants and caused tumor-regression after transfer into tumor-xenotransplanted NOD/SCID mice. The therapeutic efficiency of memory T cells was augmented upon co-transfer of TAA presenting dendritic cells.

We will provide data indicating that pre-existing memory T cells from cancer patients can be reactivated also *in situ* by antitumor vaccination when using the virus-modified live cell vaccine ATV-NDV. ATV-NDV stands for autologous tumor vaccine modified by infection with Newcastle Disease Virus (NDV). Results from clinical trials of antitumor vaccination with ATV-NDV will be presented. They demonstrate augmentation of memory T cell responses and augmentation of long-term overall survival of patients in Phase II studies.

L17

Complement resistance impairs anti-tumor therapy

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Membrane-bound complement regulatory proteins (mCRPs; CD55, CD46 and CD59) protect normal cells from accidental damage by activated complement. During neoplastic transformation cells often gain complement-resistance by overexpression of one or more mCRPs, by secretion of soluble complement inhibitors or expression on their surface of complement degrading ecto-proteases. Complement resistance of tumour cells is a main hindrance for the efficiency of antibody-based cancer immunotherapy. Understanding the complex molecular mechanisms involved in tumour cell resistance to complement is essential for the development of strategies to interfere with these evasion mechanisms and a prereqioste for effective targeting complement-mediated cytoxicity to cancer cells.

To better exploit complement for cancer cell eradication, it is therefore conceivable to reduce complement resistance either by neutralising mCRP function by specific antibodies or by gene silencing applying siRNA-technology.

First results from both research lines indicate, that targeted interference with complement regulatory mechanisms efficiently promotes antibody–mediated cytotoxicity of malignant cells.

l18

The humoral and cellular mechanisms effective for killing tumor cells in vitro

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Aim of the presentation is to discus different humoral and cellular mechanisms of surveillance and defense against tumors.

Material and methods: Rabbit antibodies directed against membrane antigens of K562 cells were produced and used to make K562 – anti K562 antibodies immune complexes. Guinea pig complement finally diluted 1:10 was used to trigger the apoptosis and the cell death of target cells. Apoptosis and cell death were detected by the flow cytometry employing Annexin V test. The same kind of experiment was used on CD20+ Raji cells. Anti CD20 humanized monoclonal antibodies were used to make immune complexes. Human complement finally diluted 1:5 was added to induce apoptosis and cell killing. Effect was detected by Annexin V test also. The same target cells were used to asses effects of nonprimed fresh human PBMC. The Annexin test was used to detect a possible synergistic action of complement and PBMC induced cell death.

Results: Clear proapoptotic and cytotoxic activity of complement was detected in both models. PBMC were cytotoxic also, but in a lesser degree than complement. Synergistic effect of both ways of cytotoxicity was not observed.

Discussion: Tumor cells induced immune response and the production of specific antibodies directed against selected tumor antigens. These antibodies together with complement induce apoptosis and cell cytotoxicity of antibody specific tumor cells. Tumor cells labeled with specific antibodies activate nonprimed PBMC obviously via ADCC mechanisms resulting in apoptosis and cell death. However, the tested mechanisms were functional and very effective in described models, several investigators reported, that tumor cells are able to escape from destruction using distinctive strategies, like changing or shedding of specific antigens, developing inhibitory regulatory molecules able to stop functioning of killer cells or complement activation, making tolerogenic surfaces etc.

l19

Classical tumor vaccine potently stimulates dendritic cells for the activation of naive T lymphocytes and forming of memory cell pool

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Background: The dendritic cells - DC represent a small subpopulation of bone marrow-derived leukocytes that have a primary role in antigen presentation. They appear to be required for the initial activation of naive T-cells. DC exist in two stages of maturation. Immature (non-activated) DC expressing a low level of co-stimulatory molecules and mature DC displaying increased levels of the cell surface co-stimulatory molecules (CD40, CD80, CD86, CD83) as well as HLA-DR molecules. The maturation of DC is primed through different stimuli (cytokines, microbial products) and consequently results in antigen presentation and T cell activation. In reply to antigen recognition, the responding T cells secrete growth promoting cytokines (e.g. IL2, IL4, IFNγ) and express cell surface molecules CD25 (IL-2-receptor), and CD69 (activation marker). After the interaction of CD4+ T helper cell (Th) with an antigen presenting cell, Th differentiate to effector Th1 – producing predominately IFNγ and TNFγ, and/or Th2 - producing IL4, IL5, IL9, IL10, IL13.

Aim: Since it has been clearly presented that DC could be stimulated in different ways, we considered that irradiated tumor cells combined with the nonspecific immunomodulator might be quite useful for their activation. The aim of this study was thus to determine the capability of DC stimulated through the irradiated tumour cells and MVE-2 to induce the activation of T cells.

Materials and Methods: For the activation of DC, irradiated B16F1 melanoma cells and nonspecific immunomodulator MVE-2 were used. The activation of T cells was assessed by the determination of expression of T-cell specific activation markers - CD25, CD69. Using the cytotoxicity assay, the cytotoxic activity of MNC co-incubated with DC was determined.

Results: Activated DC significantly increased the proportion of CD25+ and CD69+ cells among CD3+ T lymphocytes; at the same time a significant increase of cytotoxic capacity was determined in MNC co-incubated with DC that have been previously stimulated with the tumor vaccine. With the results of *in vivo* experiments, the phagocytic cells (including DC) were proved to be essential for establishing an active protection against tumor cells (tumor development), but more importantly, also for the formation of memory cell pool.

Conclusion: The tumor vaccine composed of irradiated tumor cells and a nonspecific immunomodulator provides an efficient stimulus to DC making them competent activators of T lymphocytes.

I20

Microscopic techniques approach in studying protease inhibitor cystatin in immune cells

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Dendritic cells possess a big capacity to elicit immune response. Their maturation occurs as they migrate from peripheral tissues to lymphoid organs, where they present captured antigens to T cells. The central role of endosomal/lysosomal proteolytic enzymes in generating antigenic peptides and controlling MHC II traffic defines these enzymes as an important area of investigation. The involvement of natural protease inhibitors has been supported by the study on the cystatin homologue, secreted from filiar parasite, which was shown to down-modulate MHC II-restricted antigen presentation. Our study has been predominantly focused on human cystatin C expressed in dendritic cells in response to their differentiation and activation

Dendritic cells are represented as a cell model in which a combination of confocal microscopy and quantitative electron microscopy was successfully applied in order to study endogenous and added cystatin C.

We have shown that in immature dendritic cells cystatin C content was highly elevated compared to their precursors. The low content of cystatin C in monocytes resulted from lower expression, but not from its elevated secretion. Increased expression of cystatin C and high content in Golgi apparatus were observed in immature dendritic cells. The transport of cystatin C was shown from Golgi apparatus towards the cell membrane, where cystatin C accumulated in fully mature dendritic cells. Differentiation and maturation dependence of endogenous cystatin C supports its intracellular regulatory potential and further suggests it new role in Golgi apparatus of immature dendritic cells.

L21

CpG oligonucleotides admixed with irradiated tumor cells are highly efficient for the tumor prevention but less effective for the treatment of existing tumors

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The CpG oligonukleotides could be used as immunostimulators that help the maturation of dendritic cells - DC and lymphocytes, and consequently help to trigger an antitumor immune response. The aim of our study was to assess the effectiveness of tumor vaccines composed of irradiated B-16 tumor cells and CpG when used for the prevention of tumor development or for treatment of i.p. B16 tumors. The prevention efficacy was assessed in the experiments that were performed on the intraperitonel (i.p.) B-16 mouse tumor model. The vaccines were administered once or three times i.p. The average survival (AM±SD) of control animals (mock treated) was 21.6±2.5 days. The average survival in the group of animals pretreated with irradiated tumor cells alone was 26.6±3.7 days. The 100-days-overall survival of animals pretreated once with irradiated B16 and 10 µg, 30 µg or 90 µg per animal of CpG was 57%, 30% and 28% respectively. In the very same groups, the average survival of the mice that ultimately died because of tumors was 43.3±33.0, 30.0±15.2 and 42.0±10.7 days, respectively. When the animals were pretreated with the irradiated tumor cells and 10µg, 30µg or 90µg of CpG per animal followed by two repeated injections of CpG only, the 100-days-overall survival was 88%, 100% and 100% respectively.

In the second set of experiments, the effect of the vaccine on already established tumors was followed. The animals were first *i.p.* challenged with viable tumor cells, and on the second day, treated with CpG and irradiated tumor cells. The average survival in the group of animals treated with irradiated tumor cells alone was 20.0±9.8 days. The average survival of animals treated with one injection of 30µg of CpG per animal was 21.0±7.2 days, while the average survival of animals treated with three repeated injections of 30µg of CpG was 24.0±6.7 days. Similarly to achievements in the previous groups, the treatment with one dose of irradiated tumor cells and one injection of 30µg of CpG per animal resulted with no long-term survivors. Average survival in this group was 21.0±6.4 days. Among the animals treated with the irradiated tumor cells and 30µg of CpG per animal followed by two repeated injections of CpG only, 18% of animals survived more then 100 days. The average survival of the mice that ultimately died because of tumors was 28.0±12.2 days.
In conclusion, the tumor vaccine composed of irradiated B16 tumor cells and CpG delays the B16 tumor development and, to a great extent, protects the animals against tumor development. The curative treatment capacity of this kind of vaccine is limited with the size of the tumor mass. Therefore, the tumor vaccine is more useful for the prevention of tumor development than for the treatment of existing tumors.

Electrochemotherapy of cancer – therapeutic perspectives

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Electrochemotherapy consists of chemotherapy followed by local application of electric pulses to the tumour to increase drug delivery into cells. Drug uptake can be increased by electroporation for only those drugs whose transport through the plasma membrane is impeded. Among many drugs that have been tested so far, only bleomycin and cisplatin found their way from preclinical testing to clinical trials. In vitro studies demonstrated several fold increase of their cytotoxicity after electroporation of cells. In vivo, electroporation of tumours after local or systemic administration of either of the drugs, i.e. electrochemotherapy, proved to be an effective antitumour treatment

Physico-chemical basis of this therapy allows prediction that electrochemotherapy has good antitumour effect on all tumour types, which was demonstrated in several clinical studies. The ESOPE clinical trial has demonstrated that an objective response rate of 85% (73.7% complete response rate) was achieved on the electrochemotherapy treated tumour nodules, regardless of tumour histology, and drug used or route of its administration.

The clinical experience gained so far in the ESOPE project provided evidence that electrochemotherapy is successful treatment in various clinical indications:

- Easy and effective treatment of single or multiple tumour nodules of any histology in the cutaneous and subcutaneous tissue.
- Treatment that increases quality of life in patients with progressive disease.
- Treatment of choice for tumours refractory to conventional treatments.
- Neoadjuvant treatment in form of cytoreductive therapy before conventional treatment.
- Organ sparing and function saving treatment.
- Treatment of hemorrhagic or painful nodules, since it reduces bleeding and in some cases pain level.

All these indications provide electrochemotherapy broad spectrum of use, predominantly because electrochemotherapy is effective local therapy and additionally quick and easy to perform.

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Electrochemotherapy in veterinary oncology

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The aim of our study was to introduce electrochemotherapy with cisplatin and bleomycin into veterinary oncology, where there is a need for effective, easily applied and inexpensive treatment of cutaneous and subcutaneous tumours of various histological types in domestic animals. Electrochemotherapy is an antitumour therapy that utilizes locally delivered short intense direct current electric pulses to the tumour nodule and chemotherapy. The treatment procedure consists of intratumourally injection of low doses of cisplatin or bleomycin and immediately thereafter exposure of tumour nodules to electric pulses.

In the study, 54 dogs, 6 cats and 7 horses with more than 100 cutaneous and subcutaneous tumour nodules of different sizes and histologies were included. Twenty-nine dogs had adenoma or adenocarcinoma of perianal region, 14 had cutaneous mast cell tumour, 4 mammary adenocarcinoma, 3 hemangiopericitoma, 2 hemangioma and 2 sebaceous adenocarcinoma. Four treated cats had squamous cell carcinoma and two had mammary adenocarcinoma. All 6 horses had sarcoidosis. Electrochemotherapy was performed once or repeated several times if complete response was not obtained after first treatment or if the tumour nodule regrew.

The response to treatment was followed by measuring the size of nodules at 2 to 6 weeks intervals and scored according to WHO guidelines.

Our studies show that electrochemotherapy with cisplatin or bleomycin had good antitumour effect on all tumours treated regardless of tumour histology, tumour location and animal species.

Compared to the chemotherapy treatment only, the response to treatment with electrochemotherapy was significantly better than that of the cisplatin treated group. Furthermore, there was a significant prolongation of the duration of response in electrochemotherapy treated tumours.

The main advantages of the use of electrochemotherapy in veterinary oncology are its simplicity, short duration of treatment sessions, low chemotherapeutic doses, and insignificant side effects, as well as the fact that that the patient does not have to stay in hospital.

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The effect of electrochemotherapy on microcirculatory blood flow in a subcutaneous tumour model as assessed with laser Doppler flowmetry

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Electrochemotherapy (ECT) with bleomycin (BLM) is a potent local antitumour treatment. The uptake of the drug in tumour cells is greatly enhanced by formation of transient pores in the cell membrane; a phenomenon called electroporation (EP) which is induced by local application of special very short but high-voltage electric pulses. The effectiveness of ECT has been demonstrated in numerous animal studies as well as in various clinical studies. It has been demonstrated that this antitumour effectiveness might be partially also due to the effects on tumour blood flow. In the present study we are investigating the time- and frequency-domain characteristics of microcirculatory blood flow in tumours during the first hour after treatment.

Blood flow was measured in solid subcutaneous Sa-1 fibrosarcoma tumours growing in the right flank of immunocompetent A/J mice. The mice were divided in four groups: BLM treatment (dose 20 μg/mouse, i.v. injection), EP treatment (8 pulses, duration 100 μs, amplitude 1300 V/cm, repetition frequency 1 Hz), ECT treatment (BLM treatment followed after 3 minutes by EP treatment), and control (sham) treatment. Local blood flow in tumours was monitored before, during and for one hour after therapy by means of laser Doppler flowmetry (LDF; OxyFlo2000, bare fibre sensors, Oxford Optronix, U.K.). The mice were anesthetised using isoflurane and their physiological temperature was maintained artificially throughout the experiment.

The injection of BLM alone or physiological saline (control) produced no significant change in microcirculation of tumours. On the other hand, in EP and ECT groups a practically complete shutdown of blood flow has been found within a few seconds after application of EP thus indicating a dramatic vasoconstriction. After a few minutes a partial reperfusion commenced. The increasing was relatively rapid during the first couple of minutes but it slowed down later on. In most tumours this increasing was observed for about 20 minutes after the treatment with the blood flow never reaching the pre-treatment level. In many (but not all) cases blood flow started to decrease slowly again afterwards. The level of blood flow in all EP- and ECT-treated tumours at the end of observation was significantly below the pre-treatment level in spite of relatively large heterogeneity in the pattern of blood flow changes. No systemic differences between EP- and ECT-treated tumours were found within the first hour after the treatment.

The present results are in agreement with previously reported reduced blood flow in tumours within hours following the application of EP pulses. However, no previous

studies were able to provide a detailed insight into the dynamics of blood flow immediately after EP and ECT treatments. The distinct phases in blood flow changes observed in tumours indicate that there are probably several different mechanisms involved in modification of microcirculatory pattern. The short-lived changes immedi-ately after application of EP pulses most probably reflect the direct effect of pulses on smooth muscle cells in tumour vasculature as indicated by disappearance of the vasomotion component from the LDF signals after EP application. In later stages other effects such as killing of the endothelial cells in vascular walls either by pulses alone or by ECT might be involved with a potential impact on the treatment outcome as a whole.

Treatment planning for effective electroporation

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Exposure of cells to electric field changes plasma membrane permeability without affecting cells viability or function. In this way molecules which otherwise can not cross the membrane, or the membrane represents a considerable barier for them, can enter the cell. Developments in last years proved that this approach can be successfully applied *in vivo* for improving local effectiveness of some chemoterapeutic drugs in treating tumors as well as for gene transfection. The former is referred to as electrochemotherapy and has already entered active clinical research period whereas the latter is referred to electrogenetherapy and is still waiting for its first clinical applications.

Recently electrochemotherapy clinical study involving 110 cancer patients, treating some 170 individual tumour nodules was conducted within the ESOPE – Fifth Framework Programme's funded project (FP5, 1998-2002) Quality of Life programme. Nearly three-quarters of treated nodules completely disappeared, while another 11% partially regressed, making the objective response rate to the novel treatment an impressive 85%. In addition, Standard Operating Proceedures were developed within this project which proved electrochemotherapy highly effective against cutaneous or sub-cutaneous (on or under the skin) tumours resistant to conventional cancer therapies. Electrochemotherapy is safe and effective treatment of single or multiple nodules of any histology in the cutaneous or subcutaneous tissue, which increases the quality of life of patients, with progressive disease and is an ideal treatment for tumours resistant to conventional therapies.

As the induced transmembrane voltage has to exceed certain (reversible) threshold in order to allow for molecular transport, local electric field has to exceed a given electric field threshold. This local electric field depends on electrode geometry, relative electrode geometry/tissue anatomy and passive electric tissue properties. The relevant local electric field can be estimated by means of numerical models (e.g. finite element models) which allow for calculation of electric field in tissue(s) of interest while taking into account the anatomy, tissue dielectric properties and electrode geometry and positioning. In principle, by using numerical models a treatment of target tissue can be planned in advance. Since all types of cells were demonstrated to be permeabilizable, all tissues and cells within the body can in principle be treated by properly designed and positioned electrodes.

In electrochemotherapy electropermeabilisation parameters (pulse amplitude, electrode setup) need to be customized in order to expose the whole tumour to electric field intensities above permeabilising threshold to achieve effective electrochemotherapy. A model based optimisation approach towards determi-nation of optimal electropermeabilisation parameters for effective ECT gives possibility for optimisation based on minimizing the difference between the permeabilisation

threshold and electric field intensities computed by finite element model in selected points of tumour.

We examined the feasibility of model based optimisation of electropermeabi-lisation parameters on a model geometry generated from computer tomography images, representing brain tissue with tumour. Optimisation also considered the pulse generator constraints on voltage and current.

During optimisation the two constraints were reached preventing the exposure of the entire volume of the tumour to electric field intensities above permeabilising threshold. However, despite the fact that with the particular needle array holder and pulse generator the entire volume of the tumour was not permeabilised, the maximal extent of permeabilisation for the particular case (electrodes, tissue) was determined with the proposed approach.

Model based optimisation approach can also be used for electro gene transfer, where electric field intensities should be distributed between permeabilising threshold and irreversible threshold – the latter causing tissue necrosis. This can be obtained by adding constraints on maximum electric field intensity in optimisation procedure. The approach proposed can be also useful for irreversible electroporation – suggested for tissue ablation.

Nanoscale carriers facilitate delivery of protein drugs into tumour cells

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Advances in molecular biology, biotechnology and medicinal chemistry have made available a number of new anticancer drugs, however, it is clearly recognized that inadequate delivery is the single most important factor delaying their application in clinical practice.

In our study we developed a nanoscale polymer carrier, in order to increase the bioavailability of protein drug as the carrier protects it from rapid degradation in biological environment and facilitates its intercellular delivery. Cystatin was selected as a model protein drug due to its high potential for inactivating cysteine proteases, involved in processes of tumour invasion and metastasis. As reported, extracellular and intracellular cysteine proteases participate in these processes, therefore the inhibition of both fractions would have high potential to impair malignant process.

A biodegradable copolymer of lactic and glycolic acid (PLGA) was used as a carrier material, which was transformed into nanoparticles (NPs) by using low-energy double emulsion solvent diffusion method. NPs had size of 300-350 nm diameter, and contained 1.6% (w/w) of cystatin, retaining 85% of its starting activity. To follow cellular uptake of nanoparticles, cystatin was labelled with fluorescent dye (Alexa Fluor 488) prior to its encapsulation into NPs. Image analysis showed rapid internalization of NPs into MCF-10A neoT cells as the fluorescence spots were detected immediately after treatment with NPs. On the other hand, labelled free cystatin was internalised very slowly, suggesting that NPs facilitate the delivery of its cargo into the cell.

To examine the capability of nanoparticle-delivered cystatin to inhibit its lysosomal targets a fluorogenic substrate Z-Arg-Arg Cresy Violet was used. The substrate easily penetrates cell membranes and is degraded into red fluorescent product after cleavage with cathepsin B. Living cells developed strong red fluorescence in the perinuclear region after treatment with the substrate. In case when cells were pre-treated with cystatin-loaded NPs, no red fluorescence developed, showing effective intracellular inhibition. Again, free cystatin did not inhibit cathepsin B activity as did not empty NPs.

Our results show that PLGA NPs represent a useful carrier system for rapid delivery of protein inhibitors into cells, enabling effective inhibition of intracellular proteolysis.

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Gene delivery systems in cancer gene therapy

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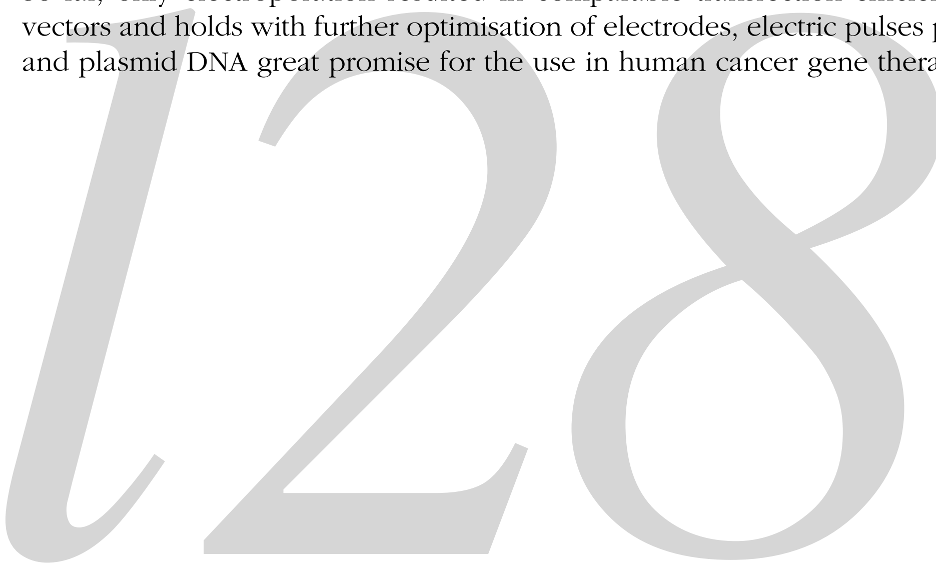
Advance in molecular biology techniques, which lead to high scale sequencing of genomes enable us to identify potential targets for gene therapy. However, to successfully apply gene therapy into human treatment several obstacles have to be overcome, one of them being successful gene delivery method. Special attention of gene therapy has been devoted to cancer. Different strategies can be applied such as suicide genes, immune system stimulating cytokines, antiangiogenesis or tumor suppressor genes. Gene delivery systems in cancer therapy can be divided into two major groups: viral and non-viral methods. Viral vectors include mostly adenoviruses, adenoassociated viruses, retroviruses and herpes viruses vector. All these vectors have been extensively studied in preclinical settings and are being tested also in clinical studies. However, due to the undesired side effects connected to the nature of viral vector, such as host immune rejection, insertional mutagenesis and viral toxicities, alternative to these methods are explored.

Non-viral delivery methods are still plagued by poor transfection efficiencies *in vivo*. However, the advantages regarding safety and patient confidence, as well as the possibility of targeted delivery, make non-viral methods attractive for further development. Non-viral methods can be further divided into chemical, mechanical, biological and physical methods. Chemical methods are mainly represented by liposomes, nanoparticles and cell penetration peptides. Mechanical methods include gene gun and liquid jet injection. Physical methods are sonoporation, electroporation, magnetofection and laser irradiation. Biological methods of gene transfection include the use of bacterial vectors.

So far, only electroporation resulted in comparable transfection efficiency to viral vectors and holds with further optimisation of electrodes, electric pulses parameters, and plasmid DNA great promise for the use in human cancer gene therapy



Gene expression regulation by siRNA electrotransfer

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In a number of evolutionary-distant organisms the introduction of a double-stranded RNA in the cell induces the degradation of homologous messenger RNA. This phenomenon, discovered in 1998 and called RNA interference (RNAi), has been widely used for post-transcriptional gene silencing in C.elegans and Drosophila. Studies on RNAi have shown that this RNA-mediated mRNA cleavage may be part of overlapping processes leading to gene silencing in a variety of organisms. The application of RNAi to mammalian cells through the use of siRNAs indicates that it represents a powerful tool for reverse genetics in mammalians and possibly for gene therapy in humans. This is supported by the development of plasmid shRNA expression vectors able to induce RNAi in vivo as well as by accumulating data from the literature describing the use of RNAi in the treatments against various diseases. Electroporation can provide siRNA delivery in various tissues.

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Electropermeabilization: a non viral method for the delivery of DNA into cells

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The use of electric pulses to deliver therapeutic molecules to tissues and organs has been rapidly developed over the last decade. A new cancer treatment modality, electrochemotherapy, has emerged [1]. Highly cytotoxic hydrophilic drugs such as the bleomycin and cisplatin that are otherwise non-permeant have been successfully used in clinical trials for cancer treatment [2]. Beside drugs, electropermeabilization can be used to deliver a wide range of potentially therapeutic agents including proteins, oligonucleotides, RNA and DNA. This, together with the capacity to deliver very large DNA constructs, greatly expands the research and clinical applications of in vivo DNA electrotransfer [3]. While the term electroporation is commonly used among biologists, the term electropermeabilization should be preferred in order to prevent any molecular description of the phenomenon. The safe and efficient use of this physical method for clinical purposes requires the knowledge of the mechanism underlying that phenomenon of electropermeabilization.
The focus of this communication is to make a report on what is known on the processes supporting the electrically mediated membrane permeabilization and the associated DNA transfer in mammalian cells. With the development of cell imaging, it becomes possible to visualize at the single-cell level the membrane regions where the electrotransfer of molecules takes place. A comparison between the uptake of small size molecules, such as anticancer drugs, and of macromolecules, such as plasmid DNA, will be done with the evidence for "competent membranes domains" [4, 5, 6].

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Electrogenetherapy in cancer

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Gene therapy basic principle is the introduction of nucleic acids into cells in order to obtain a therapeutic effect. Originally it was meant to correct hereditary monogenetic diseases but nowadays cancer diseases are the main indication, (67% of the clinical trials made between 1989 and 2006 [1]). The major limitation of gene therapy is the vector used for the transfer. Viruses, with their natural ability to infect the cells represent the most used approach in gene therapy (≈ 70% of current clinical protocols [1]). Due to adverse events in gene therapy clinical trials using adenovirus or retrovirus, there was a need for nonviral vectors. Electrogenetherapy is one of the most promising nonviral approaches.

Electrogenetherapy, or DNA electrotransfer, is the *in vivo* application of the *in vitro* cell transfection approach initiated by Neumann in 1982. Electrogenetherapy includes: (i) the injection of a DNA or RNA sequence either locally or intravenously, (ii) the delivery of electric pulses to the targeted tissue, allowing the reversible permeabilisation of the targeted cells and the penetration of the nucleic acids into these permeabilised cells, (iii) gene expression or gene silencing.

Although the main target of the therapy is the cancer cell, for electrogenetherapy in cancer there are two possible approaches: a) to transfect the tumor cells themselves, or b) to transfect muscle cells and to use the muscle as a factory to produce circulating molecules that will act on distant tissues (e.g. the tumor). Indeed, muscle is a tissue that can be very efficiently and reproducibly transfected, while transfection efficacy may vary from tumor to tumor.

Whatever the transfected tissue, we have shown that the speed and volume of the injection can affect nucleic acid distribution and transfer efficacy. We have shown that the electric pulses have two roles, a) to permeabilize the target cells and b) to electrophoretically push the nucleic acids towards the target cells [2]. The combinations of high voltage microsecond squared pulses (HV) with low voltage millisecond squared pulses (LV) allow to adjust the delivered pulses to the needs of the electrotransfer. Even though several kind of pulses have been used, we have found that very high efficacy and very reduced toxic effects (cellular stress or histological damages) are obtained using HV + LV combinations of pulses. The optimal parameters (amplitude, duration, number) of the HV and LV pulses depend on the target tissue.

A wide range of type of nucleic acid have been electrotransfected to treat cancer: from plasmid DNA expressing local suicide gene in transfected tumor cells or plasmid coding for antiangiogienic secreted factor in muscular transfected cell, to siRNA silencing known oncogene in the tumor cells. The results obtained in these preclinical studies show that electrogenetherapy is a promising approach for cancer treatment.

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Visible light microscopy, efficient use of techniques and solutions to common complications

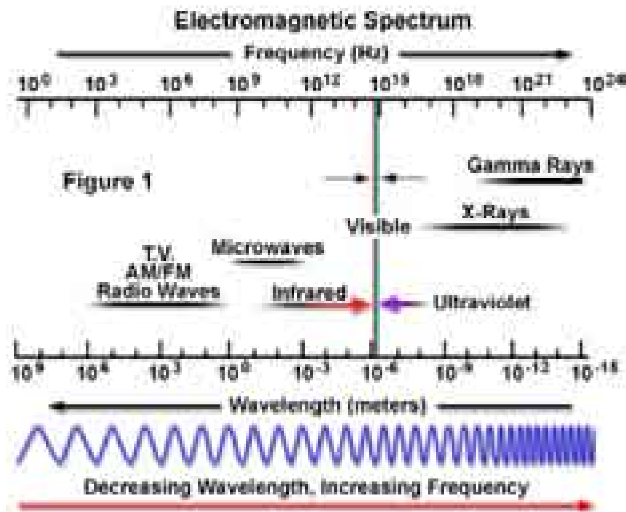
Matjaž Mencej

Olympus Slovenija d.o.o.; In cooperation with dr. Rainer Wegerhoff, Olympus Life and Material Science Europe GmBH

Historical overview

Olympus entered the world of microscopy in 1912. Today Olympus is the leading manufacturer of microscopic equipment. The range of products used in the area of life science as well as material science is amongst the largest in the world. In Slovenia there are over 2000 installed Olympus systems, which range from the simplest school microscope to motorized research systems. In Europe we are also amongst the leaders in the area of confocal microscopes.

Optics

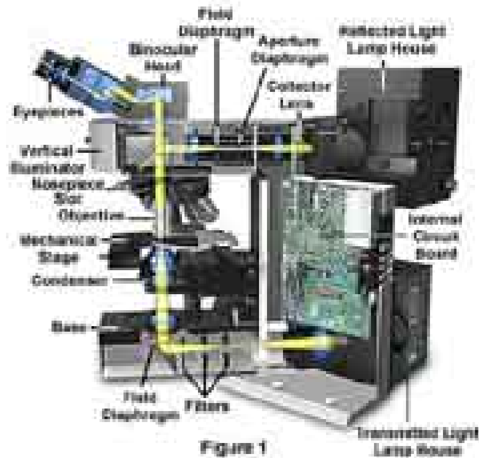


Top of the line optics with the basic systems, as well with the research systems enables the best possible illumination of the field of view and homogenous field of view in the whole range of products. The introduction of UIS (Universal Infinity System) was a large step towards our goal, that is the best possible ergonomics for the user of the microscopy system. The difference between the UIS and previous system will be presented in the presentation.

To be able to understand why we have a picture in the microscope we need a bit of physics. Light has dual nature, it can be modeled as waves and as particles (quanta). For the purposes of this presentation we will choose the wave model of light. Daylight is composed of a palette fo wavelengths. The human eye is able to detect only a small fraction of the whole spectrum of electromagnetic radiation. Picture 1. Picture 1: Spectrum of visible light.

Microscope is made of a number of optical elements which enable us to se an enlarged picture of the specimen with a magnification of up to about 2000. Later we will calculate the theoretical limit of the magnification for a visible light microscope.

Microscope structure



Microscope is made of:

a) The frame
b) The light source
c) illuminator
d) condensor
e) stage
f) objectives
g) revolving nosepiece
h) tube
i) oculars

The structure of a microscope is shown on picture 2.
Picture 2: Microscope structure

Microscopy techniques

The basic microscopy technique is the so called bright field – BF. All other techniques are based on this technique. The known techniques are shown in table 1.

Bright field	BF
Dark field	DF
Phase contrast	PH
by Zernicke	
Olympus Relief Contrast	ORC (HMC)
Differential Interference Contrast	DIC
By Nomarsky	
Polarisation	POL
Fluorescence	Fluor

Table 1: microscopy techniques
A description of each technique will be presented in the presentation.

Complications

With microscopy many complications are possible. These make work difficult or impossible. To be able to avoid such complications we will describe some of them, and also present the solutions.

The description of complications and possible solutions will be described in the presentation.

Progress in tumour vascular targeting

Gillian M. Tozer

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The blood supply of solid tumours influences the outcome of treatment via its influence on the microenvironment of tumour cells and its critical role in drug delivery. In addition, tumour blood vessels are an important target for cancer therapy because of the reliance of tumour cells on their blood supply for proliferation and survival. Over the past thirty years, major advances have been made in understanding the molecular processes associated with tumour angiogenesis, the main process by which tumours vascularize, culminating in 2004 in bevacizumab (Avastin™), a monoclonal antibody against vascular endothelial growth factor (VEGF), being accepted into clinical practice in the USA for the treatment of metastatic colorectal cancer, in combination with conventional chemotherapy.

Current research into new vascular targeting strategies can be divided into *anti-angiogenic* and *anti-vascular* approaches. Anti-angiogenic approaches aim to prevent the neo-vascularization processes in tumours, whereas anti-vascular approaches aim to cause a rapid and selective shut-down of the established tumour vasculature, leading to secondary tumour cell death. Drugs with anti-vascular properties are often known as vascular damaging agents or VDAs. Not withstanding this discrimination, individual agents may possess both anti-angiogenic and anti-vascular (vascular disrupting) actions.

The colchicine-related tubulin-binding/microtubule-depolymerizing agents, predominantly the combretastatins, constitute the largest group of low molecular weight drugs currently in clinical trial as tumour anti-vascular agents (Tozer *et al.*, 2005, Nat Rev Cancer 5: 423-435). Their potency and selectivity for the tumour vasculature is now well established and clinical trials are concentrating on their potential in combination with conventional radio- and chemo-therapy. The basis for their selective action against tumour blood vessels is now urgently sought to provide a basis for further drug development in this area.

Mouse fibrosarcoma cell lines, expressing only single VEGF isoforms ($VEGF_{120}$, $VEGF_{164}$ or $VEGF_{188}$) or all the isoforms (w/t), were developed from embryonal fibroblasts isolated from corresponding transgenic mice, in order to determine the influence of the different isoforms on tumour angiogenesis, vascular morphology and function and the corresponding vascular response to the lead combretastatin, CA-4-P. Results showed that $VEGF_{188}$ plays a significant role in the maturation phase of tumour angiogenesis, leading to the development of mature vascular walls and vessel networks. In addition, the vascular response to combretastatin A-4-P (CA-4-P) was greater in the $VEGF_{120}$ than in the $VEGF_{188}$ tumours. This work has helped identify the vascular characteristics that influence tumour susceptibility to CA-4-P.

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Tumour cell-endothelial interactions: signal pathways and therapeutic targets

Chryso Kanthou

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The interaction between a tumour and its vasculature is critical for both primary tumour growth and metastasis. Tumour blood vessels are both structurally and functionally different from those of normal tissues and these characteristics can be exploited as potential targets for cancer therapy.

Vascular disruption, as an alternative approach to anti-angiogenesis, aims to selectively destroy the existing tumour blood vessel network, thereby causing blood flow shutdown and consequently secondary tumour cell necrosis. A number of tubulin-binding agents, including the combretastatins, are under development as tumour vascular disrupting agents (VDAs). We have identified important pathways for the mechanism of action of the lead tubulin-binding VDA, combretastatin A-4-phosphate (CA-4-P), currently in phase II cancer clinical trials. We found that *rapid* responses of endothelial cells to CA-4-P are mediated by signaling between interphase microtubules, the primary cellular target of CA-4-P, and the actin cytoskeleton, and involve the GTPase RhoA and mitogen-activated protein kinases (MAPKs). Activation of these pathways leads to altered endothelial morphology and cytoskeleton, disruption of VE-cadherin junctions and a rise in monolayer permeability. A rise in tumour vascular permeability also occurs *in vivo* at early times after CA-4-P, and is strongly implicated in the decrease of tumour blood flow observed within minutes of drug administration.

The specific characteristics of the tumour vasculature that determine susceptibility to VDAs are not well understood. Nevertheless, specificity is at least in part ascribed to the relative vascular immaturity of solid tumours. A prominent role in vascular maturation is played by vascular endothelial growth factor (VEGF) and the angiopoietins (Ang 1, 2 and 4), which act on tyrosine kinase receptors specific for endothelial cells. We are currently investigating the role of VEGF and its various splice variants as well as the angiopoietins in modulating the sensitivity of the endothelial cytoskeleton toward damage by VDAs, and how this affects the extent of downstream activation of signaling pathways responsible for altering morphology and function. We have developed fibrosarcoma cell lines, expressing only single VEGF isoforms, as well as colorectal carcinoma lines genetically modified to overexpress Ang 1, Ang 2 or Ang 4 for use in co-culture studies to mimic the tumour-endothelial interactions. Such studies have demonstrated that the sensitivity of the endothelial cytoskeleton is significantly altered by VEGF and the angiopoietins. Elucidation of the molecular mechanisms responsible for regulating endothelial responses to VDAs is necessary for improving the efficacy of these agents and identifying new leads for drug development in this area.

This work was supported by Cancer Research UK

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The Fer kinas: a novel target for prostate cancer intervention

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Fer is a nuclear and cytoplasmic tyrosine kinase whose levels are increased in prostate tumors. Herein we show that Fer is required for malignant cell-cycle progression in-vitro and for tumor progression in-vivo. Decreasing the level of Fer using the RNA interference (RNAi) approach, impeded the proliferation of prostate carcinoma cells and led to their arrest at the G0/G1 phase. At the molecular level, knock-down of Fer resulted in a profound hypo-phosphorylation of the retinoblastoma protein (pRB) on both CDK4 and CDK2 phosphorylation sites. De-phosphorylation of pRB was not seen upon the direct targeting of either CDK4 or CDK2 expression, and was only partially achieved by the simultaneous depletion of these two kinases. Amino acids sequence analysis revealed two protein phosphatase 1 (PP1) binding motifs in the kinase domain of Fer and the association of Fer with PP1α in-vivo was verified using co-immunoprecipitation analysis. Down-regulation of Fer potentiated the de-phosphorylation of pRB by PP1α and over-expression of Fer decreased the phosphatase activity of PP1α. Hence, Fer is a novel regulator of cell-cycle progression in prostate carcinoma cells and this portrays it as a potential target for prostate cancer intervention.

Thus, knock-down of Fer subverts redundant G1-S promoting activities and induces cell-cycle arrest in malignant cells. Our findings portray Fer as a novel intervening target for cancer therapy.

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Gene-expression and DNA-methylation profiling in breast cancer

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High-throughput measures of gene expression and DNA methylation are promising developments in determining the prognosis of patients. We have developed a 76-gene expression profile that can predict prognosis in lymph-node negative (LNN) breast cancer patients who had not received adjuvant systemic therapy. To develop the 76-gene signature we have used a training set of 115 tumors. Validation in an independent testing set of 171 patients showed the 76-gene signature to be strongly associated with a poor metastasis-free survival (MFS) (HR: 5.7). Subsequent multicenter validation including 180 LNN patients confirmed the 76-gene signature as a strong prognostic factor for poor MFS (HR: 7.4), also in postmenopausal patients (HR: 9.8). Separately, using a training set of 72 tumors we established a 31-gene signature that with 100% sensitivity, and 50% specificity, predicted the occurrence of relapse to the bone in a testing set of 35 untreated LNN patients. Our results pointed to the involvement of the FGF signaling pathway in preference of tumor cells that relapse to bone. Further extensive pathway analysis studies including a total of 345 tumors showed highly distinctive pathways that are associated with poor prognosis in subgroups of 221 ER-positive and 124 ER-negative tumors. With respect to DNA methylation analysis, using a microarray-based technology for the analysis of promoter CpG methylation for 117 candidate genes, we found that phosphoserine aminotransferase was the strongest factor associated with failure to tamoxifen therapy in 200 patients with recurrent breast cancer. In LNN patients who were treated with adjuvant tamoxifen, PITX2 appeared the strongest prognostic factor, which was confirmed in several multicenter studies. A major advantage of DNA-methylation analysis is that it can easily be performed by methylation specific PCRs on DNA isolated from paraffin-embedded tissues as well.

**In collaboration with Veridex LLC, a Johnson & Johnson Company, San Diego, USA; the EORTC Receptor and Biomarker Group; and Epigenomics AG, Berlin, Germany, together with the EpiBreast Group.*

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Targeting of plasminogen activation in MMTV-PymT transgenic breast cancer and in skin wound healing

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uPA deficiency in mice is associated with >7-fold reduction of lung metastasis in the MMTV-PymT transgenic breast cancer model as well as reduced dissemination to brachial lymph nodes. In contrast, tumor incidence, latency, growth rate and final primary tumor burden are not significantly affected by uPA deficiency in this model (Almholt et al., 2005). In order to test whether it is possible to reduce metastasis in the MMTV-PymT model by pharmacological blocking of uPA-activity, we have developed a number of monoclonal antibodies (mAbs) directed against mouse uPA. These mAbs were generated in uPA-deficient mice and selected *in vitro* for their ability to block uPA-activity. Studies of skin wound healing in mice double-deficient in uPA and tPA have revealed that wound closure and epidermal migration are significantly impaired in uPA;tPA-deficient mice. Based on this finding we are currently testing the effect of an uPA-neutralising mAb *in vivo* in skin wound healing studies. Anti-uPA mAb was administered systemically to tPA-deficient mice with incisional wounds, which led to a dose-dependent impairment of skin repair. At the highest doses of anti-uPA mAb the effect on skin wound healing was almost identical to that seen in uPA;tPA double-deficient mice. This indicates that the present monoclonal antibody will be suitable for testing its anti-metastatic effect in the MMTV-PymT transgenic breast cancer model.

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Modelling of genomic aberrations responsible for neoplastic transformation and tumor progression in yeast

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In the yeast *Saccharomyces cerevisiae*, aneuploidy is physiologically well tolerated and the karyotype has a remarkable degree of plasticity, making this microorganism an excellent cellular model system to investigate genome dynamics. In particular, we are trying to elucidate the molecular mechanism underlying genomic homeostasis, to ultimately address human gene functions involved in the determinism of cancer. We studied the experimental feasibility of inducing chromosomal translocations between any two desired genetic loci of the genome and constructed a system for the production of site-specific non-reciprocal translocations in wild-type yeast strains. Cells were transformed with a linear DNA cassette having the KAN^R selectable marker flanked by two DNA sequences homologous to loci on two different chromosomes. Using this BIT (Bridge Induced Translocation) system, induction of targeted non-reciprocal translocations in mitosis was achieved (Tosato *et al*., 2005). In these strains we characterized the DNA rearrangement of the chromosome fragments deriving from the translocation. We obtained evidence that centromere-distal chromosome fragments may be processed by a break-induced replication (BIR) mechanism ensuing in partial trisomy, while many integrants may correctly integrate at only one DNA end. Several translocation mutant strains were obtained by our BIT technology, in which the induced chromosomal aberrations correlated with an abnormal cell and nuclear division. Some of the mutant strains revealed strange cell morphology by light and fluorescent microscopy. Especially odd, multi-branched and multi-nucleated cells were observed in old cultures (more than 24 hrs. of growth). Differences in the karyotype of these mutants was observed both, by quantitative PCR and CHEF electrophoresis. The expression of genes located close to the breakpoint regions on chromosomes VIII and XV was analyzed by quantitative RT-PCR. As could be concluded from these data, almost all genes near translocation breakpoints were 1.5 - 4.5 times more expressed then in parental strain. Experiments are in progress for chromatin immunoprecipitation (ChIP) analysis of RNA-polymerase II presence on the promoters of genes located close to translocation breakpoints and for whole-cells transcriptome profiling of wild type and mutant strains using systemic analysis of gene expression (SAGE).

The straightforward technology employed could be instrumental in elucidating the molecular mechanisms underlying Gross Chromosomal Rearrangements generated by genome DNA integration and characteristic of many types of cancer.

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Functional interdependence of natural protease inhibitor overexpression in the host: liver metastasis-promoting effects of TIMP-1 and Cystatin C counteracted by PAI-2

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Elevated expression of natural proteinase inhibitors is often correlated with poor prognosis for cancer patients, although they inhibit the activity of metastasis-associated proteinases. Also overexpression of the broad-spectrum MMP inhibitor tissue inhibitor of metalloproteinases-1 (TIMP-1), of the plasminogen activator inhibitor-2 (PAI-2), and of cystatin C, an inhibitor of papain-like cysteine proteases, is associated with poor outcome of cancer patients, although anti-metastatic activity has been demonstrated for all of these inhibitors in animal models. The aim of this study was to discover functional interdependencies between the three proteinase inhibitors TIMP-1, cystatin C, and PAI-2 when simultaneously overexpressed in the host tissue during metastasis. Overexpression of the different protease-inhibitors in CD1*nu/nu* mice was achieved by adenoviral transfer of the respective cDNA. Three days after gene transfer the mice were challenged by intravenous inoculation of 1 x 10^6 *lacZ*-tagged human fibrosarcoma cells (HT1080*lacZ*-K15), which form large metastatic foci in the lung and single cell metastasis in the liver 21 days after inoculation. Simultaneous overexpression of TIMP-1 and cystatin C led to an additive anti-metastatic effect in the lung but led to formation of large, multi-cellular, metastatic foci in the liver. This liver metastasis-promoting effect of TIMP-1/cystatin C overexpression was associated with an increase of tissue-type plasminogen activator (tPA) protein levels. To test whether tPA suppression can counteract this effect, we simultaneously overexpressed the tPA inhibitor PAI-2 together with cystatin C and TIMP-1 in the liver. PAI-2 overexpression prevented the cystatin C/TIMP-1-induced liver macrometastasis, indicating that plasminogen activators are involved in the pro-metastatic effect. This study shows the possibility of natural proteinase inhibitors-induced effects on organ-specific susceptibility to tumor cells. Combinatorial inhibition of three metastasis-associated proteolytic systems can counteract these effects. This finding may have implications on the design of combinatorial anti-proteolytic therapies.

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TrkAIII: A tumor promoting switch of potential importance in Neuroblastoma and Glioblastoma mutliforme

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The discovery of a novel hypoxia-regulated alternative TrkAIII splice variant has challenged the current concept of an exclusively tumor-suppressor role for TrkA in the aggressive paediatric tumor, Neuroblastoma (NB). TrkAIII, initial data for which suggests predominant expression in advanced stage NB, is devoid of exons 6 and 7, omitting extracellular IgGC1 domain and several N-glycosylation sites of functional importance. In NB cells TrkAIII exhibits spontaneous activity, signals through PI3K/Akt/NFκB but not Ras/MAPK, antagonises NGF/TrkAI anti-oncogenic signalling through Ras/MAPK and promotes NB tumorigenic and metastatic capacity *in vivo.* The mechanisms through which TrkAIII exerts its tumor and metastasis promoting activity will be discussed, as will the extension of our observations to Glioblastoma multiforme.

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Antiprotease therapy in cancer: hot or not ?

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There is clear evidence from experimental and clinical studies that proteases are involved in the spread of cancer. Not only do they play important roles in invasion and metastasis but also in other stages in cancer progression, namely upregulation of cell proliferation, downregulation of apoptosis, escape from immune responses, angiogenesis and the acquisition of multi-drug resistant phenotypes by the cancer cells.

Despite their known participation in tumour progression, clinical trials using protease inhibitors have been, to date, mostly disappointing. Several factors may be accountable for this failure, among them the lack of specificity of many of these compounds and the late stages of cancer of the patient populations undergoing these treatments. In addition, the genetic instability of cancer cells allows them to adapt quickly to new situations. Furthermore, regulation of the proteolytic systems is highly complex, both in terms of their activation in cascade-like processes and the effects the interactions between the tumour cells themselves and the surrounding stroma exert on the expression and, ultimately, the activity of these enzymes. Another clear obstacle to the development of antiprotease-based anti-cancer treatments is the lack of an in-depth knowledge of the specific roles played by the individual proteases and the interplay between them, in tissue- and stage-specific settings. Further work will be required in order to answer these questions. This, together with improvements on the specificity and other properties of the protease inhibitors, as well as the availability of other methods to alter protease activity, such as antibodies and genetic manipulation, are expected to aid the development of future anti-cancer therapies where antiprotease approaches may be used in conjunction with cytotoxic drugs. Promising data, already being obtained with antithrombotics, the urokinase-type plasminogen activator system, the membrane-bound membrane-type 1-MMP, cathepsin L, and the proteasome, will be discussed.

A three-dimensional *in vitro* model to study invasion

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Invasion and cell migration of tumour cells is one of the major problems in cancer treatment. In particular malignant primary brain tumours are characterized by extensive infiltration of the surrounding normal brain tissue and invasion as far as the other hemisphere. This extensive tumour spread inevitably leads to tumour recurrence even after radical surgical resection. It is, therefore, important to achieve a better understanding of the mechanisms involved in cell migration and invasion. Most animal models for brain cancer, in particular, fail to produce invasive tumours; therefore, good *in vitro* models are needed.

The three-dimensional invasion model is based on the implantation of either multicellular spheroids or tumour explants into a collagen type I matrix. Cell invasion into the surrounding collagen is monitored over several days/weeks.
This model offers the possibility

- To qualitatively **and** quantitatively study invasive behaviour of normal and tumour cells.
- To investigate the influence of extracellular matrix molecules on cell invasion,
- To test the effect of inhibitors/drugs/drug combinations on cell invasion and survival.

The gels can be fixed and embedded into paraffin for further histochemical or immunohistochemical staining.

We will present a variety of data using this three-dimensional invasion model:

- Invasion activity of fresh tumour explants in relation to their grade of malignancy
- Invasion activity of primary and established cell lines in the presence and absence of inhibitors/drug/drug combinations.

This invasion model has been tested with brain tumour explants and cell lines originating from various tumours, e.g. lung, pancreas, brain. It is a fast and easy method, which produces consistent data on cell invasion activity.

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Functional genomics and systems biology for studying oncogenesis

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In contrast to traditional molecular studies of cancer that have focused on relatively small number of genes or other biomarkers where genes have generally been analyzed one or few at a time, functional genomics and systems biology are considering a cell as a system of simultaneous interactions of thousands of molecular events in a given physiological condition. The development of DNA microarray technology has made it possible to carry out large scale parallel analyses of gene expression, allowing the simultaneous comparison of the levels of expression of several thousand genes in different cell types and/or physiological conditions. The enormous progress in proteomics, enabled by recent advances in mass spectrometry combined with sequence data correlation, has enhanced speed and accuracy in identification of proteins in complex mixtures. Both approaches represent key strategies for getting complex integrative information referred as cell 'transcriptome' and 'proteome' which by bioinformatical clustering can yield systems immages relevant for diagnostics and prediction of the disease.

By clustering a set of 160 genes/proteins that were differentially expressed in gastric cancer as compared to normal sourounding gastric mucosa we were able to distinguish clearly pathologically trasformed cells from those not yet altered. Furthermore, by principal component analysis, cells showing the same histopathological features in two patients could be resolved in two different molecular subtypes. However, high costs of differential gene expression and proteome analysis are still preventing such studies to be carried out in a larger number of samples in order to get more generalized conclusions relevant for molecular diagnostics. It is true that attention could be paid to several genes/proteins showing prominent differences in expression, but the question remains whether gene-by-gene alteration really represent the true sequence of events in oncogenesis as proposed by the conventional oncogene activation and anti-oncogene inactivation theory. Actual poor correlation of the data obtained from transcriptome and proteome analysis as well as between different experimental platforms will be overcome in future by increased number of laboratories having access to the new technologies and thus increasing the number of cases investigated. In serach of key 'master genes/proteins' more attention should also be paid to overlapping a number of different physiological 'systems', not only 'malignant' to the 'normal' cell.

Angiogenesis independent growth mediated by glioma stem cells

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A highly infiltrative cancer stem cell phenotype was established by xenotransplantation of human primary glioblastomas in immuno-deficient nude rats. These tumors coopted the host vasculature and presented as an aggressive disease without signs of angiogenesis. The malignant cells expressed neural stem cell markers and showed a migratory behavior similar to normal human neural stem cells. The cells showed self-renewal capacity *in vitro* and gave rise to tumors *in vivo*. Serial animal passages, gradually transformed the stem cell tumors into an angiogenesis-dependent phenotype. This process was characterized by a reduction in stem cells markers. Pro-invasive genes were up-regulated and angiogenesis signaling genes were down-regulated in the stem cell tumors. In contrast, pro-invasive genes were down-regulated in the angiogenesis-dependent tumors, derived from the stem cell tumors. By using gene arrays, proteomics and bioinformatics, we were able to identify two separate signaling pathways characterized by the two phenotypes. Moreover, based on our proteomics data, a set of molecular targets towards the cancer stem cells were identified. In conclusion, we describe a system where primary glioblastomas can be dissected into two separate malignant phenotypes. One that depends on angiogenesis and one that is characterized by angiogenesis independent growth. Our work points at two completely independent mechanisms that drive glioma progression. The present work underlines the need for developing therapies that specifically target the cancer stem cell pools in tumors. Examples of such targets will be shown.

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Tumor proteolysis: a multidimensional approach

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Despite the awareness that proteolysis is essential for cancer progression, and that proteases represent potential drug targets, clinical trials for cancer treatment with inhibitors of matrix metalloproteases have failed. Moreover, a broad and comprehensive strategy to identify potential protease targets has not been employed. We hypothesize that proteases are valid therapeutic and prevention targets in cancer and that imaging of protease activity and its inhibition *in vivo* will provide a means to confirm this hypothesis. In our laboratories we have developed functional optical imaging techniques to monitor tumor progression and tumor-host interactions based on proteolytic activity, both *in vitro* in live cells and *in vivo*.
In vitro, we have used a 3-dimensional assay system to study tumor-stromal interactions in real time, utilizing confocal and multiphoton microscopy to document our observations. Using this system, we have found that both pericellular and intracellular proteolysis occur during tumor invasion. Furthermore, there is a high degree of interaction between tumor and stromal cells. Our results indicate that tumor cells actively recruit stromal cells and that these cells contribute significantly to the proteolytic events occurring in the tumor environment.

In order to identify the potential proteases, their endogenous inhibitors, and genes that interact with proteases in the tumor micronenvironment, we developed a dual species custom oligonucleotide microarray in conjunction with Affymetrix, Inc. The Hu/Mu ProtIn array contains 516 and 456 custom probes sets that survey 426 and 390 unique human and mouse genes of interest, respectively. Our goal is to determine tumor (human) and host (murine) contributions to the degradome in orthotopic xenograft models of cancer and demonstrate the utility, versatility and specificity of the custom probe sets. To validate the utility of the array, we profiled human MDA231 and MDA435 breast carcinoma cells derived from *in vitro* cultures or orthotopically implanted xenografts, as well as normal mouse mammary fat pads. We also profiled MCF-10A breast cell lines, grown in the 3D Matrigel overlay model, as well as xenografts of the MCF-10DCIS.com line, a line that forms DCIS lesions that progress to invasive carcinomas in mice. We have identified genes that were either significantly up or down regulated in DCIS.com as compared with MCF10A or were derived from host cells that have infiltrated into the DCIS.com xenografts. We are currently validating genes of interest by both Q-RT-PCR and immunohistochemistry in human breast samples.

In vivo, we have utilized quenched fluorescent probes that are activated by proteases. We have found that upon injection of these probes into tumor-bearing mice, the

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probes are activated at the tumor site and the resulting fluorescence can be detected thereby revealing the position/size of the tumor *in vivo*. The probes are not specific to any particular tumor type, yet since there is increased expression of proteases in tumors, the resulting fluorescent products do accumulate at the tumor site. Our preliminary observations suggest that utilization of such probes will not only provide a sensitive method for cancer diagnosis, but will also provide a means to monitor therapeutic efficacy.

Stem cell therapy for liver insufficiency

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Advances in stem cell biology and the discovery of pluripotent stem cells have made the prospect of cell therapy and tissue regeneration a possible clinical reality. We have isolated, from mobilised and leukapheresed blood, a morphologically and phenotypically homogeneous subpopulation of CD34+ cells (~1%) that exhibits the necessary properties. We have demonstrated that these cells (OmniCytes) express genes corresponding to stem cells (Rex-1, Oct-4, Nanog), hematopoietic (CD34, CD133, CXCR4), and hepatic cell differentiation (albumin, alfa-1 antitrypsin, vimentin, HGF, HNF3-B, transferrin). Animal studies have shown that OmniCytes do home and engraft into chronically damaged nude mice liver. Furthermore, we have performed a phase I safety, toxicity and feasibility clinical study in patients with liver insufficiency. The study involved the collection of bone marrow cells by leukapheresis and subsequent infusion of stem cells. The treatment proved to be safe for the patients and no obvious toxicity was observed. We have demonstrated the feasibility and safety of G-CSF administration and mobilization, leukapheresis and intrahepatic transfer of stem cells in patients with chronic liver disease. Our study documents the existence of stem cells that can be directly and reproducibly isolated from an accessible in vivo source and have considerable promise for the clinical application of liver cell therapy.

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Experimental model and immunohistochemical analyses of U87 human glioblastoma cells xenografts in the immunosuppressed rat brains

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Background: To study the neuropathology and selected tumour markers of malignant gliomas, animal glioma model, developed by using implantation of human glioblastoma clone U87 into the brain of immunosuppressed Wistar rats.

Methods: U87 cell suspension or precultured U87 tumour spheroids were inoculated into the brain of 4- week-old rats. Resulting first generation tumours were then transferred through serial transplantations to rats to get the second and third generation tumours. Brain tumour sections were examined by routine HE staining and by immunohistochemical analyses for various known tumour markers.

Results: The tumours induced by injection of spheroids and those developed by the implantation of tumour tissue grew faster and were markedly larger as those appearing after injection of U87 cells suspension. The first generation tumours demonstrated features of an anaplastic astrocytic tumours (WHO grade III), whereas second and third generations were more malignant, glioblastoma-like tumours (WHO grade IV).
Immunohistochemical analyses showed that p53, S100 protein, GFAP and synaptophysin expression, initially present in tissue culture, were gradually lost in higher tumour generations, whereas nestin and musashi expression increased, possibly indicating progressive tumour cell dedifferentation. Persistant kallikrein, CD68 and vimentin expression in U87 cells as well as in all generation tumours may be related to preservation of the mesenchymal cell phenotype in this tumourigenesis model. Decreased cathepsins expression indicates lower invasive potential, but increasing Ki-67 expression marks higher proliferation activity in subsequent tumour generations. Strong immune reaction for FVIII in second and third generation tumours correlated with observed increased vascular proliferation in these tumours.

Conclusions: We established a simple, fast growing and well defined rat animal model of glioma, which provides a basis for further experimental studies of genetic and protein expression fingerprints during human glioma tumourigenesis.

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Increased plasma DNA concentration in patients with lung cancer

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Background: Concentration of cell-free DNA in plasma (plasma DNA) was reported to be elevated in patients with various tumors, autoimmune diseases, traumatic conditions (stroke, multiple trauma, sepsis), other benign disorders (diabetes) and prenatal complications. Increased level was proposed as a novel tumor marker reflecting tumor cell turnover (lysis of circulating cancer cells, tumor necrosis and apoptosis) or spontaneous and active release from cancer cells.

Aim of the study: Our aim was to determine reference values of plasma DNA concentration in apparently health subjects and to test the hypothesis that patient with lung cancer have increased values when compared to the reference group. To test the hypothesis we measured plasma DNA concentrations with reagents Nucleospin® and PicoGreen® in 51 apparently health subjects and in 29 patients with lung cancer. In 10 patients blood was taken on a day of tumor resection and in 19 patients 1-3 months after operation.

Results: In healthy subjects acute and chronic diseases were excluded clinically, and on the basis of patient history and values of fundamental laboratory tests within reference range. The median (25th percentiles; 75th percentiles) {minimum; maximum} of plasma DNA concentrations was 2.85 (2.05; 4.30) {0.1; 15.85} µg/L. The reference range was from 0.30 to 9.75 µg/L. In patients concentrations of DNA in plasma on a day of operation were 2.47 times higher (medians 7.05 *vs.* 2.85 µg/L; $p = 0.0270$) and after operation 3.72 times higher (medians 10.60 *vs.* 2.85 µg/L; $p < 0.0001$) when compared to the health subjects. No difference was found between the both subgroups of patients. Furthermore, plasma DNA concentration did not correlate with a stage of cancer and did not differ within individual histological type of tumor, neither within both subgroups separately, nor in all patients gathered as one group. Plasma concentration higher than 4.45 µg/L distinguished patients (considered as one group) from apparently health subjects with the sensitivity of 89.7 % and specificity of 80.4 %. The area under ROC curve was 0.870 with 95 % of confidence limit from 0.776 to 0.934.

Discussion: Patients with lung cancer have increased plasma DNA concentration when compared to apparently healthy subjects. Elevated plasma DNA concentration lacks disease specificity, but may be beneficially used for screening the patients at high risk for lung cancer.

Leveraging a flat world: Novel models for effective translational research, product generation and commercialization in oncology

Doros Platika

The Pittsburgh Life Sciences Greenhouse, Pittsburgh, PA, USA

We face critical challenges regarding how to efficiently take insights and discoveries from the laboratory at the bench and translate them into effective therapies that can be used bedside in a timely and cost effective manner. Various commercialization models have been developed to promote translational research from bench to bedside throughout the world; all with different levels of success.

This presentation will outline an effective commercialization model that has been utilized to help leverage basic research to create a viable and sustainable life sciences industry. It is an approach that can be relevant in other regions throughout the world - in both developed and emerging regions.

The presentation will also discuss unique research opportunities that pose additional challenges to commercialization. One such area that will be addressed is how to deal with both the extraordinary potential and the additional complexities created by stem cell research and stem cell based products in the fields of oncology and regenerative medicine.

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List of poster presentations

P1. *Abramović Zrinka:* Increasement of tumor oxygenation with topical application of benzyl nicotinate

P2. *Belotti Dorina:* Tumor VEGF modulates host proteolytic activity during ovarian cancer progression

P3. *Bojič Lea:* Involvement of cysteine cathepsins in apoptosis

P4. *Brožič Petra:* New inhibitors of human hydroxysteroid-dehydrogenases AKR1C1 and AKR1C3: potential agents for treatment of hormone dependent forms of cancer

P5. *Cappabianca Lucia:* Thioredoxin regulates Mta1 modulation of MMP-9 transcription in MDA-MB-231 breast cancer cells

P6. *Čemažar Maja:* Cytotoxicity and antitumour effectiveness of different platinum (II) complexes alone or combined with electroporation

P7. *Čemažar Maja:* The effect of Doxorubicin and Vinblastine in EAT tumours in CBA mice

P8. *Gabrijel Mateja:* Quantification of lysosomal fusion in electrofused hybridoma cells

P9. *Genova Petia:* In vitro anticancer and anti-proliferative effects of Rapana thomasiana hemocyanin

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P11. *Gole Boris:* Expression of cathepsins and their inhibitors in U87 spheroids embedded in collagen matrices: characterisation of migrating versus non-migrating cells

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Abstracts of posters

Increasement of tumor oxygenation with topical application of benzyl nicotinate

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Tumor oxygenation is essential parameter that can influence the outcome of radio- and chemotherapy in cancer treatment. The ability to follow the time-course of tumor pO_2 (partial pressure of oxygen) levels over the longer period of therapy and finding new approaches to improve tumor oxygenation could therefore help to optimize cancer therapy. In this study we tested a hypothesis that topical application of vasodilators, which increase blood flow and consequently partial pressure of oxygen (pO_2) in skin and immediately underlying tissues, could be efficient way to increase oxygenation of superficial tumor in skin with the ultimate goal to use this approach in combination with radiotherapy. We have investigated the effect of vasodilator benzyl nicotinate (BN), applied to the surface of the tumor in two drug delivery systems: hydrogel and microemulsion on the oxygenation of subcutaneously grown RIF-1 tumors by EPR oximetry *in vivo*.

RIF-1 tumors were grown subcutaneously in mice. When tumors reached appropriate volume (100 mm^3) an oxygen sensitive paramagnetic probe lithium phthalocyanine was implanted superficially into peripheral region of the tumors. For the next 5 days a preparation of 2.5% BN in hydrogel or microemlsion was applied on the skin surface over the tumor and pO_2 was measured for 60 minutes.

It was found that BN applied topically on the tumor surface in microemulsion and hydrogel increased tumor pO_2 above the baseline values. As a control microemulsion or hydrogel without BN was applied on the tumor skin but no change in pO_2 was observed. The efficacies of the two delivery systems on the action of BN were compared in order to find optimal formulation, which would enable the best penetration of vasodilator drug into the skin and consequently maximal increase in tumor oxygenation. Two different parameters were used to evaluate the effect of BN: t_{max} – the time required for the maximal pO_2 increase, and ΔpO_{2max} – the maximal relative increase of pO_2 with respect to the baseline oxygen concentration.

Our results show an increase in tumor oxygenation after topical application of BN in both delivery systems. Microemulsions were found to be more convenient delivery system than hydrogel. The observed increase in tumor oxygenation could potentially lead to an increase in the effectiveness of radiotherapy of hypoxic tumors. This will be tested in our future work where the efficacy of radiation therapy when irradiation is done at times of maximal increase in tumor oxygenation after topical application of BN will be determined.

Tumor VEGF modulates host proteolytic activity during ovarian cancer progression

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A mutual regulation between VEGF and MMPs has been recently reported. VEGF stimulates MMP production, and, in turn, MMPs regulate the bioavailability of VEGF. We have previously reported that MMPs are involved in the release of VEGF and in ascites formation in ovarian carcinoma. To further investigate the functional interplay between VEGF and MMPs in ovarian carcinoma progression, we have established a model of human ovarian carcinoma cells overexpressing VEGF and disseminating in the peritoneal cavity of nude mice. Xenograft variants derived from the A2780 human ovarian carcinoma (1A9), stably expressing $VEGF_{121}$ in sense (1A9-VS-1) and antisense orientation (1A9-VAS-3) were generated and the invasive and metastatic potential of tumor cells producing different levels of VEGF was evaluated. 1A9-VS-1 and 1A9-VAS-3 disseminated in the peritoneal cavity of nude mice, but only 1A9-VS-1, the $VEGF_{121}$ overexpressing tumor variant, produced ascites. Significant levels of soluble VEGF were detected in plasma of mice bearing 1A9-VS-1.

An augmentation in murine MMP9 expression was observed in tumors of mice transplanted with 1A9-VS-1 compared to mice bearing 1A9-VAS-3 tumors, indicating that VEGF produced by tumor cells stimulates host expression of the matrix-degrading enzyme. Moreover, we found that tumor VEGF modulated the proteolytic activity of stromal cells not only within the tumor but also in distant organs of tumor-bearing-mice. In particular the organs could be ordered into three typologies in relation to their proteolytic activity: 1) organs that presented a general increase in gelatinases in tumor-bearing mice compared to healthy mice, but independently from VEGF production (spleen, lung, liver, and uterus); 2) organs that showed no alteration in the levels of gelatinases (kidney and pancreas); and 3) organs that presented a VEGF-dependent increase in murine MMP-9 (ovaries). Notably, the levels of proMMP-9 in ovaries correlated with the plasma levels of VEGF.

In vitro VEGF did not induce autocrine changes in tumor cell migration and invasiveness. On the contrary, tumor derived VEGF modulated invasion and MMP2 and MMP9 expression in endothelial cells. The use of specific inhibitors of VEGF receptor demonstrated the specificity of the effects.

Altogether these findings point to a complex cross-talk between VEGF and MMPs in determining ovarian tumor progression.

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Involvement of cysteine cathepsins in apoptosis

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Cysteine cathepsins have long been thought to be involved primarily in the non-selective degradation of proteins inside lysosomal compartments, where acidic pH facilitates their optimal activity (Turk et al., 2001). Nevertheless, there is accumulating evidence for their specific non-redundant *in vivo* functions of papain-like cathepsins. In certain tissues and cell types these peptidases have been localized outside lysosomes and it was suggested that they preserve some residual proteolytic activity at neutral pH. This enables them to act as a signaling molecules in a variety of processes. Recently, we showed that lysosomal cathepsins B, L, S, K and H cleaved and activated the proapoptotic BH3-only Bcl-2 homologue Bid (Cirman et al., 2004) both *in vitro* and in HeLa cellular model following lysosomal disruption by the lysosomotropic agent LeuLeuOMe. Using LeuLeuOMe we found that lysosomal disruption, followed by translocation of lysosomal peptidases into the cytosol and subsequent cleavage of Bid, caused mitochondrial destabilization and fragmentation of cristae. Moreover, in HaCaT cell line, Bcl-XL was found to be degraded with cysteine cathepsins and degaradation could be blocked by a general inhibitor of papain-like peptidases E-64d, but not by caspase inhibitor z-VAD-fmk. Therefore, we suggest that cysteine cathepsins with the assistance of different Bcl-2 family members play a major role during apoptosis after lysosomal membrane permeabilization.

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p3

New inhibitors of human hydroxysteroid-dehydrogenases AKR1C1 and AKR1C3: potential agents for treatment of hormone dependent forms of cancer

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Hydroxysteroid dehydrogenases AKR1C1 and AKR1C3, members of the aldo-keto reductase superfamily, interconvert the active and inactive forms of steroid hormones. In this manner they are involved in hormonal regulation and function in human and represent interesting targets for development of drugs for treatment of hormone dependent forms of cancer like prostate cancer, breast cancer and endometrial cancer. Recently, non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin, flufenamic acid and related compounds have been identified as potent inhibitors of AKR1C3 and moderate inhibitors of AKR1C1. We have examined the inhibitory potencies of frequently used NSAIDs that have not been evaluated yet. In order to obtain more information about structure-activity relationship and to identify compounds with new scaffolds a series of compounds designed on the basis of NSAIDs was synthesized and tested for their inhibitory activity against the recombinant AKR1C1 and AKR1C3. We have shown that it is possible to prepare selective inhibitors of either AKR1C1 or AKR1C3. IC_{50} values that were determined for compounds with promising inhibitory potency were in low micromolar range which indicates that new leads were identified and can be used as a good starting point for future design and synthesis of new inhibitors. New and improved inhibitors would be important for treatment of hormone-dependent cancers, gastrointestinal tumors, leukaemia, depression, for maintaining pregnancy and other conditions involving AKR1C1 and AKR1C3.

p4

Thioredoxin regulates Mta1 modulation of MMP-9 transcription in MDA-MB-231 breast cancer cells

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The highly malignant human breast cancer cell line MDA-MB-231 exhibits constitutive co-expression of invasion and metastasis-associated genes thioredoxin (trx), matrix metalloproteinase (MMP)-9 and metastasis associated gene (Mta)-1, which paradoxically is a repressor of MMP-9 transcriptional. Here, we identify a functional link between trx and Mta1 proteins that affects Mta1 regulation of MMP-9 transcription. We show that Mta1 is a substrate for trx and that trx stimulates and dominant negative C32S/C35S mutated trx inhibits MMP-9 transcription in MDA-MB-231 cells by a mechanism involving the regulation of Mta1/HDAC recruitment to the MMP-9 promoter in vivo. The data helps to explain the nuclear localisation and co-expression of Mta1 with MMP-9 in this highly malignant breast cancer cell line. A potential therapeutic use for Trx inhibitors and regulators of HDAC activity in the treatment of malignant breast cancer will be discussed.

p5

Cytotoxicity and antitumour effectiveness of different platinum (II) complexes alone or combined with electroporation

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Cisplatin (CDDP), oxaliplatin (OXA) and carboplatin (CARBO) are platinum(II) complexes that are already used in the clinical practice. Because of their side effects to normal tissues and innate or acquired resistance new platinum (II) complexes are being synthesized. The aim of our study was to determine *in vitro* cytotoxic activity of two new platinum (II) complexes (3P-SK and PtAMP) in comparison with CDDP, OXA and CARBO on four different human tumour cell lines (bladder carcinoma T24, ovarian carcinoma IGROV1, ovarian resistant IGROV1/RDDP carcinoma and mammary carcinoma MCF7 cells). Cells were plated into 96-well microtiter plates and incubated with platinum complexes for 3 or 5 days at 37°C in humidified atmosphere containing 5% CO_2. Cytotoxicity of the complexes was determined by the colorimetric MTT assay. The inhibitory concentration of the drug that reduced survival of cells to 50% (IC_{50}) was determined for each cell line. In vivo we compared efficiency of CDDP and one of the new platinum complex 3P-SK in MCA tumours induced in CBA mice. Tumours were treated with different eqimolar doses of platinum compounds alone or combined with application of electric pulses to the tumour (electrochemotherapy; 8 x 100 µs pulses 1300 V/cm, 1 Hz). Tumour growth was followed by measuring three mutually orthogonal tumour diameters (e_1, e_2, e_3) with a vernier calliper. Doubling times of tumours (DT) were determined for each individual tumour and tumour cures recorded.

The results of our study showed that CDDP and OXA were the most cytotoxic in all cells used. One of the new platinum (II) complexes, 3P-SK, was more cytotoxic compared to CARBO in all cells used, except in MCF7. The most sensitive cells to all complexes, as determined by IC_{50}, were human ovarian carcinoma IGROV1. IGROV1/RDDP cells were the most resistant. Both OXA and CARBO were cross resistant with CDDP (resistance level ~20 fold) in CDDP resistant IGROV1/RDDP cells, while 3P-SK and PtAMP circumvent the acquired CDDP resistance in the resistant subcloned cell line. The levels of resistance were only 4-6 folds. Results *in vivo* showed that intratumoural injection of 3P-SK alone or combined with electroporation (electrochemotherapy) was effective treatment, inducing significantly prolonged growth delay and tumour cures, but to the lesser extent compared to the CDDP based therapy.

In conclusion, our study showed that 3P-SK, a new platinum (II) complex is less cytotoxic to human tumour cell lines that CDDP and OXA, but posses' higher cytotoxicity compared to CARBO. In experimental tumour model of mammary carcinoma, treatment with 3P-SK alone or in combination with electroporation was less effective compared to CDDP, but nevertheless resulted in tumour cures after single application.

The effect of Doxorubicin and Vinblastine in EAT tumours in CBA mice

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The design of most combined chemotherapeutic schedules used in patients is based on the data from preclinical studies, and phase I and II clinical studies. Little attention is paid to the timing of drugs or possible interaction of drugs in a particular combined schedule. Both these factors could be crucial for the clinical effect of chemotherapy. The primary objective of our study was to explore whether antitumour schedule-dependency exists for the combination of doxorubicin (Doxo) and vinblastine (VLB). The second objective was to find out whether after pretreatment with VLB the accumulation of Doxo within the tumor cells could be increased.

Intraperitoneal (i.p.) Ehrlich ascites tumours (EAT) syngeneic to CBA mice were used in experiments. Three days after tumour transplantation, animals were treated with VLB (0.006 mg/kg) or Doxo (0.9 mg/kg) alone, VLB followed by Doxo, Doxo followed by VLB and both drugs given simultaneously. The time interval between i.p. injections of the drugs was 24 h or 48h. The cell number was obtained by counting viable cells using Trypan Blue exclusion assay. The autofluorescence of Doxo was observed under fluorescence microscopy and measured by flow cytometer. After all various treatment schedules, the DNA distribution pattern was also determined using flow cytometry. In addition, morphology of the tumour samples from the animals treated with various treatment combinations was evaluated. Furthermore, animal survival was determined.

Combinations of Doxo and VLB administered at 48 h, but not at 24 h interval, regardless of the sequence of drugs resulted in significantly reduced cell number in ascites in comparison with all other treatments. After treatment with VLB alone, morphologically cells were moderately enlarged and multinucleated. After treatment with Doxo alone, cells, as well as nuclei and nucleoli were enlarged. In the combined treatment schedules, the dominant morphological changes observed in the samples were dependent on the first drug applied. DNA histograms showed increased G_2M compartment of samples from animals treated with Doxo alone or when Doxo preceded VLB or when the drugs were given simultaneously. In contrast, after VLB or when VLB preceded Doxo, DNA histograms showed less distinctive peaks, high DNA values depassing G_2M peak were also observed. Regardless of the sequence and timing of the treatment, median survival times of animals did not significantly differ between the treatment groups neither at 24 h nor at 48 h interval

The combination of VLB and Doxo is schedule dependent. For the effect was crucial time interval, but not the sequence between the drug administrations. When translating the results of preclinical studies to clinical setting, we need to be cautious, since different tumor models used can yield controversial results. Nevertheless, the data from preclinical studies should be taken into consideration when planning combined treatment schedules in clinical situation.

Quantification of lysosomal fusion in electrofused hybridoma cells

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To enhance the immune response, hybridomas between dendritic and tumor cells, representing a cellular vaccine, are currently considered to be useful for treating cancer. An important step in the production of these hybridomas is quantification of the yield. In the past, the yield of hybridomas was determined by labeling the two cell types with cytoplasmic fluorescent dyes. However, it would be ideal if the hybridoma yield could be obtained using a strategy with a more biologically relevant process, such as antigen presentation. In this study we monitored the average percentage of hybridoma cells by measuring the extent of lysosomal fusion. Lysosomes are dynamic organelles that undergo self- or homotypic fusion, which is involved in antigen presentation. By using confocal microscopy, the level of fused, fluorescently labeled (red and green) lysosomes in a hybridoma cell was determined by measuring the colocalization of red and green pixels (appearing yellow) relative to all red and green pixels in the image. The results show that lysosomal fusion occurs in electrofused cells and that this is an extensive, time- and temperature-dependent process. This approach provides a new method for optimization of the preparation of hybridoma cell vaccines, based on the intracellular process of antigen presentation.

In vitro anticancer and anti-proliferative effects of *Rapana thomasiana* hemocyanin

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Many of the marine hemocyanins (Hc) are well known as biologically active substances. Previously published data have shown that *Rapana thomasiana* hemocyanin (RtH) is a mixture of two isoforms (Idakieva et al., 2001). There are no data concerning the biomedical activities of native RtH and its functional subunits. The aim of this study was to investigate the cytotoxic and anti-proliferative effects of Hc isolated from the hemolymph of the Black sea mollusk *Rapana thomasiana*. Six cell lines were used in our experiments – five human cancer cell lines (SiHa- cervical squamous cell carcinoma, CaOV- ovarian adenocarcinoma, MIA PaCa - pancreatic carcinoma, RD-rhabdomyosarcoma, EJ- urinary bladder carcinoma) and Lep - nontumor human lung cell line. Following RtH treatment cell viability was evaluated at 24h and 48h by two methods: Cytotoxic and cytostatic effect of RtH was compared to the effect of Tamoxifen (commercially available anticancer drug). The results showed that tumor cell lines were more sensitive to the application of RtH compared to the effect on nontumor Lep cells. Significant cell growth inhibition ($P < 0.05$) was observed in three of the five cell lines tested at both time treatment intervals. These were SiHa, CaOV and MIA PaCa. The cervical cell line – SiHa exhibited a mean growth inhibition and cytopatic effect (range 37 to 59%) at 48h, whereas the ovarian cell – CaOV had a range of 4 to 44% at these same concentrations. In conclusion we might suggest the native RtH could have a potential anti-tumor activity.

p9

Spatiotemporal relevance of tumor cell-derived MMP-9 in liver metastasis by short hairpin RNAi-technology

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MMP-9 expression correlates with malignancy and poor prognosis for many cancers. In established tumors or metastases, tumor cells and even more so host cells such as fibroblasts and macrophages are the source of MMP-9 expression. However, in the dynamic consecutive events of the different steps of metastasis, the spatiotemporal relevance of tumor cell-derived MMP-9 is still unclear. We therefore monitored MMP-9 expression during the time-course of experimental liver metastasis in an aggressive syngeneic murine *lacZ*-tagged T-cell lymphoma model, which led to formation of 110-130 macrometastatic colonies (>200 µm) and secondary infiltration of single cell metastases within 7 days after tumor cell inoculation (a.t.c.i.). While the lymphoma cells did not express MMP-9 *in vitro*, we detected two peaks of tumor-cell associated MMP-9 expression, one 3 h a.t.c.i., enabling extravasation and colonization of tumor cells, the other 6 d a.t.c.i. corresponding with secondary invasion of micrometastases. Down-regulation of MMP-9 in tumor cells by retroviral transfer of MMP-9-shRNA led to significant reduction of macrometastases and micrometastatic spread, while both were augmented by overexpression of MMP-9. Although MMP-9-deprived tumor cells were found to compensate their gelatinolytic activity by *in vivo*-induction of other gelatinolytic proteases (MMP-2, cathepsins and uPA) and even activate scatter factor-signaling, secondary invasion was still blocked. We conclude that tumor cell-MMP-9 acts during extravasation and is a crucial pro-metastatic factor for secondary invasion.

p10

Expression of cathepsins and their inhibitors in U87 spheroids embedded in collagen matrices: characterisation of migrating versus non-migrating cells

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In malignant tumours, cell populations are present that differ in their migratory and invasive behaviour. Characterisation of invasive tumour cells has revealed that they also differ from non-invasive tumour cells in other crucial biological properties such as proliferation ability and resistance/susceptibility to apoptosis. We are interested in the role of cathepsins and their inhibitors in the biology of tumour cells. *In vitro* and *in vivo* data point at these proteases as important players in tumour progression. Cathepsins B, L and D have so far been the cathepsins most extensively studied in various stages in tumourigenesis and malignant progression of cancer and *in vivo* studies show an increase of their expression levels in tumours, especially at the invading front. We are currently characterising the expression patterns of Cathepsin B and Cathepsin L and their inhibitors in invasive versus non-invasive U87 cells. To do this we are preparing U87 spheroids using the hanging-drop method and then placing these spheroids into collagen matrices, which is one of the models available to study invasion of tumour cells *in vitro*. After various lengths of time in collagen the migrating cells are separated from the non-migrating fraction and possible differences in protease and inhibitor expression are sought-for, regarding both the type of cells (i.e. migrating vs. non-migrating) and the length of time the cells have been kept in collagen for. Studies at the mRNA level indicate changes in the expression levels of some of these genes when looking at the two U87 phenotypes. Analyses of the activity levels of these proteases complement the mRNA data. Using confocal microscopy we observe intracellular degradation of a quenched fluorescent substrate, DQ-collagen type IV, in agreement with previour reports.

p11

Electrogene therapy with p53 alone and in combination with electrochemotherapy using cisplatin in treatment of murine sarcomas

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Electroporation is an established method for the delivery of molecules into the cells. Biomedical application of electroporation *in vivo* is either delivery of chemotherapeutic drugs - electrochemotherapy or DNA - electrically assisted gene delivery or electrogene therapy. *p53* plays crucial role in diverse cellular pathways in response to DNA damage, one of them being apoptotic cell death. Cisplatin, a commonly used chemotherapeutic agent, causes tumor cell death by producing DNA damage and generating reactive oxygen intermediates. Our hypothesis was that increased DNA damage due to increased cellular concentration of cisplatin by electroporation e.g. electrochemotherapy would result in at least additive antitumor effect in *p53* electrogene therapy pre-treated tumors.

Aim of our study was to evaluate feasibility and therapeutic potential of electrogene therapy with *p53* alone or combined with electrochemotherapy using cisplatin in two murine sarcomas with different *p53* status.

In order to demonstrate feasibility and therapeutic potential murine subcutaneous sarcomas (LPB and SA-1) were treated either with plasmid DNA, cisplatin or electroporation and combinations of these treatments. Status of *p53* in the tumors was determined immunohistochemically, transfection efficacy by luciferase expression in the tumors and antitumor effectiveness of the treatments by tumor growth delay and determination of tumor free animals.

Antitumor effectiveness of three consecutive electrogene treatments with *p53* was dependent on the status of *p53* being more effective in wild type LPB tumor than in mutated SA-1 tumor. Pretreatment of tumors with electrogene therapy with *p53* enhanced chemosensitivity of both tumor models treated by electrochemotherapy with cisplatin. After only one treatment in LPB tumor model, tumor growth delay was prolonged for 10 days in combined treatment group compared to electrogene therapy with *p53* or electrochemotherapy with cisplatin alone, whereas in SA-1 tumors this treatment combination resulted in 32% of cured animals.

In conclusion, results of our study show that electrogene therapy with p53 alone or combined with electrochemotherapy is feasible and effective in treatment of tumors. The combination of electrogene therapy and electrochemotherapy after only one treatment resulted beside prolonged tumor growth delay also in tumor free animals.

p12

In vitro influence of Amitriptyline on glioma cell oxygen comsumption and viability using oxygen electrode assay and Vi-Cell™ automated trypan blue dye exclusion assay

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Introduction: We have previously reported that the tricyclic antidepressant Clomipramine exerts a pro-apoptotic effect on neoplastic glial cells *in vitro*. This effect is mediated via the mitochondria where cytochrome C liberation and activation of caspases precedes apoptosis. There is increasing evidence in our laboratories, however, that additional tricyclics - Amitriptyline and its metabolic product, Nortriptyline - may play a similar role.

Methods: The influence of Amitriptyline & Nortriptyline at a range of concentrations on oxygen consumption in a) non-neoplastic human astrocytes (CC-2565 Cambrex Biosciences), b) a short-term cultured of biopsy-derived human glioblastoma multiforme (UPMC) and c) an established human anaplastic astrocytoma cell line (IPSB-18) was studied using a Hansatech, Clark-type Oxytherm™ & Oxygraph™ multiple series oxygen electrode apparatus. Cell viability was then studied under similar experimental conditions using the trypan blue dye exclusion test in a Vi-Cell™ XR Cell viability analyzer.

Results: The influence of Amitriptyline was more pronounced than that of its metabolic product, Nortriptyline, both in reduction of oxygen consumption and cell viability and both were concentration-dependent. The order of the level of sensitivity between different type of cell culture to both agents was UPMC>IPSB-18>CC-2565.

Discussion: The high level of resistance of CC-2565 supports a specific anti-neoplastic role for the tricyclics in the management of primary malignant brain tumours. However, although Amitriptyline crosses the blood-brain barrier and specifically kills tumour cells, the lower efficacy of Nortriptyline (which results from conversion of Amitryptyline in the liver) may suggest that local administration of Amitriptyline to the brain may confer better therapeutic potential.

p13

Do organophosphorous pesticides play a role in cancer development?

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Organophosphorous (OP) pesticides have been widely used worldwide to help boost agricultural production. Some OP compounds have also been used as warfare agents. OPs irreversibly inhibit acetylcholine esterase and this property is exploited for their use as insecticides and as warfare agents. Although the effects and the consequences of acetylcholine esterase inhibition on the nervous system have been extensively studied, very little is known about possible chronic effects of OP pesticides on non-target tissues in humans. Long term exposure to OP pesticides has been associated with higher risk of Non-Hodgkin's lymphoma and different leukaemias. Our main aim was to investigate whether low concentrations of OP pesticides can cause DNA damage in non-target human cells and affect expression of certain genes known to be involved in cancer development.

Three OP pesticides – parathion, paraoxon and dimefox were studied in human hepatoma HepG2 cell line as *in vitro* human cell model. With the comet assay, we investigated whether the sub-cytotoxic concentrations of OP pesticides (determined by MTT assay) can induce DNA damage. Dimefox did not increase the level of strand brakes at any used concentration; parathion and paraoxon showed slightly increased level of strand breaks only at highest concentration used (100 μg/ml). Using RT-PCR, we found that exposure of HepG2 cells to parathion and paraoxon caused a signifficant increase of the expression of genes that are involved in response to genotoxic stress: P53, MDM2 and GADD45. Dimefox did not significantly change the expression of MDM2 or GADD45, whereas at the highest concentration (100 μg/ml) increased P53 expression. This data indicate that in contrast to dimefox, parathion and paraoxon may have some genotoxic potential

We further investigated the influence of the pesticides on cell proliferation of HepG2 cells. Exposure to dimefox for up to 92 hours increased cell proliferation at all concentrations (0,01 to 100 μg/ml) used. This implies that dimefox could be a potential tumour promoting agent.

In conclusion, this study has shown that OP pesticides may affect tumour development through different mechanisms. Their presence in our environment calls for further investigation of their potential long-term deleterious effects to human health.

Inhibition of cathepsin L expression by siRNA influences susceptibility of U87 cells to apoptosis

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Cathepsin L is upregulated in cancer and has been shown to be associated with invasiveness and tumorigenicity of various tumor cells. On the other hand, recent data on the implication of cathepsin L in apoptotic pathways are contradictory. Therefore, our aim was to study the effect of modulation of cathepsin L in glioblastoma cell line on the invasiveness and the susceptibility to apoptosis.

We have impaired the expression of cathepsin L in glioblastoma cell line U87 by siRNA. Transfection efficiency was determined at mRNA, protein and activity level using RT-PCR, ELISA and fluorogenic substrate-based activity assay, respectively. U87 cells, transfected with non-silencing RNA duplex served as control.

Cathepsin L mRNA level and activity were the lowest at 48 hours after transfection (10 and 39 % compared to control, respectively). As expected, cathepsin L silencing did not significantly affect cathepsin B expression or activity.

Cathepsin L downregulation did not affect the invasiveness through Matrigel, measured by modified Boyden chamber assay. Apoptosis of U87 cells, induced either with staurosporine or with tumor necrosis factor α/actinomicin D (TNFα/ActD), was determined with acridine orange/ethidium bromide staining, caspase 3/7 activity measurment and Bax/Bcl2 gene expression. In cells with impaired cathepsin L expression, induction with staurosporine resulted in a marked increase in early and late apoptotic cells compared to control cells. Similarly, after induction with TNFα/ActD, the number of late apoptotic cells was grater in cells with downregulated cathepsin L than in control cells. Activity of caspases 3 and 7 was found to be higher in cells with downregulated cathepsin L, when apoptosis was induced with staurosporine. On the other hand, there was no significant effect of cathepin L downregulation on caspase activity in TNFα/ActD-induced apoptosis.

These results suggest, that cathepsin L acts in antiapoptotic manner, but the mechanism of its action needs to be further invastigated.

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Molecular analysis of lymphoproliferative disorders using standard PCR

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Background: Demonstration of clonality and detection of specific genetic abnormalities enable distinguishing between neoplastic lesions and reactive processes and, thus, have important value in the diagnosis of lymphoid neoplasms. A clonal population of lymphoid cells can be detected by PCR amplification of the rearranged immunoglobulin heavy chain (IgH) gene in B-cell lymphomas and T-cell receptor γ chain (TcRγ) gene in T-cell lymphomas. Similarly, the chromosome translocation t(14;18)(q32;q21) in follicular lymphoma can be detected by PCR amplification of rearranged bcl-2/IgH region. We have introduced PCR-based assays for clonality analysis, as well as for the detection of t(14;18)(q32;q21) as adjunct tests in lymphoma diagnostics.

Methods: One hundred and sixty-eight biopsies of various lymphoproliferative disorders collected at the Department of Pathology, Institute of Oncology over the period 1997-2005, which could not be diagnosed using classical morphological and immunophenotypic criteria, were analysed using established molecular techniques. All tissue samples were fixed in phosphate buffered formalin and embedded in paraffin (FFPE tissue). DNA was isolated according to the standard method. Rearranged IgH and TcRγ genes were amplified using primers predicted from conserved sequences in variable (V) and joining (J) gene segments. PCR products were analysed by electrophoresis in 10% polyacrylamide gels, stained with ethidium bromide and photographed under UV light.

Results: Using molecular methods we determined the clonality of 146/167 analysed cases (87.4%). Fifty-four cases (32.3%) were monoclonal and 92 cases (55.1%) were polyclonal. We could not determine the clonality of the remaining 21 cases (12.6%): 3 cases were scored as oligoclonal, 12 as »monoclonal in a polyclonal background« and 6 cases as negative. Considering both, classical histopathological and molecular findings we determined the type of lymphoid neoplasm in 94.6% of analysed cases. There were 31.5% of B-cell neoplasms, 21.4% of T-cell neoplasms, 4.8% of Hodgkin's disease cases and 36.9% reactive lymphoid proliferations. We could not define the type of lymphoid proliferation in the remaining 5.4% of cases. Cases suspected for follicular lymphoma (26) have been analysed for the presence of t(14;18)(q32;q21). Eight cases (30.8%) were positive, 15 cases (57.7%) were negative and 3 cases (11.5%) were inconclusive.

Conclusions: Introduced PCR assays are simple, fast (the results can be obtained within 2-3 days) and, what is most important in surgical pathology, can be applied on small amounts of FFPE tissue. In the era of gene expression profiling, the detection of clonality and specific genetic abnormalities by standard PCR methods still provides important diagnostic and clinical information.

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Effect of immunomodulator biological response modifiers on murine tumors and metastases

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Increased expression, activity and secretion of cysteine, aspartic, metallo- and serine proteases by tumor cells was shown to connect with tumor growth, invasion and metastases formation. Biological response modifiers, increasing the host resistance mechanisms, are perspective in tumor treatment and prevention. *The aim*: to study effect of macrophage (Mph) stimulators with different terminal groups and interaction with Mph glucan or/and fucose receptors in antitumor treatment using cysteine proteases and their endogenous inhibitor cystatin C as possible markers of prognosis and efficacy of antitumor therapy. Water-soluble chemically modified β-1,3-glucans (Institute of Chemistry, Slovak Academy of Sciences, Bratislava) and polysaccharide fucoidan (Institute of Epidemiology and Microbiology RAMS, Vladivostok, Russia) have been used. Cathepsins B, L activity and cystatin C concentration (KRKA, Slovenia) were determined as described (Khalikova et al., 2005). β-1,3-glucans were shown to increase the positive therapeutic effect of cyclophosphamide (CPA), but did not reveal antitumor activity themselves (without CPA). Murine tumors (HA-1 hepatoma, lymphosarcoma LS, Lewis lung carcinoma) were characterized by drastically decreased cystatin C concentration in *tumor* cells with significant restoration of cystatin C level in tumor cells and serum after effective antitumor therapy by CPA or/and β-1,3-glucans. Cystatin C concentration in tumor tissue and serum can be used as an index of prognosis of tumor development and efficacy of antitumor therapy. β-1,3-D-glucans are internalized after binding to specific β-glucan (also to scavenger and dectin) receptors on Mph and reduced internalization can be responsible for down regulation of Il-11 and TNF-β secretion (and apoptosis). *Fucoidan* (alone) was shown to suppress significantly tumor growth rate and metastases into lung in mice with Lewis lung carcinoma and did not influence in combination with CPA (10-100 mg/kg). The most effective was scheme with isolated repeated administration of small dose of fucoidan (5 mg/kg, 3 times). Fucoidan (5 and 10 mg/kg) decreased cathepsin L activity (three times) in *tumor tissue*, but did not change cathepsin B and D activity, as compare to untreated tumor. Similar results (except cathepsin D) were obtained in mice treated by combination of CPA + fucoidan. One can conclude that polysaccharide fucoidan in small repeated doses revealed strong antitumor and antimetastatic activity acting, possibly, directly on tumor cells and monitoring of tumor cysteine proteases is useful in control of the efficacy of therapy.

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Humoral response in pleural space to cancer, inflammation and injury

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Pleural fluids (effusions) are formed under a variety of pathological conditions in pleural space. They deflate the lung and often require evacuation by thoracentesis. Although pleural fluids have long been a subject of biochemical investigations, unambiguous diagnostic criteria for major etiological effusion groups have not yet been established. A popular Light's criterion is only used to distinguish between exudative effusions and effusions of haemodynamic origin (transudates), in general by the analyzing total protein and lactate dehydrogenase. In the past two decades, numerous attempts have been made to introduce different markers of inflammation, acute-phase proteins, tumor markers, enzymes, including proteases and their inhibitors, cytokines and growth factors, aiming to differentiate between at least two major etiological groups, i.e. paraneoplastic and parainflammatory effusions. This effort has only been successful in part, presumably due to overlapping specificities of individual analytes.

We have recently focused our attention to gelatinase B (MMP-9), a matrix metalloproteinase associated with cancer progression, in combination with C-reactive protein (CRP), an acute-phase protein reflecting an inflammation and tissue injury. We have found significant differences among the major etiological groups in gelatinase B, with the highest concentrations in parainflammatory exudates, intermediate in paraneoplastic exudates, and the lowest in transudates. Interestingly, gelatinase B was also upregulated within hours following therapeutic talc pleurodesis and explorative thoracoscopy, an intervention causing pleural injury. In analyzed pleurodesis and thoracoscopy samples progelatinase B values significantly correlated with proinflammatory cytokines IL-6 and IL-8, with myeloperoxidase, and with neutrophil count.

Despite statistical significance of progelatinase B in differentiating pleural effusions, the analysis of paraneoplastic group (n=133) revealed a distinct heterogeneity, with a minor portion of fluids reaching values typical for inflammation-associated effusions. A subsequent sorting based on tumor histology showed increased levels particularly in exudates associated with metastatic tumors. Pleural fluid progelatinase B values in general correlated with pleural CRP. A strong correlation between pleural and plasmatic CRP points to a local reflection of a systemic host response reaction.

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Electroporation results in endothelial barrier dysfunction caused by cytoskeleton disassembly and adherens junction disruption

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Electroporation of cells and tissues obtained by application of short high voltage electric pulses provides permeabilization of cell membrane, which enables access of molecules such as antibodies, DNA and drugs to the cytoplasm. In particularly, enhanced delivery of chemotherapeutic drugs (bleomycin and cisplatin), known as electrochemotherapy, has shown good antitumour effectiveness in preclinical and clinical studies. Other application of electroporation, delivery of naked DNA, termed electrogene therapy, is currently on the preclinical level. Endothelial cells seem to be potential target of electroporation and electrochemotherapy, as evident by endothelial cell sensitivity *in vitro* and significant vascular effects in tissues exposed to the electric pulses. The aim of this study was to evaluate effects of electroporation on endothelial cell cytoskeleton and endothelial monolayer permeability.

Human umbilical endothelial cells (HUVEC) were grown on fibronectin coated polycarbonate membrane transwell inserts. In situ electroporation of adherent cells was performed using the InSitu electroporation system (EquiBio, UK) (3 square wave electric pulses, 100 µs, 1 Hz, 20-80 V). Cytoskeletal proteins were visualised by immunostaining with anti-F-actin, anti-β-tubulin, anti-vimentin and anti-VE-cadherin antibodies. Cell viability after electroporation was evaluated by trypan blue exclusion assay. Levels of phosphorylated myosin light chain (MLC), heat shock protein 70 (HSP70) and cytoskeletal proteins were determined using western blotting. HUVEC monolayer permeability was determined by diffusion of FITC-coupled dextran.

Electroporation induced immediate transient disruption of microtubules and microfilaments but not intermediate filaments. Induced changes were dependent on the voltage of electric pulses used and recovered within 1 h after electroporation. Furthermore, immediate loss and recovery of microfilaments correlated with levels of phosphorylated MLC. According to unaffected total level of cytoskeletal proteins and no increase in HSP70 within 16 h after electroporation, we speculate that electroporation interferes with the organisation of actin and tubulin monomers into filamentous structures of cytoskeleton without degradation of proteins. Those cytoskeletal rearrangements were reflected in modified cell morphology. In addition, electroporation disrupted VE-cadherin in endothelial adherens junctions. Taken altogether, immediate disruption VE-cadherin endothelial junctions, dissolution of microfilaments and microtubules resulted in rapid increase in monolayer permeability.

In conclusion, this study shows that electroporation, using electrical parameters that do not affect cell viability, induced reversible effects on actin, tubulin, but not on vimentin intracellular arrangement and disrupted VE-cadherin endothelial junctions. These changes resulted in an increase in permeability of endothelial monolayer that could potentially contribute toward transient impairment of the normal function of the microvasculature, which was observed *in vivo* after application of electric pulses to tumours and normal tissues.

Characterization of cystatin F and its possible role in immune system regulation

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Cystatin F is a cysteine protease inhibitor expressed mainly in cells important for immune response. Glycosylation, six cysteine residues instead of four and not too potent inhibition of cysteine proteases make cystatin F different from other members in the type II cystatin family. A large proportion of cystatin F resides inside the cells in lysosomes, where it might have a role in regulating cysteine protease activity in processes of antigen presentation.

In our previous study [1] we showed that monomeric cystatin F is a general inhibitor of cysteine proteases with endoprotease activity. Inhibitory potential is abrogated in disulfide bonded dimers, which are predominantly found in the cells. Dimerization is mediated by one or both cysteines in positions 7 and 43, which are additional in respect to other type II cystatins. In monomeric cystatin F these two cysteine residues might form the third intramolecular disulfide bond by stretching the N-terminal along the α-helix on the surface of the molecule. Dimerization of cystatin F may be a mechanism for the regulation of inhibitory activity of cystatin F. The presence of the inhibitor in the dimeric form results in increased cysteine protease activity, which could affect the variability of antigen epitopes presented on MHC II molecules on the surface of antigen presenting cells, a mechanism which is crucial also in anti-tumour immune response.

In the baculovirus expression system we prepared wild type cystatin F along with three mutants C7S, C43S and (C7S, C43S) and purified them to homogeneity. Cysteine to serine mutants did not prevent the inhibitor to fold. Inhibitory constants were determined for the interactions of cystatin F with cysteine proteases and the cysteine residues involved in intermolecular disulfide bond formation were identified.

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Angiogenesis correlates with cycloogygenase-2 (COX-2) expression as well as with the tumour grade in gallbladder carcinoma

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Introduction and aim: Carcinoma of the gallbladder is an agressive tumor with an overall 5-year survival rate of less than 5%. Activation of the angiogenic pathways is common and, by inducing new blood vessel formation, may have a decisive role in determining local invasion, matestasis and clinical outcome. The proliferative endothelial antigen CD105 (endoglin) essencial for angiogenesis and vascular development serves as a marker of tumour neovasculature. Enhanced expression of cyclooxygenase-2 (COX-2) was found in precancerous lessions and in gallbladder carcinoma, which is likely to be involved in carcinogenesis as well as in angiogenesis.
The aim of the study was to investigate the relationships between the expression of COX-2 and degree of vascularization, clinicopathological features and survival time of patients with gallbladder carcinoma.

Materials and Methods: A retrospective analysis was performed on 27 gallbladder adenocarcinoma tissue specimens obtained from patients operatively treated (11 male and 16 female patients, mean age 66.5 +/- 11.0 years). Gallbladder adenocarcinomas were classified according to the WHO classification of gallbladder tumours: 8 were well-differentiated, 4 moderately and 15 poorly-differentiated.
The expression of COX-2 and CD105 was assessed by immunohistochemical method. COX-2 expression was evaluated according to the percentage of positive cells and the intensity of staining. Regarding immuno-reactive score gallbladder carcinomas were divided into »COX-2 positive« and »COX-2 negative« groups. For microvessel count (MVC) assessment the areas containing a large number of microvessels were identified. Image analysis system LUCIA G (Nikon, Japan) was used for MVC quantification. The average number of microvessels in 10 light microscopic field at 200x magnification was recorded as the MVC for each tumour. Tumours were separated into hypervascular and hypovascular group.

Results and conclusions: The MVC ranged from 9 to 46 (mean 25.6 +/- 9.2).15 tumours belonged to hypervascular group (mean microvessel count >= 25) and 12 to hypovascular group (MVC<25). The percentage of COX2 positivity in our carcinoma group was 59.2% (16 cases). Nonparametric tests revealed the difference in degree of angiogenesis between COX-2 positive vs. COX-2 negative group: 11 (68.8%) out of 16 »COX-2 positive« tumours were hypervascular and 7 (63.6%) out of 11 »COX-2 negative« tumours were hypovascular (p= 0.09). High MVC was found in poorly-differentiated carcinomas (11 out of 15 cases) and low MVC in well-differentiated carcinomas (6 out of 8). In conclusion, our study presented on a small group of patients with gallbladder carcinoma shows clear tendency that COX-2 overexpression and neovascularization are connected. Augmented tumour neovascularization induced by COX-2 might be responsible for poor prognosis of human gallbladder carcinoma.

Involvement of the lysosomal cysteine peptidase Cathepsin L in progression of epidermal carcinomas

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Papain-like cysteine proteases have been implicated to work as effectors of invasive growth and neovascularisation. In order to assess the functional significance of cathepsin L (CTSL) during neoplastic progression in a transgenic mice model of multistage epidermal carcinogenesis, we adopted a genetic approach utilizing cathepsin L-knockout mice breed with transgenic Tg(K14-HPV16) mice, which express the human papillomavirus type 16 oncogenes under the control of the human keratin 14 promoter, and reproducibly show multistage development of invasive squamous cell carcinomas of the epidermis [1]. Before the intercross of the two mouse lines, CTSL-deficient mice have been backcrossed to the FVB/n genetic background for 8 generations to ensure a congenic tumor model Tg(K14-HPV16);ctsl$^{-/-}$. To analyse cathepsin expression in the tumor model we quantified mRNA expression mRNA expression of cathepsins B, L, H, D, and X at various stages of tumorigenesis by real time PCR. While CTSL was absent in Tg(K14-HPV16);ctsl$^{-/-}$ mice, no other genotype specific differences could be observed for comparison of Tg(K14-HPV16);ctsl$^{+/+}$ and Tg(K14-HPV16);ctsl$^{-/-}$ mice. Immunohistochemistry (IHC) revealed that CTSL lost its strong suprabasal epidermal staining during carcinogenesis was uniformly distributed in cancers, while CTSB maintained its staining pattern. Interestingly, IHC quantification of the proliferation marker Ki67 showed significant higher proliferation rates in epidermis of 16 and 24 weeks old Tg(K14-HPV16);ctsl$^{-/-}$ mice. Further, the progression from hyperplasia to dysplasia occurs significantly faster in tumor mice with CTSL-deficiency. However, no general differences in keratinocyte differentiation, as assessed by cytokeratin 5 and 10 IHC, and in angiogenesis, as quantified by CD31-IHC and FACS analyses, could be detected. Compared to K14-HPV16 mice that are heterozygous or wild-type for CTSL, analysis of CTSL-deficient tumor mice demonstrated significant differences in squamous cell carcinoma onset and development to a tumor volume of 1cm^3. The mean onset of the first palpable tumors in ctsl$^{-/-}$, ctsl$^{+/-}$ and ctsl$^{+/+}$ K14-HPV16 animals were at 28, 36 and 37 weeks of age, and the mean age for occurrence of a 1cm^3 tumor was at 32, 43 and 42 weeks, respectively. In addition, tumors of CTSL knockout mice developed significantly higher grade (i.e. less differentiated) carcinomas than wild-type and heterozygous controls. Further, the number of lymph node metastases significantly increased in Tg(K14-HPV16);ctsl$^{-/-}$ mice.
Taken together, CTSL deficiency promotes multistage carcinogenesis in K14 HPV16 mice, results in highly dedifferentiated cancers and an increased metastatic capacity.

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Inhibition of mouse uPA activity by monoclonal antibodies against mouse uPA

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The potential of monoclonal antibodies (mAbs) as anti-cancer therapeutics has lately been demonstrated for a number of antigens. As the clinical relevant mAbs are directed against human antigens, the initial *in vivo* studies in mice have been limited to xenotransplanted tumors, which are in many respects poor models for human cancers. To enable *in vivo* therapeutic experiments of mAbs in murine cancer models, we have utilized immunization of mice deficient for the target protein in question, thus developing murine antibodies directed against the murine antigen. In the present study, we have generated murine mAbs to murine urokinase plasminogen activator (muPA) by immunization of uPA deficient mice with recombinant muPA.

We have selected 5 mAbs (i.e. mU1-mU5) that react with muPA in ELISA, in Surface Plasmon Resonance (SPR) analysis, and in Western blotting. None of these anti-muPA mAbs recognized reduced and alkylated muPA in Western blotting. Therefore, the reactivity of the mAbs was further analyzed using recombinant amino-terminal fragment of muPA (mATF) in Western blotting. The results revealed that all but one, namely mU1, recognized epitopes in mATF. Since mU1 reacted with intact muPA and not with mATF, its epitope is located in the B-chain, which encompass the catalytic site of muPA. No cross-reactivity with human uPA was observed with any of the five mAbs. The ability of the mAbs to bind to receptor (muPAR)-bound muPA was tested by SPR analysis. One mAb, mU2, was incapable of binding to receptor-bound muPA, indicating overlapping binding sites on the growth factor domain (GFD) in the muPA A-chain. The 3 residual mATF-reacting mAbs bound muPA independently of the presence of muPAR.

The influence of these different mAbs on the uPA activity was studied by an enzyme kinetic assay measuring the uPA-dependent activation of plasminogen. One mAb, mU5, stimulated the muPA activity while the other 4 mAbs inhibited the reaction to various extend. Furthermore, all 4 mAbs induced a dose-dependent reduction of the muPA activity with mU1 having the most pronounced inhibitory effect. Thus, despite the different epitope locations on muPA, i.e. various sites on either the A- or B-chain, the mAbs inhibited the muPA activity. These mAbs will now be tested *in vivo* for their effect on tumor growth and metastasis in murine cancer models.

Electrically-assisted delivery of siRNA targeting p53: an *in vitro* study on human tumor cell lines with different p53 status

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Background: Silencing of oncogenes or other genes contributing to tumor malignancy or progressson by 20–25- nucleotide dsRNA, better known as siRNA, offers a promising approach to treat tumors. High transfection efficiencies are of crucial importance for gene silencing experiments. Electroporation is currently receiving much attention as a way to increase siRNA delivery.

Aim: The aim of our study was to determine the transfection efficiency and induction of siRNA-mediated silencing of p53 in two colorectal (HT-29 and LoVo) and two prostatic (PC-3 and DU145) carcinoma cell lines with different p53 status by varying voltage of electric pulses in *in vitro* electroporation protocol for siRNA transfection.

Materials and methods: Two commercial plasmids (Invivogen) expressing siRNA were used, therapeutic (psiRNA-hp53 directed against the p53 mRNA) and control (psiRNA-GL3 directed against the luciferase mRNA). Both of them were, together with siRNA, expressing GFP, which allowed us to assess transfection efficiency. Plasmids were delivered to cells by electroporation. Mixture of cells (1×10^6) and plasmid (10 µg) as well as cells alone were exposed to eight square wave electric pulses (600 - 1000 V/cm, 5 ms, 1 Hz). Electric pulses were delivered through two parallel stainless steel plate electrodes with 2 mm distance between them and were generated by an electroporator GT-1. To qualitatively evaluate the siRNA effects against p53 in different tumor cell lines with different p53 status the western blot analysis was performed. Cell survival after treatment was determined by clonogenic assay. GFP was detected by invert fluorescence microscopy.

Results: Transfection efficiency of electrically-assisted delivery of plasmids expressing siRNA varied depending on the cell line used and voltage of electric pulses applied. The optimal voltage of applied electric pulses were 700 V/cm for PC-3 and DU145 cell lines and 900 V/cm for HT29 and LoVo cell lines which resulted in 10 - 40 % (PC-3), 1 - 9 % (DU145), 1 - 4 % (HT29) and 1 – 7 % (LoVo) transfection efficiency. The GFP expression was detected already at 6 hours after electroporation. It remained present during 1 week with a peak in fluorescence emission at day 1 or 2. Western blot analysis showed that siRNA directed against p53 mRNA resulted in up to 40% lower p53 protein level in Du145 (*p53mt*) cells. PC-3 (*p53null*) cells were negative control. In accordance with our expectation, no silencing of p53 mRNA by control siRNA was detected. Treatment of cells with electrically-assisted delivery of siRNA targeting p53 did not result in reduced cell survival compared to the treatment with control siRNA.

Conclusions: Our results showed that electroporation is feasible and effective method for delivering of siRNA to tumor cells. Electrically-assisted delivery of siRNA directed against p53 resulted in lower p53 protein content in cell lines used in this study but it did not reduce cell survival compared to the treatment with control siRNA.

Cathepsin X interacts with integrin molecules regulating cell adhesion

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Cathepsin X displays distinctive distribution profile in the cells of the immune system. Its mature form, determined by ELISA is present in large amounts in monocyte/ macrophage cell lines U-937 and KG-1, either in cytosols or cell membrane fraction. In T cells Mo-T the levels of the mature enzyme were lower. Similarly, confocal laser scanning microscopy (CLSM) revealed intense membrane staining in differentiated U-937 and KG-1 cells and weaker staining in Mo-T cells. In these cells cathepsin X was significantly co-localised with β-integrins, molecules responsible for cell adhesion and signalling.

By using MTS test we determined the impact of cathepsin X on the adhesion of pro-monocyte U-937 cell line. Inhibitor of cysteine proteases E64, inhibitors of cathepsin X CA-074 and CA-074Me and neutralizing monoclonal antibody 2F12 against cathepsin X significantly decreased the adhesion of U937 cells, previously differentiated by phorbol 12-myristate-13-acetate (PMA). Chicken cystatin showed only a moderate decrease of adhesion, whereas monoclonal antibody against another cysteine proteinase, cathepsin B, showed no influence at all. U937 cells, differentiated by PMA adhere to plastic via $\alpha_M\beta_2$ integrin, and inhibition of adhesion triggers apoptosis (anoikis) in detached cells. Obviously, the inhibitors capable of impairing cathepsin X activity enhance apoptosis of U937 cells by decreasing the adhesion to plastic surface, indicating an important function this enzyme might have in regulation of the activity of $\alpha_M\beta_2$ integrin (Mac-1). This effect was also pronounced on fibrinogen-coated surfaces, but not on fibronectin and matrigel where Mac-1 receptor is not involved in adhesion.

Our results demonstrate that in immune cells cathepsin X interacts with integrin molecules and may activate Mac-1 receptor. Further studies, including modelling of cathepsin X expression by gene transfer and siRNA silencing will reveal more details of the function of this enzyme in immune and tumour cells.

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Cleaved uPAR; a possible predictor of response in non-small cell lung cancer treated with chemotherapy

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New cancer therapies are emerging in non-small cell lung cancer (NSCLC), and more treatment options are available even in the disseminated lung cancer setting. This leaves an urgent need for easy, non-invasive and in-expensive methods to identify patients who can expect treatment response. We have previously demonstrated the prognostic importance of components of the plasminogen activation system in NSCLC. Urokinase (uPA) cleaves its glycolipid-anchored receptor, uPAR. uPAR(I) is liberated and the cleaved uPAR(II-III) stays on the cell surface. The amounts of uPAR(II-III) and uPAR(I) may be directly related to the uPA activity and therefore provide different information than the by ELISA measured total amount of uPAR. In the present study we investigate the role of different uPAR forms as predictors of response to chemotherapy (CT) in NSCLC.

Time-resolved fluoroimmunoassays have been designed for the individual measurements of uPAR(I-III), uPAR(I-III) + uPAR(II-III), and uPAR(I), respectively. The amounts of uPAR(II-III) can be calculated. These assays were applied on pre-treatment blood plasma samples from 35 patients diagnosed with stage IIIa-IV NSCLC and treated with first line CT. Patients received 1-24 cycles of CT (median 9). Patients were divided due to response to CT (as evaluated by computed tomography scans) in 1) complete and partial remission (CR+PR) $n=14$, and 2) no change (NC) $n=21$.

We found significantly elevated levels of uPAR(I-III), $p=0.008$, in the pre-treatment samples from patients having NC to CT compared to patients having CR+PR. A similar but less significant pattern was found for uPAR(I-III) + uPAR(II-III), $p=0.03$. The correlation between uPAR(I-III) and uPAR(I-III) + uPAR(II-III) was $r=0.79$, $p<0.0001$. There was no significant value of uPAR(I) or uPAR(II+III) as predictors of response. Further investigations in larger patient materials are needed to evaluate the possible clinical importance of this observation.

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Annexin-V apoptosis analysis of human malignant glioma-derived cell cultures treated with clomipramine hydrochloride

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Background: Previous research in our laboratories has shown that Clomipramine Hydrochloride, a tricyclic antidepressant in use for over thirty years, selectively kills neoplastic cells *in vitro* whilst leaving normal brain cells unaffected (Rooprai *et al Acta Neurochirurgica*: 145: 145-148 2003). Moreover, we demonstrated that Clomipramine targets the mitochondria of tumour cells and triggers Caspase 3 mediated apoptosis (Daley *et al Biochem Biophys Res Com* 328: 623-632 2005; Meredith et al The FASEB Journal, published online May 2005). The purpose of this study was to evaluate whether a range of early passage cell cultures and established cell lines, derived from patients with malignant glioma, would display different sensitivities, with regard to apoptotic cell death, when exposed to Clomipramine.

Methods: An Annexin-V flow cytometric assay was used to determine the mechanism of cell death, either necrosis or apoptosis, according to drug concentration and period of incubation. Cells grown to 90% confluence in $25cm^3$ flasks were incubated with concentrations of Clomipramine from 20μM – 100μM, for up to 6 hours. Cells were harvested and resuspended in calcium binding buffer, which triggers translocation of calcium-regulated phosphatidylserine residues to the nuclear envelope, before removing 500μl of the single cell suspension to a FACS tube. Controls used in the analysis were performed by omission of the drug incubation in one flask (positive control), and addition of 1uM staurosporine to another flask (negative control). Annexin-V FITC and propidium iodide were added to all tubes and incubated for 15 minutes at room temperature, in the dark. Subsequent to this, binding buffer was added to each tube and analysed using a BD FacsCalibur.

Results: Of the five malignant gliomas tested, the two established cell lines had the lower apoptotic threshold, with a significantly higher percentage of apoptotic cells present at 60μM and above when compared to the control sample. The three early passage cultures, developed 'in house' from biopsy, had higher apoptotic thresholds, withstanding up to 100μM Clomipramine incubation for six hours. Normal human astrocytes were assayed in parallel, and showed that Clomipramine did not cause cell death at the concentrations tested.

Conclusions: It may be possible, in a larger study, to predict individual patient response to Clomipramine using the Annexin-V assay, alongside Bcl-2 analysis and CYP (P450) gene metabolism testing, on the patient's own tumour cells and blood plasma. The difference in sensitivities seen in this small study indicates the importance of analysing early passage cultures, which retain the original morphology and cellular heterogeneity to a greater extent, alongside established malignant glioma cell lines.

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Optimization of electric pulse parameters for efficient electrically-assisted gene delivery into canine skeletal muscle

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Skeletal muscle is an attractive target tissue for delivery of therapeutic genes, since it is usually large mass of well vascularized and easily accessible tissue with high capacity for synthesis of proteins, which can be secreted either locally or systemically. Electrically-assisted gene delivery into skeletal muscle of a number of experimental animals has already been achieved using two different types of electroporation (EP) protocols. The first one utilized only low voltage electric pulses with long duration (e.g. 100-200 V/cm, 20-50 ms). Lately it has been shown, that better transfection efficiency can be achieved using combination of high voltage (HV) electric pulses (600-800 V/cm, 100 µs), which cause permeabilization of cell membrane, followed by low voltage (LV) electric pulses to enable electrophoresis of DNA across destabilized cell membrane.

The aim of this study was to determine optimal EP protocol for delivery of plasmid DNA into canine skeletal muscle. For this purpose we injected 150 µg/150 µl of plasmid, encoding green fluorescence protein (GFP), intramusculary into *m. semitendinosus* of 6 beagle dogs. Electric pulses were delivered 20 minutes after plasmid injection, with electric pulses generator Cliniporator (IGEA, Carpi, Italy), using needle electrodes. Altogether 5 different EP protocols were utilized, each applied to two muscles. Three of these protocols utilized combination of one HV pulse (600 V/cm, 100 µs), followed by different number of LV pulses. Two protocols were performed by application of LV pulses only. The control group received only injection of plasmid without application of electric pulses. Incisional biopsies of transfected muscles were performed 2 and 7 days after the procedure and transfection efficiency was determined using fluorescence microscope on frozen muscle samples.

The highest level of GFP fluorescence in the muscle was observed in EP protocol, using either 1 HV pulse, followed by 4 LV pulses (80 V/cm, 100 ms, 1Hz) or with 8 LV pulses (200 V/cm, 20 ms, 1Hz). In both protocols significant GFP fluorescence was detectable both at 2 and 7 days after transfection. Markedly lower degree of transfection was achieved using EP protocol, utilizing 1 HV, followed by 8 LV (80 V/cm, 400 ms, 1Hz). GFP fluorescence was less pronounced compared to the first two protocols. Furthermore, it was detectable only on muscle samples, taken at day 2 after electrotransfection. No GFP fluorescence was detectable either at 2 or 7 days after electrotransfection on muscle samples, taken from control group and from groups, where 1 HV, followed by 1 LV (80 V/cm, 400 ms) or 6 LV pulses (100 V/cm, 60 ms, 1Hz) were used.

Tissue swelling at the site of electroporation, which spontaneously resolved within 2 to 3 days after the procedure, was the only observed side-effect of the procedure.

In conclusion, according to our study, it is possible to achieve good transfection efficiency of canine skeletal muscle using two different EP protocols, either combination of 1 HV (600 V/cm, 100 μs) pulse, followed by 4 LV pulses (80 V/cm, 100 ms, 1Hz), or 8 LV pulses (200 V/cm, 20 ms, 1Hz), resulting in expression of transgene, lasting at least 7 days.

Web-Based Electronic Data Collection: making clinical trial data collection simple and reliable

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Despite the fact that Electronic Data Collection (EDC) tools have been available for more than two decades, clinical trials are still mainly conducted using paper data collection as the primary tool. The answer for this can be partially contained in the fact that standards for interchange of clinical data among different parties have to be developed, and that these standards should be extended to facilitate data collection at the investigation site. Present technological applications often do not have adequate functionality to meet the current needs. In addition, available commercial applications are usually economically acceptable and the cost is reasonable only for the large-scale trials. And even then they can hardly meet special trial needs, like integration of other data sources and medical devices. It is inherent to the trial process that it can be modified during the study, and thus EDC system should be flexible to support those changes. With respect to all demands listed above it is still hardly possible to expect a single system that could suit the variety of different trials needs.

At the Laboratory of Biocybernetics (Faculty of Electrical Engineering, University of Ljubljana) we develop customized web-based EDC systems for clinical trials. We use Internet technologies to reach mobility, to enable multimedia content and to achieve wide user acceptance. This approach let us reduce clinical trial costs and duration in following ways:

- Study can be conducted on the existing computer infrastructure – investigators need just a web-browser installed on any PC with access to the Internet.
- There is no need for software installation or maintenance on the investigator's site – the system is set on our servers or on the servers at any other institution which offers web-hosting.
- Multi-center clinical trials are supported by the system.
- Data monitoring time and expenses are reduced due to anytime access to the data from any computer.
- Data cleaning time is reduced due to the integrated data validation routines which do not let investigators to enter meaningless or erroneous data.
- Trial can be followed on daily basis through the online statistics.
- Further data analysis can be done by data export to appropriate form (like Excel table or text file).

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The Bcl-2 family members: mediators of cathepsin-induced apoptosis

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Programmed cell death is the major mechanism by which multicellular organisms preserves tissue homeostasis and remove infected, damaged or potentially dangerous cells (Hengartner, 2000). Caspases, a family of cysteine peptidases, play a major role in apoptosis execution. In addition apoptosis was found to be triggered by the leakage of cathepsins from lysosomes to the cytosol (Leist et al., 2001; Turk et al., 2002). In this work we show by several methods that the neuronal cell line SH-SY5Y undergone apoptosis after selective lysosomal permeabilization induced with LeuLeuOMe. Thus included morphological changes, caspase-3 activation, PARP cleavage, presence of cathepsins in the cytosol and cytochrome c release. Specifically, lysosomal peptidases were released into the cytosol thus cleaving the proapoptotic Bcl-2 homologue Bid and degrading the antiapoptotic Bcl-2 homologue Bcl-2. Lysosomal rupture after LeuLeuOMe treatment was confirmed by LysoTracker Green-uptake method and was not prevented neither by E-64d, a general inhibitor of papain-like cysteine peptidases nor by z-VAD-fmk, a general caspase inhibitor.

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Lectins isolated from mushroom Clitocybe nebularis

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Lectins are carbohydrate-binding proteins which potentially link to cell surface glycoconjugates and elicit many physiological effects. Consequently lectins possess insecticidal, immunomodulatory, antiviral, apoptosis-inducing, antitumor activities and have antiproliferative effect on cancer cells. Galectins are a family of lectins found in animals, humans and also in fungi. These proteins exhibit an important function in tumor progression and are therefore potential targets for the diagnosis and treatment of cancer.

In our research we isolated several lectins from fruiting bodies of the mushroom *Clitocybe nebularis* by specific affinity chromatographies. They have different binding specificities towards sugars, different isoelectric points and N-terminal sequences with no similarities to any lectin or protein in the databases. We showed that these lectins have strong insecticidal properties but other activities are yet to be found out.

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Proteomics of astrocytic neoplasms - the search for tumor-vascular/matrix interactions

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The most malicious tumor of the central nervous system (CNS) is *glioblastoma multiforme* (GBM). Tumor cell infiltration together with cellular atypia, necrosis and recruitment of new blood vessels characterize the GBMs. By serial transplantation of human GBM biopsies into the CNS of immunodefficient nude rats, two different tumor phenotypes were obtained. The transplanted xenografts initially displayed a highly invasive phenotype showing no signs of angiogenesis. By serial transplantation in animals, the phenotype changed to a less invasive, predominantly angiogenic phenotype.

Extracts from the tumors were sub-fractioned to enrich for hydrophobic and membrane proteins. Samples were differentially labeled using CyDye chemistry and co-separated by two dimensional-differential gel electrophoresis (2D-DIGE). Differentially expressed spots of interest were robotically processed and the extracted peptide pools were used for protein identification by matrix assisted laser disorption ionization time-of flight mass spectrometry (MALDI TOF MS) and MALDI TOF TOF mass spectrometry. Data were analyzed using various bioinformatics tools. The functional significance of one of the differentially expressed proteins was assessed using cell migration and invasion assays where the protein was inhibited by specific inhibitors and a monoclonal antibody.

We focussed on differentially expressed proteins that are potentially involved in tumor cell invasion and angiogenesis. For two proteins identified in the invasive phenotype, increased expression was confirmed by immuno-histochemistry and Western blot. Applied protein inhibitors strongly reduced the invasiveness of cancer cells, an effect that was reversible after withdrawal of the inhibitor, indicating a specific non-toxic effect. Additional candidate proteins are validated by antibody techniques and other proteomic methodologies including quantitative MS as well as functional approaches. In conclusion, the xenotransplantation model developed clearly separates the invasive phenotype from the angiogenic phenotype and a number of potential tumor markers of the two phenotypes can be delineated by the presented proteomics approach. Proteins were identified to play an important role in glioma cell invasion, and their action was effectively inhibited by appropriate inhibitors.

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Clinical relevance and tumor biological role of the urokinase receptor mRNA splice variant uPAR-del4/5

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Strong clinical and experimental evidence has accumulated that interaction of the urokinase-type plasminogen activator (uPA) with its three-domain receptor (uPAR, CD87) facilitates extravasation and intravasation of tumor cells.

Several splice variants of uPAR, which is encoded by 7 exons, have been reported, including uPAR-del4/5 lacking exons 4 and 5. This variant encodes a uPAR form in which domain DII of uPAR is missing. Analysis of uPAR-del4/5 mRNA expression revealed that this variant is predominantly expressed in tumor cells. Furthermore, elevated uPAR-del4/5-expression is significantly associated with shorter disease-free survival of breast cancer patients (Luther et al. 2003, Thromb Haemost 89: 705-17; Kotzsch et al. 2005, Eur J Cancer 41: 2760-8).

For analysis of the tumor biological role of this uPAR variant, expression plasmids encoding the splice variant uPAR-del4/5 were generated and stably transfected into a series of breast cancer cell lines (MDA-MB-231, CAMA-1, MCF-7, and ZR-75) and into an ovarian cancer cell line (OV-MZ-6).

The transfected cells were analyzed for expression of uPAR-del4/5 using uPAR ELISA, and showed a 5-10 fold increased expression of uPAR-del4/5 as compared to the vector-transfected cells. The cells displayed no significant difference with respect to morphology and proliferation. The stably transfected cell lines have been analyzed in Matrigel invasion and in adhesion assays. Interestingly, the results demonstrate that the tumor cell lines overexpressing uPAR-del4/5 exhibit a significant decrease of invasion through Matrigel as well as a significant decrease of adhesion to several ECM component proteins in comparison to the vector-transfected control cells.

Subsequently to the investigations of the characteristics of uPAR-del4/5 in *in vitro* analysis, animal experiments will be performed to test whether differences observed *in vitro* lead to an effect in tumor growth and metastasis. Furthermore, comparison of the gene expression pattern in uPAR-del4/5 overexpressing cells versus vector transfected cells may give new insights into the tumorbiological role of uPAR-del4/5 and represent new candidate genes for prognostic markers in breast cancer.

Correlation of chromosomal abnormalities and results of microsatellite markers analysis in radiation induced T-cell lymphomas in mice

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The aim of the presented investigations was to map the genes responsible for the development of radiation induced T-cell lymphoma in recombinant congenic mouse strains (RCS) CcS/Dem. The tumors were histopathologically and immunologically classified. Morphology of mouse lymphomas was evaluated by microscopic examination. Estimating the phenotype of lymphomas was based on flow cytometry (FCM) analysis. The lymphomas were classified according to “Bethesda proposals for classification of lymphoid neoplasms in mice” (2002).

In order to map the genes responsible for radiation-induced T-cell lymphomas the neoplasms representing two from seven found immunophenotypes were selected: $CD90^{+}(CD4/CD8)^{+}sCD3^{+}$ and $CD90^{+}(CD4/CD8)^{+}sCD3^{-}$. The microsatellite analysis was performed using the PCR amplification method. Genomic DNA was obtained from the tail of mice. DNA of all animals with tumor was characterized for the 24 microsatellite loci. The PCR amplification was performed as described by Dietrich et al.(1992).

The genetic linkage has been evaluated using the SAS package, and the significance of the observed linkages has been corrected for whole genome screening as described by Lander and Kruglyak (1996). The obtained results suggested linkage of the genes responsible for radiation-induced T-cell lymphomas in CcS mice involving chromosomes 10 (D10 Mit134) and 16 (D16 Mit34).

Cytogenetic analysis of these tumors was performed according to the standard procedure. G-bands were obtained with Wright stain. The karyotypes were described according to the International Committee on Standardized Genetic Nomenclature for Mice: Rules for nomenclature of chromosome aberrations (2005) and documented with the Lucia imaging system. The analysis of the found non random chromosome abnormalities - translocations and metacentric translocations - seems to confirm the results obtained by microsatellite investigations.

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Are AKR1C1 and AKR1C3 responsible for development and progression of endometrial cancer?

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Endometrial cancer is one of the most common malignancies in developed countries. In Slovenia, this type of cancer ranks fourth among all women malignancies and the incidence (29.1 cases per 100,000 predicted for 2005) is higher than that reported for the Western Europe and USA. It is well known that estrogens unopposed by progesterone present high risk for endometrial cancer. The hormone action can be regulated at the receptor level and at the prereceptor level by enzymatic interconversions of highly active hormones (estradiol, progesterone) with their inactive counterparts (estrone, 20α-hydroxyprogesterone). The hyxdroxysteroid-dehydrogenases (HSD) AKR1C1 and AKR1C3 are multifunctional enzymes which catalyze conversion at the 3-, 17-, and 20-position of estrogens, androgens and progestins. Since AKR1C1 is predominantly 20α–HSD and AKR1C3 possesses high 17β-HSD activity these enzymes could be responsible for modulating the levels of bioactive estradiol and progesterone in endometrium.

In our study, we analyzed the mRNA levels of AKR1C1 and AKR1C3 and the presence of the AKR1C3 protein in endometrial cancer. We extracted RNA and protein fraction from 16 specimens of endometrial cancer and adjacent normal endometrium of the same patient. The same endometrial samples were formalin-fixed, paraffin embedded and sectioned for histopathological analysis and immunohistochemical staining with AKR1C3 antibody. Quantification by real-time PCR revealed higher expression of AKR1C1 in 9 and AKR1C3 in 4 specimens of endometrial cancer. Western blot analysis confirmed the latter findings at the protein level and also immunohistochemistry demonstrated staining of AKR1C3 protein in cancerous endometrium but not in normal tissue. Our recent data thus indicate that changes in AKR1C1 and AKR1C3 expression may be responsible for endometrial cancer development and progression due to increased estradiol and/or decreased progesterone levels.

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Optimization of treatment parameters for successful DNA electrotransfer into skeletal muscle of mice

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Electrically assisted gene delivery to skeletal muscles is an attractive approach in two therapeutic applications: gene therapy and DNA vaccination. Prolonged expression and secretion from skeletal muscle is crucial for systemic distribution of therapeutic proteins.

The aim of this study was to determine optimal treatment protocol for electrically-assisted delivery of plasmid DNA into murine skeletal muscle.

To determine optimal treatment parameters for successful transfection of murine skeletal muscle, evaluation of different sets of electrical parameters, time interval between plasmid DNA injection and application of electric pulses as well as different plasmid DNA concentration were determined in tibialis cranials muscle of C57Bl/6 mice using DNA plasmid encoding green fluorescent protein (GFP). We used two different sets of pulses, a combination of high voltage (HV) and low voltage (LV), LV alone and a control group without electric pulses. Electrical parameters for combinations were 1HV (HV=600 V/cm, 100 µs)+1LV (400 ms), 1HV(HV=600 V/cm, 100 µs)+4LV (100 ms, 1 Hz), 1HV (HV=600 V/cm, 100 µs)+8LV (50 ms, 2 Hz). Electrical parameters for LV pulses alone were 8LV (200 V/cm, 20 ms, 1 Hz) and 6LV (100 V/cm, 60 ms, 1 Hz). The second parameter was time between the injection of plasmid DNA and application of electric pulses. Electric pulses were applied 5 s, 5 min, 10 min, 20 min, 30 min, 1 h and 2 h after injection of DNA. Plasmid DNA concentrations tested were 1, 5, 10, 20 and 30 µg/muscle. Transfection efficiency was followed by *in vivo* imaging system using fluorescence stereo microscope, which enable us to follow up the duration of GFP expression noninvasively. In addition, transfection efficiency was assessed on 24 frozen tissue sections/muscle 1 week after the electrically-assisted gene delivery using fluorescence microscope equipped with cooled digital color camera for recording the images. The pictures were analyzed using the Visilog software tool. Transfection efficiency was defined as the percentage of muscle area expressing GFP with regard to the total muscle area.

Transfection was achieved with all sets of electric pulses applied. The combination of HV and LV pulses was more efficient than LV pulses alone. The highest transfection efficiency was achieved with the set of 1HV and 4LV pulses. The optimal time interval between the DNA injection and application of pulses was 5 s. Transfection efficiency decreased with longer time interval and reached little or no transfection at 1 or 2 hours. Transfection efficiency increased with increasing amount of plasmid DNA. The transfection was detected already with 1 µg but was the highest with 30 µg of plasmid DNA. The expression of the transferred gene was high and continuous over at least 10 weeks.

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The study has shown that electrically assisted gene delivery is a successful method for plasmid DNA transfer into skeletal muscle. The optimal treatment parameters for electrically assisted gene delivery into skeletal muscle of C57Bl/6 mice in our experiments were 1HV+4LV, time interval 5 s and plasmid DNA concentration 30 µg/muscle. These results are the groundwork for evaluating therapeutic protein efficiency in gene therapy and DNA vaccination.

The effect of xantohumol on normal and cancer cell lines and its modulatory effects on genotoxicity of heterocyclic amine (IQ) in HepG2 cells

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Xanthohumol (XN) is the principal prenylated chalcone of the female inflorescences of the hop (*Humulus lupulus* L.) and commonly used as a preservative and flavouring agent in beer. It's natural role is to protect the plants against insect feeding. XN has been reported to have a broad spectrum of inhibitory mechanisms at the initiation, promotion and progression stages of carcinogenesis. The aims of the present study were to explore: 1) the cytotoxicity of XN on normal and neoplastic human cell lines and its effects on cell adhesion and apoptosis and 2) the potential of XN to inhibit the heterocyclic amine IQ induced genotoxicity in *S. typhimurium* TA98 and in human hepatoma HepG2 cells.

Normal human umbilical vein cells (HUVEC) and diploid immortalized human breast epithelial cell line (MCF10A) with normal karyotype, and two tumour cell lines (breast MCF10AneoT and glioblastoma U87 cell line) were used to study cytotoxicity of XN and its effect on adhesion of the two cancer cell lines by MTT assay. The effect of XN on apoptosis was studied by staining the cells with DNA binding dyes acridine orange and ethidium bromide. Mutagenicity and antimutagenicity of XN was tested with *S. typhimurium* TA98 using the standard plate incorporation procedure (Ames test). Genotoxicity of XN and its potential to inhibit IQ induced DNA damage in human hepatoma HepG2 cells were determined by the comet and the potential of XN to modulate CYP1A activity was determined with the EROD assays.

XN was cytotoxic for cancer cells (U87 and MCF10AneoT) at 15 μM concentration and for the normal cells (HUVEC and MCF10A) at 50 μM concentration. At subtoxic concentrations, XN had a significant impact on adhesion of U87 cell line to Matrigel and fibronectin, but it did not affect the adhesion of MCF10AneoT. At 30 μM concentration, XN triggered apoptosis in all four cell lines. The proportion of apoptotic cells did not differ among cell lines after 24 hours, whereas after 48 hours, it was significantly higher in cancer cell lines than in the normal cells. In the Ames test with *Salmonella typhimurium* TA98, XN showed dose dependent antimutagenic activity against the indirect acting mutagen IQ. In HepG2 cells, XN reduced EROD activity and at 10 μg/ml (28.25 μM) concentration completely prevented IQ induced DNA damage, indicating that the mechanism of XN antigenotoxicity is inhibition of metabolic activation of IQ.

In conclusion, this data showed selective cytotoxicity of XN for cancer cells, which can at least partly contribute to selective induction of apoptosis. Furthermore, XN is a potent inhibitor of IQ induced genotoxicity. Therefore, XN has a potential as chemopreventive agent at the initiation and progression step of cancer development and could present a candidate for prevention and treatment of cancer.

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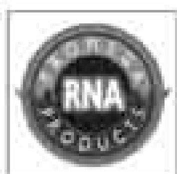

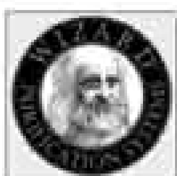

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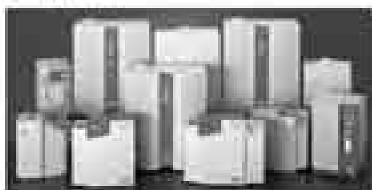

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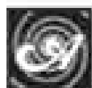

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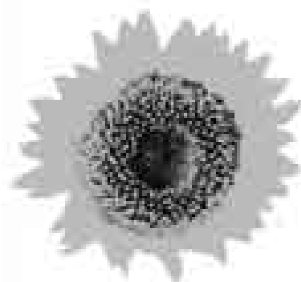

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